Universitat
de les Illes Balears

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FACTORES ALIMENTARIOS MODULADORES DE LA INFLAMACIÓN

Xavier Capó Fiol

Universitat de les Illes Balears

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2017

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FACTORES ALIMENTARIOS MODULADORES DE LA INFLAMACIÓN

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DECLARO:

Que la tesis doctoral que lleva por título *Factores alimentarios moduladores de la inflamación,* presentada por Xavier Capó Fiol para la obtención del título de doctor, ha sido dirigida bajo mi supervision.

Y para que quede constancia de ello firmo este documento.

Palma de Mallorca,

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Dr. Antonio Sureda Gomila, de la Universitat de les Illes Balears

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Palma de Mallorca,

Agraïments

Primer de tot donar les gràcies als meus directors, els doctors Antoni Pons i Antoni Sureda per tota la seva ajuda, consell i suport per dur a terme aquesta tesi doctoral. El Dr. Antoni Pons per obrir-me les portes de la investigació quan just feia una setmana que havia acabat els estudis, a més d'oferir-me totes les facilitats del món i tota la llibertat possible per dur a terme aquesta tesi doctoral i per haver-me permès fer feina amb les mostres més inimaginables. Menció especial també per el Dr. Antoni Sureda per ser el meu mestre dins el laboratori, per tenir sempre una mà estesa per ajudar en qualsevol cosa, he tengut la sensació que més d'un director eres un company més de laboratori.

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ABREVIATURAS

AA Ácido araquidónico / Arachidonic acid

AP-1 Proteína activadora 1/ Activator protein 1

CAT Catalasa/ Catalase

CK Creatina quinasa / Creatin kinase

COX Ciclooxigenasa/ Cyclooxigenase

COXIV subunidad IV de la citocromo C oxidasa/ Cytochrome c Oxidase Subunit IV i

CPR Proteina C reactiva / C-reactive protein

CSF Factores estimuladores de colonias/ Colony-stimulating factor

DAMPs Patrón molecular asociado a daño celular/ Damage-associated molecular patterns

DHA Ácido docosahexaenoico / Docosahexaenoic acid

DGLA Ácido dihomo-gamma-linolénico / Dihomo-gammalinolenic acid

EDTA Ácido etilendiaminotetraacético / Ethylenediaminetetraacetic acid

EPA Ácido eicosapentaenoico / Eicosapentaenoic acid

GLA Ácido gamma-linolénico / Gamma-linolenic acid

GM-CSF Factor estimulante de macrófagos granulocitos/Granulocyte-macrophage colony-stimulating factor

G-CSF Factor estimulante de granulocitos/ Granulocyte-colony stimulating factor

GPx Glutatión peroxidasa / Glutathione peroxidase

GRd Glutatión reductasa / Glutathione reductase

GSH Glutatión / Glutathione

GSSG Glutatión oxidado / Oxidized glutathione

5-HEPTE Ácido 5-hidro peroxieicosatetraenoico/ 5-hydroperoxyeicosatetraenoic acid

HSP-70 Proteína de choque térmico 70/ Heat Shock Protein 70

ICAM Moléculas de adhesión intercelular/ Intercellular Adhesion Molecule

IDR Ingesta diaria recomendada/ Recommended daily intake

Ig Inmunoglobulina/ Immunoglobulin

IMC Índice de Masa Corporal/Body Mass Index

IFNγ Interferón gamma / Interferon gamma

IL Interleuquina / Interleukin

iNOS Óxido nítrico sintasa / Nitric oxide synthase

LDH Lactato deshidrogenasa / Lactate dehydrogenase

LT Leucotrieno/ Leukotriene

LOX Lipooxigenasa / Lipoxygenase

LPS Lipopolisacáridos / Lipopolysaccharide

MAPK Proteína quinasa activada por mitógeno / MitogenActivated Protein Kinases

MCP-1 Proteina quimitocatica de monocitos/ Monocyte chemoattractant protein-1

MDA Malonildialdehído / Malondialdehyde

MIP-1α Proteina inflamatoria de macrófagos 1α / Macrophage Inflammatory Proteins 1α

MPO Mieloperoxidasa / Myeloperoxidase

MitND5 Subunidad V de la NADH deshidrogenasa/ Mitochondrially encoded NADH dehydrogenase 5

NFκB Factor nuclear potenciador de las cadenas ligeras kappa de las células B activadas / Nuclear factor kappa-lightchain-enhancer of activated B cells

NK Célula Natural Killer Natural killer cell

NO Óxido nítrico/ Nitric oxide

PAMPs Patron molecular asociado a patógenos/ Pathogen-associated molecular patterns

PBMC Célula mononuclear de sangre periférica / Peripheral blood mononuclear cell

PGC-1α coactivador del receptor activado por proliferadores peroxisomales 1 α/ Peroxisome proliferator-activated receptor gamma coactivator 1-alpha

PGs Prostaglandina/Prostaglandin

PMA Acetato de forbol miristato / Phorbol myristate acetate

PPAR Receptores activados de proliferación de los peroxisomas / Peroxisome proliferator-activated receptors

PRR Receptores de reconocimiento de patrones/ Pattern Recognition Receptors

ROS Especies reactivas de oxígeno / Reactive oxygen species

RONS Especies reactivas de oxígeno y de nitrógeno / Reactive oxygen and nitrogen species

Rv Resolvina/ Resolvin

SLC-2A4 Miembro 4 de la familia 2 de transportadores de solutos / Solute Carrier Family 2 Member 4)

SOD Superóxido dismutasa / Superoxide dismutase

TGF-β Factor transformante de crecimiento β/ Transforming growth factor beta

TNF-α Factor de necrosis tumoral α / Tumor necrosis factor α

TLR Receptor tipo Toll / Toll-like receptor

Trx Tioredoxina / Thioredoxin

TrxR Tioredoxina reductasa / Thioredoxin reductase

Tx Tromboxano/ Tromboxan

UCP Proteína desacopladora / Uncoupling protein

VCAM Moleculas de adhesión vascular/Vascular cell adhesion protein

RESUMEN

La inflamación es un proceso tisular caracterizado por una serie de fenómenos moleculares, celulares y vasculares con finalidad defensiva y reparadora frente a agresiones físicas, químicas o biológicas. La realización de actividad física aguda provoca una respuesta inmunológica asociada a la aparición de un estado pro-inflamatorio agudo. Sin embargo, la práctica de ejercicio regular reduce los marcadores de inflamación. Además, el ejercicio agudo incrementa la producción de especies reactivas de oxigeno (ROS) incrementando el daño oxidativo, pero a la vez estimula las defensas antioxidantes endógenas. Algunos nutrientes como los ácidos grasos omega-3, las vitaminas o los polifenoles, pueden modular el balance oxidativo y la respuesta inflamatoria. Asimismo, productos de oxidación de ácidos grasos también pueden tener propiedades anti-inflamatorias, de hecho, el sebo de cerdo, que se elabora en condiciones de oxidación de ácidos grasos, se ha utilizado tradicionalmente como tratamiento frente a procesos inflamatorios agudos.

El objetivo principal de esta tesis doctoral es estudiar los efectos de nutrientes y de alimentos funcionales enriquecidos con ácidos grasos esenciales y antioxidantes sobre marcadores de estrés oxidativo e inflamación asociados a la realización de ejercicio físico agudo y sobre las adaptaciones derivadas de un período de entrenamiento regular y evidenciar las propiedades antiinflamatorias del sebo de cerdo. Deportistas de diferentes modalidades (futbolistas y taekwondistas) y de diferentes edades (jóvenes de menos de 25 años y seniors de más de 40 años) que consumieron una bebida funcional de almendra y aceite de oliva enriquecida con ácido docosahexaenoico (DHA) y vitamina E durante un periodo determinado han participado en los estudios.

Los efectos anti-inflamatorios del sebo de cerdo se estudiaron en un modelo animal y para encontrar los componentes del sebo responsables de los mismos se utilizaron células inmunitarias humanas. El entrenamiento regular incrementa los niveles de proteínas desacoplantes 2 y 3 (UCP-2 y UCP-3) potenciando las defensas contra la producción ROS mitocondrial en células inmunitarias mononucleares periféricas (PBMCs), además provoca un cambio en la estrategia de eliminación ROS, pasando de un sistema basado en la catalasa a un sistema basado en la glutatión peroxidasa. El ejercicio regular provoca un incremento de los niveles circulantes de prostaglandina E_1 (PGE_1). La realización de un ejercicio físico intenso y prolongado incrementa los marcadores de daño oxidativo a la vez que induce una adaptación de los mecanismos antioxidantes para reducir el daño oxidativo en PBMCs. Este ejercicio incrementa los niveles plasmáticos de IL-6 en futbolistas, mientras que la suplementación de la dieta con DHA no altera los niveles de citoquinas plasmáticas. El ejercicio físico intenso incrementa la capacidad de producción de citoquinas pro-inflamatorias, además de PGE_1, PGE_2 y Resolvina D_1 (RvD_1) en PBMCs estimuladas *in vitro* con lipolisacárido (LPS). El ejercicio físico extenuante incrementa la activación del NFκB en PBMCs y los niveles plasmáticos de la molécula de adhesión intercelular 3 (sICAM3) y sL-Selectina. La suplementación con DHA potencia las capacidades antioxidantes de las PBMCs y de los neutrófilos y atenúa la respuesta inflamatoria después de la estimulación *in vitro* con LPS o *phorbol myristate acetate* (PMA) respectivamente. El consumo durante un mes de la bebida funcional atenúa el grado de activación de las células inmunes. El sebo de cerdo (por vía tópica o en forma de extracto hidroalcohólico) reduce la respuesta inflamatoria, encontrándose algunos componentes responsables de estos efectos.

En conclusión la suplementación de la dieta con nutrientes como los ácidos grasos poliinsaturados omega-3 y antioxidantes como polifenoles o vitamina C modulan la respuesta inflamatoria y el balance oxidativo asociado al entrenamiento y a la realización de un ejercicio físico. La manteca de cerdo presenta potentes efectos anti-inflamatorios atribuibles a diferentes componentes hallados en el extracto hidroalcohólico.

RESUM

La inflamació és un procés tissular caracteritzat per una sèrie de fenòmens moleculars, cel·lulars i vasculars amb finalitat defensiva i reparadora front a agressions físiques, químiques o biològiques. La realització d'activitat física aguda provoca una resposta immunològica associada a l'aparició d'un estat pro-inflamatori agut. Per contra, la pràctica d'exercici de forma regular redueix els marcadors d'inflamació. A més l'exercici agut incrementa la producció d'espècies reactives d'oxigen (ROS) incrementant el dany oxidatiu, però a la vegada estimula les defenses antioxidants endògenes. Alguns nutrients com els àcids grassos omega-3, les vitamines, o els polifenols poden modular el balanç oxidatiu i la resposta inflamatòria. S'ha demostrat que els productes d'oxidació dels àcids grassos també poden tenir propietat anti-inflamatòries , de fet, el saïm de porc, que s'elabora en condicions d'oxidació d'àcids grassos, s'ha utilitzat tradicionalment com a tractament front a processos inflamatoris aguts.

L'objectiu principal d'aquesta tesis doctoral es estudiar els efectes de nutrients i d'aliments funcionals enriquits amb àcids grassos essencials i antioxidants sobre els marcadors d'estrès oxidatiu i d'inflamació associats a la realització d'exercici físic agut i sobre les adaptacions derivades d'un període d'entrenament regular i evidenciar les propietats anti-inflamatòries del saïm de porc. Esportistes de diferents modalitats (futbolistes i taekwondistes) i de diferents rangs d'edat (joves de menys de 25 anys i sèniors de més de 40 anys) que varen consumir una beguda funcional d'ametlla i oli d'oliva enriquida amb àcid docosahexaenoïc (DHA) i vitamina E durant un període determinat han participat en els estudis.

Els efectes anti-inflamatoris del saïm de por es varen estudiar en un model animal i per a determinar els components dels saïm responsables d'aquests efectes es varen emprar cèl·lules immunitàries humanes. L'entrenament regular incrementa els nivells de proteïnes desacoblants 2 i 3 (UCP-2 i UCP-3) potenciant les defenses contra la producció de ROS a nivell mitocondrial en cèl·lules immunitàries mononuclears perifèriques (PBMCs), a més provoca un canvia en l'estratègia d'eliminació de ROS, passant-se d'un sistema basat en la catalasa a un sistema basat en la glutatió peroxidasa. L'exercici regular provoca un increment en els nivells circulants de prostaglandina E_1 (PGE_1). La realització d'un exercici físic intens i perllongat incrementa els marcadors de dany oxidatiu a la vegada que indueix una adaptació dels mecanismes antioxidants per reduir el dany oxidatiu en PBMCs. Aquest tipus d'exercici incrementa els nivells plasmàtics de IL-6 en futbolistes, per la seva banda la suplementació de la dieta amb DHA no altera els nivells de citocines inflamatòries. L'exercici físic intens incrementa la capacitat de producció de citocines pro-inflamatòries, a més de PGE_1, PGE_2 i Resolvina D_1 (RvD_1) en PBMCs estimulades *in vitro* amb lipolisacàrid (LPS). L'exercici físic extenuant incrementa l'activació de NFκB en PBMCs i els nivells plasmàtics de la molècula d'adhesió intercel·lular 3 (sICAM) i sL-Selectina. La suplementació amb DHA potencia les capacitats antioxidants de les PBMCs i dels neutròfils a més atenua la resposta inflamatòria després de l'estimulació *in vitro* amb LPS o *phorbol myristate acetate* (PMA) respectivament. El consum durant un mes de la beguda funcional atenua el grau d'activació de les cèl·lules immunitàries. El saïm de porc (per via tòpica o en forma d'extracte hidroalcohòlic) redueix la resposta inflamatòria, i s'han determinat alguns components responsables d'aquests efectes.

En conclusió la suplementació de la dieta amb nutrients amb àcids grassos poliinsaturats omega-3 i antioxidants com els polifenols o la vitamina C modulen la resposta inflamatòria i el balanç oxidatiu associat a l'entrenament i a la realització d'exercici físic agut. El saïm de porc presenta potents efectes anti-inflamatoris atribuïbles a diferents composts trobats en l'extracte hidroalcohòlic.

Universitat de les Illes Balears

Inflammation modulatory food factors

Doctoral Thesis, Xavier Capó Fiol, Research Group on Community Nutrition and Oxidative Stress (NUCOX), Department of Fundamental Biology and Health Sciences

Inflammation is a tissue process characterized by a series of molecular, cellular and vascular phenomena with defensive and restorative purpose against physically, chemistry or biological aggressions. Acute physical activity could cause an immune response, which is related to apparition of pro-inflammatory state. However, the practice of regular exercise reduces pro-inflammatory makers. Furthermore, acute exercise increases reactive oxygen species (ROS) production leading to oxidative damage, but at the same time acute exercise is able to enhance endogen antioxidant defences. Some nutrients such as omega 3 fatty acids, vitamins or polyphenols can modulate the oxidative balance and the inflammatory response. In this sense, products of fatty acids oxidation could present anti-inflammatory properties, in fact, lard which is elaborated under oxidation conditions, has been traditionally used treatment against acute inflammatory process.

The main objective of this doctoral thesis is to evaluate the effects of nutrients and functional foods enriched with essential fatty acids and antioxidants on oxidative stress and inflammation markers associated to acute and regular exercise adaptations. Additionally, to evidence the lard anti-inflammatory properties is also an aim of this doctoral thesis. Studies were performed with athletes from different modalities (football and taekwondo players) and different age (young under 25 years and seniors over 40 years) that consumed an almond and olive oil based functional beverage enriched with docosahexaenoic acid (DHA) and vitamin E during a variable period. Lard anti-inflammatory effects were studied in an animal model, and the lard components responsible of anti-inflammatory effects were tested using human immune cells.

Regular training increases uncoupling protein 2 and 3 (UCP-2 and UCP-3) levels, enhancing defences against mitochondria ROS production in peripheral blood mononuclear cells (PBMCs), besides regular exercise causes a change in ROS elimination strategy, from a system based on catalase activity to a system based on glutathione peroxidase activity. Regular exercise causes an increase in prostaglandin E_1 (PGE_1) plasma levels.

An intense and prolonged physical exercise increases oxidative stress markers, but also induces an adaptative response in order to reduce oxidative damage in PBMCs. This kind of exercise increases IL-6 plasma levels in football players, while DHA diet supplementation do not alter plasma cytokine levels. Intense physical exercise increase pro-inflammatory cytokine production at the same time intense exercise increases PGE_1, PGE_2 and Resolvin D_1 (RvD_1) production in lipolysacharide (LPS)-stimulated PBMCs. Extenuate physical exercise enhances NFκB activation in PBMCs, and also increases intercellular adhesion molecule 3 (sICAM-3) and sL-Selectin plasma levels. DHA diet supplementation enhances PBMCs and neutrophils antioxidant capabilities and reduces the inflammatory response after LPS or phorbol myristate acetate (PMA) *in vitro* stimulation. The intake of functional beverage for one month attenuates activation degree of immune cells. Lard (topically or as hydroalcoholic extract) reduces the inflammatory response and, some anti-inflammatory components have been isolated and determined.

In conclusion, diet supplementation with nutrients rich in omega 3 polyunsaturated fatty acids and antioxidant as polyphenols or vitamin C modulates the inflammatory response and oxidative balance associated to regular training and acute exercise. Lard exerts potent anti-inflammatory effects attributable to different compounds isolated in the hydroalcoholic extract.

LISTADO DE ÁRTICULOS ORIGINALES

La presente tesis se basa en los siguientes artículos originales:

I) Diet supplementation with DHA-enriched food in football players during training season enhances the mitochondrial antioxidant capabilities in blood mononuclear cells. **Capó X**, Martorell M, Sureda A, Llompart I, Tur JA, Pons A. Eur J Nutr. 2015 Feb;54(1):35-49. doi: 10.1007/s00394-014-0683-2

II) Effects of dietary almond- and olive oil-based docosahexaenoic acid- and vitamin E-enriched beverage supplementation on athletic performance and oxidative stress markers**. Capó X**, Martorell M, Busquets-Cortés C, Sureda A, Riera J, Drobnic F, Tur JA, Pons A. Food Funct. 2016 Dec 7;7(12):4920-4934.vPMID:27841405.

III) Docosahexanoic acid diet supplementation attenuates the peripheral mononuclear cell inflammatory response to exercise following LPS activation. **Capó X**, Martorell M, Llompart I, Sureda A, Tur JA, Pons A.Cytokine. 2014 Oct;69(2):155-64. doi: 10.1016/j.cyto.2014.05.026.

IV) Effects of Almond- and Olive Oil-Based Docosahexaenoic- and Vitamin E-Enriched Beverage Dietary Supplementation on Inflammation Associated to Exercise and Age. **Capó X**, Martorell M, Sureda A, Riera J, Drobnic F, Tur JA, Pons A. Nutrients. 2016 Oct 9;8(10). pii: E619

V) Effects of dietary Docosahexaenoic, training and acute exercise on lipid mediators. **Capó X**, Martorell M, Sureda A, Tur JA, Pons A. J Int Soc Sports Nutr. 2016 Apr 5;13:16. doi: 10.1186/s12970-016-0126-y

VI) Docosahexaenoic diet supplementation, exercise and temperature affect cytokine production by lipopolysaccharide-stimulated mononuclear cells. **Capó X**, Martorell M, Sureda A, Batle JM, Tur JA, Pons A. J Physiol Biochem. 2016 Sep;72(3):421-34. doi: 10.1007/s13105-016-0490-8.

VII) Effects of docosahexaenoic supplementation and in vitro vitamin C on the oxidative and inflammatory neutrophil response to activation. **Capó X**, Martorell M, Sureda A, Tur JA, Pons A. Oxid Med Cell Longev. 2015;2015:187849. doi:10.1155/2015/187849.

VIII) Composición farmacéutica que comprende 5-dodecanolida, su preparación y su uso.

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I. INTRODUCCIÓN

1. La inflamación

La inflamación es un proceso tisular constituido por una serie de fenómenos moleculares, celulares y vasculares con finalidad defensiva frente a agresiones físicas, químicas o biológicas (Kumar 2006). Se trata de un proceso focalizado en una zona concreta, aunque pueden darse excepciones como en el caso de la inflamación sistémica, además, se trata de una respuesta inmediata e inespecífica que puede facilitar el desarrollo de una respuesta especifica. La inflamación se caracteriza por una migración de las células inmunitarias hacia el foco inflamatorio. Como consecuencia de la inflamación se produce una dilatación de los vasos sanguíneos cercanos al foco inflamatorio y se incrementa la permeabilidad de los mismos con el objetivo de facilitar la llegada y transvasación de leucocitos al lugar de inflamación, además de facilitar la llegada de otras moléculas (Kumar 2006, Geering et al. 2013). El objetivo final de la inflamación es eliminar o inhibir al agente causante de la infección o daño celular para permitir que el organismo pueda regresar a las condiciones normales, restaurándose la funcionalidad del tejido u órgano afectado (Medzhitov 2008).

El proceso inflamatorio fue descrito 3000 años antes de Cristo por Celsus que enumeró los cuatro síntomas básicos de la inflamación: rubor, tumor, calor y dolor. Ya en el siglo XIX Rudolf Virchow planteó el quinto signo de la inflamación, la limitación funcional. En este mismo siglo XIX, Julius Cohnheim observó a nivel microscópico dos de los rasgos más característicos de la respuesta inflamatoria: aumento de la permeabilidad vascular y migración leucocitaria. En 1880, Metchnikoff estableció que la inflamación generaba leucocitos y anticuerpos con el objetivo de defender al organismo contra microorganismos, con lo que fue el primero en proponer la existencia de una relación entre la inflamación e infección. Años más tarde, Sir Thomas Lewis estableció que la liberación de ciertas sustancias químicas, como la histamina, median en los cambios vasculares durante el proceso inflamatorio (Kumar 2006). Hunter, en 1973, planteó que la inflamación no era una enfermedad, tal y como se creía hasta entonces, si no que se trataba de una respuesta del organismo que provocaba una respuesta beneficiosa sobre el organismo (Schleimer 1987).

Actualmente se postula que el proceso inflamatorio se divide en cinco etapas:

- Liberación de mediadores: En la mayoría de casos se trata de moléculas con estructura muy sencilla (péptidos, proteínas o lípidos sencillos) que son liberadas o sintetizadas ante la presencia de determinados estímulos. Entre los mediadores destacan aminas como la histamina o la serotonina, enzimas proteolíticas, el óxido nítrico (NO), citoquinas pro-inflamatorias y heparina. Otras sustancias de carácter lipídico constituyen un segundo grupo importante de mediadores de la inflamación. Se trata de sustancias sintetizadas a partir del ácido araquidónico (AA) a través de dos vías metabólicas, la de la enzima ciclooxigenasa (COX) que determina la producción de prostaglandinas (PG) y tromboxanos y la de la lipooxigenasa (LOX) que conduce a la formación de leucotrienos (LT).

- Efecto de los mediadores: Una vez liberadas al torrente sanguíneo, estas moléculas mediadoras producen alteraciones vasculares y efectos quimiotácticos que favorecen la llegada de moléculas y células inmunitarias al foco inflamatorio. Se produce un aumento de la permeabilidad vascular favoreciendo la llegada a la zona afectada, desde la sangre, de moléculas y células del sistema inmunitario (Schleimer 1987).

- Llegada de las moléculas y de las células inmunitarias al foco inflamatorio: Los mediadores liberados como consecuencia de un agente adverso producirán básicamente dos efectos consecutivos. En la fase inicial, se producirán toda una serie de alteraciones a nivel vascular que facilitarán la transvasación de los mediadores desde el torrente sanguíneo al foco inflamatorio, produciéndose lo que se conoce como edema. Posteriormente, las alteraciones vasculares, así como la liberación de factores quimiotácticos, provocan la llegada de células inmunitarias (basófilos, neutrófilos, macrófagos, linfocitos y eosinófilos) procedentes de la sangre y de los tejidos circundantes al foco inflamatorio.

- Regulación del proceso inflamatorio: Así como ocurre en la mayor parte de las respuestas inmunes, el proceso inflamatorio también lleva asociado toda una serie de mecanismos inhibidores que ayudan a finalizar o equilibrar el proceso. Los mediadores activadores, pueden provocar la inhibición de su propia síntesis cuando

se encuentran en elevadas concentraciones a través de mecanismos de retroalimentación negativa, consiguiendo, de esta forma, una modulación de la respuesta inflamatoria. La inflamación puede resolverse mediante la síntesis de moléculas lipídicas, como las resolvinas, los tromboxanos, o los leucotrienos a partir de los ácidos grasos poliinsaturados omega 3 de los fosfolípidos de membrana, que serían las responsables de la resolución de la inflamación (Schleimer 1987).

- Reparación: En esta fase se llevan a cabo los procesos de reparación de los tejidos dañados por el agente agresor o por la propia respuesta inflamatoria. Estos procesos conllevan la llegada a la zona de fibroblastos, que van a proliferar y sintetizar colágeno, la proliferación de células epiteliales y la proliferación de vasos dentro de la herida (Kumar 2006).

En función de la duración de la respuesta inflamatoria, la inflamación puede clasificarse en inflamación aguda e inflamación crónica.

1.1. La inflamación aguda

La respuesta inflamatoria aguda es una respuesta del organismo inducida por una infección o por daño tisular que conduce a una liberación coordinada de plasma y leucocitos al foco de inflamación (Kumar 2006). Esta respuesta está mejor caracterizada para las infecciones en las que se activan receptores del sistema inmune innato, como son los *Toll like receptors (TLR*). Esta primera respuesta es llevada a cabo por los macrófagos presentes en el tejido dañado y por los mastocitos. Ambos tipos celulares liberan una amplia variedad de productos inflamatorios como citoquinas, aminas vasoactivas, eicosanoides y proteínas de fase aguda (Medzhitov 2008). Los mediadores, liberados por los macrófagos residentes en el tejido dañado, y los mastocitos tendrán tanto efectos sobre la permeabilidad vascular como efectos quimiotácticos favoreciendo una llegada masiva de leucocitos, principalmente neutrófilos, además de otras sustancias que participarán en la respuesta inflamatoria.

Los neutrófilos se activan cuando llegan al tejido dañado bien sea por contacto directo con los microorganismos patógenos, o mediante las citoquinas liberada por las macrófagos residentes en el tejido dañado. Una vez activados, los neutrófilos

liberaran el contenido de sus gránulos, secretando proteinasas como elastasa, enzimas prooxidantes como la mieloperoxidasa (MPO) , la NADPH oxidasa y las especies reactivas de oxígeno y de nitrógeno (RONS) (Nathan 2006). Esta primera respuesta es altamente inespecífica por lo que afectará tanto al agente agresor como a la propias células del huésped pudiendo provocar daños colaterales, por dicho motivo este proceso debe estar altamente regulado y debe ser parado una vez se ha detenido la infección o el daño tisular.

Los mediadores que se liberan o sintetizan como consecuencia de la respuesta inflamatoria aguda pueden agruparse en siete categorías en función de sus propiedades bioquímicas: aminas vasoactivas, péptidos vasoactivos, fragmentos del sistema del complemento, mediadores lipídicos, citoquinas, quimioquinas y enzimas proteolíticas (Schleimer 1987, Granger and Senchenkova 2010). A continuación se detallan de forma breve sus principales características:

- Aminas y péptidos vasoactivos: Entre las primeras podemos destacar la histamina y la serotonina liberadas por los mastocitos y que tienen como función incrementar la permeabilidad vascular y la vasodilatación. Los péptidos vasoactivos, pueden estar almacenados en su forma activa en el interior de vesículas secretoras, o bien generarse por proteólisis de sus precursores que se encuentran en los fluidos extracelulares. Los péptidos vasoactivos más destacados son los fibrinopéptidos y la sustancia P que es liberada por las neuronas e induce la desgranulación de los mastocitos.

- Fragmentos del sistema del complemento: Las anafilotoxinas, que son los fragmentos C3a, C4a y C5a del sistema del complemento, pueden favorecer el reclutamiento de los granulocitos y los monocitos hacia el foco de inflamación, además de inducir la degranulación de los mastocitos (Barrington et al. 2001).

- Mediadores lipídicos: La mayoría son derivados de los fosfolípidos de las membranas celulares. Al producirse la activación celular la fosfolipasa A_2 libera AA, el cual puede ser metabolizado por las COX-1 y COX-2 generando PGs y tromboxanos o bien puede ser metabolizado por las LOXs generando lipoxinas y leucotrienos. Existen varios tipos de PGs, entre ellas cabe destacar la PGE_2 y la PGI_2 que tienen un

potente efecto vasodilatador, además la PGE_2 es un potente inductor de la fiebre (Higgs et al. 1984). Las lipoxinas al igual que algunos derivados de los ácidos grasos omega 3 como las resolvinas, las maresinas, o las protectinas participan en la resolución de la inflamación (Serhan 2014, Serhan et al. 2015).

- Citoquinas inflamatorias: Las más importantes en la repuesta inflamatoria aguda son el factor de necrosis tumoral α (TNF-α) y las interleuquinas 1 y 6 (IL-1 e IL-6). Son producidas por las células inmunitarias y juegan un papel muy importante tanto en la respuesta inmunitaria frente a la infección como en la regulación de la respuesta inflamatoria (Gabay and Kushner 1999). El papel de cada una de las citoquinas en el sistema inmune y en la respuesta inflamatoria se explicaran con detalladamente más adelante.

- Quimioquinas: Al igual que las citoquinas son producidas por diversos tipos celulares en respuesta a la inflamación, actúan como atrayentes de las células del sistema inmunitario hacia el foco inflamatorio, siendo tal vez la más destacada la IL-8 (Rossi and Zlotnik 2000).

- Enzimas proteolíticas: Tienen como función principal participar en la remodelación celular, además son una línea de defensa frente a posibles patógenos y pueden influenciar la migración de los leucocitos. Las enzimas proteolíticas más destacadas son la elastasa o la metaloproteinasas.

La inflamación aguda puede evolucionar de diferentes formas aunque lo más habitual es que se produzca una resolución completa de la inflamación, regenerándose el tejido y recuperándose la funcionalidad de la zona afectada. El proceso de resolución de la inflamación será abordado detalladamente más adelante. Aunque sería lo deseable, no siempre es posible la resolución completa de la inflamación y la regeneración del tejido dañado. En algunos casos se puede formar un absceso, especialmente en el caso de infecciones, y en otras ocasiones se puede curar el tejido dañado con tejido conjuntivo y cicatrizarse, este hecho suele ocurrir cuando el daño tisular ha sido muy importante o en casos donde el tejido afectado no tiene capacidad de regeneración. Finalmente, en el caso de que no se pueda resolver la inflamación ni reparar el tejido dañado, la respuesta inflamatoria aguda

puede evolucionar hacia una repuesta inflamatoria crónica (Nathan 2002, Lowry 2009).

La respuesta inflamatoria aguda puede tener efectos a nivel sistémico como por ejemplo el desarrollo de fiebre como consecuencia de los elevados niveles de IL-1 y TNF-α que interaccionan con los receptores de los centros termorreguladores, induciendo la producción de PGE_2 que provoca fiebre. Además, como consecuencia de la respuesta inflamatoria aguda, se produce leucocitosis, neutrofilia y eosinofilia. La inflamación aguda puede provocar cambios a nivel conductual provocando escalofríos, somnolencia y/o malestar general (Lowry 2009).

1.2. Inflamación crónica

La inflamación crónica ha recibido diversos nombres en los últimos años, puesto que se ha definido como inflamación de bajo grado, mini-inflamación, inflamación subclínica o estado micro-inflamatorio. Todos estos términos se han utilizado para indicar un estado fisiológico inflamatorio pero en el que no se encuentran los indicadores clásicos de inflamación. Este tipo de inflamación puede ser debido a una no resolución por una resolución incorrecta de un proceso inflamatorio agudo o por infecciones persistentes. No obstante, el motivo más habitual de este tipo de inflamación suele estar asociado a enfermedades metabólicas como diabetes, obesidad, síndrome metabólico, hígado graso no alcohólico, e incluso en casos de cáncer (Minihane et al. 2015).

La inflamación crónica se caracteriza por la presencia de elevadas concentraciones circulantes de IL-6, IL-1β, TNF-α, y proteína C reactiva, además de por la infiltración en los tejidos de macrófagos y en menor medida de linfocitos T citotóxicos, diferenciándose de la inflamación aguda en la que el primer tipo celular infiltrado son los neutrófilos (Weisberg et al. 2003, Chatzigeorgiou et al. 2012). Se ha evidenciado que la infiltración en el tejido de células inmunitarias durante la inflamación crónica no lleva asociado un daño tisular ni una pérdida de la funcionalidad del tejido (Weisberg et al. 2003, Feng et al. 2011).

Las personas obesas presentan elevadas cantidades de tejido adiposo por lo que son más susceptibles a sufrir lipoperoxidación, este hecho conlleva un incremento en la

producción de RONS como el anión superóxido y el NO (Murano et al. 2008). Se ha observado que elevados niveles de RONS pueden incrementar la expresión y la secreción de citoquinas proinflamatorias como el TNF-α, IL-1β e IL-6 a la vez que se reducen los niveles de citoquinas y adipoquinas anti-inflamatorias como la IL-10 o la leptina (Wolin 1996, Rahman and MacNee 2000, Murano et al. 2008). La inflamación crónica asociada a desordenes metabólicos depende de la activación de los receptores de reconocimiento de patrones (PRR), presentes en los macrófagos y otros tipos celulares. Estos receptores pueden detectar dos tipos de moléculas: las que tienen los patrones moleculares asociados a patógenos (PAMPs) y las que tienen los patrones moleculares asociados a daños celulares (DAMPs). Los encargados del reconocimiento de estos PAMPs y DAMPs son los TLRs que cuando se unen a su ligando pueden desarrollar una respuesta inflamatoria (Takeda et al. 2003, Gleeson et al. 2006). La inflamación crónica derivada de alteraciones metabólicas está asociada al reconocimiento de DAMPs (Weisberg et al. 2003), y su reconocimiento por parte de los TLRs. Una vez se produce la unión TLR-ligando se activan varias vías de señalización intracelular que finaliza con la activación del factor de transcripción nuclear κβ (NFκB), el cual activa la transcripción de diversas citoquinas pro-inflamatorias como el TNF-α, la IL-1β o la IL-8 iniciándose así una respuesta inflamatoria (Jialal et al. 2012).

La respuesta inflamatoria crónica puede estar asociada a patologías esqueléticas como la sarcopenia, la osteoporosis o la artritis (Chikanza et al. 1993, Wang et al. 2015), en estos casos el estado inflamatorio crónico se caracteriza por elevados niveles de IL-1, IL-2 y TNF-α (Nouri et al. 1984, Nouri et al. 1984, Chikanza et al. 1993). La inflamación crónica también puede aparecer en patologías como la enfermedad de Crohn o la colitis, que al igual que en los anteriores casos también se caracteriza por elevados niveles circulantes de IL-6, IL-1β, TNF-α, IL-2 e IL-4 además de proteína C reactiva (Matowicka-Karna 2016).

1.3. Resolución de la inflamación

La resolución de la inflamación es fundamental para evitar o reducir el daño provocado durante la respuesta inflamatoria. Además en el caso de no detener la respuesta inflamatoria, esta puede desembocar en una inflamación crónica desarrollando un estado patológico.

La resolución de la inflamación es un proceso activo en el cual participan varios mecanismos fisiológicos que disminuyen la proliferación y maduración de células inmunes, a la vez que favorece la apoptosis y la eliminación de leucocitos activos, además de inhibir la secreción de mediadores inflamatorios (Nathan 2002). La resolución de la inflamación se lleva a cabo en diversas etapas. La primera de todas consiste en la eliminación del estímulo o del agente agresor, a continuación se metabolizan los productos pro-inflamatorios secretados, silenciando así las vías pro-inflamatorias. El tercer paso consiste en la normalización de los gradientes de quimioquinas en el tejido afectado y la apoptosis de los neutrófilos presentes en la zona dañada, en la siguiente fase los restos apoptóticos son eliminados por los macrófagos residentes en el tejido (Buckley et al. 2014). Una vez se ha detenido la respuesta inflamatoria, aparecen los mediadores lipídicos anti-inflamatorios que son un elemento clave en la resolución de la inflamación, ya que regulan el perfil inflamatorio y promueven el retorno a la condiciones normales del tejido afectado (Serhan et al. 2008, Buckley et al. 2014). Entre estos mediadores lipídicos podemos destacar las PGs, los tromboxanos, las lipoxinas, las resolvinas, las maresinas, las protectinas y los leucotrienos, de los cuales se hablará más adelante. Durante la resolución de la inflamación participan tipos celulares específicos como los macrófagos y los linfocitos T reguladores (Buckley et al. 2014). Como ya se ha indicado, los mediadores lipídicos y los distintos tipos celulares no actúan únicamente deteniendo la respuesta inflamatoria, sino que también facilitan el retorno a las condiciones normales del tejido afectado. (Buckley et al. 2014).

1.4. Inflamación y citoquinas

La inflamación está mediada por una amplia variedad de factores solubles secretados por las células inmunitarias con efectos paracrinos y autocrinos entre los que destacan las citoquinas. Las citoquinas son los mediadores claves de la respuesta inflamatoria, se pueden definir cómo moléculas proteicas de bajo peso molecular, solubles, con un tiempo de vida media muy corto y sintetizadas por el sistema inmune normalmente frente a estímulos dañinos para el organismo. El concepto citoquina engloba una amplia variedad de mediadores, entre los que se pueden encontrar las interleuquinas, las quimioquinas, los factores de crecimiento, los factores transformantes y los interferones. Además, también participan diversos factores no proteicos, derivados principalmente de los ácidos grasos como son los eicosanoides. Clásicamente se creía que había una diferenciación muy clara entre las citoquinas que participaban en la repuesta inflamatoria aguda y la respuesta inflamatoria crónica. Así, se pensaba que la IL-1, IL-6, IL-8, IL-16, IL-17, TNF-α y los factores estimuladores de colonias (CSFs) eran los responsables de la respuesta inflamatoria aguda, mientras que las IL-2, IL-3, IL-4, IL-5, IL-7, IL-9, IL-10, IL-12, IL-13,IL-14, IL-15 además de los interferones y los factores de crecimiento eran las responsables de la repuesta inflamatoria crónica (Feghali and Wright 1997). Actualmente se sabe que la respuesta inflamatoria es un proceso de alta complejidad y muy regulado, donde las propias citoquinas influyen entre sí potenciando o reduciendo la secreción de otras citoquinas (Freitag et al. 2016). Se ha observado una distribución temporal en la producción de citoquinas, con un patrón de secreción que puede variar en función del estímulo. Por ejemplo, frente a la estimulación con lipopolisacárido (LPS) se ha visto que la primera citoquina que aumenta es el TNF-α, seguido de un aumento de IL-6 e IL-8 (Baggiolini 2001, Pedersen and Febbraio 2008), mientras que la práctica de un ejercicio físico provoca un incremento en los niveles de IL-6 y de la IL-1β seguidos de un incremento del receptor agonista de IL-1 (IL-1rα) e IL-10. Únicamente si el ejercicio físico es de muy alta intensidad se produce un incremento en los niveles plasmáticos de TNF-α (Nieman and Pedersen 1999, Steensberg et al. 2002, Petersen and Pedersen 2005, Pedersen and Febbraio 2008). En casos de inflamación crónica como en situaciones de obesidad, diabetes, artritis reumatoide, entre otros, se ha observado que las principales citoquinas que mantienen una situación de inflamación crónica son el

TNF-α, la IL-6 y la IL-1β (Weisberg et al. 2003, Alvarez-Soria et al. 2006, Ballak et al. 2015, Domingueti et al. 2016). La síntesis de estas citoquinas no es exclusiva de las células inmunitarias (Weisberg et al. 2003, Ballak et al. 2015), sino que tienen una distribución pleiotrópica, y de hecho adipocitos, miocitos, células cancerosas, etc., secretan éstas y otras citoquinas contribuyendo a la situación de inflamación crónica asociadas a estas patologías (Steensberg et al. 2002, Ballak et al. 2015, Picon-Ruiz et al. 2016).

La IL-1 en sus dos isoformas, IL-1α e IL-1β, es sintetizada por los fagocitos mononucleares, fibroblastos y por los linfocitos T y B. Ambas isoformas pueden desencadenar fiebre mediante un incremento en la síntesis de PGE_2. Además, estimulan la proliferación de linfocitos T y provocan la liberación de histamina por parte de los mastocitos. La histamina causa una vasodilatación tardía en el lugar de inflamación y un incremento de la permeabilidad vascular (Feghali and Wright 1997). Los efectos pro-inflamatorios de la IL-1 pueden ser neutralizados por el IL-1rα sintetizado por macrófagos expuestos a IL-4 y por neutrófilos estimulados con TNF-α o factor estimulante de macrófagos granulocitos (GM-CSF) (Dinarello 1992).

El TNF-α es sintetizado por los macrófagos, monocitos, mastocitos, células *Natural Killers* (*NK*) y linfocitos T (Beutler and Cerami 1988, Feghali and Wright 1997). Al igual que la IL-1 puede incrementar la síntesis de PGE_2 (Dinarello 1992), también es capaz de inducir la síntesis de proteínas de fase aguda por parte del hígado y estimular la síntesis de IL-6 en varios tipos celulares (Dinarello 1992). Además, el TNF-α es un potente inductor de la apoptosis. El TNF-β es producido por los linfocitos T y B activados, se trata de una citoquina con una gran afinidad por los receptores de TNF-α, y tiene unos efectos muy similares a los del TNF-α incluyendo la inducción de la apoptosis (Paul and Ruddle 1988).

La IL-6 es producida principalmente por los fagocitos mononucleares, los linfocitos T y por las células musculares tras la realización de un ejercicio agudo (Hirano 1992, Pedersen and Febbraio 2008). La IL-6 actúa como factor de crecimiento para los linfocitos B e induce su maduración. La IL-6 tiene funciones anti-inflamatorias ya que induce la síntesis de IL-2 y de IL-10, pero además es capaz de inhibir la producción de TNF-α limitando así la magnitud de la respuesta inflamatoria aguda

(Feghali and Wright 1997, Petersen and Pedersen 2005). Aunque, en general se dice que los efectos de las citoquinas se ejercen a nivel local, es sabido que tanto el TNF-α, el TNF-β, la IL-1 y la IL-6 actúan a nivel endocrino cuando son producidos en grandes cantidades, como en el caso de infecciones persistentes o en enfermedades inflamatorias.

La IL-11 es producida por las células de la medula ósea y por algunos fibroblastos, es capaz de inducir la liberación de proteínas de fase aguda por parte del hígado, estimular la secreción de inmunoglobulinas por parte de los linfocitos T y B, e inducir la síntesis de IL-6 por parte de los linfocitos T CD_4^+ (Baumann and Schendel 1991, Feghali and Wright 1997).

La IL-8 es producida por los monocitos, macrófagos, fibroblastos y por células endoteliales. Esta interleuquina es también llamada factor quimiotáctico de neutrófilos, puesto que su principal efecto es atraer a los neutrófilos hasta el lugar de inflamación. Junto la TNF-α y la IL-1 es uno de los principales reguladores del reclutamiento de los neutrófilos hasta el lugar de inflamación, regulan la expresión de diversas moléculas de adhesión, potencian la adhesión de los neutrófilos a las células endoteliales y facilitan la transvasación de los primeros hasta el foco de inflamación (Dinarello 1992). Además, la IL-8 tiene un potente efecto estimulador sobre la actividad de los granulocitos (Feghali and Wright 1997).

Otra citoquina importante en la respuesta inflamatoria aguda es la IL-17 que es producida por los linfocitos T activados y es capaz de estimular la síntesis de IL-6, de IL-8 y potenciar la expresión de la molécula de adhesión intercelular 1 (ICAM-1) en fibroblastos humanos (Yao et al. 1995).

Entre los CSFs podemos destacar el factor estimulante de granulocitos (G-CSF) y el GM-CSF. Tanto el G-CSF como el GM-CSF pueden ser sintetizados por los monocitos, células endoteliales y linfocitos T activados por IL-1 y TNF-α. Ambos CSF pueden activar a los neutrófilos mientras que el GM-CSF pude también activar las funciones efectoras de los eosinófilos y de los fagocitos mononucleares (Feghali and Wright 1997).

La IL-3 que es producida por los linfocitos T activados y por los mastocitos y tiene como principal función la estimulación de los eosinófilos a la vez que induce la diferenciación de los linfocitos B (Crosier and Clark 1992).

La IL-4 es producida por los linfocitos T_h CD_4^+ mastocitos y basófilos. Esta citoquina tiene como función principal inducir la diferenciación de los linfocitos T_h CD_4^+ en linfocitos T_h2, además inhibe el desarrollo de los linfocitos T_h1. La IL-4 actúa como factor de crecimiento de linfocitos B, T y de los mastocitos, y potencia la expresión del complejo mayor de histocompatibilidad en los linfocitos B. Su presencia es esencial para la síntesis de la inmunoglobulinas E y G (IgE e IgG) (Beckmann et al. 1992). En casos de algunas enfermedades inflamatorias, como la artritis reumatoide, se ha observado que la IL-4 ejerce efectos anti-inflamatorios puesto que es capaz de inhibir la producción de IL-1, IL-6, IL-8 y TNF-α.

La IL-5 es producida por linfocitos T_h CD_4^+, y por las células *NK.* Participa en la diferenciación de eosinófilos, incrementa la proliferación de los linfocitos B e incrementa la capacidad citotóxica de los linfocitos T (Yokota et al. 1988).

La IL-10, considerada una citoquina anti-inflamatoria, es producida por los linfocitos T_h CD_4^+, T_h CD_8^+ y linfocitos B activados (Chen and Zlotnik 1991). Es capaz de inhibir la síntesis de otras citoquinas proinflamatorias como el TNF-β, IL-1α, IL-1β e IL-8 (Pretolani 1999). Además, puede actuar como factor de diferenciación de los linfocitos T citotóxicos (Chen and Zlotnik 1991).

El factor transformante de crecimiento β (TGF-β) es producido por los linfocitos T, plaquetas y monocitos, es capaz de inhibir la proliferación y la activación de los linfocitos T y de las *NK,* y juega un papel clave en la regulación de la respuesta inflamatoria (Sporn et al. 1986).

La IL-2 es sintetizada por los linfocitos T_h y los linfocitos B. Su función es esencial en la regulación de la respuesta inflamatoria crónica al favorecer la diferenciación de los linfocitos T, además de incrementar la secreción de ciertas citoquinas como el interferón γ (IFN- γ), la IL-4, la IL-3, la IL-5 y del GM-CSF, y de potenciar la expresión del complejo mayor de histocompatibilidad II (Feghali and Wright 1997).

La IL-12 tiene como función principal potenciar la citotoxicidad e inducir la activación de los linfocitos T y de las *NK*, provocando la producción de IFN-γ por parte de los linfocitos T y de las *NK*. Esta interleuquina es producida por los linfocitos B activados, los macrófagos y otras células presentadoras de antígenos, y su síntesis es inhibida por la IL-4 y la IL-10 (Gately et al. 1992).

La tabla 1 resume las fuentes y las propiedades de las citoquinas anteriormente citadas.

Tabla 1. *Fuentes y funciones de las principales citoquinas relacionadas con la respuesta inflamatoria.*

Citoquina	Fuente	Función
IL-1 (IL-1α e IL-1β)	Fagocitos mononucleares, Fibroblastos y Linfocitos T y B	Estimular la proliferación de linfocitos T. Provocar liberación de histamina de los mastocitos.
TNF-α	Macrófagos, Monocitos, células *NK* y linfocitos T	Inducir síntesis de proteínas de fase en el hígado. Estimular síntesis de IL-6. Inducir apoptosis.
TNF-β	Linfocitos T y B activados	Inducir síntesis de proteínas de fase en el hígado. Estimular síntesis de IL-6. Inducir apoptosis.
IL-6	Fagocitos mononucleares, Linfocitos T, Células musculares	Actuar como factor de crecimiento para los linfocitos B. Inducir la síntesis de IL-2 y de IL-10. Inhibir la producción de TNF-α.
IL-11	Células de la medula ósea, Fibroblastos	Inducir la liberación de proteínas de fase aguda. Estimular la secreción de inmunoglobulinas por parte de los linfocitos T y B. Inducir la síntesis de IL-6 por parte de los linfocitos T CD_4^+.
IL-8	Monocitos, Macrófagos, Células endoteliales	Atraer a los neutrófilos hasta el lugar de inflamación. Estimular la actividad de los granulocitos.
IL-17	Linfocitos T activados	Estimular la síntesis de IL-6, de IL-8. Potenciar la expresión de ICAM-1.
CSFs (GM-CSF y G-CSF)	Monocitos, Células endoteliales, Linfocitos T activados	Activar a los neutrófilos (GM-CSF y G-CSF) Activar las funciones efectoras de los eosinófilos y de los fagocitos mononucleares (GM-CSF).
IL-3	Linfocitos T activados, Mastocitos	y tiene como principal función la estimulación de los eosinófilos a la vez que induce la diferenciación de los linfocitos B.
IL-4	Linfocitos T_h CD_4^+, Mastocitos, Basófilos	Inducir la diferenciación de los linfocitos T_h CD_4^+ en linfocitos T_h2,. Inhibir el desarrollo de los linfocitos T_h1. Actuar como factor de crecimiento de linfocitos B, T y de los mastocitos. Potenciar la expresión del complejo mayor de histocompatibilidad en los linfocitos B. Inducir la síntesis de la IgE e IgG.
IL-5	Linfocitos T_h CD_4^+ y Células *NK.*	Participar en la diferenciación de eosinófilos. Incrementar la proliferación de los linfocitos B. Incrementar la capacidad citotóxica de los linfocitos T.
IL-10	Linfocitos T_h CD_4^+, Linfocitos T_h CD_8^+, Linfocitos B activados.	Inhibir síntesis de TNF-β, IL-1α, IL-1β e IL-8. Inducir la diferenciación de los linfocitos T citotóxicos.
TGF-β	Linfocitos T, plaquetas y Monocitos	Inhibir la proliferación y la activación de los linfocitos T y de las *NK,* Regular la respuesta inflamatoria.
IL-2	Linfocitos T_h y Linfocitos B.	Regular de la respuesta inflamatoria crónica. Favorecer l diferenciación de los linfocitos T. Incrementar la secreción de IFN- γ, IL-4, IL-3, IL-5 y de GM-CSF. Potenciar la expresión del complejo mayor de histocompatibilidad II.
IL-12	Linfocitos B activados, Macrófagos, Células presentadoras de antígenos	Potenciar la citotoxicidad e inducir la activación de los linfocitos T y de las *NK.* Inducir la producción de IFN-γ por parte de los linfocitos T y de las *NK.*

1.5. Mediadores lipídicos e inflamación

Las citoquinas no son los únicos mediadores que actúan durante la respuesta inflamatoria sino que existen una amplia variedad de sustancias que participan y regulan el proceso inflamatorio. Entre estas sustancias destacan los mediadores lipídicos. Cuando las células del sistema inmunitario se activan, parte de sus fosfolípidos de membrana son metabolizados para generar una amplia variedad de sustancias lipídicas que mediaran en la respuesta inflamatoria (Serhan et al. 2015).

El AA es el elemento clave en la producción de mediadores lipídicos puesto que la gran mayoría derivan de él. El AA puede provenir de la conversión del ácido linoleico o bien como producto de la acción de la fosfolipasa A_2 que se activa como consecuencia de la activación de las células inmunitarias (Suram et al. 2013, Leslie 2015).

Los mediadores lipídicos derivados del AA pueden sintetizarse a partir de dos rutas diferentes, mediante la acción de las COXs que sintetizaran principalmente PGs y tromboxanos, y mediante la acción de las LOXs que sintetizan leucotrienos y lipoxinas, la figura 1 resume las principales vías de síntesis de los diferentes mediadores lipídicos así como los enzimas clave que participan en dichas vías.

- Vía de las COXs: En humanos se encuentran dos isoformas de esta enzima, la COX-1 que es constitutiva y la COX-2 que es inducible. Las COXs generan prostaglandinas, moléculas con una vida media muy corta y que pueden actuar de forma autocrina y/o paracrina actuando a través de receptores específicos (Trappe and Liu 2013). Existen varios tipos de PGs (PGD, PGE, PGF, PGG y PGH) de éstas las que tienen más importancia en la respuesta inflamatoria son la PGE_1, PGE_2, PGD_2, $PGF_{2\alpha}$, PGI_2, además del tromboxano A_2 (TxA_2). Las PGE_1 y PGE_2 tienen un potente efecto vasodilatador que permite la llegada de un mayor flujo de sangre a las zonas inflamada. Además, la PGE_1 regula las funciones de los neutrófilos reduciendo su activación y disminuye la agregación plaquetaria u leucocitaria (Weiss et al. 1998, Frassdorf et al. 2006). La PGE_2 puede ser considerada como una molécula pro y anti-inflamatoria dependiendo de su localización, por una parte puede incrementar la extravasación de los neutrófilos hacia la zona dañada pero por otra es capaz de inhibir la activación de la vía del NFκB en macrófagos, hecho que es clave en la

iniciación de la resolución de la inflamación a través de mediadores lipídicos (Liu et al. 2014, Serhan 2014). Se ha observado que elevados niveles de PGE_2 inhiben la actividad de la COX-2 y de la 5-LOX, además de activar a la 15-LOX en neutrófilos (Nathan 2002).

La PGD_2 tiene efectos inmunosupresores, se ha observado en ratones deficientes de la enzima que sintetiza dicha PG tienen una respuesta inflamatoria exacerbada que son incapaces de resolver (Trivedi et al. 2006). Además, la PGD_2 puede generar otras PGs por mecanismos no enzimáticos que pueden activar a los receptores activados de proliferación de los peroxisomas (PPAR-γ) que, a su vez, puede inhibir la síntesis de varias citoquinas proinflamatorias (Huang et al. 1999). El TXA_2 es el tromboxano que se produce en mayor cantidad, tiene un potente efecto vaso y broncoconstrictor, además de favorecer la agregación plaquetaria, pero se trata de un producto muy inestable que rápidamente se convierte en TXB_2, molécula muy estable pero sin efectos biológicos (Zipser and Laffi 1985, Williams and Higgs 1988). Asimismo, el TXA_2 es un elemento clave en la regulación de la síntesis de TNF-α e IL-1β (Caughey et al. 1997). Se ha observado también que el TXA_2 potencia la acción de los linfocitos T pudiendo incrementar el perfil pro-inflamatorio de estos (Ruiz et al. 1992).

- Vía de las LOXs: Existen varios tipos de LOXs aunque es la 5-LOX, expresada principalmente en neutrófilos, la que juega un papel más importante en la modulación de la respuesta inflamatoria (Kumar 2006). El principal producto de la 5-LOX es el 5-hidroperoxieicosatetraenoico (5-HPETE), compuesto quimiotáctico para los neutrófilos y que además sirve de precursor para la síntesis de leucotrienos. El leucotrieno B_4 (LTB_4) es tal vez el mediador lipídico más importante de esta vía, actúa como agente quimiotáctico para los neutrófilos, eosinófilos y linfocitos además de activar la respuesta de los mismos, incrementa la adhesión de los leucocitos al endotelio y potencia la producción de ROS (Samuelsson et al. 1987).

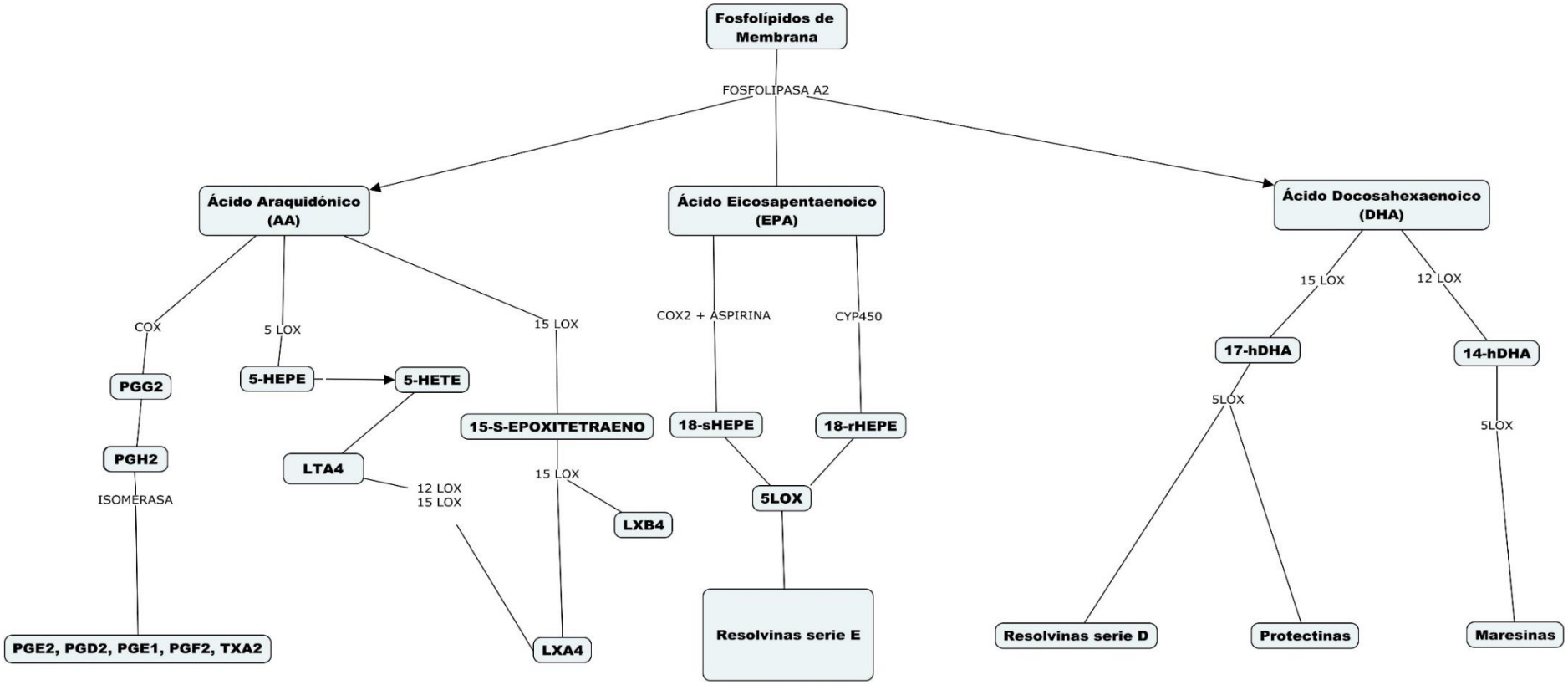
La vía de las LOXs también produce lipoxinas, como productos de la 15-LOX y son capaces de inhibir la transvasación de los neutrófilos (Nathan 2002), pero a su vez también incrementan la producción de anión superóxido en estas células inmunitarias (Samuelsson et al. 1987). Se ha observado que ratones deficientes de 15-LOX son incapaces de producir lipoxinas y a la vez presentan una respuesta

inflamatoria exagerada (Serhan et al. 2015). Se ha evidenciado que las lipoxinas son capaces de bloquear la acción de las *NK* (Ramstedt et al. 1985). Se ha demostrado también que el tratamiento con lipoxinas limita la respuesta inflamatoria, además de reducir los niveles de citoquinas pro-inflamatorias (Jin et al. 2007). Adicionalmente, la lipoxina A_4 es capaz de incrementar los niveles de IL-10, una citoquina con potentes efectos anti-inflamatorios, participando de este modo en la resolución de la inflamación (Cooray et al. 2013).

En los últimos años se han descubierto toda una serie de moléculas derivadas de la oxidación de los ácidos grasos omega 3 que tienen un potente efecto resolutivo de la inflamación. Entre estas moléculas destacan las maresinas, las protectinas y las resolvinas (Serhan et al. 2002, Serhan et al. 2015, Serhan et al. 2015). Estas moléculas provienen de la oxidación vía LOXs de los ácidos docosahexaenoico (DHA) y eicosapentaenoico (Martorell et al. 2013, Serhan et al. 2015). Las resolvinas de la serie D, así como la maresina 1 y la protectina D_1 derivan del DHA mientras que las resolvinas de la serie E proceden del EPA. Las maresinas son mediadores lipídicos sintetizados a partir del DHA por partes de los macrófagos, se trata de una molécula capaz de reducir la síntesis de leucotrienos y de favorecer la regeneración de tejidos, lo que la capacita como un potente anti-inflamatorio (Serhan et al. 2012, Serhan et al. 2015). Además, las maresinas inducen la aparición de macrófagos con fenotipo M_2 que son los macrófagos con un perfil más anti-inflamatorio. Las protectinas son sintetizadas por varios tipos celulares mediante varias oxigenaciones del DHA llevadas a cabo por LOXs (Serhan et al. 2015). Estos derivados del DHA limitan la infiltración de los neutrófilos en los tejidos (Serhan et al. 2015). Adicionalmente, las protectinas reducen los niveles de TNF-α e IFN-γ y promueven la apoptosis de los linfocitos T_h (Ariel et al. 2005). Las resolvinas son sintetizadas por las LOX y la COX-2, utilizando como substrato el DHA o el EPA, en este sentido podemos encontrar resolvinas de la serie D derivadas del DHA y resolvinas de la serie E derivadas del EPA (Serhan et al. 2002, Weylandt et al. 2012). La principal función de las resolvinas es detener la infiltración de los neutrófilos y los monocitos en el tejido afectado (Serhan et al. 2002). La resolvina E1 (RvE_1) puede interaccionar con el receptor del leucotrieno B4 de los neutrófilos actuando como agonista y deteniendo los efectos pro-inflamatorios provocados por este,

además, la RvE_1 reduce la producción de citoquinas inflamatorias y potencia la eliminación de los estímulos pro-inflamatorios (Serhan et al. 2002, Serhan et al. 2008). Parece ser que la RvD_1 tiene las mismas funciones que la RvE_1, se cree que ambas resolvinas actúan inhibiendo la activación del NFκB por un mecanismo que es dependiente del PPARγ (Arita et al. 2005, Liao et al. 2012). Las resolvinas de la serie D son capaces de inhibir la síntesis de IL-1β inducidas por el TNF-α (Serhan 2014).

Figura 1. Esquema resumen de la síntesis de mediadores lipídicos.



COX- Ciclooxigenasa; 5 LOX- 5Lipooxigenasa; 15LOX- 15 Lipooxigenasa; CYP450- Citocromo P450; 12LOX- 12 Lipooxigenasa; PGG2- Prostaglandina G2; PGH2 – Prostaglandina H2; PGE2- Prostaglandina E2; PGD2- Prostaglandina D2; PGE1- Prostaglandina E1; PGF2- Prostaglandina F2; TXA2- Tromboxano A2; 5-HEPE- Ácido 5-hidroxieicosapentaenoico; 5-HETE Ácido 5 Hidroxieicosatetraenoico LTA4 –Leucotrieno A4; LXA4- Lipoxina A4; LXB4- Lipoxina B4; 18-sHEPE – Ácido 18S-hidroxieicosapentaenoico; 18-rHEPE – Ácido 18R-hidroxieicosapentaenoico 17-hDHA- Ácido 17- Hidroxidocosahexaenoico; 14-hDHA - Ácido 14- Hidroxidocosahexaenoico.

2. Ejercicio físico y sistema inmunitario

2.1. Sistema inmunitario

El sistema inmunitario está formado por una amplia variedad de órganos (apéndice, timo, bazo, amígdalas, nódulos linfáticos), células (linfocitos, monocitos, *NK*, neutrófilos, eosinófilos etc.) y productos celulares (citoquinas, interferones anticuerpos, inmunoglobulinas) que tienen como función principal proteger al organismo frente a posibles infecciones. El organismo dispone de dos líneas de defensa frente a la entrada de microorganismos patógenos, la inmunidad innata y la inmunidad adquirida. La inmunidad innata es la primera en activarse, no es específica y tiene como objetivo principal destruir a los microorganismos patógenos, y dispara la respuesta inflamatoria intentando bloquear la expansión de la infección. Durante esta respuesta inmune se secretan IL-1, TNF-α, IL-6 e interferones. En esta respuesta inmunitaria juegan un papel muy importante los neutrófilos, los monocitos y los macrófagos. Cuando la respuesta inmune innata no es capaz de detener la infección se activa la inmunidad adaptativa, llevada a cabo por los linfocitos T y B, y las células *NK*, durante esta repuesta se producen anticuerpos que atacaran de forma específica al patógeno. En la respuesta inmune adquirida se liberan IL-2, IL-4, IL-12, IL-15 y TGF-β (Kumar 2006).

Aunque la función del sistema inmunitario es actuar contra agresiones externas que pueda sufrir el organismo, puede activarse por otros factores, como desordenes metabólicos, enfermedades autoinmunes, procesos neoplásicos entre otros (Kumar 2006).

La práctica de ejercicio físico provoca cambios a nivel metabólico y fisiológico que pueden afectar el organismo a diferentes niveles. Uno de estos cambios se produce a nivel del sistema inmunitario, se sabe que el ejercicio físico de elevada intensidad y duración puede provocar una inmunosupresión transitoria conocida como proceso de venta abierta (*Open Window*), incrementándose la susceptibilidad a padecer infecciones tras la realización de ejercicios de elevada intensidad (Nieman 1997). Por el contrario, se ha observado que la práctica de ejercicio de moderada intensidad y de forma regular puede mejorar el funcionamiento del sistema

inmunitario, reduciendo el riego de padecer infecciones en el tracto respiratorio superior (Nieman et al. 2011).

2.2. Inmunidad y ejercicio agudo

Los efectos del ejercicio de elevada intensidad sobre el sistema inmune empiezan a nivel celular. La primera consecuencia del ejercicio agudo a nivel celular es un incremento muy notable en la población de neutrófilos circulantes durante la realización e inmediatamente después de la realización del ejercicio, seguido de un descenso en la población de neutrófilos horas después de la realización del ejercicio (Robson et al. 1999, Peake and Suzuki 2004). No solo se ve afectado el número de células sino que también se ve alterada la funcionalidad de los neutrófilos, ya que se ve incrementada su capacidad para la desgranulación, la fagocitosis y la respuesta oxidativa. Estas mismas funciones se ven reducidas frente a la estimulación con un agente patógeno, como el LPS, hecho que podría estar relacionado con el procesos de *Open Window* (Peake 2002, Peake and Suzuki 2004). Parece ser que los neutrófilos juegan un papel muy importante en el daño muscular provocado por el ejercicio, ya que su infiltración en el musculo y la posterior liberación de citoquinas inflamatorias, ROS y proteasas incrementan el daño muscular. Además, se ha observado que la inhibición de los neutrófilos en el tejido muscular reduce la generación de daño (Kawanishi et al. 2016). La población de monocitos aumenta después de la realización del ejercicio agudo pero su población se normaliza a la dos hora de haber terminado el esfuerzo (Walsh et al. 2011). El fenotipo de los monocitos puede cambiar como consecuencia del ejercicio físico agudo, cambiando el perfil de secreción de citoquinas y sus proteínas de superficie. Después de la realización de un ejercicio agudo, se movilizan preferentemente los monocitos $CD14^+$ y $CD16^+$, los cuales tienen un perfil pro-inflamatorio más marcado (Steppich et al. 2000). Los efectos del ejercicio agudo no son los mismos sobre los monocitos que sobre los macrófagos. Se ha visto que tanto el ejercicio intenso como el moderado tiene un potente efecto estimulador sobre la fagocitosis, actividad antitumoral e incrementan tanto la producción de RONS como la quimiotaxis (Walsh et al. 2011). El ejercicio agudo provoca una reducción en la expresión del complejo mayor de histocompatibilidad II y en la capacidad presentadora de antígenos de los macrófagos (Ceddia and Woods 1999, Carins and Booth 2002). En repuesta a un

ejercicio de elevada intensidad, las células *NK* incrementan su número en la circulación además de aumentar su capacidad citotóxica (Shek et al. 1995, Gleeson and Bishop 2005). En cambio, esta misma actividad citotóxica se puede ver seriamente disminuida después de varias horas de ejercicio intenso pudiendo incrementar la susceptibilidad a padecer infecciones (Gleeson and Bishop 2005).

En cuanto a los linfocitos, está documentado que se produce una linfocitosis durante e inmediatamente después de la realización del ejercicio agudo, seguido de una reducción en el número de linfocitos durante el periodo de recuperación (Ferrer et al. 2009, Cordova et al. 2010, Walsh et al. 2011). Estos cambios son dependientes de la magnitud e intensidad del ejercicio y se da tanto en linfocitos B como en linfocitos T, siendo mucho más marcados en estos últimos (Shek et al. 1995). Existen evidencias de que el ejercicio agudo estimula la activación de los linfocitos T (Fry et al. 1992). El ejercicio físico intenso provoca dolor y daño muscular, sobre todo si se trata de ejercicios de muy larga duración (Cheung et al. 2003). Hace dos décadas se creía que la respuesta inmune tras un ejercicio extenuante era la misma que la que presentaba el organismo frente a una infección ya que presentaban sintomatología similar (fiebre, escalofríos, dolor de cabeza, fatiga y pérdida de apetito), sin embargo, a día de hoy conocemos que la respuesta a nivel celular y molecular es diferente (Suzuki et al. 2002).

Como ya se ha comentado, el ejercicio agudo induce una respuesta inflamatoria aguda como consecuencia del daño muscular caracterizada por un movimiento de fluidos, proteínas de fase aguda y leucocitos a la zona dañada (Pedersen et al. 1997, Nieman and Pedersen 1999). Las citoquinas juegan un papel clave en el desarrollo y regulación de dicha respuesta inflamatoria asociada al ejercicio intenso, con un incremento significativo de varias citoquinas como IL-6, IL-1β y TNF-α (Cannon and Kluger 1983, Steensberg et al. 2002). Además, se liberan quimioquinas como la IL-8, la proteína inflamatoria de macrófagos (MIP-1), GM-CSF y G-CSF también aumentan después de la realización de una maratón (Ostrowski et al. 1998, Pedersen 2000). Se ha demostrado que el ejercicio agudo reduce el porcentaje de linfocitos Th en la circulación en concreto de los Th_1, este hecho podría estar relacionado con la reducción en la síntesis de IL-2 e IFN-γ por parte de los linfocitos después de la realización de un ejercicio agudo (Steensberg et al. 2000).

Parece ser que la IL-6 es la primera citoquina que se libera como consecuencia del ejercicio físico agudo, además de ser la liberada en mayor cantidad y la que tarda más a regresar a valores basales (Evans et al. 1986, Ostrowski et al. 1998). Este hecho podría ser debido a que la principal fuente de IL-6 es el músculo en su contracción durante la realización del ejercicio (Pedersen 2000, Steensberg et al. 2002). Parece que el ejercicio físico incrementa también los niveles de IL-1β y TNF-α, aunque existe controversia respecto a este hecho ya que no siempre se observan variaciones en sus niveles plasmáticos (Suzuki et al. 2002).

Con el objetivo de evitar una respuesta inflamatoria exagerada se liberan otras citoquinas con propiedades anti-inflamatorias como la IL-1rα, IL-4 la IL-10 y el TGF-β, además también se secretan los receptores solubles de TNF-α y IL-2 reduciendo así la actividad inflamatoria de ambas citoquinas. (Suzuki et al. 2002). Así mismo, el ejercicio agudo induce la síntesis de algunos mediadores lipídicos como las prostaglandinas, sin embargo, no se sabe aún cómo puede afectar el ejercicio físico intenso a los mediadores lipídicos. Las citoquinas proinflamatorias activan el eje hipotalámico-adrenal y el sistema simpato-adrenérgico que juegan un papel muy importante en el control y regulación de la respuesta inflamatoria aguda (Nieman 1997). Además, como consecuencia del ejercicio físico extenuante se segregan grandes cantidades de cortisol que es un potente anti-inflamatorio, ya que es capaz de inhibir la síntesis de citoquinas pro-inflamatorias y activar la liberación de citoquinas anti-inflamatorias (Suzuki et al. 2002). También se ha observado que las citoquinas pro-inflamatorias, producidas durante el ejercicio físico agudo, estimulan el sistema nervioso simpático y el eje hipotalámico-adrenal, incrementando los niveles circulantes de glucocorticoides y provocando una inhibición de la repuesta inmunitaria (Mastorakos et al. 1993).

2.3. Inmunidad y entrenamiento

Contrariamente a lo que ocurre con el ejercicio agudo, no parece que un periodo de entrenamiento provoque cambios en el número de neutrófilos circulantes en condiciones basales (Gleeson and Bishop 2005). De todas formas, algunos estudios indican que el número de neutrófilos circulantes puede reducirse como consecuencia de la realización del ejercicio regular, pudiendo ser este uno de los motivos que expliquen los efectos anti-inflamatorios del ejercicio regular (Michishita et al. 2010, Walsh et al. 2011). Aun así, son necesarios más estudios para poder aclarar los efectos del entrenamiento o ejercicio regular sobre la función de los neutrófilos en condiciones basales. Se ha observado que personas que realizan deporte de forma regular presentan un menor porcentaje de monocitos $CD14^+$ y $CD16^+$, que como ya se ha indicado anteriormente, son los que presentan el perfil más pro-inflamatorio además de presentar una menor expresión de TLR (Gleeson et al. 2006, McFarlin et al. 2006, Walsh et al. 2011). Asimismo, el ejercicio físico regular reduce la infiltración de macrófagos en los tejidos pudiendo reducir la inflamación sistémica (Vieira et al. 2009). Los efectos del ejercicio regular sobre el número y la funcionalidad de las *NKs* no están todavía demasiado claros (Walsh et al. 2011), aunque parece ser que el entrenamiento regular podría incrementar la citotoxicidad de las *NKs* (Fairey et al. 2005).

No se han observado diferencias entre las poblaciones de linfocitos de personas entrenadas y las de personas sedentarias, sin embargo, sí que se ha observado que en atletas de elite bien entrenados se produce un descenso en el número de linfocitos T circulantes, además de presentar una menor tasa de proliferación de los mismos y de tener una menor producción de inmunoglobulinas por parte de los linfocitos B (Baj et al. 1994, Lancaster et al. 2004).

2.4. Entrenamiento e inflamación

Se sabe que la práctica de ejercicio físico de forma regular puede proteger al organismo frente a patologías relacionadas con el envejecimiento y puede ser un buen tratamiento frente a enfermedades inflamatorias (Petersen and Pedersen 2005, Mathur and Pedersen 2008). Se ha sugerido que los efectos anti-inflamatorios del ejercicio regular a nivel sistémico pueden ser debidos a dos factores: a la

reducción de la grasa visceral y a la creación de un entorno anti-inflamatorio (Walsh et al. 2011). La práctica regular de ejercicio físico incrementa el gasto energético reduciendo los niveles de tejido adiposo (Ross et al. 2000, Giannopoulou et al. 2005). El tejido adiposo es una fuente de citoquinas inflamatorias como el TNF-α y la IL-6 (Mohamed-Ali et al. 1998, Pedersen et al. 2003), con lo que la reducción de dicho tejido, reduce la producción de estas citoquinas inflamatorias (Troseid et al. 2004, You and Nicklas 2006). Además, el ejercicio inhibe la infiltración en el tejido adiposo de los macrófagos con fenotipo M1 a la vez que favorecen la infiltración de los macrófagos M2 con un perfil menos inflamatorio (Kawanishi et al. 2010). El ejercicio induce la liberación de adiponectina por parte el tejido adiposo que es una adipoquina con efectos anti-inflamatorios, anti-apoptóticos y antioxidantes (Hui et al. 2012, Andrade-Oliveira and Camara 2015). Parece ser que la IL-6 producida durante la contracción muscular podría jugar un papel importante en el metabolismo lipídico, siendo así una de los responsables de la perdida de tejido adiposo visceral y de otros depósitos de grasa, no obstante todavía no está claro cómo podría llevarse a cabo esta regulación (Walsh et al. 2011). La IL-15 es una citoquina secretada por el músculo durante la contracción muscular y cuyos niveles aumentan con la práctica de ejercicio regular, se ha observado que tiene efectos anabólicos y que actúa en el crecimiento muscular (Walsh et al. 2011). Además, también podría ser que jugara un papel destacado en la regulación del metabolismo lipídico, pudiendo estar implicada en la reducción de los niveles de grasa visceral (Nielsen and Pedersen 2007).

Como ya se ha indicado, el ejercicio de elevada intensidad puede desembocar en una repuesta inflamatoria de fase aguda (Shinkai et al. 1992, Nieman et al. 2000, Pedersen and Hoffman-Goetz 2000, Freidenreich and Volek 2012). Sin embargo, la práctica de ejercicio de forma regular reduce los niveles basales de varios marcadores de inflamación sistémica como el TNF-α, proteína C reactiva, INF-γ, MCP-1, IL-8, IL-18, de los receptores solubles de TNF-α e IL-6, e incrementa los niveles de citoquinas anti-inflamatorias como la IL-10, IL-12, IL-4 y TFG-β1 (Tisi et al. 1997, Troseid et al. 2004, Oberbach et al. 2006). Por otro lado, varios estudios no han demostrado efectos anti-inflamatorios a nivel sistémico (Marcell et al. 2005, Beavers et al. 2010). Existen evidencias que la IL-6 podría ser la principal

responsable de los efectos anti-inflamatorios del ejercicio regular, puesto que su liberación precede la liberación de otras citoquinas anti-inflamatorias como la IL-10 o la IL-1rα, además se ha observado que la administración de IL-6 mimetiza los efectos el ejercicio físico (Starkie et al. 2003, Walsh et al. 2011). Se ha observado también que el ejercicio físico podría inhibir de alguna manera la síntesis de TNF-α por parte del sistema inmunitario (Starkie et al. 2003).

Los que sí está claro es que los efectos anti-inflamatorios del ejercicio regular dependen de la intensidad (baja, moderada o intensa), de la frecuencia (sesiones por día, semana, mes) y de las características del individuo (sexo, edad, resistencia, estado de salud, etc.) (Sallam and Laher 2016).

3. El ejercicio como fuente de Especies Radicales de Oxigeno (ROS)

La práctica de ejercicio físico de forma regular y de una intensidad moderada tiene efectos beneficiosos para la salud, sin embargo, la realización de ejercicios físicos extenuantes puede provocar daño muscular y desencadenar procesos inflamatorios (Cordova Martinez et al. 2015). Algunos de estos efectos pueden ser consecuencia de la elevada producción de ROS que se producen durante la práctica del ejercicio.

Las principales fuentes de ROS durante la práctica del ejercicio está asociada al incremento en la actividad de la cadena transportadora de electrones durante la contracción muscular, a la xantina oxidorreductasa y a la NADPH oxidasa presente en los macrófagos. La cadena transportadora de electrones produce ROS de forma regular a un ritmo de entre dos y cuatro ROS por cada 100 moléculas de oxigeno utilizadas, se sabe que durante la práctica del ejercicio se consume una mayor cantidad de oxigeno por la tanto la producción de ROS incrementa, sin embargo se ha demostrado que la tasa de producción de ROS durante el ejercicio es solo de un 0,25% (St-Pierre et al. 2002, Gomez-Cabrera et al. 2008). Se ha hipotetizado que esta menor tasa producción de ROS durante el ejercicio es debido en parte a la acción que juegan la proteínas desacoplantes UCP-2 y UCP-3 que protegerían a la mitocondria del daño oxidativo (Brand and Esteves 2005). Otra explicación para la elevada producción de ROS durante el ejercicio es el efecto desestabilizador que puede tener el calor producido en el musculo sobre la cadena transportadora de electrones. (Sachdev and Davies 2008).

Otro proceso que explica la elevada producción de ROS durante el ejercicio es la producción de ROS por parte de los neutrófilos y otras células fagocíticas, las cuales desarrollan una repuesta inmunitaria frente a un ejercicio de elevada intensidad y duración (Nieman and Pedersen 1999, Calle and Fernandez 2010). Los neutrófilos, así como otras células fagocíticas, migran hacia el tejido muscular dañado para fagocitar a la células dañadas produciendo anión superóxido (Petrone et al. 1980). Otra fuente de ROS asociada a la realización de ejercicio es la hemoglobina (Misra and Fridovich 1972), la mayor demanda de oxigeno durante el ejercicio puede incrementar la producción de ROS, debido a que la hemoglobina puede sufrir procesos de autooxidación generándose metahemoglobina, fuente de anión

superóxido (Gwozdzinski et al. 2013). Durante la realización del ejercicio físico se sintetiza NO (Kingwell et al. 1997), ya que la realización de ejercicio incrementa la expresión de iNOS en células endoteliales (Jungersten et al. 1997) y como consecuencia de la activación del NFκB debido a otros ROS (Rahman and MacNee 2000). Se ha observado que un exceso de producción de NO puede reaccionar con el anión superóxido y producir peroxinitrito una especie altamente reactiva (Oldreive and Rice-Evans 2001).

La sobreproducción de RONS durante el ejercicio desemboca en la oxidación de biomoléculas y en la aparición de daño oxidativo. Son muchos los estudios que han demostrado el incremento en marcadores de daño oxidativo en lípidos, proteínas y ADN después de la realización de una actividad física (Tauler et al. 2008, Martorell et al. 2014, Sureda et al. 2015).

3.1. El ejercicio como antioxidante

Como ya se ha mencionado, la realización de un ejercicio intenso o prolongado incrementa la producción RONS, que puede conducir a la aparición de daño oxidativo. Sin embargo la práctica de ejercicio de intensidad moderada y de forma regular potencia las defensas antioxidantes endógenas aportando una mayor resistencia al estrés oxidativo. Dicho incremento en las capacidades antioxidantes debido a la práctica regular de ejercicio podría ser atribuido a un fenómeno de hormesis. Pequeñas cantidades de ROS pueden inducir la expresión de enzimas antioxidantes y de otros mecanismos de defensa antioxidante (Radak et al. 2005, Gomez-Cabrera et al. 2006).

Los estudios de los efectos del ejercicio físico sobre las defensas antioxidantes se han realizado principalmente en músculo, aunque se ha visto que las células inmunitarias también son un buen modelo de estudio (Tauler et al. 2003, Sureda et al. 2008, Ferrer et al. 2009). Individuos que han realizado ejercicio de forma regular son menos propensos a padecer estrés oxidativo después de la realización de un ejercicio agudo en comparación con individuos no entrenados (Cases et al. 2005, Nicks et al. 2009, Tian et al. 2010). Este hecho puede tener su origen en que los enzimas antioxidantes de individuos entrenados sean más activos o que haya niveles más elevados en comparación a los individuos no entrenados. La actividad

superóxido dismutasa (SOD) se incrementa en el músculo esquelético como consecuencia del entrenamiento, aunque esta respuesta no es de igual magnitud para todas las isoformas del enzima. La Cu/Zn-SOD incrementa su actividad sin incrementar sus niveles proteicos mientras que la Mn-SOD incrementa su actividad debido a un incremento en los niveles proteicos lo que podría indicar que la regulación de la expresión de las diferentes isoformas de la SOD está sujeta a mecanismos postraducionales (Ji 1999). La glutatión peroxidasa (GPx) es otra enzima antioxidante cuya actividad se ve incrementada por la realización de ejercicio físico de forma regular (Leeuwenburgh et al. 1997). En cuanto a la catalasa no está todavía claro cuál es su respuesta a la práctica de ejercicio físico de forma regular (Sureda et al. 2007).

Está claro que los distintos enzimas antioxidantes tienen diferentes mecanismos de regulación frente a la práctica de un ejercicio regular y podrían depender de la intensidad del ejercicio físico así como de los niveles y tipos de ROS producidos.

3.2. ROS e inflamación

Las ROS producidos durante el ejercicio y que no han podido ser eliminados por las defensas antioxidantes pueden tener efectos sobre el equilibrio redox del organismo y desarrollar una repuesta inflamatoria. El estrés oxidativo y la respuesta inflamatoria comparten varias vías de señalización, además las ROS pueden desarrollar una repuesta inflamatoria a la vez que estos son productos del procesos inflamatorio (Sallam and Laher 2016), de hecho, se ha observado que la administración de antioxidantes como la vitamina E o la vitamina C reducen el estado inflamatorio además de reducir el daño oxidativo en el músculo después de la realización de un episodio de ejercicio agudo (Thompson et al. 2003, Aoi et al. 2004). Durante el estallido oxidativo las células del sistema inmunitario generan elevadas cantidades de RONS como consecuencia de la activación de la NADPH oxidasa y de la iNOS además de liberar una amplia variedad de citoquinas proinflamtorias como TNF-α, IL-1β, IL-6 e IL-8 (Cannon et al. 1991, Sallam and Laher 2016).

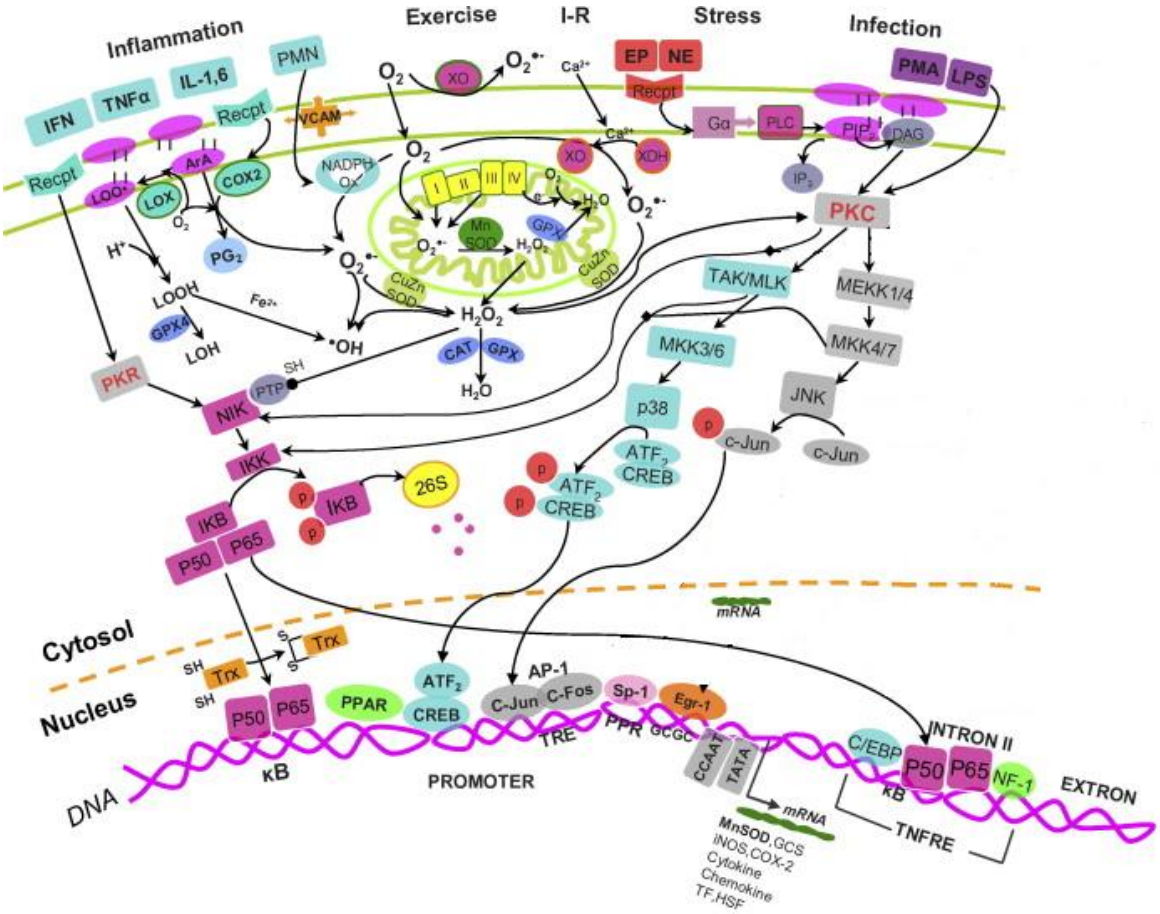
La sobreproducción de ROS puede activar varios factores de transcripción sensibles al estado redox de la célula entre ellos podemos destacar el NFκB y la proteína

activadora 1 (AP-1) que se activan vía varias quinasas (Sallam and Laher 2016). La activación de estos dos factores de transcripción incrementan la expresión de varias proteínas que participan en la repuesta inflamatoria como la metaloproteinasa 9, moléculas de adhesión intercelular (ICAMs), moléculas de adhesión vascular (VCAMs), la iNOS, la COX-2 además de varios mediadores pro-inflamatorios como el TNF-α, IL-1 e IL-8 (Rahman and MacNee 2000). Muchas de estas proteínas inflamatorias o bien sus productos (NO y PGs) son fuentes de más ROS (Wolin 1996) produciéndose un *feed back* de retroalimentación positiva que incrementa la producción de ROS y de productos inflamatorios. Además las ROS también pueden incrementar los niveles de TNF-α e IL-1β vía la activación de proteínas kinasas activadoras de mitogenos (MAPK) por parte de los macrófagos y células dendríticas (Padgett et al. 2013)

Por ejemplo, el peróxido de hidrogeno, producido por la acción de la xantina oxidasa o durante el estallido oxidativo de los neutrófilos, puede inducir la producción de IL-8 con los consecuentes efectos inflamatorios que puede provocar dicha citoquinas (Dinarello 1992, Feghali and Wright 1997, van der Vliet and Janssen-Heininger 2014). Como ya se ha indicado, la realización de un ejercicio puede activar la expresión de la iNOS incrementándose la producción de NO (Steensberg et al. 2007). Se ha observado que el NO puede ser uno de los responsables del incremento la producción muscular de IL-6 después de la realización ejercicio físico (Steensberg et al. 2007).

La figura 2 resume los efectos de la inflamación, el ejercicio y las infecciones sobre el estado redox de la célula y muestra cómo afectan estos factores sobre la expresión los principales genes envueltos en el control del estado redox de la célula.

Figura 2. Esquema resumen de los efectos de la inflamación, el ejercicio y las infecciones sobre el estado REDOX y la expresión de genes.



Modificado de Ji et al. 2007 (Ji 2007).

4. Efectos inmunomoduladores de los nutrientes

Una dieta saludable y equilibrada es básica para el mantenimiento de un buen estado de salud, pero además se ha visto que es esencial para un correcto funcionamiento del sistema inmunitario. En este sentido, se ha demostrado que son varios los nutrientes que pueden ejercer un efecto modulador sobre el sistema inmunitario. Este hecho hace pensar que a través de la dieta se puede intentar prevenir o tratar una amplia variedad de enfermedades inflamatorias.

Las vitaminas, los minerales y ciertos elementos traza son esenciales en varias rutas metabólicas ya que actúan como cofactores de muchas enzimas y también son esenciales para un correcto funcionamiento del sistema inmunitario. Vitaminas como la A, la B_6, la B_{12}, C o E, así como el ácido fólico y minerales como el hierro, el zinc y el selenio, tienen efectos moduladores sobre el sistema inmunitario e influyen sobre la capacidad de respuesta del organismo frente a infecciones (Chandra 1997, Calder and Kew 2002). Pero no solo los micronutrientes pueden alterar el funcionamiento del sistema inmunitario, algunos tipos de grasas, los carbohidratos o las proteínas pueden tener efectos moduladores sobre el sistema inmune (Calder 1998, Calder 2006).

A continuación se detallan los efectos sobre el sistema inmune.

4.1. Ácidos grasos

Dentro de los ácidos grasos hay que diferenciar entre los ácidos grasos saturados con un clara efecto inflamatorio y los ácidos grasos insaturados que tiene un potente efecto anti-inflamatorio (Serhan et al. 2000, Basu et al. 2006, Huang et al. 2012).

Los ácidos grasos saturados pueden activar diversas vías pro-inflamatorias, entre ellas pueden incrementar la expresión de la COX-2 y aumentar los niveles de TNF-α. Se ha demostrado que el ácido palmítico es capaz de incrementar la expresión de la iNOS, además de incrementar los niveles plasmáticos de TNF-α y PCR. Los mecanismos por los cuales los ácidos grasos pueden ejercer sus efecto pro-inflamatorios no están del todo claros todavía pero todo indica que podría ser debido a la interacción entre los ácidos grasos saturados y los TLRs ya que

participan en el reconocimiento de PAMPs y DAMPs activando la respuesta inmune (Huang et al. 2012, Wu et al. 2014).

Por su parte los ácidos grasos insaturados, concretamente los ácidos grasos omega 3, tienen un claro efecto anti-inflamatoria través de diversas vías (Lee et al. 1985, Lee et al. 2004, Serhan et al. 2015). Se ha observado que el DHA puede inhibir la unión entre los ácidos grasos insaturados y los TLRs, impidiendo así la activación de la respuesta inflamatoria (Lee et al. 2004). Además, se sabe que los ácidos grasos omega 3 pueden jugar un papel importante en la prevención de enfermedades cardiovasculares así como en algunas enfermedades metabólicas como la diabetes tipo 2 o la obesidad en la que se evidencia una situación de inflamación crónica (Calder 2006, Gonzalez-Periz et al. 2009). Tanto el DHA como el EPA pueden actuar como antagonistas del AA, compitiendo con este por el centro activo de la COX-2 y generando mediadores lipídicos con elevada capacidad anti-inflamatoria, que juegan un papel fundamental en la resolución de la inflamación. La suplementación de la dieta con ácidos grasos omega 3 también reduce la movilidad e infiltración en los tejidos de los leucocitos mediante una reducción en sus capacidades quimiotácticas y alterando la expresión de ciertas moléculas de adhesión como las ICAM. Además, la suplementación de la dieta con ácidos grasos omega 3 reduce la capacidad para producir ROS por parte de las células inmunes. La producción de citoquinas por parte de las células inmunitarias también se ve alterada por los ácidos grasos omega 3 ya que se ha observado que tanto el EPA como el DHA reducen significativamente los niveles de citoquinas inflamatorias como TNF-α, IL-1β, Il-6 e IL-1 (Calder and Grimble 2002, Calder 2006).

Por estos motivos se han realizado numerosos estudios que analizan los efectos de los ácidos grasos omega 3 sobre el control de enfermedades inflamatorias como la artritis reumatoide, enfermedad de Crohn, diabetes tipo 2, asma entre otras, con resultados positivos en algunas de las enfermedades (Calder 2006).

- Ácidos grasos oxidados

Algunos productos obtenidos como resultado tanto de la oxidación enzimática como no enzimática de los ácidos grasos tienen propiedades moduladoras sobre la respuesta inflamatoria (Bochkov and Leitinger 2003). Como consecuencia de la activación celular se incrementan los niveles intracelulares de Ca^{2+}, hecho que activa la fosfolipasa A_2 que rompe algunos fosfolípidos de membrana generando AA. Algunos derivados de la oxidación del AA, como 1-palmitoil-2-araquidonoil-sn-glicero-3-fosforilcolina, tienen un potente efecto anti-inflamatorio. La administración de 1-palmitoil-2-arachidonoil-sn-glicero-3-fosforilcolina reduce los efectos pro-inflamatorios del LPS, inhibiendo en gran medida la producción de TNF-α. Este derivado también reduce la inflamación en infecciones pulmonares, disminuyendo drásticamente los niveles de IL-1β e IL-6, además de reducir la infiltración de neutrófilos, e inhibir la expresión de la iNOS (Bochkov et al. 2002, Friedl et al. 2006). Se cree que dicho efectos pueden venir mediados por la inactivación de los TLR-4 (Bochkov and Leitinger 2003).

Otros derivados de la oxidación del AA por parte de las LOXs como los ácidos hidroperoxieicosatetraenoico y hidroxieicosatetraenoico, son capaces de inhibir los efectos del TNF-α sobre algunas moléculas de adhesión como la ICAM-1, E-selectina y VCAM-1, reduciendo así la migración de las células inmunitarias (Huang et al. 1997). Otros derivados del hidroxieicosatetraenoico pueden interactuar con el PPARγ pudiendo ejercer un efecto modulador sobre la respuesta inflamatoria (Huang et al. 1999).

La manteca de cerdo, producto de la cocción prolongada del tejido adiposo blanco del animal, ha sido utilizada tradicionalmente como tratamiento para golpes y procesos inflamatorios agudos pero sin que se sepa los componentes que pueden ser responsables de estos efectos anti-inflamatorios. Sin embargo, son varios los estudios que han mostrado efectos anti-inflamatorios de la manteca de cerdo, normalmente en combinación con otros ingredientes. De hecho, existen varias patentes que usan la manteca de cerdo en combinación con otros ingredientes (miel, aceite de oliva, alcanfor, bicarbonato y romero) como remedio a procesos inflamatorios, anti-eritematoso, eczemas, dermatitis atópica, pérdida de cabello,

varices, dolor articular y calambres (WO2009077635, JP2009149599, ES2087036, US2014377370A1).

4.2. Polifenoles

Los polifenoles son los metabolitos secundarios más abundantes y ampliamente distribuidos en el reino vegetal, involucrados en la defensa contra la radiación ultravioleta por su capacidad antioxidante o contra la agresión de patógenos. Además, los polifenoles tienen propiedades bioactivas que les permiten modular la actividad de algunas enzimas e interferir en mecanismos de señalización celular. Estos compuestos se pueden dividir en diferentes clases de acuerdo con el número de anillos de fenol contenidos y los elementos estructurales que unen estos anillos. Así, los polifenoles pueden clasificarse en ácidos fenólicos, flavonoides, estilbenos y lignanos.

Los polifenoles son micronutrientes que están presentes en la dieta humana, y hay evidencia que sugiere que su consumo ofrece cierta protección contra el desarrollo de cánceres, enfermedades cardiovasculares, diabetes, obesidad, enfermedades óseas degenerativas o enfermedades relacionadas con la edad, entre otros. Los efectos saludables se asocian a su acción antioxidante, anti-inflamatoria e inmunomoduladora (Serafini et al. 2010, Galleano et al. 2012)

Los polifenoles tienen un potente efecto antioxidante puesto que pueden neutralizar directamente los radicales libres y secuestrar iones metálicos, impidiendo las reacciones de multiplicación de la cadena de peroxidación lipídica y la generación de más ROS. Los polifenoles pueden activar diversas vías de señalización, incrementado la expresión y la actividad de otras defensas antioxidantes endógenas (Soobrattee et al. 2005, Rodrigo et al. 2011). Además de sus efectos antioxidantes, los polifenoles tiene efectos cardioprotectores ya que pueden disminuir la presión sistólica, mejorar la función endotelial, inhibir la agregación plaquetaria y reducir la respuesta inflamatoria (Vauzour et al. 2010).

Se han descrito efectos de los polifenoles a nivel de sistema inmunitario. El consumo de zumo de arándanos rico en polifenoles reduce el riesgo de padecer infecciones en el tracto urinario, incrementa la proliferación de los linfocitos T y de las células

NK (Nantz et al. 2013, Takahashi et al. 2013). De hecho, recientemente se ha evidenciado que un tipo de polifenol, la delfinidina, es capaz de impedir la entrada del virus de la hepatitis C en cultivos celulares (Calland et al. 2015).

La suplementación de la dieta con polifenoles reduce la inflamación sistémica en pacientes con síndrome metabólico, descendiendo de forma significativa los niveles de IL-6, IL-12 y CRP. Resultados muy parecidos se han observado en individuos con elevado riego de enfermedad cardiovascular en los que se ha observado una reducción en los niveles séricos de ICAM-1, E-selectina, IL-6 IL-16, MCP-1 y moléculas de adhesión a células vasculares (Chiva-Blanch et al. 2012, Kolehmainen et al. 2012).

4.3. Vitaminas

Las vitaminas son micronutrientes, sin valor energético, pero esenciales para el funcionamiento del organismo. A pesar de que se necesitan en cantidades muy pequeñas deben ser ingeridas con la dieta y su déficit puede conducir al desarrollo de ciertas patologías. Además se ha observado que la suplementación de la dieta con vitaminas puede ayudar a prevenir el desarrollo de algunas enfermedades. El déficit de algunas vitaminas afecta la funcionalidad del sistema inmune (Meydani and Beharka 1998, Chew and Park 2004), si bien una sobredosis de estas vitaminas no se ha visto que repercuta de forma clara en la función inmune.

El equilibrio redox influye sobre la funcionalidad de las células del sistema inmune (Kesarwani et al. 2013) y a su vez las vitaminas juegan un papel clave en este equilibrio (Calder and Kew 2002, Sureda et al. 2008, Ergin et al. 2013) al ser algunas de ellas antioxidantes, o bien ser necesarias para la funcionalidad de enzimas antioxidantes. La vitamina A además de ser esencial para un correcto funcionamiento del timo y del bazo, favorece la actividad del sistema inmune ya que su déficit se relaciona con una reducción en la actividad de las células *NK* y una menor producción de IFN-γ (Gross and Newberne 1980). La deficiencia en vitamina A incrementa la actividad de los Th número y el número de linfocitos Th1 aumentando los niveles de citoquinas proinflamatorias (Garcia 2012).

La vitamina E juega un papel importante como antioxidante al tratarse del principal antioxidante en la fase lipídica, siendo esencial para el mantenimiento del nivel de oxidoreducción de los lípidos de membrana (Cases et al. 2005, Sureda et al. 2008, Tauler et al. 2008). La disponibilidad de vitamina E puede influenciar el funcionamiento del sistema inmunitario (Meydani and Beharka 1998). Un déficit en vitamina E puede repercutir en una respuesta inmune de menor magnitud, provocada por alteraciones tanto en la inmunidad humoral como celular así como una reducción en la actividad fagocítica. La suplementación de la dieta con vitamina E mejora el funcionamiento del sistema inmunitario (Meydani and Beharka 1998, Meydani 2000). La capacidad antioxidante de la vitamina E, al eliminar ROS, puede evitar la activación del NFκB mediada por ROS, reduciendo así la producción de citoquinas pro-inflamatorias. De hecho se ha descrito que la vitamina E es capaz de reducir los niveles de IL-4 en linfocitos Th humanos (Li-Weber et al. 2002).

La vitamina C también participa de la regulación del equilibrio de oxidorreducción celular en medios acuosos (Mukai et al. 1991, Washko et al. 1993), siendo esencial para el correcto funcionamiento del sistema inmunitario. El déficit de vitamina C incrementa la susceptibilidad a padecer infecciones en deportistas (Gleeson et al. 2004). Se ha visto que la suplementación de la dieta con vitamina C en atletas que practican ejercicio de ultra fondo mejora la actividad de las células NK, y la función fagocítica y oxidativa de neutrófilos (Grimble 1997), incrementando la quimiotaxis y potenciando la actividad bactericida mediante la secreción de lisozima y protegiendo frente al anión superóxido (Leibovitz and Siegel 1978). La vitamina C puede tener un potente efecto anti-inflamatorio como se ha observado al tratar con la dicha vitamina enterocitos activados por gluten procedentes de pacientes celiacos en los que revierten los efectos inflamatorios del gluten, al reducir los niveles de TNF-α, INF-γ, INF-α e IL-6. Estos efectos anti-inflamatorios descritos podrían atribuirse a una inactivación de la vía inflamatoria iniciada por NFκB por parte de la vitamina C (Bernardo et al. 2012). Otros estudios han demostrado que dosis elevadas de vitamina C pueden inhibir la producción de IL-6 y TNF-α en monocitos, pero no afectan la producción ni de IL-1 ni de IL-8 (Hartel et al. 2004). En linfocitos se ha observado que dosis elevadas de vitamina C inhiben la producción de IL-2 pero no afectan la producción de TNF-α e IFN-γ (Hartel et al. 2004).

5. Efectos de los nutrientes en el rendimiento deportivo

Una dieta adecuada tanto en calidad como en cantidad, antes, durante y después del ejercicio y durante el período de recuperación tras la práctica de un ejercicio físico es esencial para optimizar el rendimiento. Por ello un deportista debe tener una dieta sana y equilibrada, que le suministre la suficiente energía para cubrir las necesidades del organismo y además proporcione todos los nutrientes necesarios para el correcto funcionamiento del organismo. Los elementos de la dieta del deportista también deben ayudar a mantener y reparar los tejidos dañados durante la realización del ejercicio y mantener y regular el metabolismo corporal (Burke 2007). Las demandas nutricionales para un deportista varían en función del momento de la preparación física en la que se encuentra el atleta (entrenamiento, competición o recuperación), así como del tipo de deporte que se realice. La composición en macronutrientes de la dieta de un deportista debe ser la adecuada para poder cubrir los requerimientos nutricionales del deportista con el fin de alcanzar los objetivos de rendimiento propuestos. Se debe variar la dieta para poder alcanzar los objetivos de rendimiento propuestos (Ormsbee et al. 2014, Shaw et al. 2014, Williams and Rollo 2015).

El consumo de suplementos nutricionales es una práctica ampliamente extendida entre los deportistas con el objetivo de mejorar su rendimiento deportivo, dichos suplementos son conocidos como suplementos deportivos. Se sabe que el rendimiento deportivo está influenciado por la alimentación y la hidratación (Williams 2002). Dentro de los suplementos deportivos podemos encontrar preparados alimenticios elaborados específicamente para suplementar la dieta con fines saludables y ayudar a mantener los estados fisiológicos del deportista, los más habituales son los suplementos ricos en hidratos de carbono, proteínas, vitaminas y minerales, además de aquellos suplementos ricos en nutrientes capaces de reducir la inflamación y la oxidación (Colls Garrido et al. 2015). De todas formas, en la mayoría de casos no se ha podido demostrar que el consumo de suplementos deportivos incremente el rendimiento. Además, se ha demostrado que el consumo excesivo de suplementos deportivos pueden llegar a ser perjudiciales para la salud, como por ejemplo el consumo excesivo de vitaminas, que puede llegar a tener efectos prooxidantes y reducir la capacidad antioxidante endógena (Sureda et al.

2004). Se ha observado también que los suplementos proteicos pueden provocar una perdida excesiva de calcio por la orina pudiendo provocar problemas renales e incluso osteoporosis (Martin Jimenez et al. 2015). De hecho no se han observado efectos ergogénicos en la mayoría de los suplementos nutricionales, exceptuando casos muy concretos como el beta-hidroxi-beta-metilbutirato, la creatina, la dihidroxiacetona, el piruvato y la cafeína que si han demostrado efectos ergogénicos, aunque en el caso de la cafeína puede ser considerada producto dopante en determinados deportes (Juhn 2003, Hespel et al. 2006, Maughan et al. 2007).

5.1. Ácidos grasos Omega 3

Se han realizado muchos estudios sobre los posibles efectos ergogénicos de la suplementación de la dieta con ácidos grasos omega 3 (Pedersen et al. 1997, Toft et al. 2000, Egert et al. 2012, Martorell et al. 2014, Martorell et al. 2014). La suplementación de la dieta con aceite de pescado es eficaz para incrementar el contenido en DHA y EPA en las membrana de los eritrocitos (Egert et al. 2012, Martorell et al. 2014). A este enriquecimiento de las membranas se le atribuyen los efectos ergogénicos del aceite de pescado dado que incrementa la deformabilidad de los eritrocitos. Una mayor deformabilidad eritrocitaria facilita una mayor capacidad de transporte y liberación tisular de oxigeno por parte de los eritrocitos (Jeukendrup and Aldred 2004). Algunos estudios han llegado a afirmar que la suplementación con aceite de pescado puede incrementar la VO_{2max}; sin embargo, otros estudios no han mostrado cambios en la VO_{2max} atribuibles a la suplementación con aceite de pescado (Guezennec et al. 1989, Brilla and Landerholm 1990, Oostenbrug et al. 1997).

La suplementación de la dieta con EPA o DHA o con aceite de pescado tiene efectos sobre la función cardiaca, reduce el ritmo cardiaco, disminuye la presión arterial y reduce el consumo de oxigeno (Peoples et al. 2008). Estos efectos podrían estar relacionados con un menor grado de fatiga detectado en atletas después de suplementación durante 21 días con ácidos grasos omega 3 (Lewis et al. 2015). La práctica de actividad física intensa provoca una respuesta inflamatoria en el organismo que puede afectar al rendimiento deportivo (Walsh et al. 2011, Bentley et al. 2015); los efectos anti-inflamatorios atribuidos a la suplementación de la dieta

con omega 3 podrían contribuir a contrarrestar esta respuesta inflamatoria excesiva asociada a una actividad física exhaustiva y mejorar el rendimiento deportivo. Por ello, la suplementación de la dieta con ácidos grasos omega 3 en atletas es una materia de estudio recurrente (Pedersen et al. 1997, Toft et al. 2000, Egert et al. 2012, Martorell et al. 2014, Martorell et al. 2014). El ejercicio físico intenso puede causar daño muscular (Pedersen and Hoffman-Goetz 2000, Peake et al. 2005) que está directamente asociado con la inflamación asociada al ejercicio (Nieman and Pedersen 1999, Toft et al. 2002). Se cree que las agujetas son la principal consecuencia del daño muscular y la respuesta inflamatoria asociada el ejercicio. Las agujetas se caracterizan por una inflamación aguda, dolor hinchazón y perdida de función que pueden provocar un descenso en el rendimiento (Kanda et al. 2013, Hoseinzadeh et al. 2015). Son varios los estudios que han demostrado los efectos anti-inflamatorios del aceite de pescado, DHA o EPA (Serhan et al. 2000, Calder 2006, Bloomer et al. 2009). En este sentido se ha establecido que los ácidos grasos poliinsaturados atenúan la respuesta inflamatoria relacionada con el daño muscular y por lo tanto pueden reducir las agujetas y mejorar el rendimiento de los deportistas (Tartibian et al. 2011, Corder et al. 2016).

El papel ergogénico de la suplementación de la dieta con omega 3 puede obedecer a otros mecanismos como el que involucra la eliminación muscular de lactato (Beaulieu et al. 1995). Durante la práctica de ejercicio se produce ácido láctico que es uno de los principales responsables de la fatiga, debido a que su acumulación lleva asociada una elevación de la concentración de protones aumentando la acidez muscular y provocando un descenso en la eficacia de la contracción muscular, hecho que afecta directamente al rendimiento deportivo (Beaulieu et al. 1995). El flujo de protones y ácido láctico depende de diversos factores tales como la superficie de intercambio, la velocidad de difusión entre tejidos, el gradiente de concentración de lactato y de la permeabilidad de la membrana a los iones (Roth 1991). Se ha observado que una dieta rica en ácidos grasos omega 3 puede facilitar el flujo de ácido láctico, debido a que los ácidos grasos omega 3 pueden modificar las características del sarcolema, favoreciendo así el flujo de lactato y protones des de la célula muscular al espacio intersticial, reduciendo así el grado de fatiga (Esteve 2002).

5.2. Polifenoles

La suplementación de la dieta con polifenoles, concretamente con quercetina, puede mejorar el rendimiento deportivo puesto que retrasa la aparición de la fatiga en atletas y es capaz de incrementar la VO_2max en ciclistas (Davis et al. 2010). Estos efectos pueden estar mediados por un incremento de la biogénesis mitocondrial que permitiría una mayor tasa de oxidación de ácidos grasos (Davis et al. 2010). La suplementación de la dieta con catequinas atenúa la pérdida de fuerza muscular, reduce el daño muscular asociado al ejercicio, además de reducir los indicadores de daño oxidativo - malondialdehido (MDA), creatina quinasa (CK) y lactato deshidrogensa (LDH) - en ratones (Haramizu et al. 2011). Estudios en modelos animales han mostrado que tras la suplementación con catequinas se incrementa la β-oxidación de ácidos grasos en musculo, además de hacer más eficiente el uso de lípidos como combustible (Murase et al. 2005). De todos modos, los efectos sobre el rendimiento de los polifenoles en humanos no están claros puesto que la mayoría de estudios no han observado efectos sobre la VO_2max, ni sobre la inflamación o los marcadores de estrés oxidativo (Dean et al. 2009, Bigelman et al. 2010), aunque algún estudio ha detectado un incremento en la VO_2max en adultos (Richards et al. 2010).

5.3. Vitaminas

Se ha observado que un déficit en la ingesta de vitaminas puede reducir el rendimiento deportivo ya que esta situación deficitaria puede alterar alguna vía metabólica reduciendo así la disponibilidad de energía para el deportista (Williams 2002). También se ha evidenciado que individuos que sigan dietas hipocalóricas necesitan suplementos vitamínicos para cubrir las necesidades del organismo (Williams 2002). De todas formas son pocos los estudios que hayan demostrado algún efecto ergogénico atribuible al consumo alto de vitaminas más allá de los efectos antioxidantes de algunas como la E o la C (Nikolaidis et al. 2012, Bentley et al. 2015, Paschalis et al. 2016). Incluso se ha indicado que la suplementación de la dieta con altas dosis de vitaminas antioxidantes puede ser contraproducente puesto que puede inhibir los efectos como señalizadores celulares de las ROS necesarios para inducir la adaptación al entrenamiento y generar defensas antioxidantes endógenas (Radak et al. 2005, Gomez-Cabrera et al. 2006, Gomez-Cabrera et al.

2008). De hecho, el organismo tiene mecanismos endógenos para hacer frente a una mayor producción de ROS inducida por la actividad física y el consumo excesivo de vitaminas antioxidantes bloquearía su activación (Sureda et al. 2013). El consumo excesivo de algunos suplementos vitamínicos podría alterar el equilibrio de oxidorreducción en el organismo y provocar más daños que beneficios (Nieman et al. 2004, McAnulty et al. 2005). Sin embargo, varios estudios han evidenciado efectos beneficios de la ingesta suplementaria con bajas concentraciones de vitaminas antioxidantes en la activación de las defensas antioxidantes y la disminución del daño oxidativo inducido por la actividad física (Cases et al. 2005, Sureda et al. 2008) .

Los efectos de la suplementación de la dieta con vitaminas antioxidantes sobre el rendimiento deportivo, sobre la funcionalidad del sistema inmunitario o sobre las adaptaciones del entrenamiento deportivo no están todavía del todo claros (Leibovitz and Siegel 1978, Buzina and Suboticanec 1985, Nieman et al. 1997, Bowie and O'Neill 2000). Algunos estudios afirman que la suplementación de la dieta con vitamina C produce un incremento en la VO_2max pero únicamente en atletas que presentan un déficit basal de vitamina C (Buzina and Suboticanec 1985, Paschalis et al. 2016). La mayoría de estudios no han demostrado mejoras en el rendimiento debido a la suplementación con vitamina C (Yfanti et al. 2010, Roberts et al. 2011), incluso existe algún estudio que ha mostrado efectos adversos de la suplementación con vitamina C sobre el rendimiento (Gomez-Cabrera et al. 2008). La mayoría de estudios en relación con los cambios a nivel de sistema inmunitario durante la actividad física y suplementación con vitamina C no han mostrado efectos beneficiosos de dicha suplementación (Nieman et al. 1997, Krause et al. 2001), sin embargo, otros estudios han encontrado que la suplementación con 1500 mg de vitamina C por día reduce los niveles de IL-6, IL-10, IL-1rα, e IL-8 tras una carrera de 90 km (Nieman et al. 2000). Existen evidencias que la suplementación con vitamina C reduce el dolor y el daño muscular asociado a la práctica de ejercicios intensos y puede facilitar la recuperación muscular (Jakeman and Maxwell 1993, Clarkson and Thompson 2000), además, la suplementación de la dieta con vitamina C reduce la incidencia de resfriados en personas físicamente activas, al igual que reduce las infecciones en el tracto respiratorio superior y atenúa la respuesta

inmunosupresora asociada a la práctica de ejercicio físico intenso (Peters 1997, Peters et al. 2001).

La mayoría de estudios sobre vitamina E y rendimiento deportivo se realizaron en la década de los setenta y ochenta y los resultados obtenidos no clarifican demasiado los efectos sobre el rendimiento. Existe alguna evidencia de que la suplementación con vitamina E puede mejorar la forma física en condiciones de hipoxia permanente (Simon-Schnass and Pabst 1988), aunque la mayoría de estudios no han encontrado efectos ergogénicos asociados a la suplementación con vitamina E (Oostenbrug et al. 1997, Paulsen et al. 2014). A pesar de no haberse detectado mejoras en el rendimiento debido a la suplementación con vitamina E se ha observado que el déficit de vitamina E incrementa la fatiga muscular, altera la contractilidad muscular e incrementa el daño muscular (Coombes et al. 2002). La suplementación con vitamina E reduce la secreción de IL-6 e IL-1β pero no afecta los niveles de TNF-α después del ejercicio; además la suplementación de la dieta con vitamina E puede afectar al funcionamiento de las LOX y las COX alterando el metabolismo del AA reduciendo los niveles de PGE_2 (Goetzl 1980, Meydani et al. 1990, Cannon et al. 1991).

II. OBJETIVOS Y PLANTEAMIENTO EXPERIMENTAL

1. Objetivos

La inflamación es un proceso tisular constituido por una serie de fenómenos moleculares, celulares y vasculares con finalidad defensiva frente a agresiones físicas, químicas o biológicas (Kumar 2006). Se trata de un proceso focalizado a una zona concreta, aunque como en el caso de la inflamación sistémica puede estar más generalizado. La inflamación es una respuesta aguda, inmediata e inespecífica aunque puede facilitar el desarrollo de una respuesta especifica. La inflamación también puede ser crónica o sub-clínica refiriéndose a un estado fisiológico inflamatorio en el que no se encuentran los indicadores clásicos de inflamación y responde a una no resolución o resolución incorrecta de una respuesta inflamatoria aguda, o bien a infecciones persistentes. Sin embargo, el motivo más habitual de esta inflamación sub-clínica suele estar asociado a enfermedades metabólicas como diabetes, obesidad, síndrome metabólico, hígado graso no alcohólico incluso al cáncer (Minihane et al. 2015). Los componentes de la dieta podrían participar en la modulación de la respuesta inflamatoria e inmune del organismo. Se han descrito efectos anti-inflamatorios de nutrientes como determinados ácidos grasos, vitaminas o polifenoles y antioxidantes, con capacidad de modular la respuesta inflamatoria aguda del organismo. No se conocen con claridad los mecanismos por los que estos nutrientes ejercen efectos anti-inflamatorios, ni si modulan la inflamación aguda o también modulan la inflamación crónica. La acción anti-inflamatoria de los ácidos grasos polinsaturados omega 3 se ha relacionado con su interacción directa con los TLRs interfiriendo con la unión de sus ligandos y frenando así la respuesta inflamatoria (Lee et al. 2004). Se ha indicado que los ácidos grasos poliinsaturados omega 3 pueden interaccionar también con los mecanismos de activación de factores de transcripción como NFκB o PPAR γ o bien directamente con los mismos factores de trascripción (Huffman et al. 2004) modulando la transcripción de genes inflamatorios y también de genes relacionados con el control del estrés oxidativo. Además, se ha evidenciado que los ácidos grasos omega 3 pueden generar una amplia variedad de mediadores lipídicos con unos potentes efectos anti-inflamatorios o resolutivos de la inflamación, tales como las resolvinas, maresinas, protectinas entre otros (Serhan et al. 2002, Hong et al. 2003, Serhan et al. 2015).

La realización de actividad física aguda desencadena una respuesta inmunológica que se ha asociado durante mucho tiempo con la aparición de un estado pro-inflamatorio agudo (Pedersen et al. 1996, Nieman and Pedersen 1999, Toft et al. 2002). Sin embargo, la realización de ejercicio de manera regular, a modo de entrenamiento, se prescribe para combatir enfermedades crónicas con inflamación sub-clínica dado que reduce los marcadores de inflamación (Petersen and Pedersen 2005, Walsh et al. 2011, Sallam and Laher 2016). Adicionalmente, la realización de un ejercicio físico agudo no solo afecta al estado inflamatorio del organismo sino que también incrementa la producción de ROS y de marcadores de daño oxidativo y, en contraposición, induce una mayor actividad de las defensas antioxidantes endógenas (Aoi et al. 2004, Peake and Suzuki 2004, Sureda et al. 2005). La repetición regular de ejercicios físicos agudos, evitando los hábitos de una vida sedentaria, conduce a potenciar la expresión de enzimas antioxidantes y otros mecanismo de defensa antioxidante y a minimizar el efecto de las ROS (Cases et al. 2005, Radak et al. 2005, Gomez-Cabrera et al. 2008, Nicks et al. 2009, Tian et al. 2010). Tanto el ejercicio físico practicado de forma aguda como de forma regular (entrenamiento) son un buen modelo fisiológico para estudiar los mecanismos de la respuesta inflamatoria y del estrés oxidativo y su modulación por nutrientes como los ácidos grasos poliinsaturados omega 3 y antioxidantes como polifenoles y vitamina C. De hecho, ya se disponen de estudios que han investigado los efectos del ejercicio físico y la suplementación de la dieta con ácidos grasos polinsaturados omega 3 en deportistas sobre la respuesta inflamatoria y del estrés oxidativo (Pedersen et al. 1997, Toft et al. 2000, Peake and Suzuki 2004, Sureda et al. 2008, Bloomer et al. 2009, Filaire et al. 2010, Tartibian et al. 2011, Egert et al. 2012, Martorell et al. 2014, Martorell et al. 2014, Da Boit et al. 2015, Corder et al. 2016); sin embargo, los resultados obtenidos en relación a los marcadores de inflamación y de estrés oxidativo no son claros e incluso en algunos casos contradictorios. Es preciso valorar como afecta la suplementación crónica con un ácido graso poliinsaturado omega 3, como el DHA, al balance oxidativo y a la respuesta inflamatoria a la realización de una actividad física aguda e intensa. De forma similar, la vitamina C es otro de los nutrientes que ha sido usado de forma recurrente como suplemento de la dieta para deportistas, dado sus atribuidas propiedades antioxidantes y anti-inflamatorias (Buzina and Suboticanec 1985, Nieman et al. 1997, Nieman et al. 2000, Tauler et al.

2003). Los efectos antioxidantes de la suplementación de la dieta con dosis moderadas de vitamina C en deportistas están demostrados en diversos estudios (Tauler et al. 2003, Sureda et al. 2008, Sureda et al. 2013), así como también que dosis más elevadas provocan efectos adversos en la adaptación al ejercicio al inhibir los efectos de las ROS como señalizadores celulares, necesarios para inducir la adaptación al entrenamiento y generar defensas antioxidantes endógenas (Radak et al. 2005, Gomez-Cabrera et al. 2006, Gomez-Cabrera et al. 2008). Hay estudios antiguos sobre los efectos de la vitamina C como moduladores de la respuesta inmune asociada a la infección (Hartel et al. 2004) si bien los resultados no están suficientemente validados para generalizar el uso de la vitamina C como anti-inflamatorio y, menos en deportistas activos. Es preciso aportar nuevas evidencias sobre los posibles efectos y mecanismos anti-inflamatorios de la vitamina C, administrada sola o en combinación con otros nutrientes como el DHA.

Un alimento que de forma tradicional se ha utilizado como medicamento anti-inflamatorio de uso tópico es la manteca de cerdo (Hernández Navarro 2009). La manteca de cerdo se obtiene después de triturar y cocer el tejido adiposo blanco del cerdo a una temperatura de entre 60 y 80ºC. Se trata de un alimento que ha sido utilizado tradicionalmente como tratamiento para golpes y procesos inflamatorios agudos pero sin que se sepan los elementos que pueden ser responsables de estos efectos anti-inflamatorios. Algunos productos de la oxidación enzimática y no enzimática de lípidos pueden ejercer potentes efectos anti-inflamatorios (Bochkov et al. 2002, Bochkov and Leitinger 2003, Friedl et al. 2006).

Nuestra hipótesis es que la suplementación de la dieta con nutrientes como los ácidos grasos poliinsaturados omega 3 y antioxidantes como polifenoles o vitamina C son capaces de modular la respuesta inflamatoria aguda y crónica así como el balance oxidativo asociado a situaciones fisiológicas y/o patológicas, con efectos favorables para mejorar el estado de salud o de forma física. Hipotetizamos que otros nutrientes presentes en alimentos grasos elaborados como la manteca de cerdo, productos de la oxidación de los ácidos grasos que se generan durante su elaboración, tienen capacidades anti-inflamatorias.

El objetivo principal de la presente tesis es estudiar los efectos de nutrientes y de alimentos funcionales enriquecidos con ácidos grasos esenciales y antioxidantes sobre los marcadores de estrés oxidativo e inflamación asociada a la realización de ejercicio físico de forma aguda y sobre las adaptaciones derivadas de un período de entrenamiento regular y evidenciar las propiedades anti-inflamatorias de alimentos grasos como la manteca de cerdo y de sus componentes.

Este objetivo general puede desglosarse en los siguientes objetivos específicos:

1. Determinar los efectos del consumo de una bebida funcional enriquecida con DHA y del entrenamiento regular sobre el balance oxidativo de las células inmunitarias mononucleares periféricas (PBMCs) tras la práctica de ejercicio físico aguda, y sobre los niveles plasmáticos de citoquinas pro- y anti-inflamatorias.

2. Determinar si el ejercicio físico agudo tras 8 semanas de entrenamiento y de la suplementación con DHA modula la respuesta inflamatoria de los neutrófilos y de las PBMCs para producir citoquinas frente a un estímulo exógeno, y establecer si el tratamiento agudo de con vitamina C puede modular dicha capacidad de respuesta en neutrófilos.

3. Valorar si ejercicio físico regular, el ejercicio agudo y la suplementación de la dieta con DHA afectan a los niveles plasmáticos de mediadores lipídicos de inflamación y valorar, en estas condiciones, la capacidad de las PBMCs para producir mediadores lipídicos tras su estimulación inmunológica.

4. Analizar si la suplementación de la dieta con una bebida funcional, rica en DHA, polifenoles y vitamina E y el entrenamiento durante 6 semanas pueden mejorar el rendimiento deportivo.

5. Estudiar los efectos de una suplementación con una bebida funcional enriquecida con DHA, polifenoles y vitamina E y de la actividad física sobre los marcadores plasmáticos de inflamación y de estrés oxidativo en deportistas jóvenes y sénior.

6. Demostrar que la manteca de cerdo tiene propiedades anti-inflamatorias e identificar y evaluar qué elementos de su composición son los responsables de estos efectos anti-inflamatorios.

2. Planteamiento experimental

Para poder estudiar el efecto de diversos nutrientes sobre el estado inflamatorio y oxidativo del organismo se diseña una bebida funcional elaborada con almendras, enriquecida con aceite de oliva, DHA y vitamina E, que contiene cantidades elevadas de polifenoles y, de forma paralela, también se diseña una bebida placebo a la que se sustituye el DHA por una cantidad equivalente y adicional de aceite de oliva. Estas bebidas se administran como suplemento dietético a atletas de diferentes deportes (futbolistas y taekwondistas) y de diferentes edades (atletas jóvenes y atletas sénior) según el experimento concreto que se plantee para alcanzar los objetivos previstos.

Experimento 1. Efectos de una suplementación crónica con DHA sobre el estado oxidativo e inflamatorio en respuestas al entrenamiento deportivo de futbolistas y al ejercicio agudo.

Se diseña un estudio a doble ciego en el que se lleva a cabo una intervención nutricional durante ocho semanas en el inicio de la temporada deportiva de futbolistas federados. El estudio está aprobado por el *Comité de Ètica de les Illes Balears* y está registrado también en ClinicalTrial.gov (NCT02177383). La intervención nutricional consiste en el consumo de una bebida funcional elaborada con almendras y enriquecida con DHA o una bebida placebo. Las bebidas utilizadas como vehículo están elaboradas con almendras, y presentan la misma composición con la única diferencia que una parte del aceite de oliva de la bebida placebo se sustituye por una cantidad equivalente de DHA, con lo que la bebida experimental está enriquecida con DHA. Ambas bebidas presentan un elevado contenido en vitamina E, con el objetivo de evitar su oxidación. En ambas bebidas se analiza, el contenido de lípidos (1.9%), proteínas (1.0%), azucares (6.8%), ácidos grasos individuales, de tocoferoles y de polifenoles totales.

Los criterios de inclusión y exclusión de los participantes en el estudio son: edad (entre 16-35 años), sexo (varones), no fumadores, que sigan una dieta equilibrada, cubriendo globalmente las ingestas diarias recomendadas (IDR), Índice de Masa Corporal (IMC) (entre 19-24 kg/m^2), deportista de competición: al menos 2 horas diarias (14 horas semanales) y nivel competitivo estatal en la actualidad, voluntad de participar en el estudio: consentimiento informado y firmado.

Se realiza una valoración nutricional y antropométrica de todos los participantes en el estudio para asegurar el cumplimiento de los criterios de inclusión, determinar los patrones nutricionales y comprobar que todos siguen unos patrones nutricionales y presentan unas características antropométricas similares.

Los admitidos a participar en el estudio son 22 futbolistas pre-profesionales de entre 19 y 21 años pertenecientes a la plantilla del equipo filial del Real Club Deportivo Mallorca en los inicios de la temporada 2011-2012. Dichos participantes se distribuyen de forma aleatoria entre un grupo placebo (11) y un grupo experimental (11) cuya dieta se suplementa con la bebida placebo o la bebida experimental.

La intervención nutricional consiste en administrar como suplemento dietético adicional las bebidas funcionales placebo y experimental durante ocho semanas. Los futbolistas consumen un litro de bebida funcional (placebo o experimental) cinco días a la semana, dejando los sábados y domingos sin suplementar la dieta. El consumo de las bebidas se comienza al inicio de los entrenamientos y se culmina la ingesta del litro de bebida al finalizarlos.

Únicamente completan las ocho semanas de intervención nutricional un total de 15 futbolistas, 6 del grupo placebo y 9 del grupo experimental. Los motivos por los que se producen las bajas son en todos los casos ajenos al estudio, puesto que dejan de entrenar en el equipo del RCD Mallorca B por lesión o por pasar a entrenar con la primera plantilla del RCD Mallorca.

Al finalizar las 8 semanas de intervención nutricional los futbolistas realizan un ejercicio de intensidad programada con objeto de evaluar los efectos de una actividad física aguda. Los participantes realizan una sesión de entrenamiento controlado, de una actividad física intensa durante 2 horas, de manera que durante más del 50% del tiempo de entrenamiento los futbolistas ejercitan a tasas superiores al 70% de su $VO_{2máx}$. El entrenamiento consiste en la realización del test de Léger-Boucher, para la determinación indirecta la $VO_{2máx}$ individual, la realización de ejercicios tácticos de simulación de situaciones que se producen durante un partido de futbol y que requieren de una elevada intensidad.

Se realizan tres extracciones de sangre, una en condiciones basales al inicio y las otras dos al finalizar las 8 semanas de intervención nutricional, una, en condiciones basales y la segunda, dos horas después de la realización de la sesión de entrenamiento.

Los resultados de este experimento se resumen en los manuscritos I, III, V, VI y VII.

Se realiza el recuento de eritrocitos, leucocitos, linfocitos, monocitos, eosinófilos, basófilos, PBMCs y neutrófilos en las muestras sanguíneas. Se toman muestras de sangre para la obtención de plasma, suero, eritrocitos, PBMCs y neutrófilos.

En eritrocitos se determina la concentración de ácidos grasos individuales, con objeto de efectuar el seguimiento de la intervención nutricional y valorar la incorporación de DHA en este tipo celular.

En las PBMCs aisladas se determinan marcadores de daño oxidativo y nitrosativo en lípidos (MDA) proteínas (carbonilos y nitrotirosinas) y ADN (Comet Assay). También se determinan las actividades de enzimas antioxidantes (CAT, GPx y GRd), los niveles de proteínas antioxidantes (CAT, GPx, GRd, Cu/ZnSOD, Mn-SOD, TrxR1, UCP2 y UCP3) y de iNOS y la expresión de genes antioxidantes (CAT, GPx, Cu/Zn-SOD, Mn-SOD y UCP3). Los resultados de este estudio se resumen en el manuscrito I.

En plasma se determinan los niveles de citoquinas (IL-2, IL-4, IL-6, IL-8, IL-10, IL-1α, IL-1β, Il-5, IL-15, GM-CSF, TNF-α, TNF-β, INFγ, MCP-1, MIP-1α, factor de crecimiento epitelial y factor de crecimiento vasoendotelial) y mediadores lipídicos (RvD_1, PGE_1 y PGE_2) circulantes. Los resultados de este estudio se resumen en los manuscrito III y V.

En las PBMCs se estudian la capacidad de producción de especies reactivas de oxígeno, de productos de oxidación del óxido nítrico, de citoquinas (IL-2, IL-4, IL-6, IL-8, IL-10, IL-1α, IL-1β, Il-5, IL-15, GM-CSF, TNF-α, TNF-β, INFγ, MCP-1, MIP-1α, factor de crecimiento epitelial y factor de crecimiento vasoendotelial) y de mediadores lipídicos (RvD_1, PGE_1 y PGE_2). Así mismo, se determina el efecto de la temperatura sobre la respuesta inflamatoria mediada por LPS cuantificando la tasa

de producción de citoquinas y mediadores lipídicos. Se incuban PBMCs en medio de cultivo con o sin LPS a dos temperaturas diferentes (37ºC y 39.5ºC), durante dos horas. En los sobrenadantes de centrifugación de los cultivos se analizan los niveles de nitrato, nitrito, (IL-2, IL-4, IL-6, IL-8, IL-10, IL-1α, IL-1β, Il-5, IL-15, GM-CSF, TNF-α, TNF-β, INFγ, MCP-1, MIP-1α, factor de crecimiento epitelial y factor de crecimiento vasoendotelial) y de mediadores lipídicos (RvD_1, PGE_1 y PGE_2). Los resultados de este experimento se resumen en los manuscritos , III, V y VI.

La capacidad anti-inflamatoria de la vitamina C se ensaya utilizando neutrófilos de futbolistas en condiciones basales tras las ocho semanas de intervención nutricional activados con PMA Los neutrófilos obtenidos en condiciones basales al finalizar las 8 semanas de suplementación se distribuyen en tres alícuotas diferentes y se pusieron en presencia de 3 medios de cultivo diferentes: medio control, medio control al que se le añade PMA (5 μg/mL) y medio control al que se le añade PMA y vitamina C (5 μg/mL de PMA y 3mM de vitamina C). Se incuban los neutrófilos durante dos horas a 37ºC y se centrifugan a fin de obtener las células y el sobrenadante del cultivo. En los sobrenadantes de los medios de cultivo se determinan los niveles de citoquinas, actividades enzimáticas (CAT y mieloperoxidasa (MPO)), niveles de nitrato y nitrito. En los precipitados de neutrófilos se determinan las actividades de enzimas antioxidantes y pro-inflamatorios (CAT y MPO), la expresión de genes inflamatorios (MPO, COX-2, IL-8, NFκB y TNF-α) y concentración de nitritos y nitratos. Los resultados de este experimento se resumen en el manuscrito VII.

Experimento 2. Efectos de la suplementación con una bebida funcional elaborada con almendras y enriquecida con DHA y vitamina E sobre el estado inflamatorio y oxidativo en atletas jóvenes y sénior.

Se diseña un estudio a doble ciego en el que se lleva a cabo una intervención nutricional con una bebida de almendra enriquecida con aceite de oliva, DHA y vitamina E durante 6 semanas. La intervención nutricional consiste en el consumo de lunes a viernes de 1L de una bebida funcional elaborada con almendras y enriquecida con aceite de oliva, DHA y con vitamina E. La ingesta de la bebida se realiza en dos tomas, una antes de iniciar la actividad física diaria y la otra a media mañana. En el estudio participan 10 atletas jóvenes (de entre 18 y 25 años) y 8 atletas sénior (de entre 35 y 57 años) que consumen la bebida funcional durante 6 semanas.

Antes de ser aceptados en el estudio todos los atletas se someten a un examen médico, y se les practica un electrocardiograma en reposo. El protocolo del estudio cumple con la declaración de Helsinki y es aprobado por el "*Comité Ètic per la Investigació Clínica de la Direcció General del Esport del Consell d'Esports de Catalunya*". El estudio también se registra en ClinicalTrial.gov (NCT02177383).

Los deportistas realizan una prueba de esfuerzo para determinar la VO_2máx y la frecuencia cardiaca máxima, a partir de esta determinación se calcula la velocidad correspondiente al 60% (V60), 70% (V70), 80% (V 80) y 90% (V90) de su VO_2máx se calcula por interpolación lineal de los datos de la prueba de esfuerzo máximo. Los sujetos llevan a cabo una prueba de esfuerzo, en una cámara climática con las condiciones de temperatura y humedad controladas, dicha prueba de esfuerzo se realiza en cinta ergométrica de manera continua a velocidad V60 durante 5 minutos, a V70 durante 5 minutos y otros 5 minutos a V80, todo ello durante tres episodios separados por dos minutos de recuperación. Para finalizar, los sujetos corren a V90 hasta el agotamiento. Se obtiene una muestra de 20 µl de sangre del lóbulo de la oreja de cada sujeto para medir la concentración de lactato en reposo, en los minutos 15, 32, 49 e inmediatamente después de finalizar la prueba, a la vez que se mide la frecuencia cardiaca en cada periodo tiempo y la frecuencia cardiaca máxima, en el mismo momento que se realiza la extracción de sangre para determinar el lactato se lleva a cabo el test de Borg para determinar la percepción de fatiga.

La temperatura corporal central se determina mediante la utilización de termómetros-píldora intestinales. Los sujetos ingirieren la píldora ocho horas antes de la prueba para asegurarse de que alcanzara el tracto gastrointestinal. El valor de temperatura corporal se recoge en el momento inicial (tiempo 0) y cada 5 minutos durante el ejercicio hasta el agotamiento de cada sujeto y a los 5 minutos de haber finalizado la actividad, también se determina la temperatura de la piel, que se mide de manera continua con 4 termistores de piel colocados en el pecho, brazo derecho, muslo y pantorrilla. Los valores se recogen en el momento inicial (tiempo 0) y cada 5 minutos durante el ejercicio hasta el agotamiento de cada sujeto y a los 5 minutos de haber finalizado la actividad.

Se realizan 4 extracciones de sangre, dos al inicio y dos al finalizar la intervención nutricional. La primera muestra se toma en condiciones basales y la segunda se toma 1 hora después de la finalización de la prueba de esfuerzo, tanto al inicio como al final de la intervención nutricional. La sangre se fracciona para obtener plasma, eritrocitos y PBMCs.

En el plasma se determina la concentración de ácidos grasos, la concentración de polifenoles totales, los niveles de nitratos y nitritos, marcadores de oxidación de lípidos (MDA y lipoperóxidos) y proteínas (carbonilos), concentración de lactato y marcadores de inflamación (IL-6 y TNF-α), mediadores lipídicos (PGE_1, PGE_2, lipoxina) y moléculas de adhesión (sL-Selectina e iCAM3). Los resultados de este experimento se resumen en los manuscritos II y IV,

En eritrocitos se determina la composición y la concentración de ácidos grasos, el contenido de polifenoles totales y marcadores de daño oxidativo en lípidos (MDA) y en proteínas (carbonilos y nitrotirosinas). Los resultados de este estudio se resumen en el manuscrito II.

En PBMCs se determinan los niveles de NFκB activado, la expresión de genes antioxidantes (CAT, Mn-SOD, Cu/Zn-SOD, UCP-3, Hemooxigenasa 1, GRd, y GPx), genes inflamatorios (TLR-2, TLR-4, NFκB, COX-2, 5 LOX, 15 LOX-2, IL-1β, IL-8, TNF-α, IL-10, IL-15) Heat Shock Protein 70 (HSP-70) y genes de dinámica mitocondrial (mitofusina 1 , mitofusina 2, Miembro 4 de la familia 2 de transportadores de solutos

(SLC-2A4), Subunidad V de la NADH deshidrogenasa (MtND5), la subunidad IV de la citocromo C oxidasa (COXIV), y el coactivador del receptor activado por proliferadores peroxisomales 1 α (PGC-1α).

Los resultados de este estudio se resumen en los manuscrito II y IV.

Experimento 3. Efectos de anti-inflamatorios del sebo de cerdo y determinación de los principios activos.

Se diseña un experimento con animales a los que se les ha inducido un proceso inflamatorio con objeto de comprobar los efectos anti-inflamatorios del sebo de cerdo. Tras la comprobación de los efectos anti-inflamatorios del sebo de cerdo se investiga sobre la fracción del sebo responsable de sus efectos anti-inflamatorios mediante estudios 'in vitro' con PBMCs y neutrófilos humanos estimulados con LPS. La comprobación de que la fracción de extracto hidroalcohólico del sebo es responsable de los efectos anti-inflamatorios y de que la grasa de cerdo sin procesar no tiene estos efectos, permite, mediante análisis de composición del extracto hidroalcohólico y de la grasa de cerdo no procesada detectar posibles compuestos que pudieran ser responsables de los efectos anti-inflamatorios. Finalmente, se ensayan los efectos anti-inflamatorios de los componentes del extracto hidroalcohólico del sebo de cerdo que no se encuentran en la grasa de cerdo, utilizando neutrófilos humanos estimulados con LPS.

Experimento 3.1 Comprobación de los efectos anti-inflamatorios del sebo de cerdo

Se utilizan ratas Sprague-Dawley macho de 200-220 gramos de peso. Las ratas se distribuyen en dos grupos: un primer grupo control al que se le induce un proceso inflamatorio que se trata con vaselina tópica (6 animales), y un segundo grupo experimental al que también se le induce el mismo proceso inflamatorio pero que se trata con la misma dosis de sebo de cerdo (6 animales).

Los animales se anestesian con pentobarbital sódico intraperitoneal (50 mg/Kg). Se induce el proceso inflamatorio mediante inyección subplantar en una de las patas posteriores de 0.1 ml de una solución de zymosan (10 mg/ml en suero salino). La otra pata se utiliza como control negativo de inflamación y se le inyecta suero salino (0.1 ml de NaCl al 0.9%).

Tras una hora de iniciar y visualizar el proceso inflamatorio con Zymosan, se extiende una capa de 0.5 g de vaselina a los animales del grupo control mientras que a los animales del grupo experimental se les extiende una capa de 0.5 g de sebo. Los dos tratamientos se aplican de forma uniforme de manera que quede una capa recubriendo toda la zona inflamada. El proceso inflamatorio se sigue por medición del diámetro de las patas a diferentes tiempos y, tras sacrificio, por el peso de las patas y mediante la determinación de marcadores de inflamación en plasma y neutrófilos, como las actividades de enzimas antioxidantes y pro-oxidantes y daño oxidativo. El diámetro de las patas se mide en los tiempos 0 (antes inducir la inflamación), 1 hora (antes los tratamientos), y a las 2, 3 y 4 horas. Tras 4 horas de haber iniciado la inflamación los animales se sacrifican por decapitación, se recoge sangre, se extrae el tejido subplantar y se pesan las patas de las ratas. 1 gramo de tejido se homogeneiza en tampón fosfato potasio 50m M, pH 7 con 0,5% de hexa-decil-trimetil-bromuro de amonio a 4ºC, se centrifuga 30 minutos a 8,000 xg a 4ºC. En el sobrenadante se determinan las actividades de enzimas antioxidantes (CAT y GPx), la actividad mieloperoxidasa y los niveles de nitrito y de marcadores de daño oxidativo en lípidos (MDA) y en proteínas (carbonilos). Las muestras de sangre se fraccionan para obtener los neutrófilos, en ellos se determinan las actividades de enzimas antioxidantes (CAT, GPx,) y de mieloperoxidasa.

Experimento 3.2 Obtención del extracto hidroalcohólico del sebo de cerdos y valoración de su capacidad anti-inflamatoria.

El extracto hidroalcohólico del sebo de cerdo se obtiene fundiendo el sebo de cerdo y añadiéndole una mezcla de metanol:agua (65:35, v:v) y un ácido concentrado preferiblemente HCl 1M. Se agita vigorosamente con ayuda mecánica y se separan las fases por centrifugación. Seguidamente se purifican los principios anti-inflamatorios mediante una extracción en fase sólida en la que los principios activos anti-inflamatorios se adsorben a un material hidrofóbico que se lava con agua, hexano y finalmente se desadsorben y liberan con metilformiato.

Las capacidades anti-inflamatorias del extracto hidroalcohólico se determina en estudios 'in vitro' con PBMCs y neutrófilos procedentes de sangre humana.

Se recogen muestras de sangre de 8 individuos sanos. Se aíslan las PBMCs y los neutrófilos. Las PBMCs y los neutrófilos de cada individuo se incuban en diferentes medios de cultivo durante 2 horas a 37ºC:

- Cultivo Control: contiene el medio de cultivo y las células.

- Cultivo LPS: contiene el medio de cultivo al que se le añade una solución de LPS (1 μg/mL) como aditivo y las células.

- Cultivo Extracto: contiene el medio de cultivo con un extracto hidroalcohólico de sebo de cerdo a una concentración de 1 mg/mL y las células. La concentración de extracto es equivalente a la que se encuentra en el sebo de cerdo que ha dado resultados positivos como anti-inflamatorio en el experimento con los animales.

- Cultivo Extracto + LPS: contiene el medio de cultivo con el extracto hidroalcohólico (concentración de 1 mg/mL), las células y al que se le añade LPS (1 μg/mL) al cabo de 30 minutos de haberse iniciado la incubación.

- Cultivo LPS + Extracto: contiene el medio de cultivo con LPS (1 μg/mL) y las células al que se le añade el extracto hidroalcohólico de sebo de cerdo (concentración de 1 mg/mL) al cabo de 30 minutos de haberse iniciado la incubación.

El proceso inflamatorio se sigue mediante la determinación de diferentes citoquinas en los sobrenadantes de centrifugación de los cultivos. Las citoquinas analizadas son: TNF-α, IL-6, IL-8.

Experimento 3.3 Análisis diferencial de los componentes del extracto hidroalcohólico del sebo y grasa de cerdo y valoración de la capacidad anti-inflamatoria de los mismos.

El análisis de los componentes del extracto hidroalcohólico se realiza por cromatografía de gases acoplado a un detector de masas de impacto electrónico (CG-MS). Se analizan los extractos hidoralcohólicos del sebo de cerdo y del tejido adiposo del cerdo que se utiliza para obtener el sebo de cerdo. Se identifican los componentes presentes en el sebo y no en la grasa de cerdo. Se ensaya la capacidad anti-inflamatoria de estos compuestos.

La capacidad anti-inflamatoria de los componentes del extracto hidroalcohólico del sebo de cerdo se ensaya en neutrófilos humanos activados con LPS. La respuesta inflamatoria de los neutrófilos se sigue mediante la determinación de las actividades CAT y MPO o mediante la tasa de producción de la citoquina TNF-α.

Se aíslan los neutrófilos a partir de sangre humana de individuos sanos. Los neutrófilos se distribuyen en diferentes medios de cultivo: cultivo control, cultivo con resolvina D1, cultivo con oleamida y cultivo con 5-dodecanolida siempre en presencia de LPS. Las concentraciones de resolvina D1, oleamida y 5-dodecanolida que se utilizan se calculan a partir de la composición del extracto hidroalcohólico del sebo de cerdo que es efectiva como anti-inflamatorio. En los sobrenadantes de centrifugación de los cultivos recogidos después de 2 horas de incubación se cuantifican las actividades enzimáticas de CAT y MPO como indicadores de la degranulación de los neutrófilos.

Se analiza la influencia de la concentración de la 5-dodecanolida sobre su capacidad anti-inflamatoria. Se ensaya también con neutrófilos humanos activados con LPS en los que se cuantifica la influencia de diferentes concentraciones de 5-dodecanolida (0.1, 0.06, 0.01 mg /mL) sobre la tasa de producción de TNF-α. Se utilizan neutrófilos aislados de sangre humana, se distribuyen en diferentes cultivos: Cultivo control, Cultivos con diferentes concentraciones de 5-dodecalolida a los que se añade o no LPS. En el sobrenadante de los cultivos recogido después de dos horas de incubación se determina la tasa de producción de TNF-α.

III. RESULTADOS Y DISCUSIÓN

Manuscript I

Diet supplementation with DHA-enriched food in football players during training season enhances the mitochondrial antioxidant capabilities in blood mononuclear cells

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ORIGINAL CONTRIBUTION

Diet supplementation with DHA-enriched food in football players during training season enhances the mitochondrial antioxidant capabilities in blood mononuclear cells

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Abstract
Purpose Exercise induces oxidative stress and causes adaptations in antioxidant defenses. The aim of the present study was to determine the effects of a 2-month diet supplementation with docosahexaenoic acid (DHA) on the pro-oxidant and antioxidant status of peripheral blood mononuclear cells (PBMCs) during football training and after acute exercise.
Methods Fifteen male football players, in a randomized double-blind trial, ingested a beverage enriched with DHA or a placebo for 8 weeks. Blood samples were collected in basal conditions before and after the training period and after an acute and intense exercise.
Results The training season increased the carbonyl and nitrotyrosine index but decreased the malondialdehyde (MDA) levels. Basal catalase activity decreased in both groups after 8 weeks of training, whereas glutathione peroxidase activity increased mainly in the placebo group. Protein levels of uncoupling proteins (UCP2 and UCP3) and inducible nitric oxide synthase significantly increased after the training period. Acute exercise induced redistribution in the number of circulating cells, increased the MDA levels and nitrotyrosine index, and decreased the levels of nitrate. Acute exercise also increased PBMCs reactive oxygen species (ROS) production after immune stimulation. Diet supplementation with DHA significantly increased the UCP3 levels after training and the superoxide dismutase protein levels after acute exercise, and reduced the production of ROS after acute exercise.
Conclusion Docosahexaenoic acid increased the antioxidant capabilities while reducing the mitochondrial ROS production in a regular football training period and reduced the oxidative damage markers in response to acute exercise.

Keywords DHA · PBMC · Oxidative stress · Exercise · ROS production

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Introduction

Intense acute exercise changes the oxidative balance as result of oxygen overconsumption [1]. The oxygen consumption during physical activity has been associated with high rate of production of ROS [2] which can overwhelm the antioxidant defenses, resulting in an oxidative stress status [3–5]. It has been described that the high production of ROS during exhaustive exercise causes oxidative changes in lipids, proteins and nucleic acids [6–9]. However, the practice of regular exercise leads to an adaptation of the body to high oxidant levels and improves exercise performance [10–13]. These adaptive responses include the NFkB pathway, which activates target genes related to antioxidant defenses [14–16], inducible nitric oxide synthase [17] and UCPs [18]. The changes in the enzymatic activity designated to reduce the deleterious effects of ROS coexist with oxidative damage in lipids [19]. Consequently, ROS participate in both, the effects on the antioxidant gene

expression and the effects on the oxidative changes of lipids, proteins and nucleic acids.

It has been shown that the omega-3 fatty acids are essential for various physiological processes in humans [20–24] and an increase thought the diet led to a marked incorporation of these fatty acids into membrane phospholipids [25–27]. Docosahexaenoic acid (DHA), an omega-3 fatty acid abundant in marine foods, is an important component of neuronal membranes where it has been described a clear structural function [22] and may affect the signal transduction speed [23]. Moreover, omega-3 fatty acids exert anti-inflammatory properties after eccentric exercise in untrained men [28] and promote the gene expression of key enzymes to introduce fatty acid into the mitochondria and their use as energetic fuel in the respiratory chain [29, 30]. However, the polyunsaturated fatty acids (PUFAs) are highly susceptible to oxidation, which is directly related to their unsaturation degree [31]. Concretely, studies about the prooxidant or antioxidant properties of omega-3 fatty acids are controversial. Some studies have reported that supplementation with high concentrations of omega-3 fatty acids increases oxidative stress in both animals and humans [32], whereas other studies have observed a protective effect of PUFAs against oxidative stress [33, 34] or no clear effects [35].

The aim of the present study was to determine the effects of the consumption of a functional drink enriched with moderate levels of DHA on the oxidative balance status during exercise, as well as on the adaptive response of the lymphocyte oxidative balance to training.

Materials and methods

Subjects and study design

The study group consisted of 15 male football players, at the start of the competition season. At the beginning of the study, 22 subjects were recruited, but 6 of them left the football team during the experimental time to go play with the first and professional team and one broke the anterior cruciate ligament of the knee as it was explained in a consort flow diagram (Fig. 1). There were no differences in the anthropometric characteristics and physical activity capabilities between the placebo and experimental groups of football players. Participants in the study were professional and federated football players 19.3 ± 0.4 years old (placebo group) and 20.4 ± 0.5 years old (experimental group), 76.5 ± 1.8 kg of weight (placebo group) and 76.4 ± 3.5 kg (experimental group), and 179 ± 2 cm of height (placebo group) and 180 ± 3 cm (experimental group). The waist circumference was 78.2 ± 0.8 and 78.5 ± 1.1 cm, the hip circumference was 97.0 ± 1.0 and 96.6 ± 1.4 cm, and the waist–hip ratio (WHR) was 0.805 ± 0.012 and 0.814 ± 0.012, in placebo and experimental groups, respectively. The value of systolic blood pressure was 117 ± 8 and 122 ± 3, and 56.7 ± 5.9 and 66.7 ± 3.5 mmHg for the diastolic blood pressure, in placebo and experimental groups, respectively. The body mass index (BMI) was 24.0 ± 0.6 and 23.5 ± 0.5 kg/m^2, and the football players had 92.5 ± 0.2 and 92.8 ± 0.3 % fat-free mass, in placebo and experimental groups, respectively. The VO_2max was 60.4 ± 1.8 and 62.0 ± 0.9 mL/kg min,

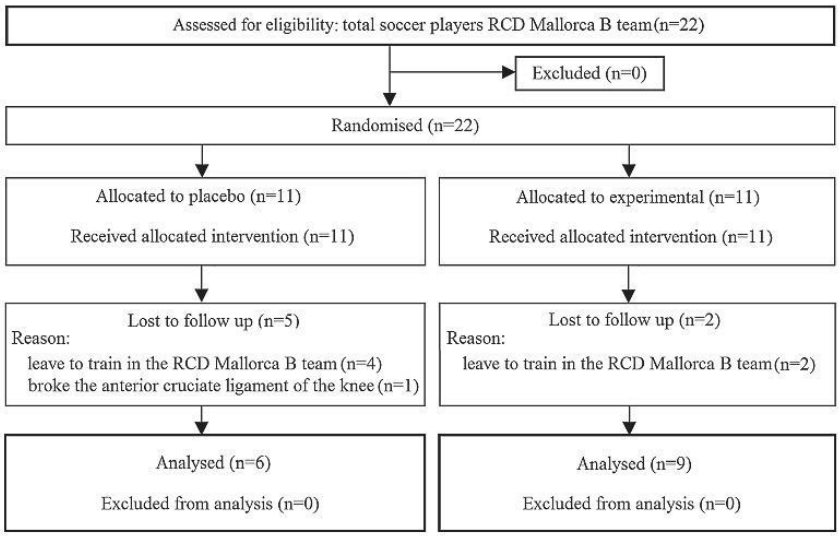

Fig. 1 Representation of a consort flow diagram showing the movement of participants in the study

in placebo and experimental group, respectively. Finally, the intense physical activity time was 96.4 ± 57.9 and 50.4 ± 13.1, and 68.6 ± 17.1 and 63.2 ± 14.6 min/day of moderate physical activity time, in placebo and experimental group, respectively. The subjects were healthy, non-smokers. Subjects were randomly selected to be included in two groups: placebo and experimental groups, and there were no differences between groups in the anthropometric characteristics and physical activity. At the end of the experimental procedure, the placebo group was made up of 6 subjects, while the experimental group was made up of 9 other subjects. Participants in the study had daily intake of 1 liter of their respective beverages for 5 days a week (excluding the match day and the day of rest), over a total period of 8 weeks. The beverages were consumed before the physical activity session. All the subjects were informed of the purpose and demands of the study before giving their written consent to participate. The study protocol was in accordance with the Declaration of Helsinki for research on human subjects and was approved by the Ethical Committee of Clinical Investigation of the Autonomic Community of the Balearic Islands No. IB 994/08 PI (Palma de Mallorca, Balearic Islands, Spain).

Drink composition

The two drinks were composed of 3.0 % almond, 0.8 % sucrose and 0.8 % of different lipids in function of placebo or experimental drink and the rest was water, flavor and vitamin E. The lipid content of the placebo drink was 0.8 % refined olive oil, and the experimental drink was 0.6 % refined olive oil and 0.2 % DHA-S Market (Market Biosciences Corporation, Columbia, EEUU). The two almond drinks were manufactured for Liquats Vegetals S.A. (Girona, Spain), and it was obtained by the following procedure: bleaching the almonds, then crushing them in water, centrifuging of the mixture to eliminate insoluble materials, and the addition of cinnamon and lemon natural flavors, sucrose, vitamin E, and the respective oil for the experimental (olive oil plus DHA-S) or placebo (olive oil) drink. Finally, the beverage was sterilized and packed. Externally, the two beverage types were identical in their taste and appearance. The concentration of vitamin E in both placebo and experimental drinks is equivalent to 0.4 mg/mL of α-tocopherol acetate.

Experimental procedure

Three blood samples were obtained from each subject. One sample was extracted in basal conditions before starting the nutritional intervention, and another two blood samples were taken at the end of the nutritional intervention, in basal and post-exercise conditions.

The exercise consisted of a 2-h habitual physical training session. After 15 min of warm-up, the players performed the Leger Boucher test. After that, the players did a recovery exercise of control-passing for 15 min. The main body of the training session was characterized by small-sided games. Briefly, the first exercise consists of a 5 versus 5 possession exercise in an area of 20 × 15 m (4 repetitions of 5 min with 30 s of recovery between repetitions); the second was a 6 versus 6 possession exercise in an area of 30 × 20 m (3 repetitions of 6 min with 1 min of recovery between repetitions), and finally, the players played a football match of 5 versus 5 in 30 × 40 m for 20 min. The exercise was performed at 70 % VO_2 max during more than 50 % of the training session in order to induce an oxidative stress situation [36].

Venous blood samples were obtained from the antecubital vein of sportsmen in suitable vacutainers with EDTA as anticoagulant. Venous blood samples were obtained after 12 h, overnight, fasted conditions (basal sample) and 2 h after finishing training. Since it is well known that the posture (hydrostatic pressure gradients) has an impact on the plasma volume after exercise [37], subjects remained seated 0.5–1 h prior to blood sample collection.

Dietary intake

Dietary habits were assessed using a 7-day dietary record questionnaire completed at the beginning of the study. A qualified dietician verified and quantified the food records. All food items consumed were transformed into nutrients by a special computerized program based on the European and Spanish food composition tables [38].

The subjects followed a Mediterranean diet during the sport season.

PBMCs purification

The PBMC fraction was purified following an adaptation by a described method [39]. Blood was carefully introduced on Ficoll in a proportion of 1.5:1 and was then centrifuged at 900×*g*, at 4 °C for 30 min. The PBMCs layer was carefully removed. The plasma and the Ficoll phases were discarded. The PBMCs slurry was then washed twice with PBS and centrifuged for 10 min at 1,000×*g*, 4 °C. This process was performed in triplicate, one of the samples was destined to obtaining RNA, another sample was lysed with distilled water, and the other was preserved with RIPA for Western blot analysis. Cell lysates were stored at −80 °C until biochemical analyses.

Fatty acids determination

The fatty acid extraction of erythrocyte samples and beverages was done by a modification of the Folch method

[40]. Initially, 250 μL of erythrocyte samples or beverages were diluted in 5 mL of Cl_3CH:CH_3OH (2:1, v/v) BHA 0.01 % containing 20 μL of *n*-heptadecanoic acid (15 mM) as internal standard for fatty acids correction and quantification. The final organic phase was evaporated under a nitrogen stream at 55 °C. The dry residue was dissolved in 225 μL of *n*-hexane and 25 μL of Meth-Prep™ II (GRACE), derivatization reagent, and 1 μL was injected into the gas chromatograph. The mobile phase consisted of helium. The gas chromatograph was a Agillent 5890 model with a flame ionization detector (FID), and the column was a Supelcowax® 10 Capillary GC column, 30 m × 0.53 mm, d_f 0.50 μm.

Malondialdehyde assay

Malondialdehyde (MDA) as a marker of lipid peroxidation was analyzed PBMCs by a colorimetric assay for MDA determination based on the reaction of MDA with a chromogenic reagent to yield a stable chromophore with maximal absorbance at 586 nm. Briefly, samples or standards were placed in glass tubes containing *n*-methyl-2-phenylindole (10.3 mM) in acetonitrile:methanol (3:1). HCl 12 N was added, and the samples were incubated for 1 h at 45 °C. Absorbance was measured at 586 nm.

Protein carbonyl and nitrotyrosine determination

Protein carbonyl derivatives and nitrotyrosine were determined by immunological methods using the OxiSelect™ Protein Carbonyl Immunoblot Kit (Cell Biolabs, INC) and OxiSelect™ Nitrotyrosine Immunoblot Kit (Cell Biolabs, INC) by following the manufacturer's details. Total protein concentrations of an aliquot of cells lysed with distilled water were measured by the Bradford method [41] using the Bio-Rad protein assay reagent (Bio-Rad, Munich, Germany). Initially, samples (10 or 150 μg of protein for carbonyl or nitrotyrosine, respectively) were transferred onto a nitrocellulose membrane by the dot blot method. For carbonyl determination, the membrane was incubated in the presence of 2,4-dinitrophenylhydrazine (DNPH) after transference. Then, the membrane was incubated with the primary antibody, specific to DNP moiety proteins in the case of carbonyl determination (1:4,000) or rabbit anti-nitrotyrosine antibody for nitrotyrosine determination (1:1,000). This step was followed by incubation with a horseradish peroxidase-antibody (goat anti-rabbit IgG) conjugate directed against the primary antibody (carbonyl 1:10,000; nitrotyrosine 1:2,000). The membrane was then treated with luminol, which is converted to a light-emitting form at wavelength 428 nm by the antigen/primary antibody/secondary antibody/peroxidase complex. The light was visualized and detected by short exposure to a Chemidoc XRS densitometer (Bio-Rad Laboratories). Image analysis was performed using Quantity One-1D analysis software (Bio-Rad Laboratories).

Hydrogen peroxide production

H_2O_2 production in PBMCs was measured before and after stimulation with phorbol myristate acetate (PMA) or lipopolysaccharide (LPS) using 2,7-dichlorofluorescin-diacetate (DCFH-DA) as indicator. A stock solution of DCFH-DA (1 mg/mL) in ethanol and PMA (1 mg/mL) in DMSO was prepared and stored at 20 °C until analysis. DCFH-DA (30 μg/mL) in PBS was added to a 96-well microplate containing 50 μL PBMCs suspension. PMA (10 ng/mL) or LPS (1 μg/mL) prepared in HBSS or HBSS alone was added to the wells, and the fluorescence (Ex, 480 nm; Em 530 nm) was recorded at 37 °C for 1 h in FL ×800 Microplate Fluorescence Reader (Bio-tek Instruments, Inc.).

Comet assay

Assessment of DNA damage was carried out using the alkaline comet assay method. Briefly, slides were prepared by adding purified PBMCs, mixed with 0.6 % low-melting-point agarose. For DNA release, cells were lysed by immersing slides in lysing solution (2.5 mol/L NaCl, 100 mmol/L EDTA disodium salt, 10 mmol/L Tris, 1 % Triton X-100 and 10 % DMSO, pH 10.0) at 4 °C for 40 min. After removal from the lysing solution, the slides were placed in an electrophoresis trough containing an alkaline electrophoresis buffer (300 mmol/L NaOH, 1 mmol/L EDTA). A current of 25 V and 300 mA was applied for 30 min. The slides were then removed, and Tris buffer 0.4 mol/L adjusted to pH 7.5 with concentrated HCl was added onto the slides to neutralize excess alkali. DNA was stained by adding ethidium bromide. Comet measurements were made by image analysis using a fluorescence microscope equipped with an excitation filter of 450–490 nm and a barrier filter of 520 nm and the Comet software (TriTek CometScoreTM). Images of 50 random nuclei were taken at 200× magnification and were analyzed for each sample. The comet measurements that were recorded and subsequently used for analysis were percentage DNA in tail (tail intensity) and tail moment (tail intensity × tail length).

Nitrate determination

An aliquote of PBMCs was used to measure nitrate (100 μL) concentration by detecting liberated nitric oxide

(NO) in a gas-phase chemiluminescence reaction with ozone using a NO analyzer (NOA 280i; Sievers) following an adaptation of the described method [42]. The area under the curve of NO peaks was recorded and processed using Liquid software.

Enzyme activities

We determined the catalase, GRd and GPx activities in PBMCs. All activities were determined with a Shimadzu UV-2100 spectrophotometer at 37 °C. Catalase activity was measured by the spectrophotometric method of Aebi [43] based on the decomposition of H_2O_2. Glutathione reductase activity was measured by a modification of the Goldberg and Spooner [44] spectrophotometric method. Glutathione peroxidase activity was measured using an adaptation of the spectrophotometric method of Flohe and Gunzler [45].

SDS-polyacrylamide gel electrophoresis and Western Blot analysis

Antioxidant enzyme protein levels were determined by Western blot. Cells were lysed with 250 μL of RIPA buffer [250 mM Tris/HCl, pH 8.0, 4.4 % NaCl, 5 % IGEPAL®, 2.5 % deoxycholic acid, 0.5 % sodium dodecylsulfate (SDS)]. Proteins of total cell extract (10–200 μg) (Bio-Rad, Munich, Germany) were separated by size using SDS polyacrylamide gel. Catalase (20 μg of protein extract), Cu/Zn-SOD (10 μg of protein extract), Mn-SOD (20 μg of protein extract), thioredoxin reductase 1 (TrxR1) (20 μg of protein extract), UCP3 (20 μg of protein extract) and GPx (200 μg of protein) were loaded on a 12 % agarose gel, UCP2 (20 μg of protein extract); iNOS (20 μg of protein extract) was loaded on 10 % agarose gel, and GRd and Cu/Zn-SOD (10 μg of protein extract) were loaded on a 15 % agarose gel. Following electrophoresis, samples were transferred onto a nitrocellulose membrane and incubated with a primary monoclonal antibody: anti-catalase antibody (1:1,000) and anti-Mn-SOD antibody (1:1,000) (Calbiochem), anti-Cu/Zn-SOD antibody (1:2,000) (Sigma), anti-GRd antibody (1:1,000), anti-GPx antibody (1:200), anti-TrxR1 antibody (1:200) and anti-UCP2 antibody (1:500) (Santa Cruz), anti-UCP3 antibody (1:500) (Chemicon Internationa), and anti-iNOS antibody (1:1,000) (Stressgen). Blots were then incubated with a secondary peroxidase-conjugated antibody (1:10,000), and the primary antibody was performed. Protein bands were visualized by Immun-Star® Western C® Kit reagent (Bio-Rad Laboratories) Western blotting detection systems. The chemiluminescence signal was captured with a Chemidoc XRS densitometer (Bio-Rad Laboratories) and analyzed with Quantity One-1D Software (Bio-Rad Laboratories).

PBMCs RNA extraction and real-time PCR assay

Catalase, Cu/Zn-SOD, Mn-SOD, GPx, UCP3, mRNA expression was determined by multiplex real-time PCR based on incorporation of a fluorescent reporter dye and using human 18S rRNA as reference. For this purpose, total RNA was isolated from PBMCs by Tripure extraction (Roche Diagnostics, Germany). RNA (1 μg) from each sample was reverse transcribed using 50 U of Expand Reverse Transcriptase (Roche Diagnostics, Germany) and 20 pmol oligo (dT) for 60 min at 37 °C in a 10 μL final volume, according to manufacturer instructions. The resulting cDNA (2.5 μL) was amplified using the LightCycler FastStart DNA MasterPLUS SYBR Green I kit (Roche Diagnostics, Germany). Amplification was performed at 55 °C and 45 cycles. The relative quantification was performed by standard calculations considering $2^{(-\Delta\Delta Ct)}$. Antioxidant enzyme levels before and after the season were normalized to the invariant control 18S rRNA. mRNA levels at the beginning of the stage were arbitrarily referred to as 1. Primers used are listed in Table 1.

Statistical analysis

Statistical analysis was carried out using the Statistical Package for Social Sciences (SPSS v.19.0 for Windows). Results are expressed as mean ± SEM, and $p < 0.05$ was considered statistically significant. A Kolmogorov–Smirnov test was applied to assess the normal distribution of the data. The statistical significance of the data was assessed by two-way analysis of variance (ANOVA). The statistical factors analyzed were beverage supplementation (*S*) and the training period (*T*) or acute exercise (*E*). The sets of data in which there was a significant interaction between the factors analyzed were tested by one-way ANOVA. When significant effects of one factor were found, a Student's t test for paired data was used to determine the differences between the groups involved.

Results

The experimental drink presented significant concentrations of the fatty acids C20:3, C22:0, C22:5 and C22:6n3 which were undetected in placebo drink. Both beverage types have a similar fatty acid composition; however, as the experimental beverage was enriched with DHA, there were slight differences between the compositions of the two drinks. The experimental drink had significantly higher concentrations of the fatty acids C20:3 (21 μM), C22:0 (76 μM), C22:5 (1,715 μM) and C22:6n3 (3,457 μM) although these fatty acids were undetected in the placebo beverage. No differences were observed in nutrient intake between the placebo

Table 1 Primers and conditions used in real-time PCRs

Gene	Primer	Conditions	
18S	Fw: 5′-ATG TGA AGT CAC TGT GCC AG-3′	95 °C	10 s
	Rv: 5′-GTG TAA TCC GTC TCC ACA GA-3′	60 °C	10 s
		72 °C	12 s
Catalase	Fw: 5′-TTT GGC TAC TTT GAG GTC AC-3′	95 °C	10 s
	Rv: 5′-TCC CCA TTT GCA TTA ACC AG-3′	60 °C	10 s
		72 °C	15 s
Cu/Zn-SOD	Fw: 5′-TCA GGA GAC CAT TGC ATC ATT-3′	95 °C	10 s
	Rv: 5′-CGC TTT CCT GTC TTT GTA CTT TCT TC-3′	63 °C	10 s
		72 °C	15 s
Mn-SOD	Fw: 5′-CGTGCTCCCACAC ATCAATC-3′	95 °C	10 s
	Rv: 5′-TGAACGTCACCG AGGAGAAG-3′	60 °C	10 s
		72 °C	12 s
GPx	Fw: 5′-TTC CCG TGC AAC CAG TTT G-3′	94 °C	10 s
	Rv: 5′-TTC ACC TCG CAC TTC TCG AA-3′	63 °C	10 s
		72 °C	15 s
UCP3	Fw: 5′-CGT GGT GAT GTT CAT AAC CTA TG-3′	95 °C	5 s
	Rv: 5′-CGG TGA TTC CCG TAA CAT CTG-3′	60 °C	7 s
		72 °C	10 s

Table 2 Fatty acid composition in erythrocytes in basal conditions

	Initial	Final	ANOVA		
			S	*T*	*S* × *T*
Eicosenoic (%)					
Placebo	1.97 ± 0.07	2.01 ± 0.12			
Experimental	1.79 ± 0.12	1.82 ± 0.17			
Eicosadienoic (%)					
Placebo	3.16 ± 0.15	2.33 ± 0.13*		X	
Experimental	2.86 ± 0.15	2.52 ± 0.13			
Eicosatrienoic (%)					
Placebo	4.97 ± 0.47	4.95 ± 0.47	X		
Experimental	3.78 ± 0.26#	3.41 ± 0.22#			
Arachidonic (%)					
Placebo	71.9 ± 0.3	71.6 ± 0.6	X	X	
Experimental	71.8 ± 1.4	68.0 ± 0.9*,#			
Docosahexaenoic (%)					
Placebo	18.0 ± 0.6	19.1 ± 0.9	X	X	
Experimental	19.8 ± 1.4	24.3 ± 0.9*,#			

Statistical analysis: two-way ANOVA. *S*, significant effect of supplementation; *T*, significant effect of training, *S* × *T* significant interaction between both factors

* Significant differences between placebo and experimental; # significant differsignificant differences between initialences between the beginning and final training period, $p < 0.05$

and experimental groups. The average energy intake of 2,518 ± 226 kcal was consumed, distributed as 108 ± 10 g of proteins, 282 ± 37 g of carbohydrates, 106 ± 9 g of fats and 1,880 ± 287 mL of water in the placebo group; meanwhile, the experimental group was distributed as 105 ± 12 g of proteins, 225 ± 26 g of carbohydrates, 99.2 ± 8.1 g of fats and 1,254 ± 173 mL of water, and the average of energy consumed was 2,215 ± 210 kcal. The DHA from the diet is between 171 and 376 mg/day. The DHA intake from the experimental beverage could triple the DHA from the diet. Daily intake of 1 liter of beverage during 5 days of the respective beverage supplied a daily intake of 1.14 g of DHA, to which must be added the intake of omega-3 in the diet, and on the other hand, the placebo group only received their omega-3 intake from the diet.

The nutritional intervention changed the lipid composition of erythrocyte membranes of footballers (Table 2). The percentage of DHA in erythrocyte membranes significantly increased in the experimental group after 8 weeks of dietary intervention, whereas the placebo group maintained the initial values. In parallel, the enrichment of the erythrocytes membrane in DHA produced a light decrease in the proportion of eicosatrienoic and arachidonic acids in the experimental group.

Blood cell distribution is reported in Table 3. The lymphocyte counts significantly increased, during the training season in both the experimental and placebo groups (Table 3A). Similarity, PBMCs counts also increased in a similar way as lymphocytes although this increase was not statistically significant in the placebo group. The basophil counts presented the highest increase after training season in both groups. Acute exercise significantly increased the number of leukocytes and the number of monocytes in both groups (Table 3B). A significant lymphopenia and a decrease in basophils were observed after exercise, in both groups.

The effects of diet supplementation with DHA, the training season and the acute exercise on the oxidative and nitrosative damage of PBMCs are shown in Table 4. No significant effects of DHA diet supplementation on the oxidative damage markers in PBMCs were observed; however, both the training season and acute exercise significantly affects some key markers of oxidative damage. The basal MDA levels of PBMCs significantly decreased in placebo and in experimental groups at the end of the training season, whereas the basal values of carbonyl index

Table 3 Cellular counts and distribution

	Initial	Final	ANOVA		
			S	*T*	*S* × *T*
A					
Leukocytes (10^3 cells/μL)					
Placebo	6.84 ± 1.14	6.52 ± 0.35			
Experimental	6.30 ± 0.38	7.01 ± 0.39			
Lymphocytes (10^3 cells/μL)					
Placebo	1.98 ± 0.18	2.48 ± 0.18#	X	X	
Experimental	2.49 ± 0.17	3.10 ± 0.33*,#			
Monocytes (10^3 cells/μL)					
Placebo	0.480 ± 0.054	0.500 ± 0.050			
Experimental	0.430 ± 0.040	0.510 ± 0.035			
Eosinophils (10^3 cells/μL)					
Placebo	0.231 ± 0.083	0.282 ± 0.078			
Experimental	0.270 ± 0.121	0.291 ± 0.083			
Basophils (10^3 cells/μL)					
Placebo	0.032 ± 0.005	0.072 ± 0.012#		X	
Experimental	0.032 ± 0.009	0.090 ± 0.010#			
PBMC (10^3 cells/μL)					
Placebo	2.79 ± 0.16	3.34 ± 0.15	X	X	
Experimental	3.23 ± 0.29	3.99 ± 0.34#			
	Basal	Post-exercise	ANOVA		
			S	*E*	*S* × *E*
B					
Leukocytes (10^3 cells/μL)					
Placebo	6.52 ± 0.35	9.96 ± 0.40#		X	
Experimental	7.01 ± 0.39	9.55 ± 0.47#			
Lymphocytes (10^3 cells/μL)					
Placebo	2.48 ± 0.18	2.21 ± 0.14#		X	
Experimental	3.10 ± 0.33	2.17 ± 0.14#			
Monocytes (10^3 cells/μL)					
Placebo	0.500 ± 0.050	0.770 ± 0.052#		X	
Experimental	0.510 ± 0.035	0.740 ± 0.051#			
Eosinophils (10^3 cells/μL)					
Placebo	0.282 ± 0.078	0.174 ± 0.041			
Experimental	0.291 ± 0.083	0.163 ± 0.050			
Basophils (10^3 cells/μL)					
Placebo	0.072 ± 0.012	0.042 ± 0.008#		X	
Experimental	0.090 ± 0.010	0.049 ± 0.011#			
PBMC (10^3 cells/μL)					
Placebo	3.34 ± 0.15	3.19 ± 0.15			
Experimental	3.99 ± 0.34	3.13 ± 0.16			

A, the initial and final data studies, in basal conditions; B, final study data, in basal and post-exercise conditions. Statistical analysis: two-way ANOVA, $p < 0.05$; *S*, significant effect of supplementation; *T*, significant effect of time, $S \times T$ significant interaction between supplementation and time; *E*, significant effect of exercise; $S \times E$ significant interaction between supplementation and exercise. One-way ANOVA, $p < 0.05$

* Significant differences between placebo and experimental; # significant differences between initial and final training period or significant differences between basal and post-exercise

and N-Tyr index significantly increased in both groups. The basal nitrate levels significantly decreased in the placebo in the experimental group at the final of training season. Training season did not alter the initial status of oxidative damage in PBMCs nucleic acids.

Acute exercise significantly increased the values of the MDA in PBMCs in placebo and experimental groups. Similarity, the nitrosative damage marker of PBMCs proteins only increased significantly in the experimental group. The nitrate levels decreased in PBMCs after

Table 4 Oxidative and nitrosative damage of PBMC

	Initial	Final	ANOVA		
			S	*T*	*S* × *T*
A					
MDA (μmol/10^9 cells)					
Placebo	0.36 ± 0.04	0.26 ± 0.02#		X	
Experimental	0.44 ± 0.06	0.20 ± 0.06#			
Carbonyl index (%)					
Placebo	100 ± 23	975 ± 95#		X	
Experimental	109 ± 19	922 ± 86#			
N-Tyr index (%)					
Placebo	100 ± 35	621 ± 117#		X	
Experimental	76.9 ± 13.8	501 ± 39#			
Nitrate (μmol/10^9 cells)					
Placebo	1.97 ± 0.73	1.03 ± 0.15		X	
Experimental	3.51 ± 0.72	1.10 ± 0.15#			
Comet assay					
% Tail					
Placebo	4.39 ± 0.34	4.17 ± 0.39			
Experimental	4.13 ± 0.32	3.94 ± 0.39			
Tail moment					
Placebo	0.32 ± 0.03	0.34 ± 0.06			
Experimental	0.34 ± 0.05	0.36 ± 0.07			
	Basal	Post-exercise	ANOVA		
			S	*E*	*S* × *E*
B					
MDA (μmol/10^9 cells)					
Placebo	0.26 ± 0.05	0.58 ± 0.11#		X	
Experimental	0.20 ± 0.02	0.40 ± 0.06#			
Carbonyl index (%)					
Placebo	100 ± 10	114 ± 4			
Experimental	94.4 ± 8.8	105 ± 4			
N-Tyr index (%)					
Placebo	100 ± 19	137 ± 18		X	
Experimental	87.0 ± 8.2	141 ± 21#			
Nitrate (μmol/10^9 cells)					
Placebo	1.03 ± 0.15	0.401 ± 0.096#		X	
Experimental	1.10 ± 0.15	0.700 ± 0.236			
Comet assay					
% Tail					
Placebo	4.17 ± 0.39	5.90 ± 0.64#		X	
Experimental	3.94 ± 0.39	5.48 ± 0.59#			
Tail moment					
Placebo	0.34 ± 0.06	0.44 ± 0.07			
Experimental	0.36 ± 0.07	0.42 ± 0.06			

A, the initial and final data studies, in basal conditions; B, final study data, in basal and post-exercise conditions. Statistical analysis: two-way ANOVA, $p < 0.05$; *S*, significant effect of supplementation; *T*, significant effect of time, *S* × *T* significant interaction between supplementation and time; *E*, significant effect of exercise; *S* × *E* significant interaction between supplementation and exercise. One-way ANOVA, $p < 0.05$

* Significant differences between placebo and experimental; # significant differences between initial and final training period or significant differences between basal and post-exercise

exercise although the decrease was only statistically significant in the placebo group. No significant influences of acute exercise or DHA diet supplementation were evidenced in the carbonyl index. Moreover, acute exercise significantly increased the percentage DNA in tail in both groups.

No significant effects of training season or DHA diet supplementation were evidenced on the ROS production by PBMCs stimulated with PMA (Fig. 2a). Acute exercise significantly increased the ROS production in both groups. In a similar way, the training season did not influence the ROS production by PBMCs when stimulated with LPS, but acute exercise and DHA diet supplementation significantly affected the PBMCs' ROS production after stimulation with LPS (Fig. 2b). Acute exercise significantly increased ROS production by PBMCs, and this increase was significantly higher in the placebo than in the experimental group.

The training season significantly influenced the basal catalase and GPx activities, the basal levels of antioxidant proteins UCP2 and UCP3, and the iNOS levels (Table 5A). The DHA diet supplementation significantly influenced the

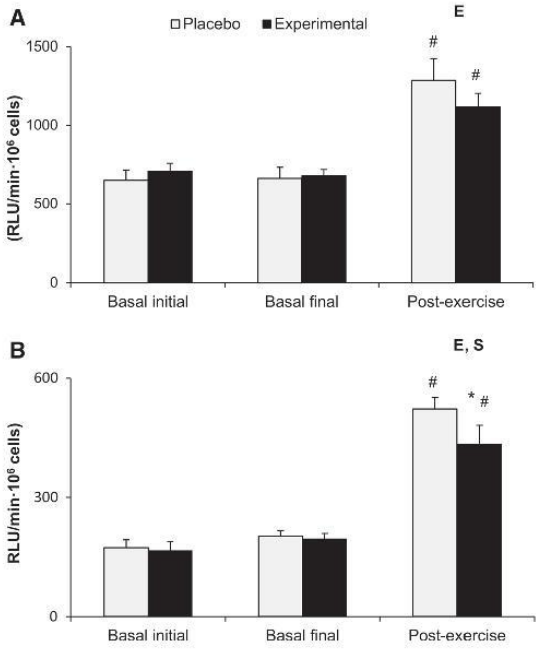

Fig. 2 ROS production by PBMCs stimulated with PMA (**a**) or LPS (**b**). Statistical analysis: two-way ANOVA, $p < 0.05$. *S*, significant effect of supplementation; *T*, significant effect of time; *S* × *T*, significant interaction between supplementation and time; *E*, significant effect of exercise; *S* × *E* significant interaction between supplementation and exercise. One-way ANOVA, $p < 0.05$. *Significant differences between placebo and experimental, #Significant differences between initial and final training period or significant differences between basal and post-exercise

basal GPx activity and the basal protein levels of UCP3 in PBMCs. The training season significantly reduced the catalase activity in PBMCs in the placebo group, whereas the reduction in the experimental group was not statistically significant. Inversely, the GPx activity significantly increased in PBMCs at the final of training season both in placebo and in experimental groups. No significant effects were evidenced in the GRd activity. These changes in the activities of catalase and GPx were not parallel to changes in the protein levels of these enzymes. The protein basal levels of catalase, Cu/Zn-SOD, Mn-SOD, GRd, GPx and TrxR1 in PBMCs did not change from the initial values during the training season and supplementation. At the end of the training season, the protein levels of UCP2, UCP3 and iNOS were higher than at the beginning in the placebo and experimental groups. DHA diet supplementation also influenced the UCP3 protein levels, with basal levels significantly higher in experimental than in placebo group.

Table 5B shows the effects of acute exercise and DHA diet supplementation on the antioxidant capabilities of PBMCs. The acute exercise significantly influenced the enzyme activities of catalase, GRd and GPx. The catalase activity significantly increased after the exercise in the placebo and in the experimental group. The GRd activity was also increased after exercise, although the increase was only statistically significant in the placebo group. The GPx increased after exercise in placebo and in experimental groups, with significant differences only in the supplemented group. The pattern of these antioxidant enzymes was very similar to the pattern of change for their respective protein levels, with the exception of the GRd enzyme, which reported not significant differences in their protein levels. Moreover, the protein levels of Cu/Zn-SOD, TrxR1, UCP2, UCP3 and iNOS, but not Mn-SOD, were significantly increased after exercise in both the experimental and the placebo groups. The protein levels of Cu/Zn-SOD in PBMCs after acute exercise were influenced by DHA supplementation, with those of the experimental group significantly higher than those of the placebo group. Gene expressions of catalase, GPx, UCP3, Cu/Zn-SOD and Mn-SOD were not significantly affected by acute exercise or by DHA diet supplementation.

Table 5 Antioxidant enzyme activities, protein levels and gene expressions on PBMC

	Initial	Final	ANOVA		
			S	*T*	*S* × *T*
A					
Enzyme activity					
Catalase (K/10^9 cells)					
Placebo	27.5 ± 8.14	8.06 ± 3.94$^{\#}$		X	
Experimental	16.7 ± 3.03	7.24 ± 4.04			
GRd (nkat/10^9 cells)					
Placebo	71.3 ± 16.7	81.5 ± 27.6			
Experimental	80.2 ± 17.9	57.7 ± 16.7			
GPx (nkat/10^9 cells)					
Placebo	74.8 ± 13.2	101 ± 5.13$^{\#}$	X	X	
Experimental	52.1 ± 6.74	88.5 ± 8.97$^{*,\#}$			
Protein levels					
Catalase (%)					
Placebo	100 ± 17	96.1 ± 25.4			
Experimental	99.3 ± 19.5	92.8 ± 16.7			
Cu/Zn-SOD (%)					
Placebo	100 ± 29	79.4 ± 14.7			
Experimental	82.9 ± 14.9	123 ± 20			
Mn-SOD (%)					
Placebo	100 ± 32	92.3 ± 47.3			
Experimental	78.3 ± 25.9	88.5 ± 39.6			
GRd (%)					
Placebo	100 ± 31	99.5 ± 6.9			
Experimental	113 ± 22	138 ± 28			
GPx (%)					
Placebo	100 ± 28	132 ± 22			
Experimental	97.2 ± 38.5	157 ± 31			
TrxR1 (%)					
Placebo	100 ± 27	83.7 ± 23.6			
Experimental	130 ± 39	79.8 ± 16.8			
UCP2 (%)					
Placebo	100 ± 22	362 ± 98$^{\#}$		X	
Experimental	126 ± 23	331 ± 53$^{\#}$			
UCP3 (%)					
Placebo	100 ± 18	464 ± 71$^{\#}$	X	X	
Experimental	179 ± 39	684 ± 98$^{*,\#}$			
iNOS (%)					
Placebo	100 ± 14	166 ± 26		X	
Experimental	90.1 ± 20.6	170 ± 17$^{\#}$			

Discussion

The footballers followed a Mediterranean diet during the stage, but in a similar way as adolescent and adult people

Table 5 continued

	Basal	Post-exercise	ANOVA		
			S	*E*	*S* × *E*
B					
Enzyme activity					
Catalase (K/10^9 cells)					
Placebo	8.06 ± 3.94	16.8 ± 3.88#		X	
Experimental	7.24 ± 4.04	33.5 ± 9.29#			
GRd (nkat/10^9 cells)					
Placebo	81.5 ± 27.6	218 ± 69.2#		X	
Experimental	57.7 ± 16.7	118 ± 40.2			
GPx (nkat/10^9 cells)					
Placebo	101 ± 5.13	120 ± 6.77		X	
Experimental	88.5 ± 8.97	153 ± 20.4#			
Protein levels					
Catalase (%)					
Placebo	100 ± 26	143 ± 17		X	
Experimental	96.6 ± 17.5	190 ± 30#			
Cu/Zn-SOD (%)					
Placebo	100 ± 18	184 ± 43	X	X	
Experimental	155 ± 25	276 ± 40#			
Mn-SOD (%)					
Placebo	100 ± 51	160 ± 74			
Experimental	95.2 ± 42.6	156 ± 54			
GRd (%)					
Placebo	100 ± 28	158 ± 32			
Experimental	178 ± 47	166 ± 41			
GPx (%)					
Placebo	100 ± 17	165 ± 25		X	
Experimental	119 ± 24	213 ± 40#			
TrxR1 (%)					
Placebo	100 ± 28	146 ± 27		X	
Experimental	95.3 ± 20	151 ± 23			
UCP-2 (%)					
Placebo	100 ± 27	158 ± 46		X	
Experimental	91.4 ± 14.6	184 ± 31			
UCP-3 (%)					
Placebo	100 ± 15	114 ± 12			
Experimental	147 ± 21	128 ± 20			
iNOS (%)					
Placebo	100 ± 12	172 ± 27		X	
Experimental	107 ± 11	138 ± 28			
Gene expresion					
Catalase					
Placebo	1.00 ± 0.25	1.18 ± 0.75			
Experimental	0.83 ± 0.39	1.01 ± 0.36			
Cu/Zn-SOD					
Placebo	1.00 ± 0.37	3.37 ± 2.70			
Experimental	1.55 ± 0.90	1.22 ± 0.35			
Mn-SOD					
Placebo	1.00 ± 0.42	1.07 ± 0.46			
Experimental	1.67 ± 0.71	0.99 ± 0.54			
GPx					
Placebo	1.00 ± 0.28	1.7 ± 1.17			
Experimental	1.49 ± 0.50	0.80 ± 0.19			
UCP-3					
Placebo	1.00 ± 0.33	1.26 ± 0.7			
Experimental	2.08 ± 1.39	1.04 ± 0.37			

A, the initial and final data studies, in basal conditions; B, final study data, in basal and post-exercise conditions. Statistical analysis: two-way ANOVA, $p < 0.05$; *S*, significant effect of supplementation; *T*, significant effect of time, *S* × *T* significant interaction between supplementation and time; *E* significant effect of exercise; *S* × *E* significant interaction between supplementation and exercise. One-way ANOVA, $p < 0.05$

* Significant differences between placebo and experimental; # significant differences between initial and final training period or significant differences between basal and post-exercise

of Balearic Islands their adherence was not complete [27]. The nutrient intake evidences a low DHA intake. The results obtained could be affected by the low omega-3 intake in the diet and, consequently, could not be directly extrapolated to the population with an adequate DHA intake. DHA diet supplementation, through enriched beverage, favors the incorporation of this essential fatty acid into the erythrocyte membranes [25–27]; therefore, the PBMCs membranes of the football players should also incorporate the DHA supplemented by the enriched beverage [46]. In the current work, beverage intake by the participants in the trial was enough to alter the erythrocyte membrane composition and increased the incorporation of DHA. This incorporation into erythrocyte membranes reduced the proportion of other fatty acids, such as eicosatrienoic and arachidonic acids, into the membranes, which could alter the availability of pro- and anti-inflammatory lipid mediators in response to an immune stimulus.

Effects of training season on oxidative stress

Regular training induced greater protection against PBMCs' lipid peroxidation in the footballers in accordance with a previous study [47], whereas no changes were observed in the percentage of DNA in tail and in the tail moment. However, the levels of nitrotyrosine and protein carbonyl derivates were increased. The higher nitrosative damage could reflect higher peroxynitrite production in PBMCs as result of the reaction between superoxide anion

and NO [48], which could have increased by the higher levels iNOS expression at the final of training season. Peroxynitrite initiates lipid peroxidation [49] and reacts with sulfhydryls [50] and methionine [51]; however, in absence of direct targets, peroxynitrite can be also discompose generating nitrate (70 %) or can undergo homolytic rupturing (30 %), generating hydroxyl radicals (OH^-) and nitrogen dioxide (NO_2^-) [52]. Lower nitrate levels, together with the higher nitrotyrosine index in PBMCs at the end of the training season, suggests that the peroxynitrite mainly reacts with proteins residues. Oxidative modifications of proteins comprise alterations of single amino acids, such as the formation of protein carbonyls and methionine sulfoxide, or the aggregation of whole proteins [53]. The reaction between MDA and amino groups of proteins also introduces carbonyls groups into the protein [54]. We suggest that the lower levels of MDA in lymphocytes at the final of the training season could be a result of the MDA reaction with proteins increasing the carbonyl marker.

The training significantly affects the activity of antioxidant enzymes, whereas no significant effects were evidenced in the antioxidant protein levels. The changes observed in the catalase and GRd activities during the training season could reflect a change in the strategy to eliminate ROS from a catalase-based system to a glutathione-based system, as observed in other works [55]. Catalase has a high Michaelis–Menten constant for the hydrogen peroxide, while for GPx is lower [56]. These adaptations could mean that lymphocytes produce ROS at lower rate that are better removed by GPx. Moreover, after training, the lymphocytes present higher levels of UCP2 and UCP3, which can contribute as an antioxidant to reduce the rate of ROS production in mitochondria [57, 58]. It has been demonstrated that an increase in ROS production and, consequently, in oxidative damage, in muscle during a single bout of exercise is accompanied by an increase in the UCP3 expression [59]. This reduction in ROS production is important as different classes of signaling actions may be regulated by different ROS levels [60]: low/moderate rates involve processes such as proliferation and differentiation, and adaptive programs, including the transcriptional upregulation of antioxidant genes, whereas higher levels involve the initiation of senescence and cell death.

Effects of acute exercise on oxidative stress

Acute exercise causes significant changes in circulating cells increasing the number of leukocytes, such as after a duathlon [61] or a diving session [5]. Specifically, it has been reported that it causes a decrease in the number of circulating lymphocytes [13, 16] and a monocytosis [62].

Acute exercise, as a result of increased oxygen demand, induces oxidative stress and increases the markers of oxidative damage [4] and, in the present work, increased the antioxidant enzyme activities and protein levels, without affecting gene expression. After the acute exercise, the MDA marker increased in both groups, although less in the experimental group, which could reflect a protective effect of omega-3 fatty acids against oxidative stress, as it has been shown to occur in rats and humans [63, 64]. No changes were detected in the index of carbonyls index due to intense physical exercise, which is contrary to the results obtained in other studies [16, 55, 65]. This response could be a consequence of the well-trained status of athletes with a higher degree of protection against oxidative damage or the intensity of exercise. The nitrotyrosine index significantly increased as a consequence of acute exercise, and these results coincide with the results obtained in neutrophils after a half marathon [66]. Moreover, nitrate levels significantly decreased in PBMCs after acute exercise, and these results are similar to those obtained in other study, carried out on plasma [67] and reinforce the idea of the distribution of peroxynitrite between its transformation into nitrate and its reaction with proteins.

Lymphocyte enzymatic antioxidant defenses have shown great adaptations to oxidative stress induced by exercise, increasing their activities and protein levels [16, 61]. In this work, a significant increase in PBMCs catalase, GPx and GRd activities was observed. This increase was reflected also in the catalase and GPx protein levels. Therefore, the increased activity of these three enzymes after exercise could be attributed to the activation of pre-existing enzymes. In accordance, exercise has been pointed out to induce the activation of some enzymes in erythrocytes and lymphocytes as a result of post-translational regulation [68–70].

Furthermore, acute exercise induced the synthesis of other antioxidant proteins such as Cu/Zn-SOD, TrxR1 and UCP2 in PBMCs to reinforce the antioxidant defenses. Thioredoxin reductase plays an important role in regenerating disulfide sites in oxidized proteins [71]. Therefore, TrxR1 has also an important role in mediating constitutive denitrosylation, thus maintaining low levels of *S*-nitrosylation in response to exogenous and endogenous NO [72]. The UCP2 protein expression is increased with the increasing amount of ROS produced in the mitochondria [8]. In this way, the ROS production was significantly increased after acute exercise than when the PBMCs were activated with PMA. It is suggested that the monocytes or other PBMCs have greater capacity of ROS production after acute exercise in response to immune stimulus as PMA or LPS [73, 74]. However, we cannot discard that the high production of ROS after activation of post-exercise PBMCs was due to a change in the type of circulating cells.

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Manuscript II

Effects of dietary almond- and olive oil-based docosahexaenoic acid- and vitamin E-enriched beverage supplementation on athletic performance and oxidative stress markers

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Effects of dietary almond- and olive oil-based docosahexaenoic acid- and vitamin E-enriched beverage supplementation on athletic performance and oxidative stress markers

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Functional beverages based on almonds and olive oil and enriched with α-tocopherol and docosahexaenoic acid (DHA) could be useful in modulating oxidative stress and enhancing physical performance in sportsmen. The aim of this work was to evaluate the effects of supplementation with functional beverages on physical performance, plasma and erythrocyte fatty acids' and polyphenol handling, oxidative and nitrative damage, and antioxidant and mitochondrial gene expression in young and senior athletes. Athletes performed maximal exercise tests before and after one month of dietary supplementation and blood samples were taken immediately before and one hour after each test. The beverages did not alter performance parameters during maximal exercise. Supplementation increased polyunsaturated and reduced saturated plasma fatty acids while increasing the DHA erythrocyte content; it maintained basal plasma and blood polyphenol levels, but increased the blood cell polyphenol concentration in senior athletes. Supplementation protects against oxidative damage although it enhances nitrative damage in young athletes. The beverages enhance the gene expression of antioxidant enzymes in peripheral blood mononuclear cells after exercise in young athletes.

Introduction

The strategies to obtain optimal endurance training effects include certain training methods, but also nutritional approaches. Physical activity, athletic performance, and recovery from exercise are enhanced by optimal nutrition.[1] Supplements containing omega 3 fatty acids, polyphenols, antioxidants and vitamins are widely consumed for the purpose of improving health and athletic achievement.[2] The enhancing effects of dietary supplementation with omega 3 polyunsaturated fatty acids (n3-PUFA) on exercise performance[3] have been described alongside oxidative balance during physical activity.[4] Docosahexaenoic (DHA) and eicosapentaenoic acid[5] diet supplementation modulates erythrocyte membrane deformability and the capacity of O_2 transport, and adapts mitochondria to use fatty acids as a fuel increasing energy efficiency.[3] Foods enriched with n3-PUFA often need to be protected from oxidation by the addition of antioxidants such as vitamin E and, for this reason, functional foods enriched with PUFA are usually enriched with vitamin E. Recently, how suitable it is to supplement the diet of athletes with isolated vitamin C and E supplements in order to ameliorate physical performance has come into question. The effects of antioxidants on physical performance are not yet entirely clear, with studies reporting positive, negative or no effects.[6] In this sense, chronic and elevated intakes of most antioxidants have been considered as having harmful effects on performance,[7] but moderate vitamin C and E dietary supplementation prevents neutrophil protein oxidation without affecting the adaptive response to exercise.[8] Polyphenol supplementation has been tested as a countermeasure against oxidative stress induced by exercise training.[9] Almonds and olive oil are representative products of the Mediterranean diet, which can be used as a source to enrich food with polyphenols. A Mediterranean diet supplemented with olive oil or nuts increases the total polyphenol intake and also correlates with a rise in plasma nitric oxide (NO) and a reduction in

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systolic and diastolic blood pressure.[10] Almonds are an important source of essential nutrients such as arginine, calcium, potassium, niacin, α-tocopherol, fibre, monounsaturated fatty acids and polyphenols.[11] A diet supplemented with almonds exerts protective effects against oxidative stress.[11] A functional beverage based on almond and olive oil enriched with α-tocopherol acetate and DHA may be a good vehicle to supplement the diet of athletes in order to counteract oxidative damage and enhance physical performance. However, the efficacy of such a functional food in ameliorating exercise-induced physiological and oxidative stress and as an enhancer for athletic performance needs to be evaluated. New information related to this topic could contribute to the clarification of the controversy on the need for antioxidant use in well-trained athletes to improve physical performance and the body redox status.[12]

The oxidative stress associated with intense exercise in a warm environment is a good human model for studying the possible functional effects of consuming foods enriched with n3-PUFA, vitamin E and polyphenols.[13] Ageing is an additional factor influencing physical performance and oxidative stress that could influence the demands of n3-PUFA and vitamins.[14,15] It has been reported that elderly people are more susceptible than young people to oxidative damage in muscles after exercising.[15] In fact, n3-PUFAs are now identified as potential key nutrients, safe and effective in the treatment and prevention of several negative consequences of ageing.[16]

The aim of the present study was to evaluate the effects of diet supplementation with an almond and olive oil based beverage enriched with DHA and vitamin E on physical performance, plasma and erythrocyte fatty acids' and polyphenol handling, oxidative and nitrative damage and antioxidant gene expression induced by acute exercise in a warm environment in young and senior athletes. The main hypothesis was based on polyunsaturated fatty acids, given their pro-oxidative character and also due to their action on transcription factors together with vitamin E and other polyphenols which can modulate the antioxidant response and oxidative and nitrative damage induced by physical activity. The overall objective of the study is to establish situations improving athletic performance and study the oxidative balance by manipulation of feeding patterns and consumption of nutritional supplements and investigate the involved mechanisms.

Materials and methods

Subjects and anthropometric characteristics

Ten young male taekwondo athletes and eight well-trained senior athletes related to sports competitions (trainers and sport medical doctors) volunteered to participate in this study. All the subjects were informed of its purpose, requirements and possible risks before giving their written consent to take part. Before being accepted to participate in the research, each subject underwent a complete medical examination, which included a medical history and resting ECG, to prevent any medical problem that would contraindicate participation in the study. The protocol complied with the Declaration of Helsinki for research on human subjects, and was approved by the Ethical Committee for Clinical Research at the Direcció General de l'Esport of the Catalonian Sports Council. The study was registered at ClinicalTrials.gov (NCT02177383). The participants were not acclimatised to heat and the study was conducted in the months of May and June with an average temperature of 18.2 °C and 22.8 °C respectively. The participants were split into two groups depending on their age. All participants began the nutritional diet trial, but only five young and five senior athletes completed it. The reason for abandoning the nutritional trial was participation in sporting competitions.

Beverage composition

The nutritional intervention consisted of daily supplementation of the diet with one liter of almond and olive oil based beverage enriched with a DHA functional beverage five days a week. Beverages were isotonic (278 mOsm kg^{-1}) and made up of 3.0% almond and 0.8% sucrose and the rest was water, flavour, and the added oils and α-tocopherol acetate (vitamin E). The added oils were 0.6% olive oil and 0.2% (wt%) DHA-S (DSM, Columbia, USA). DHA-S is a nutritional oil derived from the marine algae, *Schizochytrium* sp., a rich source of DHA with soy lecithin and rosemary (*Rosmarinus officinalis*) extract as the flavour, and tocopherols and ascorbyl palmitate as antioxidants. The procedure for obtaining the beverage was bleaching of the almonds; crushing of the almonds in water; centrifuging of the mixture to eliminate insoluble materials; and the addition of cinnamon and lemon natural flavours, sucrose, vitamin E, and olive oil plus DHA-S. Finally, the beverage was sterilized and packed. The functional beverage was elaborated by Liquats Vegetals S.A. (Girona, Spain).

The fatty acid composition of the beverage was determined following the same procedure used to determine the erythrocyte fatty acid composition as described below. Similarly, the polyphenol content of the beverage was determined following the same procedure used to determine the polyphenol content of plasma, erythrocytes and blood described below. The total fat content of the functional beverage was 2.6%, taking into account 60% of the fat content of the almonds, olive oil and DHA-S added.

Nutritional intervention

The nutritional intervention comprised ingesting one litre per day (five days per week) of the isotonic functional beverage instead of water for five weeks. Half of the beverage was taken in the morning and the other half before the daily training session. The participants maintained their nutritional and exercise habits alongside the study. Each subject performed two exercise tests separated by a 5-week period during which they all took the nutritional supplement. Before the first exercise test athletes take water habitually; this fact was changed during the nutritional intervention when athletes consumed the functional beverage until the second exercise test. At the

end of the nutritional intervention, all participants performed the same exercise test as at the beginning of the intervention. The results obtained after the nutritional intervention (supplemented groups) were compared with those obtained at the beginning of the intervention (control groups).

Experimental procedure

Two weeks before the first test, each subject performed an incremental maximal test until exhaustion on a motorised treadmill (EG2, Vitoria, Spain) to determine their maximal oxygen consumption (VO_2max) using a computerised metabolic chart (Master Screen CPX, Erich Jaeger, Würzburg, Germany). The velocity corresponding to 60% (V60), 70% (V70), 80% (V80) and 90% (V90), of their VO_2max was calculated by linear interpolation of data from the maximal exercise test. On day 1 of the study, subjects arrived at the laboratory at 9:00 AM after an overnight fast and having drunk a minimum of 500 cc of water since wakening. Dry nude body weight was measured before and after the experiment after the subjects had emptied their urinary bladder. The subjects equipped with a heart rate transmitter and skin thermistors entered into the climatic chamber set at 30 °C temperature and 70% humidity; after 10 minutes the baseline core temperature, skin temperature and heart rate (HR) values were collected. Subjects continuously ran on the treadmill at the speed of V60 for 5 minutes, V70 for 5 minutes and V80 for 5 minutes for three consecutive bouts with two minutes of recovery between bouts. Finally, the subjects ran at V90 until exhaustion, and this time was measured as a quantity of exercise performance. Subjects were required to wear the same clothes and shoes in the two exercise sessions. Water was provided *ad libitum* in 50 cl bottles at room temperature and the amount of water consumed was measured. The percentage of dehydration was calculated from the weight difference corrected by drinking water during the test. The Polar® heart watch system (Polar Electro Inc., Kempele, Finland) was used to measure the basal HR every 5 minutes during the test and after 5 minutes of recovery time. A microsample of blood (20 μl) was taken from the ear lobe to measure lactate concentration. The Borg scale was used to assess subjective perception of effort at minutes 15, 32, 49 and after concluding the test.[17]

Venous blood samples were obtained from the antecubital vein of participants with vacutainers containing EDTA (ethylenediaminetetraacetic acid) as an anticoagulant for blood count analyses (2 mL), to obtain erythrocytes and plasma (6 mL) and purify peripheral blood mononuclear cells (PBMCs) (6 mL). Venous blood samples were obtained after 12 hours, overnight, under fasting conditions (basal sample), and 2 hours after finishing training, which is consonant with the increased circulating immune cells and significant changes in antioxidant enzyme activities and in markers for oxidative damage.

The erythrocyte fraction was obtained after centrifugation at 900*g*, 30 min, 4 °C. Then, erythrocytes were washed with phosphate buffered saline (PBS), centrifuged at 900*g*, 20 min, 4 °C and lysed with distilled water at the initial blood volume. Cell lysates were stored at −80 °C until biochemical analyses thereof.

PBMCs were obtained by following a method previously described.[18] Blood was carefully introduced on Ficoll in a proportion of 1.5 : 1 and was then centrifuged at 9009*g*, at 4 °C for 30 min. The PBMC layer was carefully removed. The plasma and the Ficoll phases were discarded. The PBMC slurry was then washed twice with PBS and centrifuged for 10 min at 1000*g*, 4 °C. This process was performed in triplicate, with one of the samples used to obtain RNA, and another being lysed with distilled water. Cell lysates were stored at −80 °C until biochemical analyses thereof.

Fatty acid determination

Erythrocyte, plasma and beverage fatty acids were extracted in duplicate with chloroform/methanol (2 : 1 v/v) by a modified method of Folch,[19,20] containing 0.01% butylated hydroxyanisole as the antioxidant and 20 μL of *n*-heptadecanoic acid (15 mM) as the internal standard. The resultant organic phase was evaporated under a nitrogen stream at 55 °C. The dry residue was dissolved in 225 μL of *n*-hexane and 25 μL of Meth-Prep™ II (Grace Davison Discovery Sciences, Columbia, MD, USA) and the derivatization reagent was added. The gas chromatograph was an Agilent 5890 model (Agilent Technologies, Santa Clara, CA, USA) with a flame ionization detector (FID) and the column was a Supelcowax® 10 Capillary GC column, 30 m × 0.53 mm × 0.50 μm (Supelco, Bellefonte, PA, USA).

Plasma triglyceride determination

Plasma triglycerides were determined in duplicate using an enzymatic colorimetric assay kit (Spinreact®) following the manufacturer's instructions for use. The intra-assay coefficient of variation was 1.57% and the inter-assay coefficient of variation 3.15%.

Malondialdehyde determination

Malondialdehyde (MDA) as a marker of lipid peroxidation was analyzed in duplicate in 1/100 diluted erythrocytes and in plasma using a colorimetric assay kit (Calbiochem®). Briefly, samples and standards were placed in 1.5 mL tubes containing *n*-methyl-2-phenylindole (10.3 mM) in acetonitrile/methanol (3 : 1, by vol). 12 N HCl was added and the samples were incubated for 1 h at 45 °C. The absorbance was measured at 586 nm. The method used is specific for MDA determination.[20]

Nitrotyrosine and protein carbonyl determination

Protein carbonyl derivatives and nitrotyrosine (N-Tyr) in proteins were determined in duplicate by immunological methods using the OxiSelect™ Protein Carbonyl Immunoblot Kit (Cell Biolabs, Inc.) and OxiSelect™ Nitrotyrosine Immunoblot Kit (Cell Biolabs, Inc.) following the manufacturer's instructions. Total protein concentrations were measured by a method previously described.[18,20] The light was visualized and detected by short exposure to a Chemidoc XRS densitometer (Bio-Rad Laboratories). Image analysis was

performed using Quantity One-1D analysis software (Bio-Rad Laboratories). The coefficient of variation has been calculated to be 10% for the carbonyl index and 12% for the nitrotyrosine index.

Nitrite and nitrate determination

Duplicate serum samples were centrifuged (15 000*g*, 30 min, 4 °C) in 10 K filters (Amicon® Ultra; Millipore) to remove proteins. The 10 K filters were previously cleaned twice with distilled water to eliminate nitrite and nitrate residues in the filters. The supernatants were recovered and used to measure nitrite and nitrate concentrations by detecting the synthesized NO in the gas phase chemiluminescence reaction with ozone, using a NO analyzer (NOA 280i; Sievers, GE Power & Water, Boulder, CO).[10]

Lipoperoxide determination

Plasma lipid peroxide was determined in duplicate using the human lipid peroxide (LPO) ELISA kit from Cusabio® following the manufacturer's instructions for use. The intra-assay coefficient of variation was calculated to be lower than 8% and the inter-assay coefficient of variation was lower than 10.0%.

Vitamin E determination

Total vitamin E determination for the functional beverage was attained by extracting the liposoluble vitamins using *n*-hexane after deproteinization with ethanol containing 0.2% butylated hydroxytoluene (BHT). The vitamin E concentration was determined after drying the samples under a nitrogen current and dissolving them with ethanol. The mobile phase consisted of acetonitrile–tetrahydrofuran–water (550 : 370 : 80, v/v/v). The HPLC was a Shimadzu (Canby, OR, USA) equipped with a diode array detector and a Nova Pak C_{18}, 3.9 × 150 mm column, and α-tocopherol was determined at 290 nm. Quantification was performed with an external standard (Sigma-Aldrich®).

Polyphenol determination

The total polyphenol content of the functional beverage, plasma and washed blood cells was determined through the Folin–Ciocalteu method[10] in the supernatants of deproteinized samples with cold acetone (1 : 1.2) using L-tyrosine as the standard. The results are expressed as mmoles of L-tyrosine per L. The blood concentration of polyphenols was calculated from the plasma and blood cell concentration, and haematocrit (Hm) values following the formula:

$$\text{Blood concentration} = \text{Plasma concentration} \times (100 - \text{Hm}/100) + \text{Blood cells concentration} \times (\text{Hm}/100)$$

PBMCs' RNA extraction and real-time PCR assay

Catalase, Mn-superoxide dismutase (Mn-SOD), uncoupling protein-3 (UCP3), hemoxigenase-1 (HO1), glutathione reductase (GRd), glutathione peroxidase (GPx), Cu/Zn-superoxide dismutase (Cu/Zn-SOD), mitofusin-1 (Mtf1), mitofusin-2 (Mtf2), solute carrier family 2 facilitated glucose transporter member 4 (SLC), mitochondrial NADH dehydrogenase subunit 5 (MitND5), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PCG1α) and cytochrome c oxidase subunit IV (COXIV) mRNA expression were determined by multiplex real-time PCR based on the incorporation of a fluorescent reporter dye and using human 18S rRNA as the reference. For this purpose, total RNA was isolated from PBMCs by Tripure extraction (Roche Diagnostics, Germany). RNA (1 μg) from each sample was reverse transcribed using 50 U of Expand Reverse Transcriptase (Roche Diagnostics, Germany) and 20 pmol oligo (dT) for 60 min at 37 °C in a 10 μL final volume, according to the manufacturer's instructions. The resulting cDNA (2.5 μL) was amplified using the LightCycler FastStart DNA MasterPLUS SYBR Green I kit (Roche Diagnostics, Germany). Amplification was performed at 55 °C and 45 cycles. The relative quantification was performed by standard calculations considering $2^{(\Delta\Delta Ct)}$. Antioxidant enzyme levels before and after the season were normalized to the invariant control 18S rRNA. mRNA levels at the basal young control group were arbitrarily referred to as 1. The primers used are listed in Table 1.

Statistical analysis

Statistical analysis was carried out using the Statistical Package for Social Sciences (SPSS v.21.0 for Windows). Results are expressed as mean ± SEM and $p < 0.05$ was considered statistically significant. A Kolmogorov–Smirnov test was previously applied to assess the normal distribution of the data. The statistical significance of the data was assessed by a three-way analysis of variance (ANOVA). The statistical factors analysed were beverage supplementation (S), ageing (A) and exercise (E). For the sets of data where there was a significant SxExA, SxE, SxA, AxE interactions were tested by the ANOVA one-way test.

Results

There were no differences in the anthropometric characteristics and physical activity capabilities between the young and senior groups (Table 2) except for the mean age of the young (22.8 ± 1.6 years old) and senior (45.6 ± 3.8 years old) groups. The mean weight, height, % body fat, % fat-free mass, body surface area, VO_2max and the VO_2max kg^{-1} and maximal rate attained during the exercise test for athletes from the young group were similar to athletes from the senior group. These values did not change after five weeks of nutritional intervention with the functional beverage (data not shown).

Effects on exercise performance

The exercise test significantly increased the values of skin and central body temperature, blood lactate and the Borg index but significantly decreased heat storage (ΔHS) (Fig. 1A–D) in a similar way, both for the control and functional beverage supplement and in the young and senior groups. Functional beverage diet supplementation did not influence any of the measured parameters, whereas age significantly decreased

Table 1 Primer sequences and conditions

Gene	Primer		Conditions	
18S	Fw:	5′-ATG TGA AGT CAC TGT GCC AG-3′	95 °C	10 s
	Rv:	5′-GTG TAA TCC GTC TCC ACA GA-3′	60 °C	10 s
			72 °C	12 s
CAT	Fw:	5′-TTT GGC TAC TTT GAG GTC AC-3′	95 °C	10 s
	Rv:	5′-TCC CCA TTT GCA TTA ACC AG-3′	60 °C	10 s
			72 °C	15 s
CuZn-SOD	Fw:	5′-TCA GGA GAC CAT TGC ATC ATT-3′	95 °C	10 s
	Rv:	5′-CGC TTT CCT GTC TTT GTA CTT TCT TC-3′	63 °C	10 s
			72 °C	15 s
GPx	Fw:	5′-TTC CCG TGC AAC CAG TTT G-3′	94 °C	10 s
	Rv:	5′-TTC ACC TCG CAC TTC TCG AA-3′	63 °C	10 s
			72 °C	15 s
UCP3	Fw:	5′-CGT GGT GAT GTT CAT AAC CTA TG-3′	95 °C	5 s
	Rv:	5′-CGG TGA TTC CCG TAA CAT CTG-3′	60 °C	7 s
			72 °C	10 s
HO-1	Fw:	5′-CCA GCG GGC CAG CAA CAA AGT GC-3′	95 °C	10 s
	Rv:	5′-AAG CCT TCA GTG CCC ACG GTA AGG-3′	60 °C	5 s
			72 °C	10 s
Mn-SOD	Fw:	5′-GAG AAG TAC CAG GAG GCG TTG-3′	95 °C	10 s
	Rv:	5′-CAA GCC AAC CCC AAC CTG AGC-3′	64 °C	10 s
			72 °C	15 s
GRd	Fw:	5′-TCA CGC AGT TAC CAA AAG GAA A-3′	95 °C	10 s
	Rv:	5′-CAC ACC CAA GTC CCC TGC ATA T-3′	64 °C	10 s
			72 °C	15 s
COX IV	Fw:	5′-AGAAGCACTATGTGTACGGCCC-3	95 °C	10 s
	Rv:	5′-GGTTCACCTTCATGTCCAGCAT-3′	60 °C	10 s
			72 °C	15 s
MitDN5	Fw:	5′-CGGCTGAGAGGGCGTAGG-3′	95 °C	10 s
	Rv:	5′-GATGAAACCGATATCCGGCCGA-3′	60 °C	10 s
			72 °C	15 s
Mfn1	Fw:	5′-TGTTTTGGTCGCAAACTCTG-3′	95 °C	10 s
	Rv:	5′-CTGTCTGCGTACGTCTTCCA-3′	60 °C	10 s
			72 °C	15 s
Mfn2	Fw:	5′-ATGCATCCCCACTTAAGCAC-3′	95 °C	10 s
	Rv:	5′-CCAGAGGGCAGAACTTTGTC-3′	60 °C	10 s
			72 °C	15 s
SLC2A4	Fw:	5′-CTGCTCCTGGCCTCACAG-3′	95 °C	10 s
	Rv:	5′-CCCCTCGAGATTCTGGATGAT-3′	64 °C	10 s
			72 °C	15 s
PGC1α	Fw:	5′-CACTTACAAGCCAAACCAACAACT-3′	95 °C	10 s
	Rv:	5′-CAATAGTCTTGTTCTCAAATGGGGA-3′	60 °C	10 s
			72 °C	15 s

fatigue perception during the exercise test in senior athletes compared to the young athletes (Fig. 1D). Dietary functional beverage supplementation did not influence the duration of exercise at a speed representing a 90% value of VO_2max until exhaustion; nor does the age of the athletes influence this parameter.

Functional beverage composition

The composition of the functional beverage is shown in Table 3. The functional beverage contained around 2.6% (wt%) fat, 2.85 ± 0.29 mM (51 ± 5 mg per 100 mL as L-tyrosine equivalents) of polyphenols and 4.6 ± 2.8 mg per 100 mL of vitamin E. The daily intake of one litre of beverage five days a week implies a daily supplement to diet with a mean of 18.6 g day^{-1} of fat (of which 820 mg day^{-1} is DHA), 32.6 mg day^{-1} vitamin E, and 36.4 mg day^{-1} polyphenols. The fatty acid content of the beverage was mainly monounsaturated (51.7 ± 5.0%) and polyunsaturated (38.3 ± 4.4%) with a low percentage of saturated fatty acids (9.90 ± 1.15%). The most abundant

Table 2 Subject's anthropometric characteristics and physical activity time

	Young	Senior
Age (years)	22.8 ± 3.8$	45.6 ± 1.6
Weight (kg)	71.0 ± 4.8	76.1 ± 2.9
Height (cm)	176 ± 3.8	177 ± 3.8
Body mass index (BMI, kg m^{-2})	22.9 ± 0.5	24.3 ± 0.9
Fat mass (%)	47.0 ± 1.6	44.8 ± 1.1
Fat-free mass (%)	9.50 ± 1.1	11.6 ± 1.0
Body surface (m^2)	1.86 ± 0.05	1.93 ± 0.07
Max speed (km h^{-1})	16.1 ± 0.6	14.9 ± 0.9
VO_2 (l min^{-1})	4029 ± 255	3819 ± 329
VO_2 max (l min^{-1} kg^{-1})	58.8 ± 2.5	50.4 ± 3.4

Statistical analysis: two-way ANOVA, $p < 0.05$. $ Differences between young and master groups. Results are the mean ± sem.

Table 3 Beverage fatty acid composition

Fatty acid	Concentration
C16:0 (μM)	3607 ± 620
C16:1 (μM)	591 ± 115
C18:0 (μM)	1928 ± 423
C18:1 (μM)	22 503 ± 5605
C18:2 (μM)	12 177 ± 2996
C18:3n6 (μM)	298 ± 152
C18:3n3 (μM)	455 ± 44
C20:0 (μM)	116 ± 7
C20:1 (μM)	35.9 ± 2.3
C20:2 (μM)	282 ± 14
C20:3 (μM)	20.9 ± 2.2
C20:4n6 (μM)	140 ± 9
C22:0 (μM)	75.6 ± 1.7
C22:5 (μM)	1715 ± 70
C22:6n3 (μM)	3457 ± 117
Total fatty acids (μM)	47 400 ± 9586
SFA (%)	9.90 ± 1.15
MUFA (%)	51.7 ± 5.0
PUFA (%)	38.3 ± 4.4
Vitamin E (mg L^{-1})	45.7 ± 27.7
Polyphenols (mM)	2.85 ± 0.29

Values are the average of 6 samples of the beverage.

fatty acids were C18:1 and C18:2 followed by C16:0, C22:6, C18:0 and C22:5, whereas C18:3n3, C18:3n6, C20:2, C20:0, C20:4 and C22:0 represented under 1% of the total fat content of the functional beverage.

Effects on plasma triglycerides and plasma and erythrocyte fatty acid composition

Dietary supplementation with the functional beverage influenced the composition of plasma fatty acids (Table 4) for age but not acute exercise. The young group had significantly higher plasma MUFA levels than the senior group after exercising in both the placebo and supplemented group. Dietary functional beverage supplementation significantly decreased C16:0, C16:1 C18:3n3 and C18:3n6 but significantly increased C20:3, C20:4n6 and C22:6n3 in plasma. The senior group had more C22:6n3 and lower C18:1n9 and C18:3n3 in their plasma than the young group.

As with plasma, dietary supplementation with the functional beverage influenced the fatty acid composition of erythrocytes (Table 5) for age but not acute exercise. The percentages of erythrocytes C16:0, C16:1, C18:0, C18:1, C18:2, C18:3n6, C20:4, SFAs, MUFAs, and PUFAs were not impacted by functional beverage dietary supplementation, age and acute exercise. Nevertheless, the percentages of C18:3n3 and

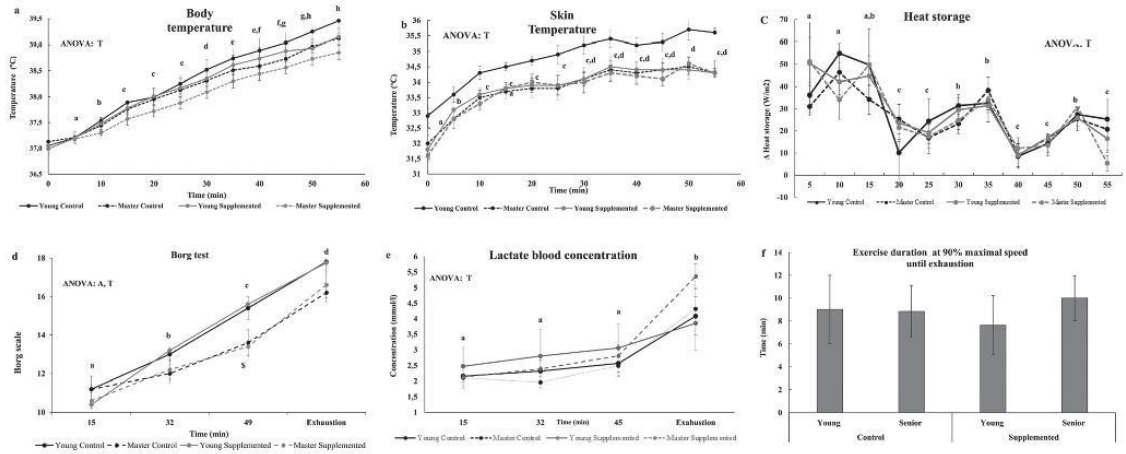

Fig. 1 Statistical analysis: three-way ANOVA, $p < 0.05$. S, supplementation effect; A, age effect, T, stress test effect, SxA, interaction between supplementation and age effects, SxT, interaction between supplementation and stress test effects, TxA, interaction between stress test and age effects, AxTxS, effects of interaction between three factors. S, A, T, SxA, TxA, SxT or AxTxS indicates a significant effect of each statistical factor. * indicates significant differences between the dietary control and functional beverage dietary supplementation plasma levels; different letters reveal significant differences during the stress test; $ indicates significant differences between the young and senior groups. Different letters reveal significant differences during the stress test.

Table 4 Effects of supplementation with the functional beverage, acute exercise and age on plasma fatty acid content

		Pre-exercise		Post-exercise		
		Young	Senior	Young	Senior	ANOVA
C16:0 (%)	Control	20.9 ± 0.72	21.2 ± 0.73	19.7 ± 0.43	21.3 ± 1.23	
	Supplemented	19.3 ± 0.66	20.4 ± 3.08	20.1 ± 1.63	18.4 ± 1.03	
C16:1 (%)	Control	4.80 ± 0.09	4.39 ± 0.53	4.91 ± 0.42	4.93 ± 0.64	**S**
	Supplemented	3.18 ± 0.44*	2.83 ± 0.63	4.39 ± 0.89	3.23 ± 0.53*	
C18:0 (%)	Control	14.9 ± 1.49	13.5 ± 0.90	13.2 ± 1.19	13.7 ± 0.63	
	Supplemented	12.9 ± 0.53	14.6 ± 0.71	13.0 ± 0.87	13.4 ± 0.51	
C18:1 (%)	Control	17.8 ± 0.63	14.1 ± 0.67	19.1 ± 1.39	15.4 ± 1.37	**A**
	Supplemented	15.8 ± 1.68	15.4 ± 0.85	20.4 ± 1.52	14.9 ± 1.29$^{\$}$	
C18:2 (%)	Control	19.9 ± 1.69	23.9 ± 2.34	22.7 ± 2.14	22.6 ± 1.35	**S**
	Supplemented	24.7 ± 1.97*	26.4 ± 1.38	23.9 ± 1.09	24.0 ± 1.38	
C18:3n6 (%)	Control	1.80 ± 0.37	1.63 ± 0.17	1.87 ± 0.13	1.94 ± 0.26	**S**
	Supplemented	1.25 ± 0.31	1.11 ± 0.13	1.24 ± 0.38	0.78 ± 0.12*	
C18:3n3 (%)	Control	2.30 ± 0.80	0.96 ± 0.19$^{\$}$	1.57 ± 0.15	1.21 ± 0.15	**S, A**
	Supplemented	0.98 ± 0.08*	0.63 ± 0.18	0.88 ± 0.28	0.78 ± 0.10	
C20:3 (%)	Control	1.97 ± 0.19	1.70 ± 0.29	1.96 ± 0.17	1.71 ± 0.18	**S**
	Supplemented	2.23 ± 0.26	2.15 ± 0.27	2.15 ± 0.28	2.19 ± 0.18	
C20:4 (%)	Control	11.5 ± 1.75	13.4 ± 1.72	12.1 ± 0.78	12.9 ± 1.39	**S**
	Supplemented	14.8 ± 0.77*	15.4 ± 1.11	13.7 ± 0.44	14.6 ± 0.57	
C22:6 (%)	Control	4.09 ± 0.71	5.69 ± 2.31	2.93 ± 0.32	4.30 ± 0.98	**S**
	Supplemented	6.73 ± 1.36	8.22 ± 0.22	6.35 ± 1.07*	7.71 ± 0.75*	
Triglycerides (mg dL^{-1})	Control	306 ± 109^{a}	111 ± 26.2^{b}	110 ± 26.4^{b}	186 ± 27.8^{a,b}	**SxExA**
	Supplemented	109 ± 35.0^{b}	143 ± 26.6^{b}	189 ± 42.7^{a,b}	118 ± 27.7^{b}	
SFA (%)	Control	36.4 ± 2.13	35.3 ± 0.29	33.4 ± 1.53	35.7 ± 1.62	
	Supplemented	32.2 ± 0.75	35.4 ± 3.67	33.0 ± 2.32	32.0 ± 1.02	
MUFA (%)	Control	22.6 ± 0.66	18.5 ± 0.87	23.9 ± 1.54$^{\$}$	20.3 ± 1.44	**A**
	Supplemented	19.0 ± 2.09	18.2 ± 1.22	24.8 ± 2.33$^{\$}$	18.1 ± 1.60	
PUFA (%)	Control	41.6 ± 1.74	46.8 ± 0.91	43.0 ± 2.23	44.6 ± 0.86	
	Supplemented	48.9 ± 1.81	46.8 ± 4.62	42.1 ± 4.10	50.2 ± 1.78	
TOTAL (μM)	Control	100 ± 19.2	100 ± 9.30	100 ± 19.1	100 ± 8.49	
	Supplemented	100 ± 32.0	100 ± 31.3	100 ± 41.0	100 ± 22.2	

Results are the mean ± sem. Statistical analysis: three-way ANOVA, $p < 0.05$. S, supplementation effect; A, age effect, E, exercise effect, SxA, interaction between supplementation and age effects, SxE, interaction between supplementation and exercise effects, ExA, interaction between exercise and age effects, AxExS, effects of interaction between three factors. S, A, E, SxA, ExA, SxE or AxExS indicates a significant effect of each statistical factor. * indicates significant differences between the dietary control and functional beverage dietary supplementation; # indicates significant differences between pre-exercise and post-exercise; $ indicates significant differences between the young and senior groups. Where interaction exists between different statistical factors, different letters reveal significant differences.

C22:6n3 in erythrocytes were significantly higher after dietary supplementation with the functional beverage than in the control situation, mainly in the young group after exercise for the C18:3n3 value. Furthermore, a significant age effect was observed in the percentage of C22:0 and C20:3, which was significantly higher in the senior than in the young group after exercising in the control situation.

An interaction between the functional beverage, acute exercise and age influenced plasma triglyceride levels. Pre-exercise levels of the young group in the control situation were significantly higher than all groups and situations; no differences were observed in plasma triglyceride values attributable to functional beverage dietary supplementation, acute exercise or age except for the significantly higher values of the young group in the pre-exercise and control situations.

Effects on blood polyphenol levels, haematological parameters, and plasma NOx

Blood haemoglobin is affected by the three analysed factors; haematocrit is influenced only by age and the number of

Table 5 Effects of supplementation with the functional beverage, acute exercise and age on erythrocyte fatty acid content

		Pre-exercise		Post-exercise		
		Young	Senior	Young	Senior	ANOVA
C16:0 (%)	Control	14.7 ± 0.5	14.7 ± 0.5	14.6 ± 0.5	14.7 ± 0.8	
	Supplemented	14.7 ± 0.7	14.8 ± 0.8	14.5 ± 0.2	14.9 ± 0.8	
C16:1	Control	0.81 ± 0.12	0.79 ± 0.06	0.75 ± 0.10	0.80 ± 0.15	
	Supplemented	0.56 ± 0.03	0.80 ± 0.15	0.63 ± 0.03	0.82 ± 0.15	
C18:0 (%)	Control	19.4 ± 0.5	19.2 ± 0.1	19.6 ± 0.5	19.3 ± 0.3	
	Supplemented	20.5 ± 0.6	19.3 ± 0.2	19.8 ± 0.3	19.7 ± 0.7	
C18:1 (%)	Control	11.9 ± 0.2	11.4 ± 0.3	11.4 ± 0.4	11.2 ± 0.4	
	Supplemented	11.7 ± 0.4	11.5 ± 0.3	12.0 ± 0.3	11.2 ± 0.3	
C18:2 (%)	Control	11.6 ± 0.4	11.8 ± 0.4	13.0 ± 1.2	12.4 ± 0.7	
	Supplemented	11.6 ± 0.5	11.9 ± 0.3	12.0 ± 1.4	11.7 ± 0.6	
C18:3n6 (%)	Control	0.80 ± 0.13	0.54 ± 0.12	0.64 ± 0.03	0.49 ± 0.06	
	Supplemented	0.85 ± 0.18	0.62 ± 0.50	0.55 ± 0.12	0.64 ± 0.08	
C18:3n3 (%)	Control	0.39 ± 0.03	0.41 ± 0.04	0.44 ± 0.03	0.43 ± 0.04	S
	Supplemented	0.49 ± 0.03	0.40 ± 0.05	0.77 ± 0.19*	0.53 ± 0.10	
C20:3 (%)	Control	1.72 ± 0.11	1.84 ± 0.07	1.69 ± 0.13	1.79 ± 0.09$	A
	Supplemented	1.69 ± 0.17	1.52 ± 0.07	1.78 ± 0.17	1.51 ± 0.05	
C20:4 (%)	Control	28.6 ± 0.7	28.4 ± 1.2	28.2 ± 0.9	27.0 ± 0.7	
	Supplemented	28.3 ± 0.8	26.5 ± 1.4	28.3 ± 0.5	25.9 ± 1.4	
C22:0 (%)	Control	0.61 ± 0.12	0.68 ± 0.09	0.48 ± 0.09	0.73 ± 0.07$	A
	Supplemented	0.50 ± 0.04	0.64 ± 0.04	0.45 ± 0.08	0.61 ± 0.06	
C22:6 (%)	Control	9.73 ± 0.5	9.27 ± 0.3	8.81 ± 1.3	9.09 ± 1.0	S
	Supplemented	11.2 ± 1.1	12.1 ± 1.5	9.32 ± 0.7	12.7 ± 1.9	
SFA (%)	Control	34.7 ± 0.6	34.6 ± 0.5	34.7 ± 0.7	34.7 ± 0.8	
	Supplemented	35.6 ± 1.0	34.7 ± 0.9	34.8 ± 0.2	35.2 ± 1.1	
MUFA (%)	Control	12.7 ± 0.2	12.2 ± 0.3	12.2 ± 0.5	12.0 ± 0.5	
	Supplemented	12.1 ± 0.3	12.3 ± 0.3	12.6 ± 0.4	12.1 ± 0.4	
PUFA (%)	Control	52.6 ± 0.7	53.2 ± 0.6	53.1 ± 0.4	53.3 ± 1.4	
	Supplemented	52.3 ± 1.2	53.0 ± 1.0	52.6 ± 0.6	52.7 ± 1.1	

Results are the mean ± sem. Statistical analysis: three-way ANOVA, $p < 0.05$. S, supplementation effect; A, age effect, E, exercise effect, SxA, interaction between supplementation and age effects, SxE, interaction between supplementation and exercise effects, ExA, interaction between exercise and age effects, AxExS, effects of interaction between three factors. S, A, E, SxA, ExA, SxE or AxExS indicates a significant effect of each statistical factor. * indicates significant differences between the dietary control and functional beverage dietary supplementation; # indicates significant differences between pre-exercise and post-exercise; $ indicates significant differences between the young and senior groups. Where interaction exists between different statistical factors, different letters reveal significant differences.

circulating erythrocytes was influenced in all of them (Table 6). The senior group had significantly lower haematocrit and blood haemoglobin levels than the young group. Both acute exercise and functional beverage dietary supplementation significantly increased blood haemoglobin levels.

Age and acute exercise and interaction between these two factors influenced the plasma and blood polyphenol levels (Table 6). Pre-exercise plasma and blood polyphenol levels were significantly higher in the senior group than in the young one, both under the control and supplemented conditions, and acute exercise decreased plasma and blood polyphenol levels in the senior group. No differences between the senior and young groups were evidenced after acute exercise under both control and supplemented conditions. Exercise had a similar influence on blood cell polyphenol, lowering post-exercise levels; age and dietary beverage supplementation interactions also affect this parameter. The dietary supplementation with the functional beverage increased pre-exercise polyphenol levels in blood cells of the senior group but not of the young one, reaching significantly higher values.

Nitrate and nitrite are used as markers of nitric oxide production, in addition to their use as a marker of nitrate intake. Plasma nitrite levels were not altered by acute exercise, age or functional beverage dietary supplementation (Table 5). In contrast to this, plasma nitrate levels were influenced by exercise and by an interaction between age, exercise and functional

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Table 6 Effects of supplementation with the functional beverage, acute exercise and age on polyphenol levels, haematological parameters, NOx plasma levels, plasma and erythrocyte oxidative stress markers

		Pre-exercise		Post-exercise		
		Young	Senior	Young	Senior	ANOVA
Haematological parameters						
Erythrocytes (10^6 cells per μl)	Control	4.85 ± 0.27	4.60 ± 0.18	4.84 ± 0.13	4.63 ± 0.17	
	Supplemented	4.96 ± 0.16	4.44 ± 0.14	4.96 ± 0.21	4.36 ± 0.11	
Haemoglobin (g per 100 mL)	Control	15.1 ± 0.2	14.5 ± 0.4	15.7 ± 0.2	14.6 ± 0.3$^{\$}$	**E, A, S**
	Supplemented	15.8 ± 0.1	14.5 ± 0.2$^{\$}$	16.4 ± 0.3	15.2 ± 0.3$^{\$}$	
Haematocrit (%)	Control	44.8 ± 0.6	42.9 ± 1.4	46.6 ± 0.6	43.3 ± 1.1	**A**
	Supplemented	45.7 ± 0.9	41.6 ± 0.7	47.1 ± 0.5	42.6 ± 0.8	
Polyphenol levels						
Blood polyphenols (μM)	Control	454 ± 45^{a}	1007 ± 313^{b}	551 ± 103^{a}	552 ± 69^{a}	
	Supplemented	486 ± 66^{a}	940 ± 177^{b}	429 ± 54^{a}	456 ± 54^{a}	**E, A, ExA**
Plasma polyphenols (μM)	Control	513 ± 49^{a}	1532 ± 531^{c}	814 ± 211^{a,b}	779 ± 96^{a}	**A, ExA**
	Supplemented	607 ± 77^{a}	1230 ± 237^{b,c}	597 ± 100^{a}	488 ± 35^{a}	
Blood cell polyphenols (μM)	Control	377 ± 48^{a,b}	289 ± 58^{b}	257 ± 43^{b}	262 ± 47^{b}	**E, AxS**
	Supplemented	341 ± 51^{b}	525 ± 114^{a}	240 ± 26^{b}	364 ± 72^{a,b}	
NOx plasma levels						
Nitrite (nM)	Control	41.2 ± 3.0	48.7 ± 3.4	40.5 ± 4.0	42.7 ± 3.9	
	Supplemented	40.5 ± 3.0	37.3 ± 2.4	33.8 ± 2.8	43.5 ± 3.0	
Nitrate (μM)	Control	30.0 ± 3.5^{a}	35.3 ± 2.1^{a,b}	42.4 ± 3.8^{b,c}	36.0 ± 2.7^{a,b}	**E, A*E*S**
	Supplemented	32.8 ± 2.0^{a}	30.6 ± 3.5^{a}	38.2 ± 2.5^{a,b,c}	44.8 ± 3.0^{c}	
Plasma oxidative damage markers						
MDA concentration (μM)	Control	2.93 ± 1.67	4.24 ± 2.31	3.41 ± 1.09	1.79 ± 0.64	**S**
	Supplemented	1.40 ± 1.13	0.53 ± 0.30*	1.92 ± 0.62	0.77 ± 0.26	
Carbonyl index (%)	Control	100 ± 5.0	98.0 ± 7.1	93.4 ± 5.0	125 ± 12.0$^{\$}$	**E, A**
	Supplemented	78.6 ± 7.5	88.3 ± 10.9	88.1 ± 7.4	97.9 ± 8.8#	
Nitro-tyrosine (%)	Control	100 ± 7^{a}	105 ± 6^{a}	77.7 ± 9.3^{b}	93.4 ± 5.6^{a}	**S*A**
	Supplemented	95.6 ± 6.2^{a,b}	95.1 ± 10.5^{a}	103 ± 3^{a}	111 ± 6^{a}	
Lipoperoxide (ng ml^{-1})	Control	13.1 ± 6.0	17.1 ± 1.2	19.3 ± 7.4	11.3 ± 3.1	
	Supplemented	24.9 ± 9.9	5.6 ± 10.4	19.0 ± 5.4	13.5 ± 5.4	
Erythrocytes oxidative damage markers						
MDA (μmoles × 10^6 erythrocytes)	Control	20.3 ± 0.5	19.1 ± 0.8	23.4 ± 4.2	17.7 ± 1.1	
	Supplemented	19.2 ± 1.2	18.3 ± 1.3	18.4 ± 0.5	17.4 ± 2.4	
Carbonyl index (%)	Control	100 ± 5	103 ± 7	108 ± 12	99.7 ± 5.2	
	Supplemented	113 ± 4	113 ± 8	101 ± 7	107 ± 9	
Nitro-tyrosine (%)	Control	100 ± 15	95.7 ± 13.7	75.8 ± 10.0	128 ± 23$^{\$}$	**E**
	Supplemented	106 ± 19	124 ± 18	80.8 ± 6.6	140 ± 28$^{\$}$	

Results are the mean ± sem. Statistical analysis: three-way ANOVA, $p < 0.05$. S, supplementation effect; A, age effect, E, exercise effect, SxA, interaction between supplementation and age effects, SxE, interaction between supplementation and exercise effects, ExA, interaction between exercise and age effects, AxExS, effects of interaction between three factors. S, A, E, SxA, ExA, SxE or AxExS indicates a significant effect of each statistical factor. * indicates significant differences between the dietary control and functional beverage dietary supplementation; # indicates significant differences between pre-exercise and post-exercise; $ indicates significant differences between the young and senior groups. Where interaction exists between different statistical factors, different letters reveal significant differences.

beverage dietary supplementation. Acute exercise increased plasma nitrate levels with regard to pre-exercise levels, mainly in the young control group and in the senior supplemented group. The highest plasma nitrate levels were reported in the senior and supplemented groups after exercise.

Effects on oxidative and nitrative damage markers

Table 6 shows the effects of supplementation with the functional beverage, acute exercise and age on plasma and erythrocyte oxidative stress markers. The oxidative damage markers of

erythrocytes were not influenced by age, acute exercise or functional beverage dietary supplementation with the exception of the nitrative damage of erythrocyte proteins that was influenced by acute exercise. Nitrotyrosine levels increased significantly after acute exercise in the senior group but not in the young group both under control and supplemented conditions. Nitrotyrosine serves as a marker for peroxynitrite action. Nitric oxide reacts with superoxide to form peroxynitrite, which in turn nitrates tyrosine residues in proteins. Functional beverage dietary supplementation significantly decreased MDA plasma levels (Table 7), mainly in the senior group under pre-exercise conditions. Acute exercise or age did not influence MDA plasma levels. No effects of acute exercise, age or dietary functional beverage supplementation were observed in lipoperoxide levels. However, acute exercise and age significantly influenced the levels of the carbonyl index, mainly in the senior group. The plasma carbonyl index was significantly higher after acute exercise than at pre-exercise in the senior group after supplementation. In addition, the plasma carbonyl index was significantly higher in the senior group than in the young group after exercise in the control situation. No effects of functional beverage dietary supplementation were observed in the marker for protein carbonyl modification in plasma. Nitrotyrosine plasma levels were not affected by age, acute exercise or the functional beverage but an interaction between age and functional beverage dietary supplementation was observed. The plasma nitrotyrosine levels were significantly lower in the young group after acute exercise in the control situation than all other groups and situations studied.

Table 7 Effects of supplementation with the functional beverage, acute exercise and age on antioxidant and mitochondrial PBMC gene expression

		Pre-exercise		Post-exercise		
		Young	Senior	Young	Senior	ANOVA
Mn SOD	Control	1.00 ± 0.33	1.16 ± 0.37	1.22 ± 0.38	1.39 ± 0.32	
	Supplemented	1.24 ± 0.39	1.25 ± 0.42	2.62 ± 1.36	0.70 ± 0.06	
GPx	Control	1.00 ± 0.13^{a}	1.19 ± 0.25^{a}	1.21 ± 0.28^{a}	0.98 ± 0.10^{a}	**S** (0.080) **E*A** (0.068)
	Supplemented	1.23 ± 0.28^{a}	1.56 ± 0.52^{a,b}	2.33 ± 0.89^{b}	1.12 ± 0.17^{a}	
CAT	Control	1.00 ± 0.23^{a}	0.94 ± 0.17^{a}	0.80 ± 0.07^{a}	0.88 ± 0.17^{a}	**S*E*A** (0.066)
	Supplemented	0.88 ± 0.17^{a}	1.50 ± 0.46^{a,b}	1.94 ± 0.69^{b}	0.82 ± 0.11^{a}	
GRd	Control	1.00 ± 0.22	1.09 ± 0.31	1.17 ± 0.25	0.96 ± 0.16	**S** (0.099)
	Supplemented	1.68 ± 0.67	1.74 ± 0.72	1.97 ± 0.69	0.96 ± 0.13	
UCP-3	Control	1.00 ± 0.13	1.16 ± 0.28	1.86 ± 0.59	1.26 ± 0.29	
	Supplemented	2.36 ± 1.23	1.46 ± 0.44	2.96 ± 1.49	1.08 ± 0.16	
HO-1	Control	1.00 ± 0.15	1.12 ± 0.28	1.49 ± 0.48	1.11 ± 0.24	
	Supplemented	1.63 ± 0.59	1.86 ± 0.66	1.88 ± 0.62	1.01 ± 0.15	
Cu–Zn SOD	Control	1.00 ± 0.13	1.01 ± 0.17	1.02 ± 0.15	1.91 ± 0.59	**E** (0.08)
	Supplemented	1.08 ± 0.18	1.06 ± 0.21	3.42 ± 1.81#	1.23 ± 0.22	
COXIV	Control	1.00 ± 0.14	2.64 ± 1.15	1.35 ± 0.44	2.05 ± 0.69	**S*A** (0.067)
	Supplemented	2.22 ± 0.94	1.33 ± 0.35	1.86 ± 0.94	1.32 ± 0.44	
Mtf1	Control	1.00 ± 0.24	2.72 ± 1.30	2.67 ± 1.45	1.33 ± 0.42	
	Supplemented	1.41 ± 0.50	1.49 ± 0.61	1.20 ± 0.52	1.47 ± 0.59	
Mtf2	Control	1.00 ± 0.28	1.72 ± 0.65	1.65 ± 0.75	1.05 ± 0.34	
	Supplemented	1.07 ± 0.29	2.24 ± 1.16	1.75 ± 0.62	1.32 ± 0.49	
SLC	Control	1.00 ± 0.28	2.56 ± 1.30	0.82 ± 0.18	0.99 ± 0.24	
	Supplemented	1.21 ± 0.43	1.44 ± 0.57	1.13 ± 0.34	0.68 ± 0.12	
MitND5	Control	1.00 ± 0.45	1.04 ± 0.49	0.64 ± 0.22	0.64 ± 0.26	
	Supplemented	0.48 ± 0.19	0.34 ± 0.08	0.54 ± 0.17	1.07 ± 0.66	
PGC1α	Control	1.00 ± 0.40	1.17 ± 0.42	0.52 ± 0.07	1.06 ± 0.50	
	Supplemented	2.78 ± 1.53	1.14 ± 0.40	0.85 ± 0.21	1.49 ± 0.64	

Results are the mean ± sem. Statistical analysis: three-way ANOVA, $p < 0.1$. S, supplementation effect; A, age effect, E, exercise effect, SxA, interaction between supplementation and age effects, SxE, interaction between supplementation and exercise effects, ExA, interaction between exercise and age effects, AxExS, effects of interaction between three factors. S, A, E, SxA, ExA, SxE or AxExS indicates a significant effect of each statistical factor. * indicates significant differences between the dietary control and functional beverage dietary supplementation; # indicates significant differences between pre-exercise and post-exercise; $ indicates significant differences between the young and senior groups. Where interaction exists between different statistical factors, different letters reveal significant differences.

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Effects on antioxidant and mitochondrial turnover genes in PBMCs

Table 7 shows the effects of the functional beverage, age and acute exercise on antioxidant and mitochondrial PBMC gene expression. Functional beverage dietary supplementation, acute exercise and age did not influence the gene expression of Mn-SOD, UCP3, HO1, Mtf1, Mtf2, SLC2A4, MitND and PCG1α. However, the dietary supplementation significantly ($p < 0.1$) increased GRd and GPx gene expression; furthermore an interaction between exercise and age influenced the GPx expression ($p < 0.1$). GPx significantly increased in the young group after exercise in the supplemented group whereas the senior group maintained pre-exercise levels. Acute exercise increased Cu/Zn-SOD gene expression, mainly in the young group in the functional supplementation situation. The catalase gene expression was influenced by an interaction between the three analysed factors. Similar to the GRd, GPx and Cu/Zn-SOD expression, the CAT expression was significantly ($p < 0.1$) higher in the young group after exercise under the functional beverage dietary supplementation conditions than all other groups and situations. Dietary supplementation interacts with age influencing the COXIV gene expression. COXIV expression was higher in the senior group than the young group in the control situation, both under pre- and post-exercise conditions. Functional beverage dietary supplementation increased COXIV expression in the young group whereas it decreased COXIV expression in the senior group, both in pre-exercise and post-exercise situations.

Discussion

The composition of the functional beverage allows supplementing the diet mainly with monounsaturated and polyunsaturated fatty acids, including DHA, polyphenols and vitamin E. The nutritional intervention regime (1 L day^{-1} for five days per week) provides a 2.6× higher dose than the recommended dietary vitamin E allowance for the general population; a polyphenol dose similar to that provided by orange juice,[21] but lower than commercial polyphenol-rich juices; an additional 17% to the total fat ingested by athletes in the Balearic Islands[20] and a 3.2× higher dose of DHA than recommended for the general population.[22] The estimated polyphenol daily intake is variable depending on the age, gender and country.[23] The beverage supplementation provides a dietary polyphenol intake of around 20% more than the total polyphenol intake for populations in European countries.[23]

Effects on physical performance parameters

Heat storage, body and skin temperature, blood lactate, time spent until exhaustion at 90% VO_2max and fatigue perception (Borg index) during a maximal exercise test in a hot environment are considered markers of physical performance. It has been evidenced that heat tolerance tests are feasible measures of physiological strain.[24] During exercise, the vast majority of the chemical energy of food is converted to heat in contracting muscles increasing the body heat content. These changes are modulated by the rate of relative heat production, and represent the rate of change in body heat storage, which in turn reflects the balance between metabolic heat production, heat absorbed from the environment and total body heat loss.[25] The excess of heat production that is reflected in the central body temperature during exercise in a hot environment is an important determinant of behaviour and, therefore, performance.[26] The increase in the body heat content and, thus, core temperature will activate autonomic heat-loss responses including skin vasodilation and sweating,[27] but also will reduce the rate of metabolic heat production by behavioural means.[26] The heat storage, body and skin temperature, and blood lactate measured during exercise tests are influenced by exercise but not by age or dietary supplementation. The exercise test increased both the skin and core body temperature and decreased heat storage change in a similar way in the young and senior groups, irrespective of their control or dietary supplemented conditions. Similarly, neither nutritional intervention with the functional beverage nor the age of athletes influenced the transition to the anaerobic phase of exercise or lactate accumulation during the exercise test in heat. Nonetheless, the perceived exertion Borg index was influenced by both the exercise test and age. The perceived exertion may be the ultimate modulator of behaviour during self-paced exercise in heat.[28] The results reveal the existence of an age-related fatigue perception resistance in accordance with a previous study,[29] although no differences in the rating of perceived exertion to exercise immediately after a maximal exercise test at 25 °C between a young group of athletes and a senior group of athletes have been noted.[30]

The lack of influence of beverage dietary supplementation on exercise performance contrasts with other studies that pointed out the enhancing effects of dietary n3-PUFA supplementation on exercise performance.[3] At the same time, our results contrast with a reduction in endurance performance induced by chronic (more than a week) high fat consumption.[31] The antioxidant content of the functional beverage, in terms of vitamin E and polyphenols, also induces no effect on the physical performance. Although, it has been reported that the chronic intake of most antioxidants can block physiological adaptations to training[7] some other studies evidenced a positive or non-significant effect.[6]

Effects of the functional beverage, exercise and age on circulating polyphenols

The functional beverage is rich in polyphenols and vitamin E (also a polyphenol). The functional effects of the almond and olive oil-based beverage are mediated by the bioavailability of their active components as polyphenols and vitamin E. Polyphenol bioavailability studies involve feeding volunteers a single supplement and monitoring the levels of polyphenol in plasma and urine over a 24-hour period.[32] Diet supplementation with the functional beverage does not influence basal plasma and blood levels of total polyphenols but the senior athletes did see an increase in the total polyphenol

concentration in blood cells as a result of functional beverage supplementation. It has been stated that the consumption of nuts (rich in polyphenols) increases postprandial plasma concentration and total antioxidant capacity, and reduces plasma lipid peroxidation.[33] In general, consuming phenolic-rich fruits increases the post-prandial antioxidant capacity of blood and when they are consumed with high fat and carbohydrates, as 'pro-oxidant and pro-inflammatory' meals, they may counterbalance their negative effects. We evidenced that increasing the dietary polyphenol intake also increases the basal blood cell polyphenol levels, mainly in senior athletes. To date, there are no studies on the polyphenol content of blood cells, although these cells could transport polyphenols into the blood. Circulating polyphenol levels are influenced by age and acute exercise. Senior athletes have higher basal polyphenol levels than young athletes in their blood, plasma and blood cells, but in the latter case only after dietary supplementation with the functional beverage. Acute exercise decreased polyphenol levels in all compartments in senior athletes but maintained basal levels in young athletes. This picture is compatible with polyphenol use as an antioxidant[34] to counteract higher exercise-induced oxidative damage in senior rather than young athletes.

Effects on plasma and erythrocytes lipid profile

Functional beverage dietary supplementation changed plasma fatty acid composition, increasing polyunsaturated and lowering saturated fatty acids. Dietary fatty acid consumption influences human plasma fatty acid profiles.[19] The plasma fatty acid profile of asymptomatic men and women with moderate hypercholesterolemia changed after supplementation with a Mediterranean-type diet for four weeks, increasing plasma MUFA and PUFA.[35] Despite the effects of dietary supplementation on the lipid profile, there is no correlation between the amount of individual fatty acids supplemented with the functional beverage and the increased individual fatty acid concentration in plasma, probably due to the selective use and purpose of dietary fatty acids for oxidation or phospholipid or triacylglyceride incorporation.[36] In addition, the response of the plasma fatty acid profile to dietary functional beverage supplementation is age-dependent; senior group plasma fatty acids were more polyunsaturated and less monounsaturated than in the young group. It has been pointed out that plasma non-esterified SFA, PUFA and MUFA increase with age; senior people have more plasma non-esterified DHA than young people.[36] The different plasma fatty acid profile is most likely to be a consequence of altered cellular uptake, metabolism and biosynthesis associated with ageing. In fact, it has been confirmed that methylation of the fatty acid elongase 2 gene is altered during ageing[37] and delta 6 desaturase activity also declines with age.[36]

Dietary supplementation influenced the erythrocyte fatty acid profile in a different way than the plasma fatty acid profile. The supplementation only altered the percentage of C18:3 and DHA in the erythrocyte membranes. The dietary fatty acids are incorporated at a different rate into different cellular and molecular pools;[20] a selective process has to occur in the incorporation of dietary fatty acids into erythrocytes. The increased DHA content in erythrocytes after dietary functional beverage supplementation is similar to what has been observed in other studies.[20] This increase indicates that athletes followed the prescribed beverage intake during the trial and it was effective in incorporating C18:3 and DHA into erythrocytes. The fatty acid composition of erythrocyte membranes is slightly age-dependent because the senior group had a higher proportion of C20:3 and C22:0 than the younger group, although these fatty acids are in a very low proportion in the erythrocyte membrane and the difference disappears after dietary supplementation with the beverage.

Effects on oxidative damage and antioxidant status

Dietary functional beverage supplementation for one month makes erythrocytes and plasma fatty acids more unsaturated and they could be more susceptible to oxidation. However, lipid oxidation measured as plasma MDA (a product of unsaturated fatty acid peroxidation) reported lower levels in supplemented than in control groups. Previous dietary supplementation trials with this functional beverage analysing lipid oxidative damage in lymphocytes[18] or consuming normal or high polyphenol concentrations in orange juice are in line with a protective role of the supplement against lipid peroxidation.[21] The protective direct or indirect antioxidant effects of functional beverage dietary supplementation could be attributed to DHA, vitamin E or polyphenol.[38,39] However, the DHA dietary supplementation for eight weeks did not alter the basal plasma and erythrocyte MDA levels in trained footballers.[20] It reinforces the role of vitamin E and polyphenols as antioxidants in the functional beverage. In fact, supplementing the diet with vitamins E and C reduces oxidative damage markers in exercising athletes.[8] Similarly, it has been stated that the high polyphenol content of orange juice is responsible for protection against DNA damage and lipid peroxidation, modifying several antioxidant enzymes in overweight or obese non-smoking adults.[21] In our study, the physical test was performed under fasting conditions and the functional beverage consumption had no effect on basal plasma polyphenol levels but did lower lipid oxidative damage.

Dietary supplementation protected plasma proteins against oxidative damage induced by both acute exercise and age. Senior athletes are more susceptible to plasma protein oxidative damage than younger athletes are, in accordance with several studies that demonstrated the existence of a positive correlation between age and protein-carbonyl levels.[34] Similarly, acute exercise damaged plasma proteins, according to another study,[40] but the dietary consumption of the functional beverage avoided protein oxidation. Protein carbonylation occurs with the reaction between MDA or glyoxal and the amino groups of proteins;[41] one could speculate that functional beverage dietary supplementation reduces MDA or glyoxal production or their reaction with plasma proteins induced by acute exercise and ageing.

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Erythrocytes are more protected than plasma from oxidative damage because acute exercise increases protein oxidative damage markers in the latter but not in the former. Whereas plasma is more protected than erythrocytes from the nitrative damage of protein, exercise increased nitrative protein damage in the erythrocytes of senior athletes but not in young athletes, irrespective of functional beverage supplementation. Acute exercise enhances nitric oxide and superoxide anion production, and nitrite levels in erythrocytes;[10] protein nitration results from the reaction between protein-tyrosine and peroxynitrite (a product of the reaction between nitric oxide and a superoxide anion). In this sense, the nitrotyrosine index could reflect peroxynitrite production. This suggests that the senior group has a lower erythrocyte capacity for avoiding peroxynitrite production than the younger group during exercise. This situation was not affected by the dietary supplementation. However, dietary functional beverage supplementation influences the nitrative damage of plasma proteins in an age-dependent way. Whereas young athletes presented a lower plasma nitrotyrosine index than senior athletes after acute exercise, these differences disappeared after dietary functional beverage supplementation. In this sense, functional beverage consumption could enhance the nitrative damage of plasma proteins induced by an exercise test in young athletes. One can speculate that dietary functional beverage supplementation enhances peroxynitrite formation in young athletes during exercise. Growing evidence shows that dietary polyphenol enhances endothelial synthesis and the plasma levels of nitric oxide, and reduces systolic and diastolic blood pressure protecting the heart and blood cells.[10] The nitrative damage of plasma proteins in young athletes during exercise and after dietary supplementation with the functional beverage could reflect the higher plasma nitric oxide and superoxide anion production induced by exercise in a situation of higher polyphenol intake.

Effects on antioxidant and mitochondrial turnover gene expression

The suitability of supplementing the diet of athletes with antioxidants such as vitamins C and E or polyphenols in order to ameliorate physical adaptations to training has been scarcely questioned.[6,7,9] Although our results did not show differences in antioxidant gene expression, this fact could be explained because our experimental models are highly trained athletes who are adapted to high intensity training seasons. Furthermore, the intake of the functional beverage with antioxidants could eliminate the production of ROS, blocking their activity as cellular messengers for important cellular functions related to antioxidant defences, cellular proliferation and differentiation. However, there exist studies where variations in antioxidant gene expression on PBMCs due to exercise and supplementation are observed.[42,43] In a similar way, we did not observe significant changes in mitochondrial turnover gene expression in PBMCs due to exercise or supplementation beverage, however there exist studies where these effects were evidenced, but not in PBMCs.[44,45] Neither acute exercise nor age or functional beverage diet supplementation influenced the expression of mitochondrial turnover and antioxidant genes. However, our results pointed to an enhanced role of dietary functional beverage supplementation rich in antioxidants in the gene expression of antioxidant enzymes induced by exercise, albeit age-dependent. The gene expression of PBMC glutathione-dependent antioxidant enzymes (GPX and GRd) and antioxidant enzymes eliminating a superoxide anion and hydrogen peroxide (Mn-SOD, Cu–Zn SOD, and CAT) is enhanced after acute exercise only in the young group supplemented with the dietary functional beverage. Moderate antioxidant supplementation prevents neutrophil protein oxidation without affecting the adaptive response to exercise.[8] Dietary functional beverage supplementation avoids plasma oxidative damage and enhances adaptive PBMC antioxidant response to exercise in young athletes but does not affect senior athletes.

Conclusion

In summary, the almond- and olive oil-based, DHA and vitamin E-enriched beverage enables dietary supplementation mainly with monounsaturated and polyunsaturated fatty acids, including DHA, polyphenols and vitamin E. The dietary functional beverage supplementation for one month did not alter the performance parameters such as heat storage, body and skin temperature, blood lactate and the Borg scale for fatigue during a maximal exercise test with regard to a control non-supplemented situation. The supplementation did not influence the basal plasma and blood levels of total polyphenols but in senior athletes did increase the basal total polyphenol concentration in blood cells. Senior athletes have higher basal polyphenol levels than young athletes in blood plasma and in blood cells, but in the latter instance only after dietary supplementation with the functional beverage. The functional beverage changed the plasma fatty acid composition with higher individual polyunsaturated and lower individual saturated fatty acid levels after one month of dietary supplementation. These changes are age-dependent as the senior group plasma fatty acids were less monounsaturated than in the young group. The functional beverage only increased the percentage of both C18:3 and DHA in erythrocytes in contrast to the changes observed in plasma. Dietary functional beverage supplementation for one month protected plasma lipid oxidative damage, although it can enhance nitrative damage in young athlete erythrocytes after exercise. The gene expression of PBMC antioxidant enzymes was enhanced after acute exercise only in the young group supplemented with the functional beverage. Dietary functional beverage supplementation evaded plasma oxidative damage and enhanced the adaptive PBMC antioxidant response to exercise in young athletes but had no effect for senior athletes.

Conflict of interest disclosure

The authors declare no conflict of interest.

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Manuscript III

Docosahexaenoic acid diet supplementation attenuates the peripheral mononuclear cell inflammatory response to exercise following LPS activation

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Docosahexanoic acid diet supplementation attenuates the peripheral mononuclear cell inflammatory response to exercise following LPS activation

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ABSTRACT

Exercise induces changes in circulating pro- and anti-inflammatory cytokines. The aim was to investigate the effect of docosahexaenoic acid (DHA) diet supplementation on the plasma cytokine levels and on the peripheral mononuclear (PBMCs) cells cytokine production after a training season or an acute bout of exercise. Fifteen male soccer players were randomly assigned to a placebo or an experimental group. The experimental group consumed an almond-based beverage enriched with DHA, whereas the placebo group consumed the same beverage without DHA. Three blood samples were taken: in basal conditions at the beginning of the nutritional intervention and after eight weeks of training season in basal and post-exercise conditions. The DHA content increased in erythrocytes after 8 weeks of training and supplementation. Neither diet supplementation with DHA nor training season altered the basal plasma cytokines and growth factors. Only acute exercise significantly increased plasma IL6 in experimental and placebo groups. Lipopolysaccharide (LPS) activation induced the inflammatory response in PBMCs, with a significant production rate of TNFα, IL6 and IL8 mainly after acute exercise. DHA supplementation significantly reduced the rate of TNFα and IL6 production by stimulated PBMCs. Acute exercise increased the Toll-like receptor 4 (TLR4) protein levels in PBMCs, although the increase was only statistically significant in the placebo group. In conclusion, a training season does not induce significant changes in the circulating cytokine profile in well-trained soccer players. Exercise increases the PBMCs cell capabilities to produce cytokines after TLR4 stimulation with LPS and this rate of cytokine production is attenuated by diet DHA supplementation.

1. Introduction

Regular exercise exerts a protective effect against diseases associated with chronic inflammation [1]. Acute and chronic exercise alters the number and function of circulating cells of the immune system [2] and also induces a cytokine response with a role that not only controls exercise-induced inflammation, but also regulates the availability of metabolites for muscle contraction [3]. The prophylactic activity of regular exercise has been partially attributed to anti-inflammatory and antioxidant effects which are mediated by a reduction in visceral fat mass and by an induction of an anti-inflammatory environment after the exercise bouts [4]. However, severe and excessive physical activity provokes a situation analogous to the clinical sepsis, with tissue damage resulting from an excessive inflammatory reaction [5]. The anti-inflammatory effects of exercise could be partially related to muscle cytokine production profile induced by muscular contraction [6]; in fact, the appearance of IL6 in the circulation precedes other cytokines, such as TNF-α, and is the cytokine showing the most marked increase after exercise [3,6–8]. Moreover, some cytokines and other peptides are generated, expressed and released by muscle fibers exerting paracrine or endocrine effects on fat and glucose metabolism [6]. Cytokines secreted by contracting muscle, like IL6, have direct anti-inflammatory effects or stimulate immune cell production of anti-inflammatory components such as the IL1-receptor antagonist and IL10 [2,9]. It has been shown that a soccer match caused an increase in a large number of both pro- and anti-inflammatory cytokines and inflammatory cells in females [10].

Several studies have focused on the effect of various nutritional interventions on inflammatory and anti-inflammatory patterns

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associated with football practice [11,12]. It has been reported that some essential nutrients such as omega-3 fatty acids also influence cytokine production and inflammation in some pathological situations in which a chronic inflammation is present [13–16]. However, diet supplementation with omega-3 fatty acids does not affect the plasma cytokine response to strenuous exercise [17], although a previous study reported that fish oil supplementation reduces the increase in peripheral blood mononuclear cells (PBMCs) IL2 production three hours after exercise but has no effects on IL4 or IFNγ production [18]. Moreover, some results from animal studies reported that omega-3 fatty acid-rich oils reduce the production of pro-inflammatory cytokines in response to endotoxin [19]. The *ex vivo* production of TNFα, IL1, IL2 and IL6 are altered in animals fed with food supplements of oils rich in omega-3 fatty acids [20,21]. Diet supplementation of healthy volunteers with fish oils results in a reduction of *in vitro* production of IL1, IL6, TNFα and IL2 by PBMCs [22]. These contradictory observations could be attributed to the different sources of cytokines available during exercise, the presence of chronic inflammation and also due to a specific pathway involved in inducing the cytokine release. Cytokines released during exercise are mainly produced and secreted by the muscle, whereas the cytokines released during chronic inflammatory diseases are derived from activated immune cells [23]. However, both acute and chronic exercise alters the number and function of circulating cells of the innate immune system [2] and the omega 3 diet supplementation could exert additional anti-inflammatory effects in response to acute or chronic exercise. The main route of synthesis of the cytokines in immune cells is NFκβ-dependant, which is also connected with the function of toll-like receptors (TLRs) [24]. The immune system, in addition to detecting microbial products, can recognize endogenous molecules released following cell injury and/or death, named endogenous "danger" molecules (DAMPs) which also induce inflammation via toll-like receptor activation. The omega 3 fatty acids or their derivates can interact with these pathways by regulating NFκβ activation. Although the mechanism of omega 3 fatty acids action is still not completely defined, recent evidence suggest that these fatty acids alter immune cell membrane lipid microdomain properties inhibiting the dimerization and recruitment of TLR4 [25]. Consequently, omega 3 fatty acids downregulate the expression of target gene products of the TLR4 pathway such as co-stimulatory molecules and cytokines [26].

We hypothesized that exercise induces an activation of the capabilities of peripheral mononuclear cells (PBMCs) to produce cytokines against stimuli like DAMPs that could be modulated by the consumption of foods rich in omega-3 fatty acids. Moreover, most of studies on omega-3 supplementation used a mixture of DHA and EPA or fish oils. The observed results could be a consequence of the different proportion of the fatty acids or other components included in the fish oil. In this way, we decided to evaluate the effects of only one fatty acid incorporated in a functional food to facilitate the interpretation of results. The aims of the present study were to analyze the effects of docosahexanoic acid (DHA) diet supplementation on the circulating levels of multiple related cytokines in response to acute and chronic exercise, and to evaluate the effects of diet supplementation with DHA, on the capabilities of PBMCs to produce cytokines as results of the TLRs stimulation by LPS after acute exercise or a training season.

2. Materials and methods

2.1. Subjects and anthropometric characteristics

Fifteen male soccer players from the Real Mallorca B team volunteered to participate in this study. At the beginning of the study, 22 subjects were recruited and divided in two groups of 11 players each. However, 6 of the players left the football team during the experimental time to go play with the first and professional team and one broke the anterior cruciate ligament of the knee. 6 subjects took one liter of a placebo beverage five times a week and the other 9 subjects were given an experimental beverage rich in DHA also five times a week, for a period of 8 weeks. All subjects gave their written informed consent after an explanation of the experimental procedures and before commencement of the study. The study protocol was in accordance with the Declaration of Helsinki for research on human subjects and was approved by the Ethical Committee of Clinical Investigation of the CAIB (Palma de Mallorca, Balearic Islands, Spain).

DHA supplementation was administered to the athletes using an almond-based isotonic (278 mOsm/kg) beverage. The almond beverage drink was a carbohydrate–electrolyte solution with almond and orange juice in its recipe. It contained 203 kJ/100 ml, 1.9% lipids, 6.8% total sugars, 1.0% proteins, 1.8 mg/100 ml calcium, 4.2 mg/100 ml magnesium, 12.8 mg/100 ml sodium, 33.7 mg/100 ml potassium, 69.7 mg/100 ml iron, and 44.5 mg/100 ml. The placebo beverage was supplemented with 0.8% of olive oil and the experimental beverage with 0.6% of olive oil plus 0.2% of DHA-S Market (Market Biosciences Corporation, Columbia, USA) rich in C22:6 and C22:5. The two almond drinks were elaborated by *Liquats Vegetals S.A.* and was obtained by the following procedure: almonds were bleached, then crushed in water and the mixture was centrifuged to remove the insoluble materials. Natural cinnamon and lemon flavors, sucrose, and the respective oil for the experimental (olive oil plus DHA) or placebo (olive oil) were then added. Finally, the beverage were sterilized and packed. Externally the two beverage types were identical in taste and appearance. The fatty acid composition (%) and vitamin E content of the beverages are given in Table 2.

Height was determined using a mobile anthropometer (Kawe 44444, Asperg, Germany) to the nearest millimeter, measuring the subject's head held at the Frankfurt plane. Body weight was determined to the nearest 100 g using a digital scale (Tefal, sc9210, Rumilly, France). The subjects were weighed in bare feet and light underwear. Waist and hip perimeters were measured to the nearest 0.1 cm, using a non-stretchable measuring tape (KaWe, 43972, France). Triceps, subscapular, biceps, iliac crest, supraspinal, abdominal, thigh and leg skinfold thickness were measured using a Holtain skinfold caliper (Tanner/Whitehouse, Crosswell, Crymych, UK), and the mean of three measurements was use [27]. The subjects were asked to stand erect in a relaxed position with both feet placed together on a flat surface. The different anthropometric indexes were calculated using these measurements: body mass index [BMI = mass (kg)/squared height (m)]; waist-hip index [waist perimeter (cm)/hip perimeter (cm)]; fat free mass [FFM = 100 − BF]. Body fat (BF) was measured from skinfold thickness according to Carter-Yuhasz equation [28]. All anthropometric measurements were performed by one observer to avoid inter-observer variation.

2.2. Dietary intake

Dietary habits of subjects were assessed using a 7-day dietary record questionnaire completed at the beginning of the study and in the week before the exercise test. A qualified dietician verified and quantified the food records. All food items consumed were transformed into nutrients using a special computerized program based on the European and Spanish food composition tables [29]. The consumed DHA diet was calculated from the fish consumption taken into account the high or low DHA content of different fish types.

2.3. Experimental procedure

For each subject, three different blood samples were obtained. One blood sample was taken in basal conditions after overnight fasting at the beginning of the nutritional intervention. Another two blood samples were taken at the end of the nutritional intervention, in basal conditions and after performing a soccer training session. After the sample of the training day in basal conditions, the players ingested a half liter of the corresponding beverage. The exercise consisted of a 2 h habitual physical training session. After a 15 min warm-up, the players performed the Leger Boucher test to indirectly determine the VO_2max. After that, they practiced a recovery exercise of control-passing for 15 min. The main body of the training session was characterized by small-sided games. Briefly, the first exercise consists of a 5 vs 5 possession exercise in an area of 20 × 15 m (4 repetitions of 5 min with 30 s of recovery between repetitions); the second was a 6 vs 6 possession exercise in an area of 30 × 20 m (3 repetitions of 6 min with 1 min of recovery between repetitions), and finally, the players played a soccer match 5 vs 5 in 30 × 40 m during 20 min. The exercise was designed to allow players to work more than 50% of the match above their 80% VO_2max, ensuring that in these conditions the exercise caused oxidative stress. The post-exercise sample was taken 2 h after the exercise because is coincident with a significant increment in circulating neutrophils and in pro- and anti-oxidant markers [30,31].

Blood samples were centrifuged at 900*g* at 4 °C for 30 min and the plasma was recovered. The erythrocyte phase at the bottom was washed with PBS, centrifuged as above and finally the erythrocytes were reconstituted with distilled water. The PMN cell fraction was purified from whole blood following an adaptation of the method described by Boyum [32,33] using Ficoll-Paque PLUS reagent (GE Healthcare, Chalfont St Giles, UK). An aliquot of PMN cells was used for LPS incubation and another aliquot was treated with RIPA lysis buffer for Western blotting analyses.

The whole blood aliquot was analyzed in an automatic flow cytometer analyzer Technicon H2 (Bayer) VCS system to determine cell counts.

2.4. Erythrocyte fatty acid determination

The extraction of fatty acids from the erythrocyte samples was done by a modification of the Folch method [34]. Briefly, 250 μL of erythrocyte samples were diluted in 5 mL of Cl3CH:CH3OH (2:1, v/v) BHA 0.01% containing 20 μL of n-heptadecanoic acid (15 mM) as internal standard. Then 1 mL NaCl 0.9% was added to form two phases, and the aqueous phase was eliminated. Methanol was added to the organic phase to reform the initial 5 mL, then 1 mL NaCl 0.9% was added to form two phases and the aqueous phase was again discarded. The organic phase was cleaned three times with Cl3CH:CH3OH:H2O (3:48:47, v/v/v). Methanol was added again until the initial 5 mL was recovered and it finally washed with NaCl 0.9% and the aqueous phase was discarded. The organic phase was evaporated under a nitrogen stream at 55 °C. The dry residue was dissolved in 225 μL of n-hexane and 25 μL of the derivatization reagent Meth-Prep™ II (GRACE), and 1 μL was injected into the gas chromatograph. The mobile phase consisted of helium. The gas chromatograph was a Agillent 5890 model with a flame ionization detector (FID) and the column was a Supelcowax® 10 Capillary GC column, 30 m × 0.53 mm, df 0.50 μm. For the identification of chromatography peaks individual fatty acid and mix of fatty acid methyl ester (Supelco®) standards were used.

Quantification of all fatty acids was done from the internal standard (C17:0) and was corrected by a response factor calculated from the areas of the fatty acids from different chromatograms with different fatty acid concentrations.

2.5. PBMCs incubation with LPS

Incubation of PMN cells in the presence of the bacterial stimuli was performed in RPMI 1640 culture media containing 2 mM L-glutamine. PMN cells obtained from the subjects after the 8-week beverage supplementation in basal conditions and after exercise were diluted with the culture media to 2×10^6 cells/ml and were activated by addition of the bacterial stimuli LPS from *Escherichia coli* (1 μg/ml). The samples were incubated in polypropylene tubes at 37 °C for 2 h. Then, after shaking, the cells were pelleted by centrifugation (900×*g*, 5 min, 4 °C) and the cell-free supernatants were stored at −70 °C for cytokine determinations.

2.6. Cytokine determination

The plasma cytokine determinations were performed using the Randox Biochip Array technology for simultaneous quantitative detection of multiple analytes from a single sample. The core technology is the Randox Biochip, a solid-state device containing an array of discrete test regions of immobilized antibodies specific to different cytokines and growth factors. A sandwich chemiluminescent immunoassay was employed for the cytokine array. Increased levels of cytokine in a specimen will lead to increased binding of antibody labeled with horseradish peroxidase and thus, an increase in the chemiluminescent signal was emitted. The light signal generated from each of the test regions on the biochip was detected using digital imaging technology and compared to that

Table 1
Subject's anthropometric characteristics and physical activity time.

	Placebo		Experimental	
	Mean	SEM	Mean	SEM
Age (years)	19.3	0.4	20.4	0.5
Weight (kg)	76.5	1.8	76.4	3.5
Height (cm)	179	2	180	3
Waist circumference (cm)	78.2	0.8	78.5	1.1
Hip circumference (cm)	97.0	1.0	96.6	1.4
Systolic blood pressure (mmHg)	117	8	122	3
Diastolic blood pressure (mmHg)	56.7	5.9	66.7	3.5
Body mass index (BMI, kg/m^2)	24.0	0.6	23.5	0.5
Waist-hip ratio (WHR)	0.805	0.012	0.814	0.012
Fat mass (Yuhasz, %)	7.53	0.24	7.21	0.25
Fat-free mass (%)	92.5	0.2	92.8	0.3
Intense physical activity time (min/day)	96.4	57.9	50.4	13.1
Moderate physical activity time (min/day)	68.6	17.1	63.2	14.6

Statistical analysis: Student's *t*-test for unpaired data, $p < 0.05$.
* No significant differences between placebo and experimental groups were found.

Table 2
Beverages composition.

	Placebo		Experimental	
	Mean	SEM	Mean	SEM
C16:0 (%)	9.35	1.34	7.62	0.97
C16:1 (%)	1.45	0.23	1.20	0.14
C18:0 (%)	6.50	1.31	4.24	0.70
C18:1 (%)	49.5	3.5	42.5	4.8
C18:2 (%)	26.3	0.7	22.9	2.5
C18:3n6 (%)	0.778	0.321	0.949	0.550
C18:3n3 (%)	2.25	0.46	1.21	0.35
C20:0 (%)	0.653	0.158	0.368	0.105
C20:1 (%)	0.136	0.033	0.113	0.032
C20:2 (%)	1.93	0.47	0.884	0.252
C20:3 (%)	ND		0.065	0.019*
C20:4n6 (%)	1.11	0.27	0.434	0.124*
C22:0 (%)	ND		0.262	0.075*
C22:5 (%)	ND		5.68	1.62*
C22:6n3 (%)	ND		11.6	3.3*
Vitamin E (mg/L)	41.6	±17.8	45.7	27.7

Statistical analysis: Student's *t*-test for unpaired data, $p < 0.05$.
* Significant differences between placebo and experimental groups.

from a stored calibration curve. The concentration of cytokines present in the sample was calculated from the calibration curve. The determinations were made following the manufacturer's instructions. Two Randox Biochips were used: the Cytokine Array III and The Cytokine & Growth Factors Array.

The cytokines determined using the Randox Biochip Array technology were: Interleukin-5 (IL5), Interleukin-15 (IL15), Tumor Necrosis Factor-beta (TNFβ), Granulocyte Macrophage Colony-Stimulating Factor (GMCSF), Macrophage Inflammatory Protein-1α (MIP1α), Interleukin-1α (IL1α), Interleukin-1β (IL1β), Interleukin-2 (IL2), Interleukin-4 (IL4), Interleukin-6 (IL6), Interleukin-8 (IL8), Interleukin-10 (IL10), Vascular Endothelial Growth Factor (VEGF), Interferon γ (IFNγ), Epidermal Growth Factor (EGF), Monocyte Chemotactic Protein-1 (MCP1) and Tumor Necrosis Factor-alpha (TNFα).

IL8 and TNFα from supernatants were determined using individual ELISA kits because of the high PBMN cells production levels of these cytokines overranged the calibration curve of the Biochip array. IL8 production in lymphocytes after activation with LPS was determined by the Human IL8 ELISA kit for RAyBio following the manual instructions. The intra-assay and the inter-assay reproducibility were lower than 10% and 12%, respectively. TNFα was determined using the Diaclone TNFα ELISA kit for GEN-PROBE following its instructions for use. The overall intra-assay coefficient of variation has been calculated to be 3.3%; the calculated overall inter-assay coefficient of variation was 9.0%. IL6 from supernatants was determined with the Randox Biochip Array technology.

2.7. Western blot analysis

Toll-Like Receptor (TLR) 4 protein levels were determined in lymphocytes by Western blot. Protein extracts were analyzed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE). Total protein concentrations were measured by the method of Bradford [35]. 80 μg of total protein were loaded on a 12% agarose gel. Following electrophoresis, samples were transferred onto a nitrocellulose membrane and incubated with a primary monoclonal anti-TLR4

Table 3
Lipid composition of erythrocytes.

		Initial		Final		ANOVA		
		Mean	SEM	Mean	SEM	*S*	*T*	*S* × *T*
18:0 (%)	Placebo	30.2	1.3	26.2	1.1		X	
	Experimental	32.7	4.0	27.1	0.8#			
18:1 (%)	Placebo	10.3	0.4	10.3	0.2			
	Experimental	10.0	0.2	10.0	0.2			
18:2 (%)	Placebo	12.7	0.3	13.7	0.6	X		
	Experimental	11.1	0.7*	12.1	0.4*			
18:3n6 (%)	Placebo	1.09	0.08	1.09	0.15			
	Experimental	1.06	0.11	1.08	0.08			
18:3n3 (%)	Placebo	3.13	0.38	2.89	0.50			
	Experimental	3.33	0.24	3.45	0.48			
20:0 (%)	Placebo	0.450	0.061	0.332	0.028			
	Experimental	0.498	0.065	0.452	0.079			
20:1 (%)	Placebo	0.797	0.031	0.857	0.052			
	Experimental	0.677	0.025	0.806	0.074			
20:2 (%)	Placebo	1.27	0.07	1.00	0.06#		X	
	Experimental	1.18	0.07	1.07	0.05			
20:3 (%)	Placebo	2.03	0.20	2.16	0.22	X		
	Experimental	1.42	0.14*	1.46	0.10*			
20:4n6 (%)	Placebo	29.0	0.2	31.0	0.5			
	Experimental	27.6	3.2	29.7	0.6			
22:0 (%)	Placebo	0.209	0.018	0.299	0.025	X		
	Experimental	0.393	0.073*	0.381	0.052			
22:5 (%)	Placebo	1.69	0.10	1.90	0.20			
	Experimental	1.50	0.15	1.76	0.22			
22:6n3 (%)	Placebo	7.21	0.26	8.23	0.40	X	X	
	Experimental	8.48	0.58	10.6	0.5*,#			

Statistical analysis: Two-way ANOVA, $p < 0.05$. (*S*) Significant effect of supplementation, (*T*) significant effect of time, ($S \times T$) significant interaction between both factors. One-Way ANOVA, $p < 0.05$.
* Significant differences between placebo and experimental groups.
# Significant differences between initial and final training period.

Table 4
Effects of training season, acute exercise and DHA supplementation on peripheral mononuclear cell counts.

		Initial		Final		ANOVA		
		Mean	SEM	Mean	SEM	*S*	*T*	*S* × *T*
A								
PMNCs (10^3 cells/μL)	Placebo	2.79	0.16	3.34	0.15			
	Experimental	3.23	0.29	3.77	0.20			
Lymphocytes (%)	Placebo	71.2	6.2	74.3	5.4			
	Experimental	77.1	5.3	77.7	8.3			
Monocytes (%)	Placebo	17.2	1.93	14.9	1.49			
	Experimental	13.3	1.24	12.8	0.88			
Eosinophils (%)	Placebo	8.24	2.87	8.38	2.09			
	Experimental	8.36	3.72	7.27	2.08			
Basophils (%)	Placebo	1.14	0.18	2.16	0.36#		X	
	Experimental	0.99	0.28	2.26	0.25#			
		Basal		Post-exercise		*S*	*E*	*S* × *E*
		Mean	SEM	Mean	SEM			
B								
PMNCs (10^3 cells/μL)	Placebo	3.34	0.15	3.19	0.15		X	
	Experimental	3.77	0.20	3.13	0.16#			
Lymphocytes (%)	Placebo	74.3	5.4	69.3	4.38#		X	
	Experimental	77.7	8.27	68.5	4.47#			
Monocytes (%)	Placebo	14.9	1.49	24.1	1.56#		X	
	Experimental	12.8	0.88	23.6	1.59#			
Eosinophils (%)	Placebo	8.38	2.09	5.33	1.56			
	Experimental	7.27	2.08	5.11	1.55			
Basophils (%)	Placebo	2.16	0.36	1.32	0.25#		X	
	Experimental	2.26	0.25	1.57	0.32#			

(A) Effects of training season and DHA diet supplementation determined in basal conditions and (B) effects of acute exercise and diet supplementation determined in basal and post-exercise conditions. Statistical analysis: Two-way ANOVA, $p < 0.05$. (*S*) Significant effect of supplementation, (*T*) significant effect of time, (*S* × *T*) significant interaction between supplementation and time, (*E*) significant effect of exercise, (*S* × *E*) significant interaction between supplementation and exercise. One-way ANOVA, $p < 0.05$.
* Significant differences between placebo and experimental groups.
# Significant differences between initial and final training period or significant differences between basal and post-exercise.

antibody (Santa Cruz Biotechnology) and a secondary anti-mouse IgG peroxidase-conjugated antibody. Protein bands were visualized by Immun-Star® Western C® Kit reagent (Bio-Rad Laboratories) Western blotting detection systems. The chemiluminiscence signal was captured with a Chemidoc XRS densitometer (Bio-Rad Laboratories) and analyzed with Quantity One-1D Software (Bio-Rad Laboratories).

2.8. Statistical analysis

The sample size calculation was performed taking into account a statistical power of 80% and, the DHA enrichment of the erythrocyte membranes was estimated as 25% for the experimental group. The required sample size was estimated in 7 subjects each group. We estimated that only 75% of subjects that begin the study will finish it and, consequently the recruitment of 20 subjects will be enough to fulfill the minimal requirement of sample size.

Statistical analysis was carried out using Statistical Package for Social Sciences (SPSS v.18.0 for Windows). Results are expressed as mean ± SEM and $P < 0.05$ was considered statistically significant. A Kolmogorov–Smirnov test was applied to assess the normal distribution of the data. The statistical significance of the data was assessed by two-way analysis of variance (ANOVA). The statistical factors analyzed were beverage supplementation (*S*) and exercise (*E*). The sets of data in which there was a significant $S \times E$ interaction were tested by the ANOVA one-way test. When significant effects of *S* or *E* factor were found, a Student's *t* test for paired data was used to determine the differences between the groups involved.

3. Results

No significant differences were reported between both placebo and supplemented soccer players characteristics (Table 1). No statistical differences exist between placebo and experimental groups in the nutrient intake at the beginning of the training season (data not shown). The DHA consumption of soccer players by the diet ranged from 68.5 mg/day to 150 mg/day according to the 7-day dietary record questionnaire analysis.

The percentage of fatty acid and vitamin E beverage contents are reported in Table 2. The experimental beverage had a significantly higher content of C20:3, C20:4n6, C22:0, C22:5 and C22:6n3 compared to the placebo beverage. No significant differences between beverages were evidenced in vitamin E content. The five times a week intake of the 1L of the respective beverage supposed a daily intake of about 1.14 g of DHA additional to the basal DHA intake by the diet in the experimental group, whereas the placebo only took up the DHA from the diet.

Fatty acid composition of erythrocytes before and after diet supplementation with omega 3 is presented in Table 3. A training period of 8 weeks induced a decrease in the percentage of 18:0 and 22:2 of erythrocyte membranes, although this decrease was only significant in the experimental group for 18:0 and in the placebo group for 22:2. The percentage of 22:6 was increased after 8-weeks of training and supplementation and this increase was significantly higher in the supplemented group (a 36% increase) respect to the placebo group (a 14% increase).

The effects of DHA diet supplementation, the training season and the acute exercise on blood cell counts are shown in Table 4.

Table 5
Effects of training season and diet supplementation with DHA on cytokine and growth factors plasma levels.

		Initial		Final		ANOVA		
		SEM	Mean	Mean	SEM	*T*	*S*	*T* × *S*
IL2 (μg/L)	Placebo	0.708	0.121	0.986	0.234			
	Experimental	0.920	0.110	0.800	0.116			
IL4 (μg/L)	Placebo	1.05	0.06	0.971	0.064			
	Experimental	1.08	0.08	1.17	0.11			
IL6 (μg/L)	Placebo	0.558	0.181	0.427	0.050			
	Experimental	0.321	0.082	0.404	0.097			
IL8 (μg/L)	Placebo	1.17	0.17	2.80	1.46			
	Experimental	1.22	0.22	1.51	0.27			
IL10 (μg/L)	Placebo	0.387	0.124	0.301	0.052			
	Experimental	0.300	0.043	0.384	0.045			
VEGF (μg/L)	Placebo	5.53	0.44	6.50	0.88			
	Experimental	8.02	2.16	9.25	2.18			
INFγ (μg/L)	Placebo	0.273	0.057	0.341	0.076			
	Experimental	0.280	0.054	0.265	0.042			
TNFα (μg/L)	Placebo	1.12	0.11	1.10	0.08			
	Experimental	1.22	0.29	1.12	0.12			
IL1α (μg/L)	Placebo	0.095	0.022	0.136	0.052			
	Experimental	0.131	0.026	0.116	0.014			
IL1β (μg/L)	Placebo	0.645	0.271	0.623	0.271			
	Experimental	0.740	0.082	0.734	0.136			
MCP1 (μg/L)	Placebo	45.9	7.8	60.5	10.7			
	Experimental	50.5	11.2	52.7	10.9			
EGF (μg/L)	Placebo	0.620	0.138	3.43	0.82			
	Experimental	3.38	2.81	7.50	3.66			
IL5 (μg/L)	Placebo	1.13	0.109	1.23	0.12			
	Experimental	1.11	0.154	1.21	0.25			
IL15 (μg/L)	Placebo	0.558	0.064	0.602	0.065			
	Experimental	0.533	0.050	0.595	0.066			
TNFβ (μg/L)	Placebo	1.50	0.92	1.74	0.67			
	Experimental	1.50	0.50	0.565	0.186			
GMCSF (μg/L)	Placebo	0.607	0.147	0.768	0.213			
	Experimental	0.844	0.360	0.577	0.286			
MIP1α (μg/L)	Placebo	3.39	0.52	3.77	0.58			
	Experimental	4.51	0.85	5.37	0.83			

Statistical analysis: Two-way ANOVA, $p < 0.05$. (*T*) Significant effect of time of training, (S) significant effect of supplementation, ($T \times S$) significant interaction between both factors. One-way ANOVA, $p < 0.05$.
* Significant differences between placebo and experimental groups.
# Significant differences between initial and final training period.

No significant effects on PBMCs counts were reported by the training season or by the DHA diet supplementation. The training period only reported a significant increase in the percentage of basophils in both placebo and experimental groups. Acute exercise significantly influenced the PBMCs counts and the contribution of the different cell types to this pool. The PBMCs counts significantly decreased in the experimental group after acute exercise, whereas placebo maintained the basal PBMCs counts. These lower PBMCs counts after exercise in the experimental group are attributable to a significant decreased contribution of lymphocytes and basophils and to a significant increase in the contribution of monocytes.

Neither the diet supplementation with DHA nor the training season altered the basal levels of plasma cytokines and growth factors during the nutritional intervention (Table 5). Acute exercise realized after eight weeks consuming the placebo or DHA rich beverages lightly alters the plasma levels of cytokines (Table 6). IL6 was the only cytokine that significantly increased its plasma values 2 h after acute exercise. The plasma levels of IL6 after exercise were 4 times and 4.6 times higher than basal levels in placebo and supplemented groups, respectively. MIP1α levels were significantly higher in the experimental group after exercise.

The capability to synthesize cytokines by PBMCs before and after acute exercise was determined after the PBMCs stimulation with LPS (Fig. 1). LPS induced an inflammatory response in PBMCs with an important rate production of TNFα, IL6 and IL8, which was significantly enhanced after exercise. DHA supplementation significantly reduced the rate of TNFα and IL6 production by LPS-stimulated PBMCs.

Cytokine production by LPS-stimulated PBMCs determined at the beginning and at the end of the training season and nutritional intervention as well as after the end of acute exercise is shown in Fig. 2. The TLR4 protein levels in PBMCs remained at basal conditions after 8 weeks of training sessions and they were similar in placebo and DHA supplemented groups. Acute exercise significantly increased the TLR4 protein levels in PBMCs, although the increase in TLR4 protein levels was only statistically significant in the placebo group.

4. Discussion

Exercise training has systemic anti-inflammatory effects [36–39]; although the local inflammatory response within skeletal

Table 6
Effects of exercise and diet supplementation with DHA on cytokine and growth factors plasma levels.

		Basal		Post-exercise		ANOVA		
		Mean	SEM	Mean	SEM	*E*	*S*	*E* × *S*
IL2 (μg/L)	Placebo	0.986	0.234	0.883	0.249			
	Experimental	0.800	0.116	0.792	0.155			
IL4 (μg/L)	Placebo	0.971	0.064	1.00	0.058			
	Experimental	1.17	0.11	1.13	0.17			
IL6 (μg/L)	Placebo	0.427	0.050	1.69	0.88[#]	X		
	Experimental	0.404	0.097	1.85	0.63[#]			
IL8 (μg/L)	Placebo	2.80	1.46	2.11	0.97			
	Experimental	1.51	0.27	2.25	0.85			
IL10 (μg/L)	Placebo	0.301	0.052	0.409	0.125			
	Experimental	0.384	0.045	0.464	0.086			
VEGF (μg/L)	Placebo	6.50	0.88	5.20	0.73			
	Experimental	9.25	2.18	7.81	2.47			
INFγ (μg/L)	Placebo	0.341	0.076	0.256	0.045			
	Experimental	0.265	0.042	0.271	0.052			
TNFα (μg/L)	Placebo	1.10	0.08	1.26	0.14			
	Experimental	1.12	0.12	1.16	0.15			
IL1α (μg/L)	Placebo	0.136	0.052	0.164	0.053			
	Experimental	0.116	0.014	0.151	0.025			
IL1β (μg/L)	Placebo	0.623	0.271	0.804	0.324			
	Experimental	0.734	0.136	0.862	0.131			
MCP1 (μg/L)	Placebo	60.5	10.7	61.2	7.9			
	Experimental	52.7	10.9	58.4	13.3			
EGF (μg/L)	Placebo	3.43	0.82	2.19	0.92			
	Experimental	7.50	3.66	7.49	5.82			
IL5 (μg/L)	Placebo	1.23	0.12	0.908	0.082			
	Experimental	1.21	0.25	1.20	0.14			
IL15 (μg/L)	Placebo	0.602	0.065	0.478	0.034			
	Experimental	0.595	0.066	0.605	0.064			
TNFβ (μg/L)	Placebo	1.74	0.67	1.71	0.79			
	Experimental	0.565	0.186	1.51	0.503			
GMCSF (μg/L)	Placebo	0.768	0.213	0.588	0.386			
	Experimental	0.577	0.286	0.715	0.214			
MIP1α (μg/L)	Placebo	3.77	0.58	2.69	0.34		X	
	Experimental	5.37	0.83	4.69	0.50[*]			

Statistical analysis: Two-way ANOVA, $p < 0.05$. (*E*) Significant effect of exercise, (*S*) significant effect of supplementation, ($E \times S$) significant interaction between both factors. One-way ANOVA, $p < 0.05$.
[*] Significant differences between placebo and experimental groups.
[#] Significant differences between basal and post-exercise.

muscle after eccentric exercise is predominantly pro-inflammatory [40]. It was classically thought that the exercise-induced increase in cytokines was a consequence of an immune response due to local damage in the working muscles [41] and that the immune cells are responsible for this increase, as occurs during sepsis [42]. However, it is nowadays well established that the contracting skeletal muscle *per se* is the main source in some cytokines like IL6 in the circulation in response to exercise [43]. This increase of IL6 levels after acute exercise is independent of DHA diet supplementation, which is in accordance with other studies consuming higher omega 3 doses in exercise-trained men [17,44]. However, it is known that the omega 3 fatty acids have a therapeutical value in some pathologies in which an inflammatory component exists, such as obesity [45]. Similarly, two different studies with dietary omega 3-fatty acid supplementation in non-trained men reported effective results in ameliorating eccentric exercise induced inflammatory markers [46,47]. The main feature of the present results is that exercise also affects the PBMCs capabilities to synthesize cytokines, evidenced when PBMCs were stimulated with LPS. In accordance with our initial hypothesis, the diet supplementation with DHA reduced the rate of cytokine production by LPS-stimulated PBMCs mainly after exercise. The lack of influence of omega 3 fatty acids on the circulating cytokine pattern could be explained since muscle IL6 secretion after exercise comes from a pathway independent of their anti-inflammatory effects or due to the well-training status of the participants. However, the local pro-inflammatory response to tissue damage mediated by immune cells is probably dependent of nuclear translocation of NFκβ and could be influenced by omega 3 fatty acids in a similar way as omega 3 fatty acids reduce colitis inflammation in rats [9,16].

No significant effects were observed by 8-week training or DHA diet supplementation on basal circulating cytokine levels. It was previously reported that regular training has anti-inflammatory effects, although there is a lack of studies performed in well-trained and healthy subjects determining changes in plasma cytokine levels. The present study was performed at the beginning of the soccer competition season, consequently all participants are physically active and in a good health, which could limit the beneficial effects of training on inflammation. Moreover, DHA diet supplementation also showed no effects on any of the parameters analyzed in basal conditions, which is in accordance with previous studies [17,48]. Only in one study EPA/DHA supplementation (2.22 g EPA + 2.20 mg DHA for 6 weeks) was associated with a decrease in resting CRP and TNF-α in well trained men [44]. Our

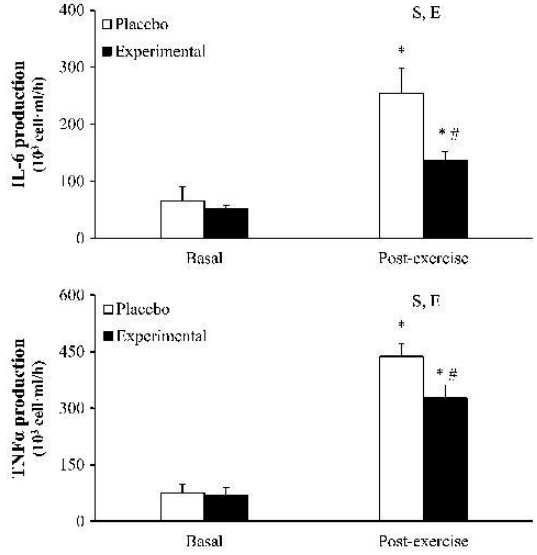

Fig. 1. IL6 and TNFα PBMc production. Statistical analysis: Two-way ANOVA, $p < 0.05$. (E) Significant effect of exercise, (S) significant effect of supplementation, ($E \times S$) significant interaction between both factors. One-way ANOVA, $p < 0.05$. (*) Significant differences between placebo and experimental groups, (#) significant differences between basal and post-exercise.

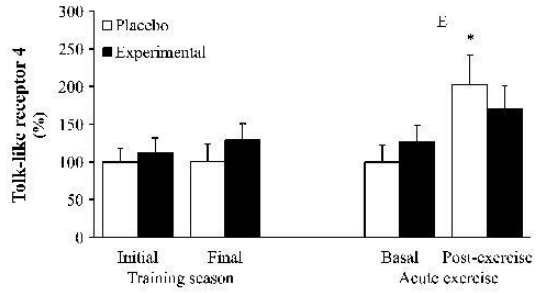

Fig. 2. Effects of training and acute exercise on TLR4 protein levels in PMN cells. Statistical analysis: Two-way ANOVA, $p < 0.05$. (S) Significant effect of supplementation, (T) significant effect of time, ($S \times T$) significant interaction between supplementation and time, (E) significant effect of exercise, ($S \times E$) significant interaction between supplementation and exercise. One-way ANOVA, $p < 0.05$. (*) Significant differences between placebo and experimental groups, (#) significant differences between initial and final training period or significant differences between basal and post-exercise.

study was performed with half of that quantity of DHA, and thus the DHA intake could not have been sufficient enough to induce significant effects in the basal cytokine levels.

Acute exercise performed by soccer players only increased the IL6 levels in plasma, whereas the other determined cytokines and growth factors maintained the pre-exercise values. Muscle in contraction secretes cytokines and their appearance in plasma depends on the duration, intensity of exercise, and time after the cessation of exercise [49]. IL8 and IL15 are also secreted by contracting muscle although it is difficult to detect increases in their circulating levels after exercise [50]. Several authors reported increases in IL15 levels after exercise, whereas in other studies its levels were unchanged [43,51]. The discrepancies found with IL15 are probably because this cytokine remains intracellular, localized to non-endoplasmic muscle regions [9]. In the present results, the basal plasma concentration of IL8 and IL15 was maintained 2 h after the cessation of exercise by the soccer players which is in accordance with previous reports [9]. The appearance of IL6 in the circulation is the most evident and precedes the appearance of other cytokines. However, no changes in the circulating levels of anti-inflammatory cytokines like IL10 were observed after the acute exercise performed by the soccer players, although IL6, probably produced by contracting muscle, was increased. Moreover, glycogen depletion as a consequence of muscle function may also play a role in elevating IL6 levels [52]. In fact, the different results obtained could be attributed to different type and intensity of exercise and different sampling time after exercise [51,53]. Strenuous and eccentric exercise increases the concentration of IL8, IL15, MIP1α and MIPβ [50,54,55]; whereas moderate exercise intensity such as bicycle ergometer [56] or rowing [57] did not increase plasma IL8 concentration [56–58].

The effects of omega 3 diet supplementation on the control of cytokines and growth factors production by PBMCs depends on the type of stimulus that activates the cells. Exercise produces some stimuli that could induce the immune cell cytokine production such as the increase of the body central temperature [59], the increase in the endotoxin plasma levels{Hurst, 2009 #128}, an increase of the plasma levels of stress hormones, energy crisis and oxidative stress [60], and the possible appearance of DAMPs [38]. In the current work, the lack of changes in cytokine plasma levels after exercise could indicate that this stimulus was not enough to activate the cytokine production by the PBMCs. LPS, a molecule that mimics the bacterial membrane, interacts with TLR4 to activate an immune response to eliminate the possible bacterial infection [61]; the LPS produces the activation of NFκβ, which is influenced by omega3 fatty acids [16] and we evidenced a clear effect of omega 3 diet supplementation that reduces the cytokine production by PBMCs, mainly after exercise. It was reported that omega 3 fatty acids can interfere with TLR4 activation by LPS inhibiting signaling components downstream from TLR4 but also can directly prevent the NFκβ activation by impeding I-κβ phosphorylation and therefore prevent NFκβ translocation into the nucleus [26,62]. The control by omega 3 fatty acids of the capabilities of cytokine production by LPS stimulated PBMCs could be important to the cells attracted and infiltrated into the damaged tissue.

The debris or DAMPs signals such as heat shock proteins, fibrinogen, uric acids crystals, oxidized LDL or noxious exogenous factors [26,61,63] from the damaged muscles could activate the cytokine production by infiltrated immune cells in the muscle in the same way that LPS activates the cytokine production by PBMCs [64,65]. The local inflammatory response within skeletal muscle after exercise is predominantly pro-inflammatory [40,66]. LPS-activated whole blood produced more IL6 and IL1β after exercise than previous to exercise; although trained groups presented a significantly lower cytokine production than non-trained groups [67]. We also detect higher rate of cytokine production by LPS-stimulated PBMCs after exercise, indicating that the PBMCs are primed to produce cytokines after acute exercise. This could be related to the level of the TLR4 receptor present in PBMCs cells. The TLR4 levels are higher after exercise than in basal condition in accordance with a previous study reporting increased TLR4-mediated NF-κβ and MAPK activation after eccentric exercise [24]. However, several studies have pointed out that both acute aerobic and chronic resistance exercise and training decreases monocyte cell-surface expression of TLRs and cytokine production [38,68] that could be in disagreement with our results. The TLR4 levels observed after exercise could be attributed to increased presence of TLR4 in the monocytes or to increased proportion of monocytes respect to PBMCs after exercise or to both causes. However, the increase in the TLR4 observed in the present work was higher than the

increase observed in monocyte counts. It could be suggested that after exercise increases TLR4 density in the PBMCs. Additionally, it has also been found an increased number of monocytes that respond to stimulation with LPS two hours after a marathon race [69]. Acute exercise increases TLR4 levels in the PBMCs that may represent a beneficial effect against bacterial infection but in turn it may also increase the pro-inflammatory capabilities of PBMCs.

It has been also described that the synthesis by PBMCs stimulated *in vitro* with LPS of IL1β, IL1α, and the TNFα can be ameliorated by dietary supplementation with long-chain n3 fatty acids [22]. In accordance, we have showed a significant reduction in the production rate of TNFα and IL6 after LPS activation in the post exercise sample from the DHA supplemented group. PBMCs contribute to the degradation of exercise-induced muscle damage by the release of ROS and this process is regulated by pro-inflammatory cytokines [40,70]. The reduction in the cytokine production by PBMCs after activation with LPS in the supplemented group shows that DHA influences the inflammatory response and function of these cells [47,71]. The increase of IL8 production in response to LPS stimulation is enhanced after exercise but it is not significantly influenced by DHA diet supplementation. IL8 is a known chemokine that attracts primarily neutrophils, but also acts as an angiogenic factor [72]. The effects of exercise as stimuli on blood myokines and angiogenesis factors such as IL8 in the metabolic process of angiogenesis and the formation of muscle have been reported [73]. The absence of differences in IL8 production between groups suggests that the regulatory pathway of this cytokine is not affected by DHA diet supplementation.

In conclusion, regular training for eight weeks and acute exercise induced few changes in plasma cytokine levels in well-trained soccer players, without any effects on circulating cytokines and growth factors induced by DHA diet supplementation. Acute exercise significantly increased the capability of PBMCs to produce cytokines after LPS stimulation and this production was attenuated by DHA diet supplementation, suggesting its anti-inflammatory function. The diet supplementation with DHA could contribute to reduce the possible inflammatory response to damaging exercise but at the same time it also could contribute to regulate the response to the infection. More *in vitro* studies are necessary to determine the specific effects of DHA on cytokine metabolism.

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Manuscript IV

Effects of Almond- and Olive Oil-Based Docosahexaenoic- and Vitamin E-Enriched Beverage Dietary Supplementation on Inflammation Associated to Exercise and Ag

nutrients

MDPI

Article

Effects of Almond- and Olive Oil-Based Docosahexaenoic- and Vitamin E-Enriched Beverage Dietary Supplementation on Inflammation Associated to Exercise and Age

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Abstract: *n*-3-polyunsaturated fatty acids and polyphenols are potential key factors for the treatment and prevention of chronic inflammation associated to ageing and non-communicable diseases. The aim was to analyse effects of an almond and olive oil beverage enriched with α-tocopherol and docosahexaenoic, exercise and age on inflammatory plasma markers, and immune gene expression in peripheral blood mononuclear cells (PBMCs). Five young and five senior athletes who were supplemented for five weeks with a functional beverage performed a stress test under controlled conditions before and after beverage supplementation. Blood samples were taken immediately before and 1 h after each test. Plasma, erythrocytes and PBMCs were isolated. Beverage supplementation increased plasmatic Tumour Necrosis Factor α (TNFα) levels depending on age and exercise. Exercise increased plasma non esterified fatty acids (NEFAs), soluble Intercellular adhesion molecule 3 (sICAM3) and soluble L-selectin (sL-Selectin), and this increase was attenuated by the supplementation. Exercise increased PGE2 plasma levels in supplemented young and in senior placebo athletes. Exercise increased NFkβ-activated levels in PBMCs, which are primed to a pro-inflammatory response increasing pro-inflammatory genes expression after the exercise mainly in the young group after the supplementation. The functional beverage supplementation to young athletes enhances a pro-inflammatory circulating environment in response to the exercise that was less evident in the senior group.

Keywords: inflammation; docosahexaenoic acid; cytokines; physical performance; polyphenol; vitamin E

1. Introduction

Functional foods enriched with specific nutrients present in natural foods are a good tool in functional food design. Nuts such as almonds are an important source of essential nutrients, such as arginine, calcium, potassium, niacin, α-tocopherol, fibre, monounsaturated fatty acids and

polyphenols [1,2]. Nut consumption has been reported to be inversely correlated with the incidence of cardiovascular disease, diabetes and some types of cancer [2,3] and with protective effects against oxidative stress [2,4]. Olive oil is an emblematic ingredient of the Mediterranean diet and is the most important differentiating factor compared to other countries as a source of polyphenols [5]. Olive oil consumption, specifically the extra-virgin variety, is associated with a reduced inflammation and a lower risk of atrial fibrillation and cardiovascular disease and mortality in individuals at high risk for cardiovascular disease [6–8]. Supplementing a Mediterranean diet with olive oil or nuts increases the total polyphenol intake, which correlates with higher plasma nitric oxide (NO) and lower systolic and diastolic blood pressure [9]. Supplements containing omega-3 fatty acids, polyphenols, antioxidants and vitamins are widely consumed for better health and athletic achievement [10]. Beneficial effects of dietary supplementation with omega-3 polyunsaturated fatty acids (*n*-3-PUFAs) on exercise performance [11] and on physical activity oxidative balance [12] have been evidenced. Dietary supplementation with *n*-3-PUFAs reduces the inflammatory response against pathogen-associated molecular patterns (PAMPs) in vitro [13,14], although the effects on the immune response associated with intense physical activity are questioned [15–17]. The use of functional foods enriched with several nutrients to provide synergic benefits for health and performance is a rising trend [18]. A beverage based on almonds and olive oil, enriched with docosahexaenoic acid (DHA) and vitamin E, could be a good vehicle to supplement athletes' diet with *n*-3-PUFAs, vitamin E and polyphenols.

Exercise is a good human model for studying the effects of functional food consumption on inflammation. Habitual exercise has been shown to result in an augmented cellular and plasma antioxidant defence system [19,20], reduced lipid peroxidation [21,22] and a protective effect against diseases associated with chronic inflammation [23,24]. Exercise induces a vascular anti-inflammatory response that contributes to counteracting chronic inflammation associated with sedentary habits [25]. Moreover, acute exercise also primes the immune cells for an inflammatory response to PAMPs [13,14].

Ageing is a complex process related to increased inflammation and oxidative stress, with the latter contributing to several age-related changes [26,27]. It has been reported that elderly people are more susceptible than younger people to suffer from oxidative damage in muscles after acute exercise [28]. Age is an additional factor influencing the inflammatory status and demands of *n*-3-PUFA and vitamin E supplementation [29]. *N*-3-PUFAs have now been identified as potential key nutrients that are safe and effective in the treatment and prevention of several adverse consequences of ageing [30]. The effects of supplementing the diet of athletes with functional foods based on almonds enriched with *n*-3-PUFA, olive oil and vitamin E, of plasma markers for inflammation, of markers for immune cell activation, and of the response to acute exercise in young and senior athletes are not known yet.

The aims of this study were to evaluate the effects of both diet supplementation with an almond and olive oil-based beverage enriched with docosahexaenoic and vitamin E, and acute exercise, on erythrocyte fatty acid composition, on plasmatic markers of inflammation and markers of immune cell activation in young and senior athletes.

2. Materials and Methods

2.1. Subjects and Anthropometric Characteristics

Ten young male taekwondo athletes and eight well-trained male senior athletes related to sport competitions (trainers and sport medical doctors) volunteered to participate in this study. All subjects were informed of its purpose, requirements and possible risks before giving their written consent to take part. Inclusion/exclusion criteria were: Age (18–25 years in young group and 35–57 years in senior group), sex (male), non-smokers, balanced diet, body mass index (19–25 kg/m^2) and physical activity of 1–2 h daily 5–7 day/week. Before being accepted to participate in the research, each subject underwent a complete medical examination, which included a medical history and resting electrocardiogram (ECG), to prevent any medical problem that would contraindicate the inclusion in the study. The protocol complied with the Declaration of Helsinki for research on human subjects,

and was approved by the Ethical Committee for Clinical Research at the Direcció General de l'Esport of the Catalonian Sports Council. The study was registered at ClinicalTrial.gov (NCT02177383). The participants were not acclimatised to heat and the study was conducted in the months of May and June with an average temperature around 18.2 °C and 22.8 °C, respectively. The participants were split into two groups depending on their age. All participants began the nutritional diet trial, but only five young and five senior athletes completed it. The need to participate in sport competitions was the cause to leave the nutritional intervention.

There were no differences in the anthropometric characteristics and physical activity capabilities between the young and senior groups (Table 1).

Table 1. Subject's anthropometric characteristics and physical activity time.

	Young	Senior
Age (years)	22.8 ± 3.8 $	45.6 ± 1.6
Weight (kg)	71.0 ± 4.8	76.1 ± 2.9
Height (cm)	176 ± 3.8	177 ± 3.8
Fat-free mass (%)	9.50 ± 1.1	11.6 ± 1.0
Body surface (m^2)	1.86 ± 0.05	1.93 ± 0.07
VO_2 max (L/min*kg)	58.8 ± 2.5	50.4 ± 3.4

Statistical analysis: Two-way ANOVA, $p < 0.05$. $ Differences between Young and Master groups. Results are the mean ± Standard Error Mean (SEM).

2.2. Beverage Composition

The nutritional intervention consisted of daily supplementation of the diet with one litre of almond and olive oil based functional beverage five days a week for five weeks in place of mineral water that was intake before nutritional intervention in the control situation. The beverage was isotonic (278 mOsm/kg) and made up of 3.0% almond and 0.8% sucrose and the rest was water, flavour, and the added oils and α-tocopherol acetate (vitamin E). Added oils were 0.6% olive oil and 0.2% DHA-S (wt %) (DSM, Columbia, SC, USA). DHA-S is nutritional oil derived from the marine alga *Schizochytrium* sp., a rich source of (DHA) with soy lecithin and rosemary (*Rosmarinus officinalis*) extract as flavour, and tocopherols and ascorbyl palmitate as antioxidants. The procedure for obtaining the beverage was bleaching of the almonds; crushing of the almonds in water; centrifuging of the mixture to eliminate insoluble materials; and the addition of cinnamon and lemon natural flavours, sucrose, vitamin E, and olive oil plus DHA-S. Finally, beverage was sterilized and packed. Functional beverage was elaborated by Liquats Vegetals S.A. (Girona, Spain). The fatty acid composition of the almond beverage enriched with DHA and vitamin E is shown in Table 2. The almond beverage is enriched with olive oil, DHA and a vitamin E contains 2.6% (w %) of fat, 2.85 ± 0.29 mM (51 ± 5 mg/100 mL as L-tyrosine equivalents) of total polyphenols and 4.6 ± 0.3 mg/100 mL of vitamin E (α-tocopherol acetate). The fatty acid content of the beverage is mainly monounsaturated (51.7% ± 5.0%) and polyunsaturated (38.3% ± 4.4%) with a low percentage of saturated fatty acid (9.90% ± 1.15%). The more abundant fatty acids were C18:1 and C18:2 followed by C16:0, C22:6, C18:0 and C22:5, whereas C18:3n3, C18:3n6, C20:2, C20:0 C20:4 and C22:0 were under 1% of total fat content of the functional beverage.

The fatty acid composition of beverage was determined following the same procedure used to determine erythrocyte fatty acid composition as described below. Similarly, polyphenol content of beverage was determined following the same procedure used to determine polyphenol content of plasma, erythrocytes and blood described below. Total fat content of functional beverage was 2.6%, taking into account 60% of fat content of almonds, the olive oil and DHA-S added.

Table 2. Beverage fatty acid composition.

Fatty Acid	Composition
C16:0 (%)	7.62 ± 0.97
C16:1 (%)	1.20 ± 0.14
C18:0 (%)	4.24 ± 0.7
C18:1 (%)	42.5 ± 4.8
C18:2 (%)	22.9 ± 2.5
C18:3n6 (%)	0.949 ± 0.55
C18:3n3 (%)	1.21 ± 0.35
C20:0 (%)	0.368 ± 0.105
C20:1(%)	0.113 ± 0.032
C20:2 (%)	0.884 ± 0.252
C20:3 (%)	0.065 ± 0.019
C20:4n6 (%)	0.434 ± 0.124
C22:0 (%)	0.262 ± 0.075
C22:5 (%)	5.68 ± 1.62
C22:6n3 (%)	11.6 ± 3.3
Total Fatty Acids (μM)	47400 ± 9586
SFA (%)	9.90 ± 1.15
MUFA (%)	51.7 ± 5.0
PUFA (%)	38.3 ± 4.4
VITAMIN E (mg/L)	45.7 ± 27.7
POLYPHENOLS (mM)	2.85 ± 0.29

Values are the average of six samples of the functional beverage.

2.3. Experimental Procedure

Athletes performed a stress test in controlled conditions at the beginning of the nutritional intervention and after 5 weeks of beverage supplementation (Figure 1).

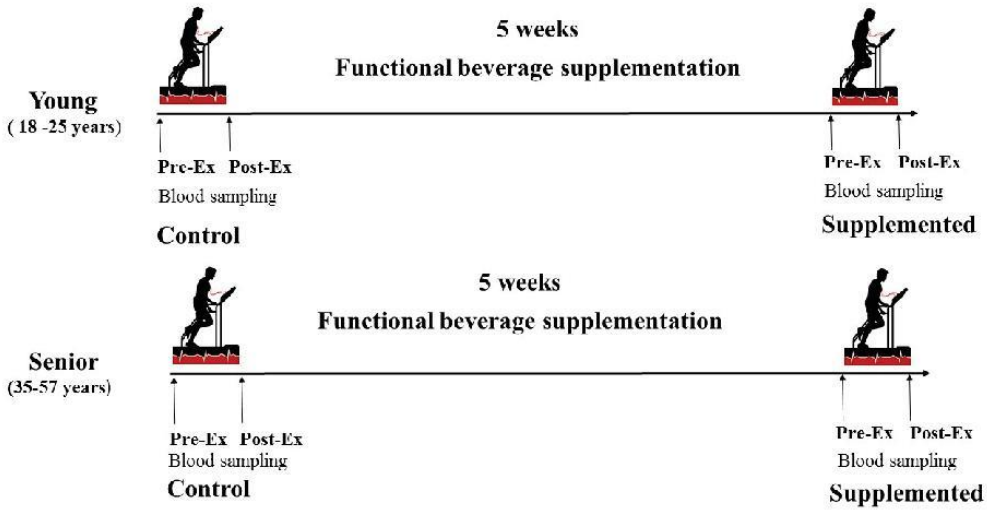

Figure 1. Diagram of the treatment time line.

Stress test consisted in incremental maximal test until exhaustion on a motorised treadmill (EG2, Vitoria, Spain) to determine their maximal oxygen consumption (VO_{2max}) using a computerised metabolic chart (Master Screen CPX, Erich Jaeger, Würzburg, Germany). The velocity corresponding to 60% (V_{60}), 70% (V_{70}), 80% (V_{80}) and 90% (V_{90}), of their VO_{2max} was calculated by linear interpolation of data from the maximal exercise test. Subjects arrived at the laboratory at 9:00 a.m. after an

overnight fast and having drunk a minimum of 500 cubic centimetre (cc) of water since wakening. Dry nude body weight was measured before and after the stress test after the subjects had emptied their urinary bladder. The subjects equipped with a heart rate transmitter and skin thermistors entered into the climatic chamber set at 30 °C temperature and 70% humidity; after 10 min the baseline core temperature [31], skin temperature and heart rate (HR) values were collected. Subjects continuously ran on the treadmill at the speed of V_{60} for 5 min, V_{70} for 5 min and V_{80} for 5 min for three consecutive bouts with two minutes of recovery between bouts. Finally, the subjects ran at V_{90} until exhaustion, and this time was measured as a quantity of exercise performance. Subjects were required to wear the same clothes and shoes in the two exercise sessions. Water was provided ad libitum in 500 mL bottles at room temperature and the amount of water consumed was measured. The percentage of dehydration was calculated from the weight difference corrected by drinking water during the test. The Polar® heart watch system (Polar Electro Inc., Kempele, Finland) was used to measure basal HR every 5 min during the test and after 5 min of recovery time. A microsample of blood (20 µL) was taken from the ear lobe to measure lactate concentration, at rest at minutes 15, 32, 49 and immediately at the end of the last bout to exhaustion (Dr. Lange®, Berlin, Germany). The Borg scale was used to assess subjective perception of effort at Minutes 15, 32, and 49, and after concluding the test [32].

Venous blood samples were obtained from the antecubital vein of participants with vacutainers containing EDTA (ethylenediaminetetraacetic acid) as an anticoagulant for blood count analyses (2 mL), to obtain erythrocytes and plasma (6 mL) and purify peripheral blood mononuclear cells (PBMCs) (6 mL). Venous blood samples were obtained after 12 h, overnight, in fasting conditions (basal sample), and 1 h after finishing training, which is consonant with increased circulating immune cells and significant changes in antioxidant enzyme activities and in markers for oxidative damage.

Erythrocyte fraction was obtained after centrifugation at 900× *g*, 30 min, 4 °C. Then, erythrocytes were washed with phosphate buffered saline (PBS), centrifuged at 900× *g*, 20 min, 4 °C and lysed with distilled water at the initial blood volume. Cell lysates were stored at −80 °C until biochemical analyses thereof.

PBMCs were obtained following a method previously described [33]. Blood was carefully introduced on Ficoll in a proportion of 1.5:1 and was then centrifuged at 900× *g*, at 4 °C for 30 min. The PBMCs layer was carefully removed. The plasma and the Ficoll phases were discarded. The PBMCs slurry was then washed twice with PBS and centrifuged for 10 min at 1000× *g*, 4 °C. This process was performed in triplicate, with one of the samples used to obtain RNA, and another being lysed with distilled water. Cell lysates were stored at −80 °C until biochemical analyses were performed.

2.4. Fatty Acid Determination

Erythrocyte and beverage fatty acids were extracted in duplicate with chloroform/methanol (2:1 v/v) by a modified method of Folch [12,34], containing 0.01% butylated hydroxyanisole as antioxidant and 20 µL of n-heptadecanoic acid (15 mM) as the internal standard. The resultant organic phase was evaporated under a nitrogen stream at 55 °C. The dry residue was dissolved in 225 µL of n-hexane and 25 µL of Meth-Prep™ II (Grace Davison Discovery Sciences, Columbia, MD, USA) and the derivatization reagent was added. The gas chromatograph was an Agilent 5890 model (Agilent Technologies, Santa Clara, CA, USA) with a flame ionization detector (FID) and the column was a Supelcowax® 10 Capillary GC column, 30 m × 0.53 mm × 0.50 µm (Supelco, Bellefonte, PA, USA).

2.5. Polyphenols Determination

Total polyphenol content of the functional beverage was determined through the Folin–Ciocalteau method [35] in the supernatants of deproteinized samples with cold acetone (1:1.2) using L-tyrosine as standard. The results are expressed as mmols of L-tyrosine/L.

2.6. Cytokine, Eicosanoids and Adhesion Molecules Determination

Prostaglandin E1 (PGE_1) and Prostaglandin E2 (PGE_2) were measured in plasma using ELISA kits (Enzo Life Sciences®, Farmingdale, NY, USA). Intra-assay and inter-assay reproducibility for PGE_1 were lower than 10% and 12%, respectively, while intra-assay and inter-assay reproducibility for PGE_2 were lower than 6% in both cases.

Lipoxin A4 was measure in plasma using ELISA kit (CUSABIO®, Baltimore, MD, USA). Intra-assay and inter-assay reproducibility were lower than 8% and 10% respectively.

Interleukin-6 (IL-6) and Tumour Necrosis Factor α (TNFα) were measured in plasma using ELISA kits (DIACLONE®, Besançon cedex, France). Intra-assay and inter-assay reproducibility for IL-6 were calculated to be 3.3% and 9.1% respectively, while intra-assay and inter-assay reproducibility for TNFα were calculated to be 4.4% and 9.0% respectively.

sL-Selectin and sICAM-3 were measured in plasma using ELISA kits (DIACLONE®, Besançon cedex, France). Intra-assay and inter-assay reproducibility for sL-Selectin were calculated to be 4.6% and 3.22% respectively, while intra-assay and inter-assay reproducibility for sICAM-3 were calculated to be 3.49% and 1.99%, respectively.

2.7. Non Esterified Fatty Acids Determination

Non esterified fatty acids were determinate in plasma using an enzymatic kit (Wako®) based on the specificity of acyl-CoA synthetase for the free fatty acids.

2.8. Nuclear Factor κβ (NFκβ) Activation Quantification

An isolated suspension of PBMCs was subjected to whole-cell protein extraction for the determination of NFκβ p50 activation, which was performed using an ELISA method according to the manufacturer's instructions TransAM NF-kB p50 Chemi (Active Motif®). Briefly, the primary antibody used to detect NFκβ recognizes an epitope on p50 that is accessible only when NFκβ is activated and bound to its DNA target.

2.9. Gene Expression

Toll like receptor 2 (TLR2), Toll Like receptor 4 (TLR4), Nfκβ, Cyclooxygenase 2 (COX2), 5 Lipoxygenase (5 LOX), 15 Lipoxygenase 2 (15 LOX 2), Interleukin 1β (IL1β), Interleukin-8 (IL-8), Tumour Necrosis Factor (TNFα), Interleukin-10 (IL-10), Interleukin-15 (IL-15) and Heat Shock Protein 70 (HSP70) mRNA expression was determined by multiplex real-time PCR based on incorporation of a fluorescent reporter dye and using human 18S rRNA as reference. For this purpose, total RNA was isolated from PBMCs by Tripure extraction (Roche Diagnostics, Germany). RNA (1 μg) from each sample was reverse transcribed using 50 U of Expand Reverse Transcriptase (Roche Diagnostics, Germany) and 20 pmol oligo (dT) for 60 min at 37 °C in a 10 μL final volume, according to manufacturer instructions. The resulting cDNA (2.5 μL) was amplified using the LightCyclerFastStart DNA MasterPLUS SYBR Green I kit (Roche Diagnostics, Germany). Amplification was performed at 55 °C and 45 cycles. The relative quantification was performed by standard calculations considering $2^{(\Delta\Delta Ct)}$. Antioxidant enzyme levels before and after the season were normalized to the invariant control 18S rRNA. mRNAlevels at basal young control group were arbitrarily referred to as 1. Primers used are listed in Table 3.

2.10. Statistical Analysis

Statistical analysis was carried out using the Statistical Package for Social Sciences (SPSS v.21.0 for Windows). Results are expressed as mean ± SEM and $p < 0.05$ was considered statistically significant. A Kolmogorov–Smirnov test was previously applied to assess the normal distribution of the data. The statistical significance of the data was assessed by a three-way analysis of variance (ANOVA). Bonferroni test was used in order to make a multiple comparison. The statistical factors analysed

were beverage supplementation (S), ageing (A) and exercise (E). For the sets of data where there was a significant S × E × A, S × E, S × A, and A × E interactions were tested by the ANOVA one-way test.

Table 3. Primer sequences and conditions.

Gene		Primer	Conditions	
18S	Fw:	5′-ATG TGA AGT CAC TGT GCC AG-3′	95 °C	10 s
	Rv:	5′-GTG TAA TCC GTC TCC ACA GA-3′	60 °C	10 s
			72 °C	12 s
TLR2	Fw:	5′-GGGTTGAAGCACTGGACAAT-3′	95 °C	10 s
	Rv:	5′-TTCTTCCTTGGAGAGGCTGA-3′	60 °C	10 s
			72 °C	15 s
TLR4	Fw:	5′-GGTCACCTTTTCTTGATTCCA-3′	95 °C	10 s
	Rv:	5′-TCAGAGGTCCATCAAACATCAC-3′	60 °C	10 s
			72 °C	15 s
TNFα	Fw:	5′-CCCAGGCAGTCAGATCATCTTCTCGGAA-3′	94 °C	10 s
	Rv:	5′-CTGGTTATCTCTCAGCTCCACGCCATT-3′	63 °C	10 s
			72 °C	15 s
NFκβ	Fw:	5′-AAA CAC TGT GAG GAT GGG ATC TG-3′	95 °C	10 s
	Rv:	5′-CGA AGC CGA CCA CCA TGT-3′	60 °C	10 s
			72 °C	15 s
COX2	Fw:	5-TTG CTG GCA GGG TTG CTG GTG GTA-3′	95 °C	10 s
	Rv:	5′-CAT CTG CCT GCT CTG GTC AAT GGA A-3′	67 °C	10 s
			72 °C	15 s
15 LOX 2	Fw:	5′-GCA TCC ACT GAT TGG ACC TT-3′	95 °C	10 s
	Rv:	5′-GCT GGC CTT GAA CTT CTG AC-3′	61 °C	10 s
			72 °C	15 s
IL1β	Fw:	5′-GGA CAG GAT ATG GAG CAA CA-3′	95 °C	10 s
	Rv:	5′-GGC AGA CTC AAA TTC CAG CT-3′	58 °C	10 s
			72 °C	15 s
5 LOX	Fw:	5′-GGG CAT GGA GAG CAA AGA AG-3	95 °C	10 s
	Rv:	5′-ACC TCG GCC GTG AAC GT-3′	59 °C	10 s
			72 °C	15 s
IL-8	Fw:	5′-GCTCTGTGTGAAGGTGCAGTTTTGCCAA-3′	95 °C	10 s
	Rv:	5′-GGCGCAGTGTGGTCCACTCTCAAT-3′	63 °C	10 s
			72 °C	15 s
IL-10	Fw:	5′-AGAACCTGAAGACCCTCAGGC-3′	95 °C	10 s
	Rv:	5′-CCACGGCCTTGCTCTTGTT-3′	60 °C	10 s
			72 °C	15 s
IL-15	Fw:	5′-CCGTGGCTTTGAGTAATGAGAATTTCGAA-3′	95 °C	10 s
	Rv:	5′-CCTGCACTGAAACAGCCCAAAATGAA-3′	60 °C	10 s
			72 °C	15 s
HSP72	Fw:	5′-CCGGCAAGGCCAACAAGATC-3′	95 °C	10 s
	Rv:	5′-CCTCCACGGCGCTCTTCATG-3′	62 °C	10 s
			72 °C	15 s

3. Results

3.1. Effects on Exercise Performance Parameters

Neither functional beverage diet supplementation nor age altered the exercise performance parameters (Table 4). Maximum exercise tests increased core and skin temperature in a similar way in all groups and situations until a maximum core temperature of about 39.4 °C and a maximum skin temperature of about 34.8 °C were attained. Heat storage during the maximum exercise test was similar in all groups and situations. The heart rate attained during the exercise test was about 97.4% of maximum heart rate and similar in all groups and situations. The physiological strain index attained during the exercise test was about 9.88 and similar in all groups and situations, which points to very high heat stress induced by the exercise test. Similarly, the Borg index of fatigue also indicates very fatiguing exercise with no influence from the functional beverage on this perception, although the senior group did perceive a significantly lower fatigue sensation than the younger group during the exercise test. The time spent running at 90% VO_{2max} until exhaustion was similar in the young and senior groups, regardless of the control or functional beverage supplemented situation. The maximum

blood lactate level, water intake and weight loss during the exercise tests were similar in all groups and situations. In summary, the exercise test was highly fatiguing for athletes showing a very high heat stress who attained the anaerobic exercise phase with high core and skin temperature values and a moderate weight loss not influenced by either age or supplementation.

Table 4. Effects of Age and dietary beverage supplementation on stress test.

				ANOVA		
		Young	Senior	S	A	S × A
Maximum Borg Index (Borg scale)	**Control**	17.8 ± 0.49	16.2 ± 0.49			
	Supplemented	17.8 ± 0.48	16.6 ± 0.68			
Time running at 90% of VO_{2max} (min)	**Control**	9.02 ± 2.99	8.84 ± 2.28			
	Supplemented	7.62 ± 2.59	10.0 ± 1.97			
Initial body temperature (°C)	**Control**	37.0 ± 0.18	37.1 ± 0.14			
	Supplemented	37.0 ± 0.07	37.0 ± 0.05			
Maximum body temperature (°C)	**Control**	39.8 ± 0.26	39.4 ± 0.15			
	Supplemented	39.4 ± 0.12	39.3 ± 0.20			
Body temperature 5 min after the end (°C)	**Control**	39.9 ± 0.23	39.5 ± 0.16			
	Supplemented	39.4 ± 0.15	39.3 ± 0.18			
Maximum skin temperature (°C)	**Control**	35.1 ± 0.21	34.7 ± 0.36			
	Supplemented	34.8 ± 0.19	34.8 ± 0.21			
Skin temperature 5 min after the end (°C)	**Control**	35.2 ± 0.24	34.5 ± 0.70			
	Supplemented	35.1 ± 0.15	34.7 ± 0.12			
Maximum Lactate (mM)	**Control**	4.08 ± 0.61	4.32 ± 0.68			
	Supplemented	3.86 ± 0.85	5.36 ± 0.41			
Maximal heart rate (beats/min)	**Control**	186 ± 4.60	175 ± 7.46			
	Supplemented	183 ± 6.39	175 ± 6.56			
% of maximal heart rate during the exercise	**Control**	97.4 ± 0.81	98.0 ±2.69			
	Supplemented	96.3 ± 0.68	98.0 ± 1.91			
Water intake (L)	**Control**	0.42 ± 0.06	0.41 ± 0.17			
	Supplemented	0.50 ± 0.09	0.42 ± 0.17			
Absolute weight loss (%)	**Control**	1.63 ± 0.08	1.41 ± 0.19			
	Supplemented	1.52 ± 0.17	1.66 ± 0.19			
Wight loss without water intake (%)	**Control**	2.21 ± 0.13	1.93 ± 0.09			
	Supplemented	2.22 ± 0.09	2.15 ± 0.13			
Physiological Strain Index [36]	**Control**	10.5 ± 0.50	9.73 ± 0.31			
	Supplemented	9.79 ± 0.24	9.49 ± 0.41			
Heat Storage(W/m^2)	**Control**	348 ± 34.1	325 ± 19.2			
	Supplemented	318 ± 18.7	344 ± 47.8			

Results are the mean ± SEM. Statistical analysis: Two-way ANOVA, $p < 0.05$. S, supplementation effect; A, age effect, S × A, interaction between supplementation and age effects. * Indicates significant differences between the dietary control and functional beverage dietary supplementation plasma levels; $ indicates significant differences between Young and Senior groups. When interaction exists between different statistical factors, different letters reveal significant differences.

3.2. Effects on Fatty Acids Composition

Age and beverage supplementation altered the fatty acid composition of erythrocytes (Figure 2). No age or supplementation effect was observed in the percentage of C16, C16:1, C18, C18:1, C18:2, C18:3n6, C18:3n3, C20:3, C20:4, and C22:0 of erythrocytes. The percentage of C22:6 was significantly higher after dietary supplementation with the functional beverage than in the control situation in both the young and senior groups. The nutritional intervention with one litre of the functional beverage for five days a week was followed by all participants and was effective at enriching erythrocytes with DHA. The plasmatic NEFAs concentration was influenced by acute exercise and age, (Figure 3). The young athletes evidenced significant higher plasma NEFAs after acute exercise, both in the control and experimental situations, whereas in the senior group, the increase was only significant in the control situation. Furthermore, an interaction between supplementation and exercise was observed, resulting in an attenuated response in the supplemented situation respect to the control.

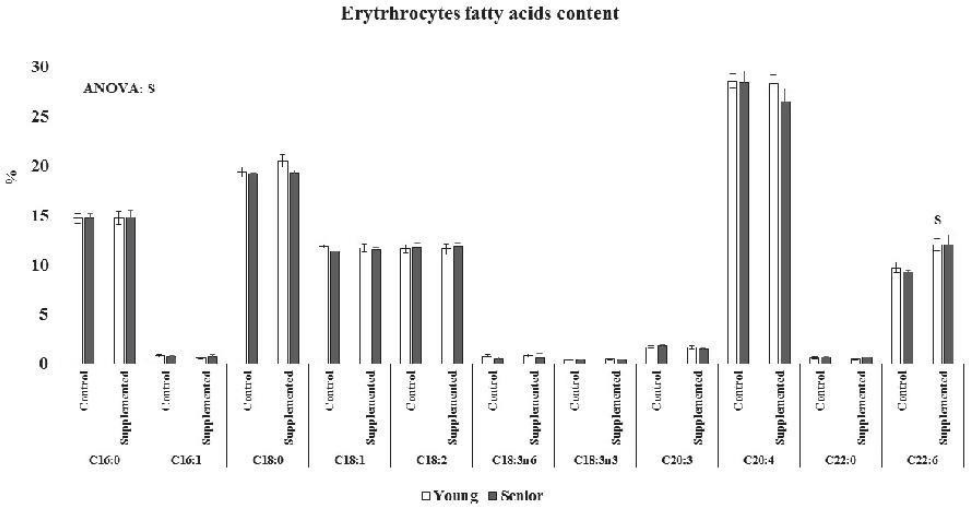

Figure 2. Effects of age and beverage supplementation on erythrocytes fatty acids composition. Results are the mean ± SEM. Statistical analysis: Two-way ANOVA, $p < 0.05$. S, supplementation effect; A, age effect, S × A, interaction between supplementation and age effects. * Indicates significant differences between the dietary control and functional beverage dietary supplementation plasma levels; $ indicates significant differences between Young and Senior groups. When interaction exists between different statistical factors, different letters reveal significant differences.

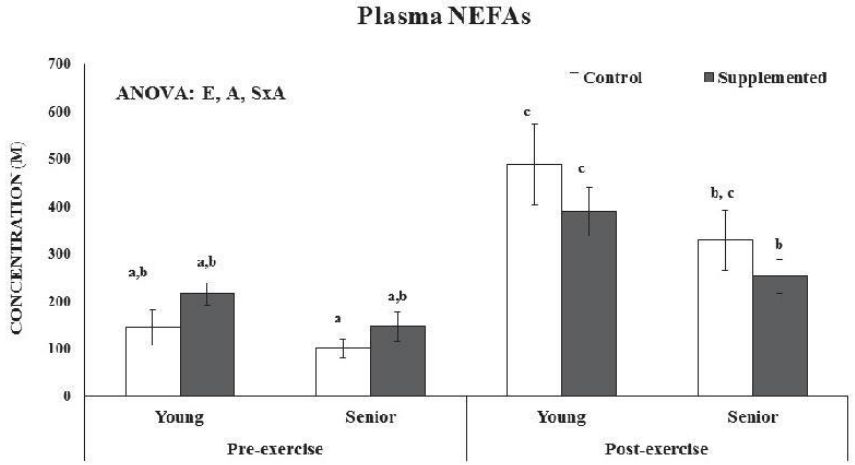

Figure 3. Age, dietary beverage supplementation and acute exercise effects on NEFAs plasma levels. Results are the mean ± SEM. Statistical analysis: Three-way ANOVA, $p < 0.05$. S, supplementation effect; A, age effect, E, exercise effect, S × A, interaction between supplementation and age effects, S × E, interaction between supplementation and exercise effects, E × A, interaction between exercise and age effects, A × E × S, interaction between three factors effects. S, A, E, S × A, E × A, S × E or A × E × S indicates a significant effect of each statistical factor. * Indicates significant differences between the dietary control and functional beverage dietary supplementation plasma levels; # indicates significant differences between pre-exercise and post-exercise plasma levels; $ indicates significant differences between Young and Senior groups. When interaction exists between different statistical factors, different letters reveal significant differences.

3.3. Effects on Inflammatory, Heat Stress and Immune Priming Response Markers

The effects of age, acute exercise and dietary functional beverage supplementation on plasmatic markers of inflammation and heat stress and on inflammatory priming of PBMCs are shown in Table 5. The functional beverage significantly influenced the immune markers: sL-selectin, sICAM3 and TNFα, whereas age influenced sICAM3, and acute exercise influenced lipoxin, PGE2 and the activation of NFkβ in PBMCs. Acute exercise influenced adhesion molecules sL-selectin and sICAM3 plasma levels, depending on the functional beverage dietary supplementation, and it also influenced cytokines IL-6 and TNFα plasma levels in an age- and supplementation-dependent manner. Activated NFkβ levels in PBMCs were significantly higher after acute exercise in both the young and senior groups, mainly after the functional beverage dietary supplementation.

Table 5. Age, dietary beverage supplementation and acute exercise effects on markers of inflammation and heat stress in plasma and inflammatory priming of peripheral blood mononuclear cells.

		Pre-Exercise		Post-Exercise		ANOVA
		Young	Senior	Young	Senior	
sL-Selectin (ng × mL plasma)	**Control**	106 ± 2.8^{ab}	80.1 ± 9.7^{ac}	146 ± 26^{b}	89.1 ± 11.9^{ac}	**S, S × E**
	Supplemented	77.7 ± 8.1^{ac}	92.4 ± 23.1^{ac}	61.4 ± 6.2^{ac}	47.8 ± 13.4^{c}	
ICAM3 (ng of × mL plasma)	**Control**	458 ± 122^{ade}	507 ± 55^{acd}	670 ± 104^{c}	705 ± 43^{c}	**A, S, S × A, S × E**
	Supplemented	324 ± 65^{ab}	604 ± 46^{cd}	206 ± 73^{b}	618 ± 72^{ce}	
HSP 70 (Pg × mL of plasma)	**Control**	5.12 ± 0.31	5.01 ± 0.19	5.09 ± 0.33	4.97 ± 0.22	
	Supplemented	4.98 ± 0.42	5.03 ± 0.18	4.87 ± 0.47	4.74 ± 0.09	
IL-6 (pg × mL of plasma)	**Control**	4.51 ± 0.35^{ac}	4.27 ± 0.46^{ac}	4.56 ± 0.40^{ac}	5.39 ± 0.49^{ab}	**S × E × A**
	Supplemented	3.54 ± 0.13^{c}	4.87 ± 0.36^{ac}	7.38 ± 1.21^{b}	4.24 ± 0.36^{ac}	
TNFα (pg × mL of plasma)	**Control**	627 ± 43^{afd}	679 ± 67^{acfd}	697 ± 52^{acfd}	524 ± 35^{f}	**S, S × E × A**
	Supplemented	831 ± 66^{cg}	781 ± 32.4^{abcd}	758 ± 43^{gd}	936 ± 140^{gb}	
Lipoxina (pg × mL of plasma)	**Control**	54.8 ± 11.7	67.9 ± 19.9	65.2 ± 12.5	33.5 ± 10.6 #	**E**
	Supplemented	104 ± 29	83.4 ± 18.5	67.2 ± 9.9	36.7 ± 8.8 #	
PGE2 (pg × mL of plasma)	**Control**	286 ± 63	398 ± 168	315 ± 71	1541 ± 579 #	**E**
	Supplemented	390 ± 112	781 ± 231	1557 ± 585 #	993 ± 476	
PGE1 (pg × mL of plasma)	**Control**	1976 ± 168	2598 ± 735	3087 ± 681	5645 ± 3038	
	Supplemented	3593 ± 995	3273 ± 1204	2465 ± 427	3947 ± 980	
NFκβ (U.A.)	**Control**	2628 ± 140	2720 ± 249	2877 ± 135	2889 ± 194	**E**
	Supplemented	2437 ± 210	2730 ± 210	3099 ± 343 #	3400 ± 167 #	

Results are the mean ± SEM. Statistical analysis: Three-way ANOVA, $p < 0.05$. S, supplementation effect; A, age effect, E, exercise effect, S × A, interaction between supplementation and age effects, S × E, interaction between supplementation and exercise effects, E × A, interaction between exercise and age effects, A × E × S, interaction between three factors effects. S, A, E, S × A, E × A, S × E or A × E × S indicates a significant effect of each statistical factor. * Indicates significant differences between the dietary control and functional beverage dietary supplementation plasma levels; # indicates significant differences between pre-exercise and post-exercise plasma levels; $ indicates significant differences between Young and Senior groups. When interaction exists between different statistical factors, different letters reveal significant differences.

The supplementation with the functional beverage significantly decreased sL-Selectin, aside from the fact that there is an existing interaction between exercise and supplementation; in this sense, acute exercise significantly increased sL-selectin plasma levels mainly in the young group, whereas dietary functional beverage supplementation eliminates this exercise effect and sL-selectin levels post-exercise were similar to pre-exercise plasma levels.

Acute exercise significantly increased sICAM3 plasma levels only in the young group, whereas dietary functional beverage supplementation eliminates this exercise effect and sICAM-3 plasma levels post-exercise for the young group were three times lower than pre-exercise ones. Neither acute exercise nor functional beverage dietary supplementation influenced sICAM3 plasma levels in the senior group. These different patterns of change in the young and senior groups were reflected in pre-exercise and post-exercise sICAM3 plasma levels that were, significantly, about three times lower in the young group than in the senior group after supplementation, but not in the control situation.

No impact from supplementation, acute exercise or age was observed on HSP70 or PGE1 plasma levels.

Acute exercise influenced the plasma levels of lipoxin and PGE2. Exercise tended to decrease lipoxin plasma levels, mainly in the senior group after functional beverage supplementation. In turn, exercise significantly increased PGE2 plasma in the young group after functional beverage supplementation and in the control dietary situation for the senior group.

The effects of the functional beverage supplementation on IL-6 and TNFα plasma levels depended on age and exercise. Plasmatic IL-6 was maintained at the control level in all conditions in the senior group. However, post-exercise plasma levels in the young group after dietary functional beverage supplementation were significantly higher respect to pre-exercise and control levels. Functional beverage supplementation enhanced IL-6 plasma levels in response to exercise in young athletes but not in senior athletes. Dietary functional beverage supplementation also increased plasmatic TNFα levels depending on age and exercise. Pre-exercise plasma TNFα levels were significantly higher after supplementation compared to control in the young group, and the post-exercise TNFα plasma levels in the senior group were significantly higher than in control. Plasma TNFα remained at the same level regardless of the age or exercise situation in control.

3.4. Effects on Inflammatory Genes Expression in PBMCs

Neither dietary functional beverage supplementation nor exercise or age influenced the gene expression of TLR4, NFκβ 5 LOX, IL-10, IL-15, HSP72 in PBMCs (Table 6). The supplementation significantly influenced TNFα gene expression in PBMCs, being significantly higher after dietary supplementation than in control for the young group in a pre-exercise situation. Functional beverage supplementation and age significantly increased the 15LOX2 gene expression; 15LOX2 gene expression in the senior group was significantly higher than in the young group, both in pre-exercise and post-exercise situations after dietary supplementation with the functional beverage, whereas no differences were seen in 15LOX2 values in the control situation. Similarly, COX2, IL1β and IL-8 gene expression were influenced by an interaction between dietary functional beverage supplementation and age. The expression of IL1β and IL-8 was enhanced in PBMCs after dietary functional beverage supplementation in the young group, mainly post-exercise, but not in the senior group.

Furthermore, an interaction between acute exercise and age was detected on TLR2 gene expression.

Table 6. Effects of dietary functional beverage supplementation, exercise and age on the expression of inflammatory and related genes.

		Pre-Exercise		Post-Exercise		ANOVA
		Young	Senior	Young	Senior	
TLR2	**Control**	1.00 ± 0.28	1.59 ± 0.56	0.99 ± 0.29	0.77 ± 0.10	**E** × **A**
	Supplemented	1.13 ± 0.37	1.01 ± 0.26	2.51 ± 1.11	0.84 ± 0.13	
TLR4	**Control**	1.00 ± 0.24	1.18 ± 0.41	1.25 ± 0.47	0.86 ± 0.12	
	Supplemented	1.46 ± 0.55	1.15 ± 0.29	2.62 ± 1.19	0.84 ± 0.11	
NFκβ	**Control**	1.00 ± 0.21	1.12 ± 0.34	1.11 ± 0.30	0.89 ± 0.12	
	Supplemented	1.75 ± 0.79	1.74 ± 0.67	1.39 ± 0.46	0.87 ± 0.09	
COX2	**Control**	1.00 ± 0.09	1.07 ± 0.17	1.39 ± 0.38	1.50 ± 0.41	**A** (0.094)
	Supplemented	2.31 ± 1.09	1.36 ± 0.36	3.99 ± 1.96	1.20 ± 0.24	**S** × **A** (0.066)
5LOX	**Control**	1.00 ± 0.19	1.21 ± 0.40	1.14 ± 0.28	1.10 ± 0.28	
	Supplemented	1.15 ± 0.34	1.57 ± 0.53	3.59 ± 1.97	0.96 ± 0.13	
15LOX2	**Control**	1.00 ± 0.33	1.63 ± 0.45	0.96 ± 0.22	2.14 ± 0.77	**A** (0.035)
	Supplemented	2.14 ± 0.77	6.86 ± 4.21 $^{*,\$}$	1.13 ± 0.36	7.65 ± 2.52 $^{*,\$}$	**S** (0.048)
IL1β	**Control**	1.00 ± 0.14 ^{a}	0.99 ± 0.17 ^{a}	1.17 ± 0.25 ^{a}	1.68 ± 0.73 ^{a}	**S** (0.084)
	Supplemented	1.92 ± 0.59 ^{a}	1.10 ± 0.22 ^{a}	3.86 ± 1.54 ^{b}	1.28 ± 0.34 ^{a}	**S** × **A** (0.043)

Table 6. *Cont.*

		Pre-Exercise		Post-Exercise		ANOVA
		Young	Senior	Young	Senior	
IL-8	**Control**	1.00 ± 0.28^{a}	0.92 ± 0.20^{a}	0.74 ± 0.11^{a}	1.74 ± 0.75^{a}	**S × A** (0.029)
	Supplemented	2.39 ± 1.19^{ab}	0.94 ± 0.29^{a}	4.10 ± 2.48^{b}	0.73 ± 0.11^{a}	
TNFα	**Control**	1.00 ± 0.11	1.06 ± 0.19	1.07 ± 0.21	1.19 ± 0.39	**S** (0.032)
	Supplemented	1.16 ± 0.21	1.21 ± 0.42	1.72 ± 0.58	2.37 ± 0.59	
IL-10	**Control**	1.00 ± 0.23	1.56 ± 0.60	1.45 ± 0.59	1.15 ± 0.37	
	Supplemented	1.71 ± 0.75	1.51 ± 0.45	3.41 ± 1.66	1.54 ± 0.57	
IL-15	**Control**	1.00 ± 0.25	0.90 ± 0.23	0.91 ± 0.17	1.35 ± 0.52	
	Supplemented	1.18 ± 0.35	1.16 ± 0.35	1.97 ± 0.89	0.84 ± 0.15	
HSP72	**Control**	1.00 ± 0.28	1.07 ± 0.31	1.01 ± 0.31	0.81 ± 0.13	
	Supplemented	2.48 ± 1.39	1.23 ± 0.49	2.53 ± 1.17	0.79 ± 0.12	

Results are the mean ± SEM. Statistical analysis: Three-way ANOVA, $p < 0.1$. S, supplementation effect; A, age effect, E, exercise effect, S × A, interaction between supplementation and age effects, S × E, interaction between supplementation and exercise effects, E × A, interaction between exercise and age effects, A × E × S, effects of interaction between three factors. S, A, E, S × A, E × A, S × E or A × E × S indicates a significant effect of each statistical factor. * Indicates significant differences between the dietary control and functional beverage dietary supplementation; # indicates significant differences between pre-exercise and post-exercise; $ indicates significant differences between Young and Senior groups. Where interaction exists between different statistical factors, different letters reveal significant differences.

4. Discussion

The composition of the functional beverage enables dietary supplementation with the omega 3-fatty acid DHA, vitamin E and polyphenols. The daily intake of one litre of the beverage, five days a week, represents daily supplementation with an average of 18.6 g/day fat (of which 820 mg/day corresponds to DHA), 32.6 mg/day of vitamin E, and 36.4 mg/day of polyphenols. The fat intake with the functional beverage represents about 17% of recommended fat consumption for the general population [37]; however, the DHA and vitamin E intake with the same amount of the functional beverage are about three times higher than Recommended Dietary Allowances (RDA) for the general population. The dietary recommendation of vitamin E for active athletes is based on the daily energy consumed [38]; thus, the vitamin E content in the functional beverage would supply the vitamin E requirements for active athletes expending the energy content amount in the functional beverage itself. In addition, the fatty acid content of the beverage is mainly monounsaturated (51.7% ± 5.0%) and polyunsaturated (38.3% ± 4.4%) with a low percentage of saturated fatty acid (9.90% ± 1.15%). In this light, its consumption could contribute to a shift towards a more unsaturated plasma and cell membrane fatty acid profile. Accordingly, the fatty acid composition of erythrocytes is more unsaturated, mainly due to C22:6 after supplementation. The functional beverage also supplements the diet with polyphenols. The polyphenol content in the beverage is similar to the supplied by an orange juice, the consumption of which decreases basal oxidative damage in the elderly [39].

The exercise test undertaken was highly fatiguing and represents a very high heat stress for athletes, so much so that they attained an anaerobic exercise phase with high core and skin temperature values and moderate weight loss. Neither age nor dietary functional beverage supplementation influenced exercise performance parameters, including no influence from time at 90% VO_{2max} spent to exhaustion. The null influence of the functional beverage intake on exercise performance is in accordance with the null influence of vitamin E and C and polyphenol diet supplementation on the VO_{2max} values for athletes [40]. In turn, the high fat content of the beverage has no negative influence on exercise performance, in contrast to other studies where fat intake reduced physical performance [41]. It does, however, contrast with the positive impact of omega-3 fatty acid consumption on physical performance as described in other studies [42,43]. The exercise test conditions were designed to induce an immune response priming neutrophils and PBMCs to an inflammatory response [14,44], although plasmatic markers of inflammation such as IL-6, IL-10, TNFα, IL1β, etc. point to a more post-exercise anti-inflammatory condition.

5. Effects on Inflammatory Markers

Dietary fat could influence inflammatory status [45]. The anti-inflammatory effects of omega-3 fatty acids have long been reported [13,14,46] whereas saturated fatty acids have been linked to inflammation [46]. It has also been demonstrated that almond intake could decrease some inflammatory markers such as protein C reactive and E-selectin, although no effects on IL-6 have been observed yet [47]. It has been pointed out that exercise and ageing influence the degree of inflammation [48–50]. The present results showed that exercise, regardless of age or functional beverage supplementation, activates NFκβ in PBMCs that drives the cell to gene expression of pro-inflammatory cytokines, anti-oxidant enzymes and other genes [51,52]. The plasma levels of sL-selectin, sICAM3, Lipoxin, IL-6, TNFα, PGE2 are influenced by exercise [24,49], although their response to exercise are age and beverage dependent. It has been stated that an induction of biosynthesis of pro-inflammatory prostaglandins, leukotrienes and inflammation pro-resolving mediators occurs during the early hours (1–2 h) of post-exercise recovery [53]. Similarly, increased IL-6, TNFα and PGE2 plasma levels after intense exercise have been noted [13,23,54,55], although this response is age- and functional beverage supplementation-dependent. IL-6 increased after exercise indietary supplemented young athletes; PGE2 increased after exercise both in dietary supplemented young athletes and in control senior athletes; the TNFα post-exercise levels of dietary supplemented seniors are higher in than in control senior athletes. TNFα and IL-6 are considered pro-inflammatory cytokines and PGE2 a pro-inflammatory prostaglandin [23,54]. However, it has been suggested that IL-6 exerts anti-inflammatory actions via traditional signalling by binding to its cellular receptor, whilst it exerts pro-inflammatory effects, such as recruitment of mononuclear cells, through trans-signalling by binding to its soluble receptor [56]. The increased IL-6 plasma levels after exercise results from muscular secretion and exerts an anti-inflammatory action [23,24]. In turn, increased PGE2 after exercise could also be considered an anti-inflammatory effector [12,54]. TNFα is considered a pro-inflammatory cytokine that increases in plasma as result of dietary functional beverage supplementation and as result of exercise in senior athletes, in any event. The measured TNFα plasma levels are similar to other studies performed with a similar beverage but in this study, no effects from exercise or supplementation were observed [13]. The excess of dietary saturated fatty acids such as palmitic acid induce peripheral inflammation [57] but polyunsaturated fatty acids such as DHA, whilst fish oil fatty acid consumption induces a peripheral anti-inflammatory response [58]. The functional beverage presents both saturated and polyunsaturated fatty acids, with predominance of unsaturated fatty acids; we detect a possible pro-inflammatory action of supplementation in young and senior athletes' diet. In fact, dietary functional beverage supplementation increases the TNFα gene expression in PBMCs, enhancing the biosynthesis of this pro-inflammatory cytokine.

Information about the exercise effects on soluble adhesion molecule plasma levels such as sL-Selectin and sICAM 3 is scant. Resistance training does not affect the serum concentrations of the cell adhesion molecules VCAM1, ICAM1, E-selectin, sL-selectin and P-selectin [59]. On the contrary, an increase in ICAM1 after endurance training has been observed [60]; this fact could be interpreted as a protective mechanism against infections yet our results point to exercise increasing plasma sL-Selectin and sICAM3 levels whilst dietary functional beverage supplementation eliminates this exercise effect. The presence of soluble adhesion molecules in plasma indicates the degree of vascular endothelial activation or dysfunction [61], and it can reflect the status of the immune system [62]. In fact, ICAM3 is lost from neutrophils after activation [63]. It is suggested that an increase in adhesion molecules in plasma could attenuate the immune response by competing with the corresponding cell-bound adhesion molecules by cellular binding sites with leucocytes adhesion and transmigration with the endothelium response [61,64,65]; however, it has also been observed that high levels of adhesion molecules is linked to a higher cardiovascular disease risk in heart attack patients [59]. Indeed, physical fitness attenuates leukocyte-endothelial adhesion in response to exercise [66]. A dietary functional beverage abolishing the impact of exercise on sL-selectin and ICAM3

could increase leukocyte capability to adhere the endothelium but it could also reflect a reduction in leukocyte activation after exercise, attributable to dietary functional beverage consumption.

It was demonstrated that diets in rich antioxidants such as olive oil, fruits, and vegetables lower IL-6 levels in PBMCs [67]. PBMCs are primed to pro-inflammatory response after exercise, as indicated by increased NFκβ active levels in PBMCs, mainly after dietary functional beverage supplementation. PBMCs enhanced the expression of pro-inflammatory IL1β and IL-8 genes after exercise mainly in the young group after dietary functional beverage supplementation. This reinforces the pro-inflammatory impact of functional beverage consumption in young athletes partitioning exercise. Senior athletes have enhanced 15LOX gene expression after dietary functional beverage supplementation; this demonstrates higher capabilities to synthesize prostaglandins and resolvins in this situation.

In conclusion, athletic performance was not altered by dietary supplementation with a functional almond-based beverage enriched with olive oil, DHA and vitamin E for this reason it would not be necessary to supplement athletes diet with the functional beverage. Nonetheless, supplementation with a functional beverage increased erythrocyte DHA content, exercise increased plasma NEFAs (although this increase was attenuated by the supplementation with a functional beverage) and, in turn, exercise increased NFκβ activation in PBMCs. Consequently, PBMCs are primed to a pro-inflammatory response post-exercise. In the same way, exercise increased sICAM3 and sL-Selectin, but this increase was weaker after supplementation with a functional beverage. Moreover, supplementation with a functional beverage enhanced a pro-inflammatory circulatory environment in response to exercise, although this effect was less evident in senior athletes. Exercise increased PGE2 plasma levels in young supplemented athletes and in senior placebo athletes.

Conclusions of this study are limited because only five athletes of each group finished the nutritional intervention. Accordingly, the variation in human genetic background could influence the results, taking into account the small size of young and senior groups.

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Manuscript V

Effects of dietary Docosahexaenoic, training and acute exercise on lipid mediators

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Effects of dietary Docosahexaenoic, training and acute exercise on lipid mediators

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Abstract

Background: Eicosanoids mediate initiation and resolution of inflammation. Our aim was evaluating the effects of training, exercise and docosahexaenoic (DHA) supplementation on plasma eicosanoids levels and peripheral blood mononuclear cells (PBMCs) eicosanoids production.

Methods: Fifteen male footballers were distributed to placebo and experimental groups. Experimental group consumed DHA-enriched beverage (1.16 g DHA/day) for 8 weeks, placebo group consumed a placebo beverage. Blood samples were taken before and after the nutritional intervention in basal conditions and 2 h after acute exercise.

Results: Training increased basal Prostaglandin E1 (PGE_1) plasma levels and PBMCs cyclooxygenase 2 (COX-2) protein levels in both groups, but COX-1 protein levels only in the experimental group. Acute exercise increased plasma PGE_2 and PBMCs active NFκβ levels. Lipopolysaccharide (LPS)-stimulated PBMCs increases eicosanoids production (PGE_1, PGE_2, RvD1) in both groups and increased LPS-stimulated PBMCs active NFκβ. DHA supplementation increased COX-2 levels but decreased LPS-stimulated PBMCs PGE_1 and PGE_2 production. Neither DHA supplementation nor acute exercise altered the expression of NFκβ, COX-2, 15-LOX2, 5-LOX, or IL-1β genes in PBMCs.

Conclusions: The increase of PGE_1 plasma levels after training promoted systemic anti-inflammatory and vasodilator environment. Exercise and DHA supplementation acted synergistically by increasing plasma PGE_2 with anti-inflammatory effects. Exercise primed PBMCs to enhance PGE_1, PGE_2 and RvD1 production in response to LPS.

Trial registration: The project was registered at ClinicalTrial.gov (NCT02177383).

Keywords: Inflammation, Docosahexaenoic acid, PGE_1, PGE_2, RvD1, Lipid mediators, Exercise

Background

The practice of regular physical exercise has many health benefits, reducing the risk of suffering from several diseases such as cardiovascular disease, cancer, diabetes and osteoporosis [25]. Exercise exerts anti-inflammatory effects mediated by reduction of visceral fat mass [25], but may also be related to decreased levels of Toll-Like Receptors (TLRs) in monocytes [12] and to muscle production of bioactive compounds during contraction [29]. Each bout of acute exercise results in alterations in the pro/anti-inflammatory cytokine balance [6, 7, 36]. Moreover, the increased production of anti-inflammatory cytokines during exercise counteracts the production of pro-inflammatory cytokines associated to muscle damage, resulting in the instauration of an anti-inflammatory environment [28]. In addition, acute exercise alters the number and function of circulating immune cells [36], which are primed for their extravasation into tissues after exercise, thereby contributing to repair the damaged tissue [19].

Polyunsaturated omega-3 fatty acids (ω-3 PUFA) contribute to regulate inflammation associated with chronic diseases including obesity, arthritis and atherosclerosis [4]. The anti-inflammatory effects of ω-3 PUFA are evidenced by the inhibition of endotoxin-stimulated production of IL6 and IL8 in endothelial cells [18]. However,

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ω-3 PUFA diet supplementation does not affect plasma cytokine response to strenuous exercise, which produces muscle damage and inflammation [6, 7, 36]. The anti-inflammatory effects of ω-3 PUFA are mediated by their participation in the regulation of the nuclear factor κβ (NFκβ) signalling pathway and/or peroxisome proliferator-activated receptor (PPARγ) [3, 13]. The expression of pro-inflammatory genes via the NFκβ signalling pathway decreases after twelve weeks of ω-3 PUFA diet supplementation [31]. ω-3 PUFA diet supplementation also alters the function of immune cells [3], modulating chemotaxis and leukotriene generation in neutrophils by reducing arachidonic acid (AA) concentrations [20]. Eicosapentaenoic acid and docosahexaenoic acid (DHA) can compete with AA as a substrate for cyclooxygenase 2 (COX-2) and 5-lipoxygenase (5-LOX), thus reducing the production of inflammatory mediators derived from AA. This process plays a critical role in the initial inflammatory response [6, 7, 37]. Prostaglandins (PGs) mediators arising from the COX-1 and COX-2 cascade from AA or its precursors are central to vascular responses, permitting neutrophils and monocytes to leave post capillary venules. PGs also play a role in the regulation of muscle protein metabolism [2], vasodilation, human skeletal muscle microcirculation, febrile and inflammatory responses [39], and initiation and timely resolution of inflammation [32]. Prostaglandin E1 (PGE_1) and prostaglandin E2 (PGE_2) are potent vasodilators which account for the increased blood flow in inflamed areas but PGE_1 also regulates neutrophil function by reducing neutrophil activation [41]. Because of these vasodilator and immune properties, PGs are used as treatment for diseases derived from ischemia-reperfusion, such as arterial occlusive disease and venous ulcers [11, 22, 41].

The response of PGs to exercise has not been clearly established yet. Skeletal muscle produces PGs in response to muscular work, and this production is blocked by the intake of COX inhibitors, [22, 38]. Plasma PGE_2 concentration increases in response to exercise or to muscle damage [1]. In other words, exercise increases PGE_2 production as part of the inflammatory response, triggered by micro trauma occurring in skeletal muscles, [1]. In addition, PGE_2 synthesis by infiltrating macrophages in the inflamed muscle increases 24–48 h after an exercise session [40]. It is also noticeable that downhill running for 45 min at 75 % VO_2max increases circulating monocyte production of PGE_2 [5]. The effects of PGE_2 can be described as pro-inflammatory or anti-inflammatory depending on location, since PGE_2 enhances LTB4-mediated neutrophil extravasation and tissue injury, but it can also inhibit the NFκβ signalling pathway in macrophages [21]. This inhibition plays a critical function in the initiation of resolution via lipid mediator class switching [32].

A new family of lipid mediators produced from the oxidation of ω-3 PUFA including resolvins (Rvs), maresins and protectins has been described as pro-resolving mediators of inflammation [33]. The synthesis of these products involves COX and LOX pathways from DHA and EPA [33]. Rvs have been reported to reduce inflammation in chronic inflammatory diseases. Specifically, RvE1 and RvD1 inhibit trans-endothelial migration of neutrophils, preventing the infiltration of neutrophils into sites of inflammation, and RvD1 also inhibits IL1β production [34]. Plasma RvE1 and RvD1 have also been demonstrated to increase after acute exercise [22] but the participation of these lipid mediators in the anti-inflammatory effects of acute exercise has not been studied.

The aim of this study was to evaluate the effects of regular training and DHA diet supplementation on plasma lipid mediators. The effects of DHA diet supplementation and acute exercise on plasma prostaglandins and on PBMC capabilities to produce cytokines, prostaglandins and pro-resolving mediators in response to lipopolysaccharide (LPS) stimulation were also analysed.

Methods

Subjects and anthropometric characteristics

Fifteen male soccer players from the Real Mallorca B team volunteered to participate in this study, 6 subjects took one liter of a placebo beverage five times a week and the other 9 an experimental beverage rich in DHA, over a period of 8 weeks. The subjects and study design was the same described previously [6, 7, 23, 24]. At the beginning of the study, 22 subjects were recruited, but 6 of them left the football team during the experimental time and joined the first and professional team and one broke the anterior cruciate ligament of the knee. All subjects gave their written informed consent after an explanation of the experimental procedures and before commencement of the study. The study protocol was in accordance with the Declaration of Helsinki for research on human subjects and was approved by the Ethical Committee of Clinical Investigation of the CAIB (Palma de Mallorca, Balearic Islands, Spain). The project was registered at ClinicalTrial.gov (NCT02177383).

Height was determined using a mobile anthropometer (Kawe 44444, Asperg, Germany) to the nearest millimetre, with the subject's head in the Frankfurt plane. Body weight was determined to the nearest 100 g using a digital scale (Tefal, sc9210, Rumilly, France). The subjects were weighed in bare feet and light underwear, and the mean of three measurements was used [6, 7, 23, 24]. Body mass index was calculated using the following equation: [BMI = mass (kg)/squared height (m)]. Participants in the study were 19.7 ± 0.4 years old, 76.5 ± 2.5 kg weight, and 179.5 ± 2.5 cm height. The waist

circumference was 78.4 ± 0.9 cm, hip circumference was 97.4 ± 1.2 cm, waist-hip ratio (WHR) was 0.81 ± 0.01. The systolic blood pressure 119.5 ± 6.5 mmHg, and 61.7 ± 4.7 mmHg for the diastolic blood pressure. The Body Mass Index (BMI) was 23.7 ± 0.55, kg/m^2. The soccer players had 92.6 ± 0.2 % fat free mass. The VO_2max determined following the test of Leger-Boucher, was 61.4 ± 1.35 mL/kg min. There were no significant differences between groups in the anthropometric characteristics of players.

DHA supplementation

DHA was administered to the athletes using an almond-based isotonic beverage. Both placebo and supplemented beverages were made up of 3.0 % almond and 0.8 % sucrose and the rest was water, flavour, and added oils and vitamin E. Moreover, the placebo drink contained 0.8 % refined olive oil whereas the experimental drink contained 0.6 % refined olive oil and 0.2 % wt % DHA-S (DSM, Columbia, USA). The two almond beverages were manufactured by Liquats Vegetals S.A. (Girona, Spain) following an industrial process. Two beverages were made-up by: cinnamon and lemon natural flavours, sucrose, vitamin E, and the respective oil for the experimental (olive oil plus DHA-S) or placebo (olive oil) drink [6, 7, 23, 24]. Finally, the beverages were sterilized and packed. Both types of beverages were identical in taste and visual appearance. Beverages fatty acid composition was determined following the same procedure previously described [23, 24]. The experimental beverage had a significantly greater content of C20:3 (0.640 mg/100 ml of beverage), C22:0 (2.57 mg/100 ml of beverage), C22:5 (56.1 mg/100 ml of beverage) and C22:6n3 (114 mg/100 ml of beverage) compared to the placebo beverage, in which these fatty acids were not present. No significant differences between beverages were evidenced in vitamin E content (41.6 mg/L of beverage n placebo drink, and 45.7 mg/L of beverage in the experimental drink).

Dietary intake and nutritional intervention

Dietary habits of subjects were assessed using a 7-day dietary record questionnaire completed at the beginning of the study and in the week before the exercise test as previously described [6, 7, 23, 24]. A qualified dietician verified and quantified the food records. All food items consumed were transformed into nutrients using a special computerized program based on the European and Spanish food composition tables [6, 7, 23, 24]. DHA intake in the placebo group from the diet was 109 ± 40.7 mg/day, and DHA intake in the experimental group was 1209 ± 40.7 mg/day. The five times a week intake of 1 L of the supplemented beverage supposed a daily intake of about 1.14 g of DHA additional to the basal DHA intake through the diet in the experimental group, whereas the placebo only took up DHA from the diet.

This nutritional intervention with the respective beverages for eight weeks is reflected in the erythrocytes fatty acid composition [23, 24].

Experimental procedure

For each subject two blood samples were taken at the end of the nutritional intervention, in basal conditions and after performing a soccer training session. The exercise consisted in a 2 h of habitual physical training session. After a 15 min warm-up, the players performed the Leger Boucher test. After that, players practiced a recovery exercise of control-passing for 15 min. The main body of the training session was characterized by small-sided games. Briefly, the first exercise consists of 5 vs 5 possession exercise in an area of 20 x15 m (4 repetitions of 5 min with 30 s of recovery between repetitions); the second was a 6 vs 6 possession exercise in an area of 30 x 20 m (3 repetitions of 6 min with 1 min of recovery between repetitions), and finally, the players played a soccer match 5 vs 5 in 30 x 40 m for 20 min. The training session was designed to perform exercise intensity at more than 70 % VO_2max over 50 % duration of the session in order to induce an oxidative stress situation [35]. These conditions were followed by all participants in the study.

Blood samples used for analysis were collected from the same athletes, at the same time, using the same exercise protocols as previous described [6, 7, 23, 24]. The blood samples used to measure prostaglandins and Rvs plasma levels, cytokine production and expression were the same as those previously used [6, 7].

PBMCs purification

Blood samples were centrifuged at 900 g at 4 °C for 30 min and the plasma was keep. The erythrocyte phase at the bottom was washed with PBS, centrifuged as above and finally erythrocytes were reconstituted with distilled water. The PBMCs fraction was purified from whole blood following an adaptation of the method previously described [6, 7] using Ficoll-Paque PLUS reagent (GE Healthcare®). An aliquot of PBMCs was used for LPS incubation.

PBMC incubation with LPS

Incubations of PBMCs were performed in RPMI 1640 culture media containing 2 mM l-glutamine in the presence of the bacterial stimuli LPS. PBMCs obtained after 8-week beverage supplementation in basal conditions and after exercise were diluted with RPMI 1640 culture media to 2x10^6 cells/ml and activated by addition of LPS from *Escherichia coli* (1 μg/ml). Samples were incubated in polypropylene tubes at 37 °C for 2 h. In a parallel

experiment, an aliquot of PBMCs obtained after exercise were activated with LPS and incubated at either 37 °C or 39.5 °C for 2 h. Then, after shaking, the cells from both experiments were pelleted by centrifugation (900 g, 5 min) and the cell-free supernatants were stored at -70 °C for cytokine determinations.

Active NFκβ determination

An isolated suspension of PBMCs was subjected to whole-cell protein extraction for the determination of NFκβ p50 activation, which was performed using the ELISA method TransAM NF-kB p50 Chemi according to the manufacturer's instructions (Active Motif®). Briefly, the primary antibody used to detect NFκβ recognizes an epitope on p50 that is accessible only when NFκβ is activated and bound to its DNA target.

Cytokine and lipid mediator determination

IL1β and MCP1 were measured in culture medium supernatant using ELISA kits. IL1β and MCP1 kits (RayBio®); intra-assay and inter-assay reproducibility for both kits were lower than 10 % and 12 %.

PGE_1 and PGE_2 were measured in plasma and in culture medium supernatant using ELISA kits (Enzo Life Sciences®). Intra-assay and inter-assay reproducibility for PGE_1 were lower than 10 % and 12 %, respectively, while intra-assay and inter-assay reproducibility for PGE_2 were lower than 6 % in both cases.

RvD1 concentration in culture medium supernatants was determined using an RvD1 EIA Kit (Cayman®), following the instructions manual. Intra-assay reproducibility was 10 %.

PBMCs RNA extraction and real time PCR assay

COX-2, NFκβ, 15-LOX2, IL-1β, 5-LOX mRNA levels were determined by multiplex real time PCR based on incorporation of a fluorescent reporter dye and using human 18S rRNA as reference. For this purpose, total RNA was isolated from PBMCs by Tripure extraction (Roche Diagnostics®). RNA (1 μg) from each sample was reverse transcribed using 50 U of Expand Reverse Transcriptase (Roche Diagnostics, Germany) and 20 pmol oligo for 60 min at 37 °C in a 10 μL final volume, according to manufacturer instructions. The resulting cDNA (2.5 μL) was amplified using the Light-Cycler FastStart DNA MasterPLUS SYBR Green I kit (Roche Diagnostics®). Amplification was performed at 55 °C and 45 cycles. The relative quantification was performed by standard calculations considering 2(-ΔΔCt). Inflammatory gene expression levels before and after the session were normalized to the invariant control 18S rRNA. mRNA levels at the beginning of the stage were arbitrarily referred to as 1. Primers used are 18S forward (Fw): 5′-ATG TGA AGT CAC TGT GCC AG-3′ and Reverse: 5′-GTG TAA TCC GTC TCC ACA GA-3′ annealing temperature 60 °C; COX-2 Fw: 5-TTG CTG GCA GGG TTG CTG GTG GTA-3′, and Rv: 5′-CAT CTG CCT GCT CTG GTC AAT GGA A-3′ annealing temperature 67 °C; NFκβ, Fw: 5′-AAA CAC TGT GAG GAT GGG ATC TG-3′ Rv: 5′-CGA AGC CGA CCA CCA TGT-3′ annealing temperature 60 °C; 15-LOX2, Fw: 5′-GCA TCC ACT GAT TGG ACC TT-3′ and Rv: 5′-GCT GGC CTT GAA CTT CTG AC-3′ annealing temperature 61 °C; IL-1β Fw: 5′-GGA CAG GAT ATG GAG CAA CA-3′, and Rv: 5′-GGC AGA CTC AAA TTC CAG CT -3′ annealing temperature 58 °C; 5-LOX, Fw: 5′-GGG CAT GGA GAG CAA AGA AG-3′ and Rv: 5′-ACC TCG GCC GTG AAC GT-3′ annealing temperature 59 °C.

SDS-polyacrylamide gel electrophoresis and western blot analysis

Cells were lysed with 250 μL of RIPA buffer [250 mM Tris/HCl, pH 8.0, 4.4 % NaCl, 5 % IGEPAL®, 2.5 % deoxycholic acid, 0.5 % sodium dodecylsulfate (SDS)]. 20 μg of proteins of total cell extract was loaded on 12 % polyacrylamide gel, and were separated by size using SDS polyacrylamide gel. Following electrophoresis, samples were transferred onto a nitrocellulose membrane and incubated with a primary monoclonal antibody anti-COX-1 (1:1000) and anti-COX-2 (1:1000) (Cayman®).

Blots were then incubated with a secondary peroxidase-conjugated antibody (1:10,000), and the primary antibody was performed. Protein bands were visualized by Immun-Star® Western C® Kit reagent (Bio-Rad Laboratories) Western blotting detection systems. The chemiluminescence signal was captured with a Chemidoc XRS densitometer (Bio-Rad Laboratories®) and analyzed with Quantity One-1D Software (Bio-Rad Laboratories®).

Statistical analysis

Statistical analysis was carried out using Statistical Package for Social Sciences (SPSS v.15.0 for Windows). Results are expressed as mean ± SEM. and $P < 0.05$ was considered statistically significant. A Kolmogorov-Smirnov test was applied to assess the normal distribution of the data. The statistical significance of the data was assessed by two-way analysis of variance (ANOVA). The statistical factors analysed were beverage supplementation (S), acute exercise (E), training season (T) and LPS stimulation (A). The data sets with a significant SxE, SxT, ExA, SxA and SxAxE interactions were tested by the ANOVA one-way test in order to identify groups who were different. When significant effects of S, E, T or A factor were found, a Student's t test for paired data was used to determine the differences between the groups involved.

Results

Normality of the data was assessed using the Kolmogorov-Smirnov test and the dependent variable was normally distributed in each group that was compared in the ANOVA test.

Regular training and DHA diet supplementation effects

Training season and DHA diet supplementation significantly influenced PBMC counts. The training season increased circulating PBMCs from $2.79 \pm 0.16x10^3$ to $3.34 \pm 0.15x10^3$ cells/μL in the placebo group, and from $3.23 \pm 0.29x10^3$ to $3.99 \pm 0.34x10^3$cells/μL in the experimental group. This increase only was significantly in the experimental group.

DHA diet supplementation for 8 weeks caused a variation in the DHA percentage on erythrocytes membrane (results not shown). The initial percentage of DHA respect to erythrocyte total fatty acid in placebo group (7.37 ± 0.27 %) was maintained after the diet supplementation with placebo beverage (8.40 ± 0.41 %), whereas the initial percentage of DHA in experimental group (7.93 ± 0.54 %) was significantly increased (10.8 ± 0.50 %) to attain a significantly higher content than the placebo group.

Regular training of soccer players for 8 weeks significantly influenced the basal plasma levels of PGE_1 whereas DHA diet supplementation significantly changed the basal plasma levels of PGE_2 (Fig. 1). Plasma PGE_1 significantly increased after 8 weeks of training in

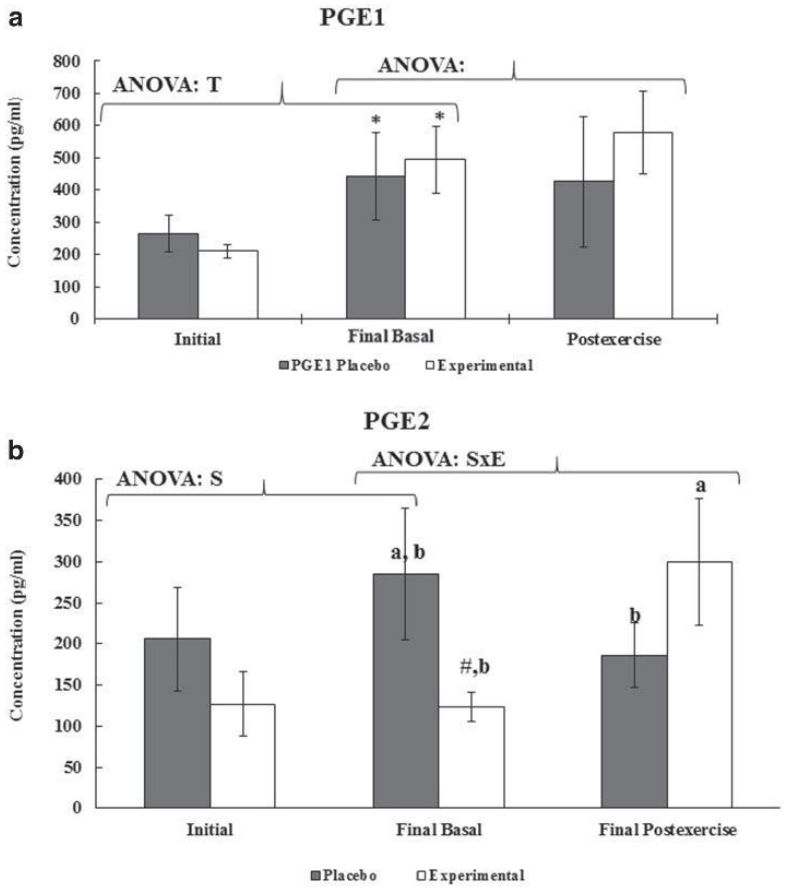

Fig. 1 Effects of acute exercise, DHA diet supplementation and training season on PGE1 and PGE2 plasma levels. Statistical analysis: Two-way ANOVA, $p < 0.05$. (T) Significant effect of Training season, (S) Significant effect of Supplementation, (E) Significant effect of Acute Exercise, (ExT) Significant interaction between both factors. One-way ANOVA, $p < 0.05$. (*) Significant difference between Initial and Final, (#). Significant differences between Placebo and Experimental groups, ($) Significant differences between the Basal and Post-exercise. When interaction exists between different groups, different letters reveal significant differences. Results are the mean ± SEM

both placebo and experimental groups, while plasma PGE_2 maintained the initial basal levels. No significant differences were reported in PGE_2 at the beginning of the intervention although its levels in the experimental group were lower than in the placebo. This trend was magnified after 8 weeks training reporting significant differences due to DHA diet supplementation.

Regular training but not DHA diet supplementation significantly influenced COX-1 and COX-2 protein levels in PBMCs (Fig. 2). Eight weeks of regular training significantly increased COX-1 and COX-2 protein levels in experimental group but not in placebo group.

Acute exercise and DHA diet supplementation effects

Acute exercise did not influence PBMC counts, maintaining the basal values both in placebo and experimental groups (Placebo group: 3.34 ± 0.15 in basal conditions, 3.19 ± 0.15 after acute exercise; Experimental group: 3.99 ± 0.34 in basal conditions, 3.13 ± 0.16 after acute exercise, all PBMC results are expressed as 10^3cells/μL).

The effects of acute exercise and DHA diet supplementation on PGE_1 and PGE_2 plasma levels are shown in Fig. 1. Acute exercise and DHA diet supplementation did not influence PGE_1 plasma levels, whereas a statistically significant interaction between acute exercise and

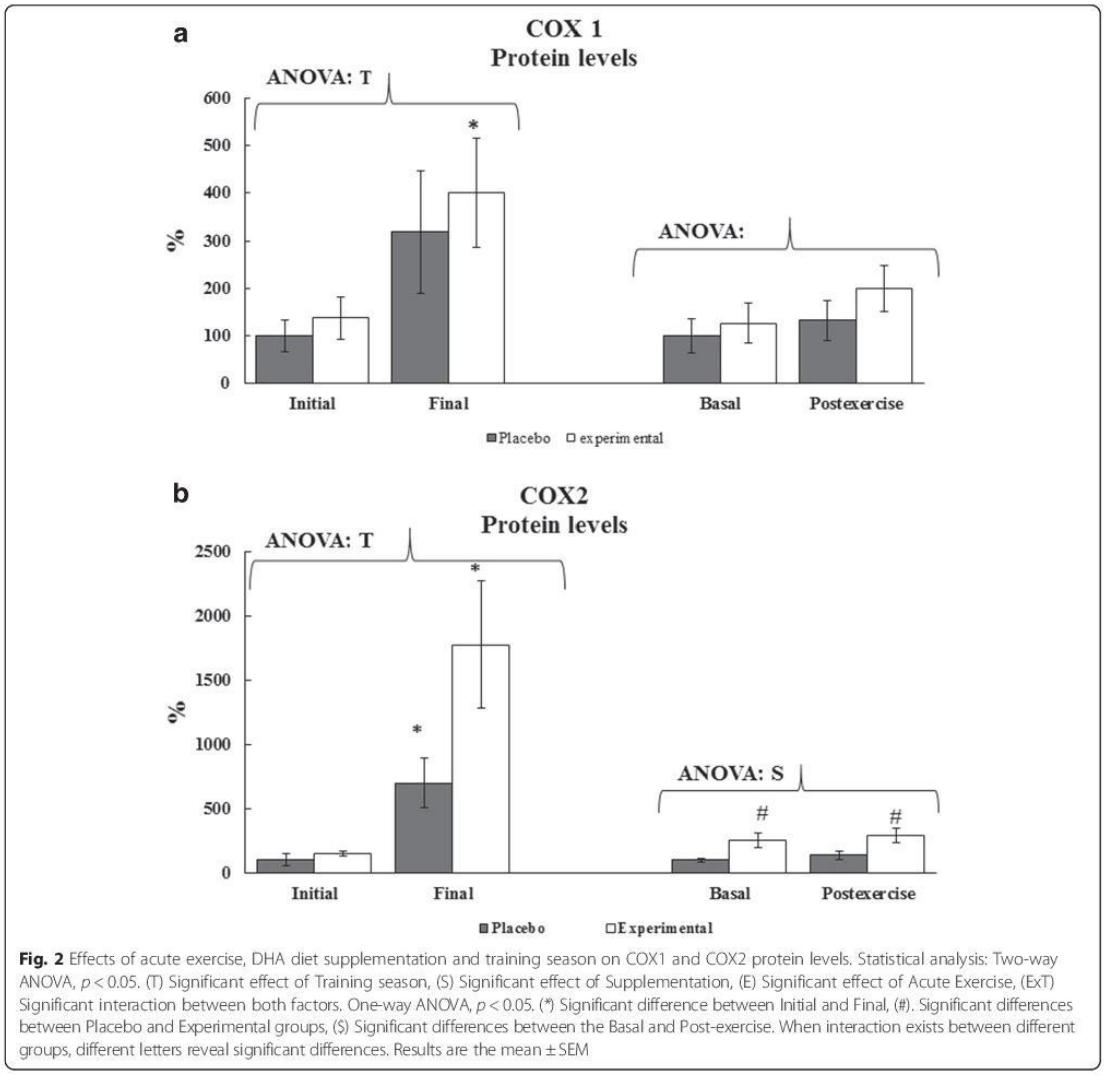

Fig. 2 Effects of acute exercise, DHA diet supplementation and training season on COX1 and COX2 protein levels. Statistical analysis: Two-way ANOVA, $p < 0.05$. (T) Significant effect of Training season, (S) Significant effect of Supplementation, (E) Significant effect of Acute Exercise, (ExT) Significant interaction between both factors. One-way ANOVA, $p < 0.05$. (*) Significant difference between Initial and Final, (#). Significant differences between Placebo and Experimental groups, ($) Significant differences between the Basal and Post-exercise. When interaction exists between different groups, different letters reveal significant differences. Results are the mean ± SEM

DHA diet supplementation was reported in plasma levels of PGE_2. The experimental group showed significantly increased levels (about 2.4 times) of plasma PGE_2 after acute exercise, whereas the placebo group maintained basal levels. This response resulted in higher PGE_2 plasma levels in the experimental group (about 1.6 times) with respect to the placebo one.

The effects of DHA diet supplementation and acute exercise on the production rates of lipid mediators by LPS-stimulated PBMCs at 39.5 °C were also evaluated (Table 1). Acute exercise caused a significant increase in PGE_1 and PGE_2 production by LPS-stimulated PBMCs both in placebo and experimental groups. DHA diet supplementation significantly ameliorated the increase in both PGs production rate by LPS-stimulated PBMCs. The RvD1 production rate by LPS-stimulated PBMCs at 39.5 °C increased after acute exercise in both groups, but the increase was only significant (about 2.2 times) in the experimental group,

The effects of LPS-stimulation of PBMCs, acute exercise, and DHA diet supplementation on PBMC cytokine production are reported in Table 2. LPS-stimulation of PBMCs significantly increased IL1β basal production rate in both placebo (about 2.2 times) and experimental (about 1.8 times) groups, without any effect by DHA diet supplementation or acute exercise. No significant effects induced by LPS-stimulation or DHA diet supplementation were observed in MCP1 production rate. However, acute exercise significantly increased the MCP1 production rate by non-stimulated PBMCs, mainly in the experimental group; while no significant effects between groups of DHA diet supplementation were observed.

In order to evaluate the participation of NFκβ activation in the response to LPS-stimulation and to acute exercise, active NFκβ levels were determined in PBMCs (Fig. 3). Active NFκβ levels significantly increased about 20 % respect to the basal value after acute exercise. Basal PBMC incubation with LPS induced a significant increase of 27 % and 35 % in NFκβ activation at 37 °C and 39.5 °C, respectively.

The effects of acute exercise and DHA diet supplementation on the expression of inflammatory and anti-inflammatory genes, and COX-1 and COX-2 protein levels in PBMCs were determined (Table 3). No significant changes were observed in the expression of any of the evaluated genes due to acute exercise or DHA diet supplementation. Neither acute exercise nor DHA diet supplementation altered COX-1 protein levels in PBMCs (Fig. 2). COX-2 protein levels in PBMCs of the experimental group were significantly higher (about 2 times) than in the placebo group in both basal and post-exercise conditions; although no effects were evidenced due to the acute exercise.

Discussion

Effects on plasma prostaglandin levels

PGs are relatively transient molecules with a half-life of only seconds to minutes, and they work in an autocrine and paracrine approach through specific receptors [38]. Despite this signalling role and short half-life, basal plasma levels of PGE_1 and PGE_2 observed in soccer players are relatively high. Both molecules show similar values to those of untrained male university students [16], higher than those of ironman sportsmen [1], and lower than post-menopausal or young women [9]. Regular training for 8 weeks practically duplicated the basal plasma levels of PGE_1, but did not affect basal plasma levels of PGE_2. Treatments with PEG_1 are used to ameliorate several diseases related to ischemia-reperfusion [11]. In these studies, PGE_1 treatment induced vasodilatation, inhibited platelet and leukocyte aggregation and decreased oedema formation during reperfusion. The dose of PGE_1 used in these treatments increased plasma PGE1 levels up to values similar to the basal plasma PGE_1 levels observed in soccer players after a training season. The anti-inflammatory and health effects of regular exercise [25, 29] could be related to increasing plasma basal PGE_1 levels, taking into account the anti-inflammatory and vasodilator role of PGE_1 [11, 39]. There are several possible origins of plasma PGs as a

Table 1 Effects of acute exercise and DHA diet supplementation on lipid mediator PBMCs-stimulated production rates

		Basal	Post-exercise	ANOVA			
				S	E	SxE	Fd
PGE1 (pg/h 10^6 cells)	Placebo	51,3 ± 8,19	159 ± 25[b]	.000 (*F* = 12.32)	.002 (*F* = 25.786)	.102 (*F* = 2.917)	(3, 22)
	Experimental	22,6 ± 6,47	76,2 ± 11,1[ba]				
PGE2 (pg/h 10^6 cells)	Placebo	263 ± 51,7	615 ± 114[a]	.000 (*F* = 11.07)	.003 (*F* = 22.787)	.813 (*F* = .057)	(3, 22)
	Experimental	46,5 ± 14,7[b]	365 ± 40,5[ab]				
RvD1 (pg/h 10^6 cells)	Placebo	4.24 ± 0.74	7.24 ± 1,69	.956 (*F* = .003)	.007 (*F* = 8.949)	.0668 (*F* = .189)	(3, 22)
	Experimental	3.79 ± 0,39	7.81 ± 1,11[b]				

Statistical analysis: Two-way ANOVA, $p < 0.05$., (S) Significant effect of Supplementation, (E) Significant effect of Acute Exercise, (ExS) Significant interaction between both factors. One-way ANOVA, $p < 0.05$. ([a]) Significant differences between Placebo and Experimental groups, ([b]) Significant differences between Basal and Post-exercise. When interaction exists between different groups, different letters reveal significant differences. Results are the mean ± SEM. Fd means Freedom degree

Table 2 Effects of LPS-stimulation, DHA diet supplementation and exercise on cytokine production rate by PBMCs

		Basal		Post-exercise		ANOVA
		NO LPS	LPS	NO LPS	LPS	
IL1β (pg/h 10^6 cells)	Placebo	6.22 ± 1.46	16.3 ± 4.79$	6.68 ± 1.51	21.1 ± 7.34$	A (.002)
						(*F = 10.673*)
	Experimental	9.35 ± 2.24	17.6 ± 6.28$	8.34 ± 2.19	21.7 ± 8.07$	Fd (7,41)
MCP1 (pg/h 10^6 cells)	Placebo	32.1 ± 9.79	36.1 ± 4.07	36.2 ± 6.14	45.4 ± 3.87	E(.002)
						(*F = 10.437*)
	Experimental	22.7 ± 5.31	29.6 ± 4.98	49.6 ± 4.73#	38.3 ± 3.81	Fd (7,41)

Statistical analysis: Two-way ANOVA, $p < 0.05$. S, Supplementation effect; A, LPS-stimulation effect, E, Exercise effect, SxA, interaction between Supplementation and LPS-stimulation effects, SxE, interaction between Supplementation and Exercise effects, ExA, interaction between Exercise and LPS activation effects, AxExS, interaction between effects of three factors. *Differences between Placebo and experimental groups; #difference between Basal and Post-exercise conditions p; $differences between NO LPS-stimulated and LPS-stimulated groups. When interaction exists between different groups, different letters reveal significant differences. Results are the mean ± SEM. Fd means Freedom degree

result of the ubiquitous presence of COX enzymes, including muscle, immune cells and platelets; therefore muscle could be a source of PGs during exercise [38]. Regular exercise training *per se* influences the phospholipid fatty acid composition of muscle membranes, but has no effect on the composition of fatty acids stored in triacylglycerol within the muscle [15]. A training season was able to induce changes in the muscle phospholipid composition in such a way as to enhance the capabilities to PGE_1 synthesis, in accordance with the increased plasma PGE_1 levels present after 8 weeks of training. The different pattern for plasma PGE_1 and PGE_2 in response to training and DHA diet supplementation is not surprising, given the different biosynthesis pathways of these two PGs [30]. PGE_2 is synthesized from AA (C20:4 Δ5, 8, 11,14) [30], whereas PGE_1 is synthesized from dihomo-γ-linolenic acid (DGLA) (C20:3 Δ8,11,14), a precursor of AA [30]. The relative differences of plasma PGE_2 versus PGE_1 levels is not merely a function of the relative abundance of AA versus DGLA in tissues, but could also be related to the different cellular metabolism of these two fatty acids. Chronic exercise induces changes in the enzyme activities related to eicosanoid metabolism [26]; elongase activity increases but Δ5- and Δ9-desaturase activities have no consistent changes attributed to chronic exercise [26], although Δ5-desaturase was found to be generally lower in muscles of trained rats [14]. Due to the limited activity of Δ5-desaturase, most DGLA is inserted into membrane phospholipids at the same C-2 position as for AA [30]. This picture is in accordance with the effects of a training season on basal plasma PGE_1 levels enhancing DGLA availability in muscle phospholipids, in order to facilitate PGE_1 synthesis via an increase in COX-1 and COX-2 muscle activities. It is also in accordance with a lack of effects of exercise on the fatty acid composition of muscle lipids

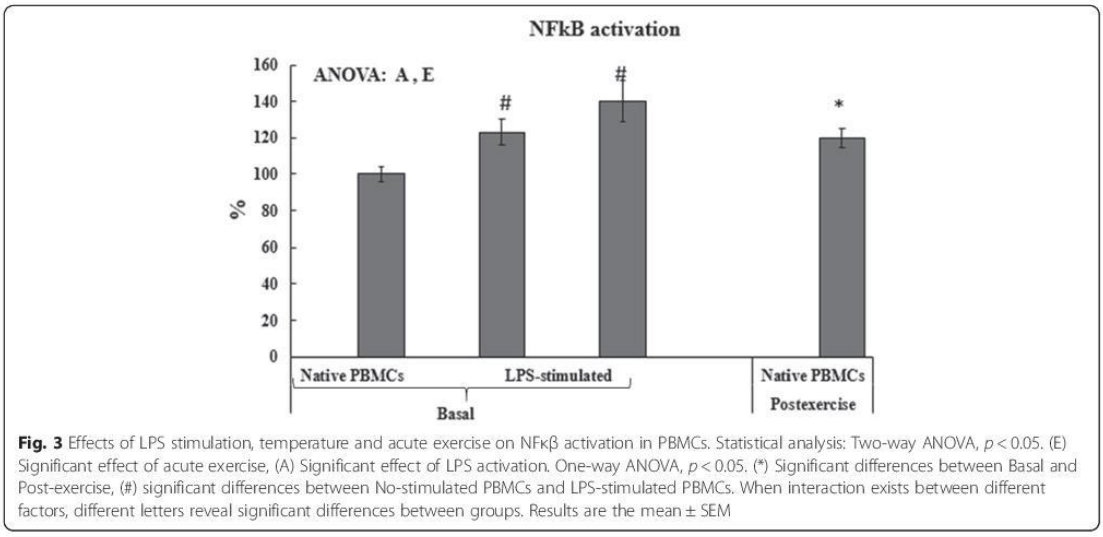

Fig. 3 Effects of LPS stimulation, temperature and acute exercise on NFκβ activation in PBMCs. Statistical analysis: Two-way ANOVA, $p < 0.05$. (E) Significant effect of acute exercise, (A) Significant effect of LPS activation. One-way ANOVA, $p < 0.05$. (*) Significant differences between Basal and Post-exercise, (#) significant differences between No-stimulated PBMCs and LPS-stimulated PBMCs. When interaction exists between different factors, different letters reveal significant differences between groups. Results are the mean ± SEM

Table 3 Effects of DHA diet supplementation and exercise on PBMCs gene expression

		Basal	Post-exercise	ANOVA			
				S	E	SxE	Fd
NFκβ (AU)	Placebo	1.00 ± 0.22	1.21 ± 0.31	.576 (*f* = .373)	.369 (*f* = .142)	.906 (*f* = .006)	(3,42)
	Experimental	1.33 ± 0.33	1.46 ± 0.33				
COX-2 (AU)	Placebo	1.00 ± 0.28	1.99 ± 0.86	.355 (*f* = .026)	.726 (*f* = .592)	.513 (*f* = .142)	(3,42)
	Experimental	1.46 ± 0.45	1.80 ± 0.51				
15-LOX2 (AU)	Placebo	1.00 ± 0.37	1.43 ± 0.76	.402 (*f* = .717)	.973 (*f* = .001)	.983 (*f* = .000)	(3,45)
	Experimental	1.03 ± 0.30	1.43 ± 0.43				
5-LOX (AU)	Placebo	1.00 ± 0.28	1.10 ± 0.42	.694 (*f* = .109)	.742 (*f* = .157)	.956 (*f* = .003)	(3,52)
	Experimental	0.89 ± 0.21	1.02 ± 0.18				
IL1β (AU)	Placebo	1.00 ± 0.34	0.84 ± 0.17	.906 (*f* = .000)	.998 (*f* = .014)	.496 (*f* = .472)	(3,42)
	Experimental	0.80 ± 0.22	1.03 ± 0.29				

Statistical analysis: Two-way ANOVA. (S) Significant effect of supplementation, (E) Significant effect of exercise, (SxE) Significant interaction between both factors. (#)Significant differences between Placebo and Experimental groups. (*)Significant differences between the Pre-exercise and Post-exercise. $p < 0.05$. When interaction exists between different groups, different letters reveal significant differences. Results are the mean ± SEM. Fd means Freedom degree. AU means arbitrary units referred to Pre-exercise Placebo values

reported by a nutritional intervention [26], because no effects of DHA diet supplementation are observed on the plasma levels of PGE_1.

Acute exercise increases intramuscular PGE_2 levels [17], and also increases or does not affect PGE_2 plasma levels [9], which are attributed to muscle PGE_2 production. Neither DHA diet supplementation nor acute exercise altered. PGE_2 plasma levels, but these two factors interact resulting in a significant increase in plasma PGE_2 levels after acute exercise in the experimental group. PGE_2 has pro-inflammatory or anti-inflammatory effects depending on location: it enhances LTB4-mediated neutrophil extravasation and tissue injury, but it inhibits the NFκβ signalling pathway via the EP4 receptor, the LPS-induced IL6 release [26], and plays a critical role in the initiation of lipid mediator class switching [32]. PGE_2 has been evidenced to suppress lymphocyte proliferation and natural killer cell activity, and to inhibit the production of TNFα, IL1, IL6, IL2 and IFNγ [4]. Acute exercise and DHA diet supplementation have synergistic effects against inflammation by enhancing PGE_2 levels in plasma. These results could reinforce the idea that practising regular exercise results in health benefits and reduces the risk of inflammation related diseases [25].

Effects on PBMC capabilities to produce lipid mediators and cytokines

It is well established that dietary ω-3 PUFA alters the fatty acid composition of immune cells [36]. This altered fatty acid composition could affect immune cell function [6, 7] and the capability to produce cytokines and lipid mediators. Similarly, training *per se* alters the fatty acid composition of phospholipids and enzyme activities of the eicosanoid metabolism [26]. Protein levels of COX-1 and COX-2 significantly increased in PBMCs after 8 weeks of a training season, mainly in the DHA supplemented group. In addition, this is the first time that an enhancing effect of DHA diet supplementation on COX-2 levels has been described in PBMCs. This could indicate greater capabilities of PBMCs after training and after acute exercise in the DHA supplemented group to transform DGLA into PGE_1 or AA into PGE_2, which in fact could increase basal plasma PGE_1 and PGE_2 levels; however, the changes in PGE_1 and PGE_2 plasma levels are not consistent with these pictures. This suggests that PBMCs are not responsible for plasma PGE_1 and PGE_2 levels, even after acute exercise. COX-2 protein levels were significantly higher in the DHA diet supplemented group than in the placebo group, both in basal and post-exercise conditions. These results are in accordance with previous studies, in which an increase in both COX2 activity and protein levels was observed after resistance exercise, and this increase was maintained among 24 h [8]. The COX-2 isoform is inducible and is involved in febrile and inflammatory responses [38], whereas the COX-1 isoform is constitutively expressed in most cells and catalyzes the production of PGs involved in homeostatic control and cell maintenance. Synergistic effects of DHA diet supplementation and acute exercise on PBMC COX-2 levels are not reflected in the rate of PGE_1 and PGE_2 production by LPS-stimulated PBMCs. It seems that increased levels of COX-2 have no functional consequences, because DHA diet supplementation decreases the production rate of PGE_1 and PGE_2 by LPS-stimulated PBMCs. Discordances between COX protein levels and COX activity have been previously described [8]. Acute exercise transiently increased COX-1 activity in muscle independently of COX-1 protein levels; in contrast, both COX-2 activity and protein levels were

elevated with exercise and this rise persists until at least 24 h after resistance exercise [8]. In contrast, DHA diet supplementation did not alter gene expression of NFκβ, COX-2, 15-LOX2, IL1β or 5-LOX in PBMCs, even after acute exercise.

LPS-stimulated PBMCs also synthesize RvD1. Rvs are lipid mediators produced from ω-3 PUFA such as EPA (Resolvin E) and DHA (Resolvin D) [33]. RvD1 is produced from DHA through a pathway initiated by COX-2 [33, 34]. Oil fish diet supplementation has been demonstrated to increase leucocyte capabilities to produce Rvs [33]. RvD1 has pro-resolving and anti-inflammatory properties by inhibiting trans-endothelial migration of neutrophils, preventing their infiltration into sites of inflammation and inhibiting IL1β production [33, 34]. The RvD1 production rate by LPS-stimulated PBMCs was significantly increased after exercise, mainly in the DHA supplemented group. Rvs are synthesized by immune cells after initiation of inflammation in order to contribute to its complete resolution and returning to homeostasis [32]. These results are in accordance with previous studies showing an increase in RvD1 serum levels after acute exercise [22], which could be related in part to the anti-inflammatory effects of regular exercise [25]. LPS-stimulated PBMCs simulate the effects of an infection. In self-limited *Escherchia coli* infections, resolution programs were activated in mice, and RvD1 and RvD5 were elevated [10] in accordance with the effects of LPS activation on RVD1 production rate by PBMCs. The greater capabilities to produce RVD1 by LPS-stimulated PBMCs at post-exercise respect to basal could indicate that post-exercise circulating immune cells are switching in order to resolve inflammation to a greater extent than basal samples. More studies are needed to assess whether other lipid mediators influence the anti-inflammatory effects associated with physical exercise.

LPS stimulates PBMC basal production rate of cytokines such as IL6, IL8, and TNFα [6, 7]. LPS links to TLR4 by activating the NFκβ pathway and inducing an inflammatory response [27], which was evidenced by increased NFκβ activation after LPS-stimulation of PBMCs at 37 °C and 39.5 °C and increased basal rate of IL1b production. Acute exercise also increased active NFκβ in PBMCs, but it was in parallel with a lack of acute exercise effects on the expression of inflammatory genes. Active NFκβ translocates to the nucleus allowing the expression of inflammatory genes but additional stimulus must be operative to enhance the expression of NFκβ, COX-2, 15-LOX2, IL1β and 5-LOX in PBMCs after acute exercise. Acute exercise increased active NFκβ levels priming PBMCs for inflammatory response.

Acute exercise increased PBMC production rate of MCP1 and also primes PBMCs to respond to LPS-stimulation in order to enhance their PGE_1, PGE_2, RvD1 and MCP1 production. These results are in accordance with increased levels of TLR4 found in PBMCs after acute exercise, and with the higher rate of cytokine production after LPS-stimulation of post-exercise PBMCs [6, 7]. DHA diet supplementation attenuated the response of acute exercise to stimulation of PBMCs with LPS. The anti-inflammatory effects of DHA-diet supplementation have been described in previous studies [6, 7, 36]. DHA-supplementation attenuates the effects of acute exercise by increasing TLR4 levels in PBMCs [6, 7]. ω-3 PUFA can interfere with TLR4 activation by LPS; this process inhibits signalling components downstream from TLR4 but can also directly prevent NFκβ activation by impeding I-κβ phosphorylation and therefore prevent NFκβ translocation into the nucleus [27].

Conclusion

In summary, regular training promotes systemic anti-inflammatory and vasodilator effects by increasing PGE_1. Acute exercise and DHA diet supplementation act synergistically by increasing plasma PGE_2 and also with anti-inflammatory effects. Acute exercise primes PBMCs to enhance PGE_1, PGE_2 and RvD1 production rates in response to LPS, with a higher anti-inflammatory and resolute profile for PBMCs after exercise. The differences between eicosanoid production by PBMCs and plasma eicosanoid levels reinforce the idea that PBMCs are not the main contributors to plasma PGE_2 and PGE_1 after acute exercise.

Finally, prescription of regular exercise, similar to football training, could produce anti-inflammatory effects associated to an increase in PGE1 and PGE2 levels. Furthermore, the enhancement of muscular prostaglandins synthesis, probably trough exercise, could subserve these anti-inflammatory and vasodilator effects. In addition, acute exercise and DHA supplementation can act synergistically in the induction of anti-inflammatory response, but also both factors may reduce the inflammatory response induced by pathogen-associated molecular pattern (PAMPs) as LPS.

Limitation of the study

The experimental procedure was designed to perform exercise at intensity higher than 70 % VO_2max for more than 50 % duration of the session in order to induce an oxidative stress situation. These conditions were followed by all participants in the study; however, we did not realized any quantification of the effort using portable metabolic carts, activity monitors or video analysis to confirm energy expenditure or movement pattern, and this fact could be the main limitation of the study. Another limitation was low number of participant in each group. Twenty-two athletes, eleven in each group, participate in the trail at the beginning of the study;

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Manuscript VI

Docosahexaenoic diet supplementation, exercise and temperature affect cytokine production by lipopolysaccharide-stimulated mononuclear cells

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ORIGINAL PAPER

Docosahexaenoic diet supplementation, exercise and temperature affect cytokine production by lipopolysaccharide-stimulated mononuclear cells

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Abstract Acute exercise induces changes in peripheral mononuclear cells' (PBMCs) capabilities to produce cytokines. The aim was to investigate the effect of docosahexaenoic acid (DHA) diet supplementation on cytokine production, by lipopolysaccharide (LPS)-stimulated PBMCs after exercise, and the in vitro influence of temperature. Fifteen male soccer players were randomly assigned to a placebo or an experimental group. The experimental group consumed an almond-based beverage enriched with DHA (1.16 g DHA/day) for 8 weeks, whereas the placebo group consumed a similar non-enriched beverage. Blood samples were taken before and after the nutritional intervention in basal conditions and 2 h after acute exercise. Nutritional intervention significantly increased the DHA content in erythrocytes only in experimental group (from 34±3.6 to 43 ±3.6 nmols DHA/10^9 erythrocytes). Exercise significantly increased Toll-like receptor 4 (TLR4) in PBMCs but only in the placebo group (203 %). Exercise also significantly increased IL6, IL8, VEGF, INFγ, TNFα, IL1α, IL1β, MCP1, and EGG production rates by LPS-stimulated PBMCs, and this response was attenuated by DHA supplementation. Temperature but not DHA also affected the pattern of cytokine production increasing IL6, IL8, IL1β, and MCP1 synthesis. The higher change was evidenced in IL1β increasing the production rate at 39.5 °C from 3.19±0.77 to 22.4±6.1 pg/h 10^6 PBMC in placebo and from 2.36±0.11 to 10.6±0.38 pg/h 10^6 PBMC in the supplemented group. The profile of affected cytokines differs between temperature and exercise, suggesting a different PBMC activation pathway. DHA diet supplementation only attenuated cytokine production after exercise and not that induced by temperature.

Keywords Cytokine · Inflammation · Docosahexaenoic acid · Temperature · Exercise

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Introduction

Regular exercise exerts a protective effect against diseases associated to chronic inflammation [56, 71]. It is well established that acute and chronic exercise alters the number and function of circulating cells in the innate immune system [71] and produces a cascade of cytokines with an important role, not only in controlling exercise-induced inflammation but also in regulating

the release and necessary flow of metabolites [2, 33, 57]. The prophylactic activity of exercise has been in part attributed to the anti-inflammatory effect mediated through reduction in visceral fat mass and/or induction of an anti-inflammatory environment with each bout of exercise [41]. The anti-inflammatory effects of exercise have been related to decreased Toll-like receptor (TLR) expression in monocytes [27], with muscle IL6 production during muscle contraction [23] which, in turn, stimulates the production of several anti-inflammatory components such as IL1-receptor antagonist and IL10 [55, 71].

Peripheral blood mononuclear cells (PBMCs) are mobilized during and after intense exercise to several stressors such as several hormones as catecholamine and stress hormones [18, 27, 43, 59], cytokines [59], or central body temperature [27, 43] which could alter their immune function by interfering with the oxidative and inflammatory response to the immune stimulus [43, 59, 71]. IL6, metallprotease-9, and TNFα are mediators released into plasma during exercise that have been reported to promote leukocyte adherence and chemotaxis and to increase the expression of adhesion molecules on leukocytes and endothelial cells [59] Moreover, IL1α, INFγ, and TNFα prime lymphocytes and neutrophils to respond against the immune stimulus [51]. It has been shown that cytokines can be released by PBMCs modulating the inflammatory response of the body [34]. Exercise has also been evidenced to alter PBMC response to different immune effectors such as gram-negative bacterial lipopolysaccharide (LPS) and phorbol myristate (PMA) [55]. LPS presents a pathogen-associated molecular pattern (PAMP) that binds to TLR4 [31, 58]. Muscle in exercise produces heat, thereby increasing the body temperature [43, 55]. Body temperature affects leukocyte mobilization, cytokines, and markers of neutrophil activation during and after exercise, stimulating greater systemic mobilization in a hot environment [53]. However, a limited effect of temperature on monocyte function and natural killer cell activity and mucosal immunity has also been reported [42]. In accordance, whole body hyperthermia combined with chemotherapy induces a strong but reversible anti-inflammatory stress response in cancer patients during therapy [1]. Therapeutical hyperthermia might activate the immune system in a reproducible manner and influence positively the course of diseases [16]. However, heat stress over 41 °C of body temperature is also associated with lymphopenia [9] and with lymphocyte apoptosis [17]. The hyperthermia induced by exercise could participate in priming PBMCs in response to intense exercise.

Omega-3 fatty acids have also been reported to influence cytokine production and inflammation in some pathological situations in which chronic inflammation is present [10, 28–30]. Diet supplementation of healthy volunteers with fish oils results in a reduced in vitro production of IL1, Il6, TNFα, and IL2 by PBMCs [21]. It has been pointed out that the anti-inflammatory effects of omega-3 fatty acids are mediated via regulation of NFκβ pathway signaling [29]. However, diet supplementation with omega-3 fatty acids does not affect plasma cytokine response to strenuous exercise [11, 48]. The effects of DHA diet supplementation on PBMC priming in response to intense exercise are not well known.

The effects of acute exercise, training session, and DHA supplementation on the cytokine plasma levels were reported in a previous study [11]. Acute intense exercise causes few changes in plasma levels of a large number of cytokines and only IL6 significantly increased 2 h after acute exercise. Moreover, acute exercise significantly increases the capability of PBMCs to produce IL6, IL8, and TNFα after LPS stimulation, and this production was attenuated by DHA diet supplementation, supporting its anti-inflammatory function [11]. The previous results lack to study the effects of DHA diet supplementation, body temperature, and acute exercise on the production rate of a great number of cytokine and growth factor responses to immune stimulation with LPS. We wanted to study if the increased body temperature produced during acute exercise could influence the PBMC response to LPS. It would be advisable to study how acute exercise and dietary supplementation with DHA affect the capability of PBMCs to produce a large number of cytokines and their relationships.

We hypothesize that exercise enhances the PBMCs' capability to produce cytokines and growth factors against LPS as immune stimulus. This immune response could be modulated by body temperature and by consumption of food rich in omega-3 fatty acids such as DHA. The aim of this study was to evaluate PBMC production of cytokines after induction by LPS stimulus in order to analyze the possible mechanisms underlying this response and to observe whether the increased body temperature during acute exercise could influence PBMC response to LPS. The influence of DHA diet

supplementation and the effect of temperature on cytokine production were also determined. The blood samples used to measure cytokine production and TLR4 protein levels in the present article were the same as those in our clinical trial (NCT02177383).

Materials and methods

Subjects and anthropometric characteristics

The study was carried out with 15 male soccer players from the Real Mallorca B team; 6 subjects took 1 L of a placebo beverage five times per week and the other 9 an experimental beverage enriched with DHA during 8 weeks. At the beginning of the study, 22 subjects were recruited, but 6 of them left the football team during the experimental time and joined the first and professional team and another broke the anterior cruciate ligament of the knee, as it was explained in a consort flow diagram (Fig. 1). All subjects volunteered to participate in the study and gave their written informed consent after an explanation of the experimental procedures and before the start of the study. The study protocol was in accordance with the Declaration of Helsinki for research on human subjects and was approved by the Ethical Committee of Clinical Investigation of the CAIB No. IB 994/08 PI (Palma de Mallorca, Balearic Islands, Spain). The project was registered at ClinicalTrial.gov (NCT02177383). The cells were collected from the same athletes, at the same time, using the same exercise protocols [11, 12, 40].

DHA supplementation was administered to the players using an almond-based isotonic (278 mOsm/kg) beverage. Both placebo and supplemented beverages were made up of 3.0 % almond and 0.8 % sucrose and the rest was water, flavor, and the added oils and vitamin E. Moreover, the placebo drink contained 0.8 % refined olive oil whereas the experimental drink contained 0.6 % refined olive oil and 0.2 % wt% DHA-S (DSM, Columbia, USA). The two almond beverages were manufactured by Liquats Vegetals S.A. (Girona, Spain), and the procedure for obtaining them was bleaching of the almonds; crushing of the almonds in water; centrifuging of the mixture to eliminate insoluble materials; and the addition of cinnamon and lemon natural flavors, sucrose, vitamin E, and the respective oil for the experimental (olive oil plus DHA-S) or placebo (olive oil) drink. Finally, the beverages were sterilized and packed. Externally, both types of beverages were identical in both taste and visual appearance. The fatty acid composition of beverages was determined following the same procedure used to determine erythrocyte fatty acid composition as described below. The experimental beverage had a significantly greater content of C20:3 (0.640 mg/100 mL of beverage), C22:0 (2.57 mg/100 mL of beverage), C22:5 (56.1 mg/100 mL of beverage), and C22:6n3 (114 mg/100 mL of beverage) compared to the placebo beverage, in which these fatty acids were not present. No significant differences between beverages were evidenced in vitamin E content (41.6 mg/L of beverage in placebo drink, and 45.7 mg/L of beverage in the experimental drink) [11, 12, 40].

Height was determined using a mobile anthropometer (Kawe 44444, Asperg, Germany) to the nearest millimeter, with the subject's head in the Frankfurt plane. Body weight was determined to the nearest 100 g using a digital scale (Tefal, sc9210, Rumilly, France). Subjects were weighed in bare feet and light underwear, and the mean of three measurements was used [22]. Different anthropometric indexes were calculated using these measurements: body mass index [BMI = mass (kg) / squared height (m)].

Dietary intake

Dietary habits of subjects were assessed using a 7-day dietary record questionnaire completed at the beginning of the study and in the week before the exercise test. A qualified dietician verified and quantified the food records. All food items consumed were transformed into nutrients using a special computerized program based on European and Spanish food composition tables [68]. The DHA consumption of soccer players from diet ranged from 68.5 to 150 mg/day according to the 7-day dietary record questionnaire analysis. The five-times-a-week intake of 1 L of the respective beverage amounted to a daily intake of about 1.14 g of DHA in addition to the basal DHA intake through the diet in the experimental group, whereas the placebo only took up DHA from the diet.

To assess the nutritional intervention, the initial and final fatty acid composition of erythrocytes was determined.

Experimental procedure

For each subject, three blood samples were taken, one at the beginning and two at the end of the nutritional

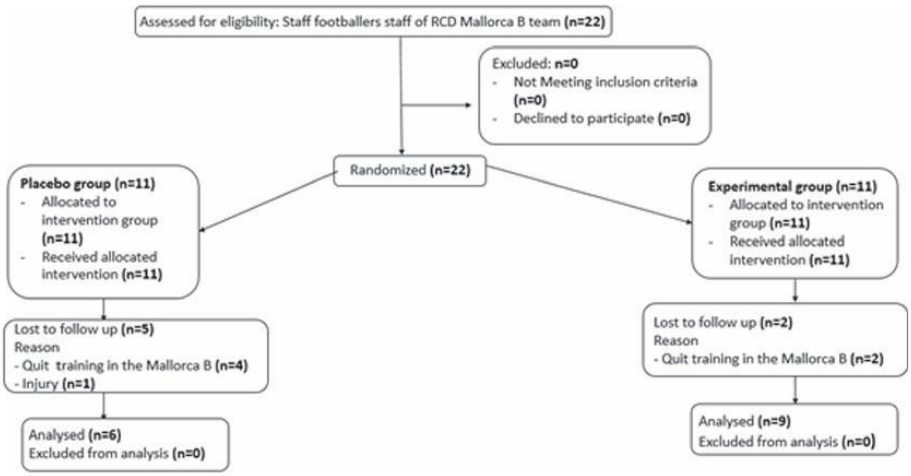

Fig. 1 Representation of a consort flow diagram showing the movement of participants in the study

intervention, in basal conditions and after performing a soccer training session. The training session was planned to perform exercise intensity at more than of 70 % VO_2max for more than 50 % duration of the session in order to induce an oxidative stress situation [65]; these conditions were followed by all participants in the study. The exercise consisted of a 2-h habitual physical training session. After a 15-min warm-up, the players performed the Leger Boucher test [38] to indirectly determine the VO_2max. After that, players practiced a recovery exercise of control-passing for 15 min. The main body of the training session was characterized by small-sided games. Briefly, the first exercise consisted of 5 vs 5 possession exercise in a 20 × 15-m area (four repetitions of 5 min with 30 s of recovery between repetitions); the second was a 6 vs 6 possession exercise in a of 30 × 20-m area (three repetitions of 6 min with 1 min of recovery between repetitions); and finally, the players played a 5-a-side soccer match in 30 × 40 m for 20 min. The training exercises prescribed by the trainer during the training session were followed by all participants.

PBMC purification

Venous blood samples were obtained from the antecubital vein of sportsmen in suitable vacutainers with EDTA as anticoagulant. Venous blood samples were obtained after 12 h, overnight, fasted conditions (basal sample), and 2 h after finishing training. Since it is well known that posture (hydrostatic pressure gradients) has an impact on the plasma volume after exercise, subjects remained seated 0.5–1 h prior to blood sample collection. The PBMC fraction was purified from whole blood following an adaptation of the method described by Boyum [7, 24] using Ficoll-Paque PLUS reagent (GE Healthcare, Chalfont St Giles, UK). An aliquot of whole blood was analyzed in an automatic flow cytometer analyzer Technicon H2 (Bayer) VCS system to determine lymphocyte, eosinophil, basophil, and monocyte counts.

Erythrocyte fatty acid determination

Initial and final nutritional intervention blood samples were obtained, and erythrocytes were purified by blood centrifugation at 900*g* at 4 °C for 30 min. The erythrocyte phase at the bottom was washed with PBS, centrifuged as above, and finally, erythrocytes were reconstituted with distilled water.

Fatty acid extraction of erythrocyte samples was carried out by a modification of the method of Folch [25]. The method for individual fatty acid determination in erythrocytes was previously described [40].

PBMC incubation with LPS

Incubations of PBMCs in the presence of the bacterial stimuli were performed in RPMI 1640 culture media containing 2 mM L-glutamine. PBMCs obtained after an

8-week beverage supplementation in basal conditions and after exercise were diluted with RPMI 1640 culture media to 2×10^6 cells/mL and were activated by addition of the bacterial stimuli LPS from *Escherichia coli* (1 μg/mL). The samples were incubated in polypropylene tubes at 37 °C for 2 h. In a parallel experiment designed to determine the influence of temperature, an aliquot of PBMCs obtained in basal conditions was activated with LPS and incubated at 39.5 °C for 2 h. Then, after shaking, the cells from both experiments were pelleted by centrifugation (900*g*, 5 min, 4 °C) and the cell-free supernatants were stored at −70 °C for cytokine determinations.

Cytokine determination

Cytokine determinations, in plasma and in supernatants of the culture medium, were performed using Randox Biochip Array technology. It was used to perform simultaneous quantitative detection of multiple analytes from a single sample. The core technology is the Randox Biochip, a solid-state device containing an array of discrete test regions of immobilized antibodies specific to different cytokines and growth factors. A sandwich chemiluminescent immunoassay was employed for the cytokine array. Increased levels of cytokine in a specimen will lead to increased binding of antibody labeled with horseradish peroxidase and thus an increase in the chemiluminescent signal emitted. The light signal generated from each of the test regions on the biochip was detected using digital imaging technology and compared to a stored calibration curve. The concentration of cytokines present in the sample was calculated from the calibration curve. The determinations were made following the manufacturer's instructions. We used Randox Biochips: the Cytokine Array I. The intra-assay coefficient of variation was 6.9–15.0 % and the inter-assay coefficient of variation was 8.0–16 %.

The cytokines determined using Randox Biochip Array technology were as follows: interleukin-1α (IL1α), interleukin-1β (IL1β), interleukin-2 (IL2), interleukin-4 (IL4), interleukin-6 (IL6), interleukin-8 (IL8), interleukin-10 (IL10), vascular endothelial growth factor (VEGF), interferon γ (IFNγ), epidermal growth factor (EGF), monocyte chemotactic protein-1 (MCP1), and tumor necrosis factor-α (TNFα).

In order to evidence the stimulation effects of LPS and temperature, we analyzed basal IL6 production by PBMCs in the presence and absence of LPS at 37 and 39.5 °C. The assay was performed using Human IL6 High Sensitivity ELISA Kit (Diaclone, Besaçon, Cedex, France), following the manufacturer's instructions. Intra-assay and inter-assay reproducibility were 4.4 and 9.1 %, respectively.

The rate of cytokine and growth factor production by its determination in the cell culture after 2 h of incubation was calculated. The amount of cytokine present divided by the counts of PBMCs and by time of incubation was considered the rate of cytokine production.

Western blot analysis

TLR4 protein levels were determined in duplicate in PBMCs by Western blot. Protein extracts were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Total protein concentrations were measured by the method of Bradford [8]. Eighty micrograms of total protein was loaded onto a 12 % SDS-PAGE gel. Following electrophoresis, samples were transferred onto a nitrocellulose membrane and incubated with a primary monoclonal anti-TLR4 antibody (1:200) (Santa Cruz Biotechnology) and a secondary anti-mouse IgG peroxidase-conjugated antibody (1:10000). Protein bands were visualized by Immun-Star® Western C® Kit reagent (Bio-Rad Laboratories) Western blotting detection systems. The chemiluminescence signal was captured with a ChemiDoc XRS densitometer (Bio-Rad Laboratories) and analyzed using Quantity One 1D Software (Bio-Rad Laboratories).

Statistical analysis

Statistical analysis was carried out using Statistical Package for Social Sciences (SPSS v.15.0 for Windows). Results are expressed as mean ± SEM and $P < 0.05$ was considered statistically significant. A Kolmogorov–Smirnov test was applied to assess the normal distribution of the data. The statistical significance of the data was assessed by two-way analysis of variance (ANOVA). The statistical factors analyzed were beverage supplementation (S) and exercise (E). When significant differences were reported in any of the factors, one way ANOVA with Bonferroni post hoc test was performed.

Results

No significant differences were reported in the anthropometric characteristics of players: age (19.3 ± 0.4 years in the placebo group and 20.4 ± 0.5 years in the experimental group), weight (76.5 ± 1.8 kg in the placebo group and 76.4 ± 3.5 kg in the experimental group), height (179 ± 2 cm in the placebo group and 180 ± 3 cm in the experimental group), and body mass index (24.0 ± 0.6 kg/m^2 in the placebo group and 23.5 ± 0.5 kg/m^2 in the experimental group).

Nutritional intervention resulted in significant differences in DHA intake between the placebo and experimental groups. DHA intake in the placebo group from the diet was 109 ± 40.7 mg/day, and DHA intake in the experimental group was 1209 ± 40.7 mg/day. Nutritional intervention was reflected in the erythrocyte fatty acid composition (Fig. 2). The initial content of DHA in erythrocytes in the placebo group was maintained after 8 weeks of diet supplementation with placebo beverage (from 29.0 ± 1.3 to 33.6 ± 3.1 nmols DHA/10^9 erythrocytes), whereas the initial DHA content in the experimental group was significantly increased after the intervention (from 34 ± 3.6 to 43 ± 3.6 nmols DHA/10^9 erythrocytes) to attain a significantly higher content than the placebo group.

PBMC counts did not change as a consequence of acute exercise or DHA diet supplementation (Table 1). Acute exercise, but not DHA diet supplementation, influenced the cellular subpopulations of circulating immune cells. A significant effect of acute exercise on the PBMC cellular populations was observed. Acute exercise significantly increased the percentage of monocytes and decreased the percentage of lymphocytes and basophils both in placebo and experimental groups.

Protein levels of TLR4 before and after acute exercise after 8 weeks of diet intervention are presented in Table 2. No differences were found between the placebo and experimental groups in TLR4 levels in basal conditions. However, acute exercise significantly influenced PBMC TLR4 levels. Acute exercise caused a significant increase in the TLR4 protein levels in the placebo group, whereas this increase was not statistically significant in the experimental group.

The effects of 2 h incubation with or without LPS at 37 or 39.5 °C on the production of IL6 by basal placebo PBMCs are shown in Fig. 3. The rate of IL6 production by PBMCs was significantly influenced by both LPS and temperature and by an interaction between these two factors. Temperature had no effect on the rate of production of IL6 without LPS activation. LPS caused a significant increase in IL6 production rate at both 37 and 39.5 °C, and this increase was significantly higher at 39.5 °C when compared with 37 °C.

The production of cytokines and growth factors by PBMCs was determined after stimulation with LPS. PBMC cytokine and growth factor production is influenced by both acute exercise and DHA diet supplementation (Table 2). The rate of cytokine and growth factor production by LPS-stimulated PBMCs followed the sequence IL8 > TNFα > IL6 > EGF > VEGF > IL1α > IL4 = IL10 = MCP1 > IL2 = IL1β > INFγ. Acute exercise significantly increased IL6, IL8, VEGF, INFγ, TNFα, IL1α, IL1β, MCP1, and EGG production in response to LPS. The rates of IL6, VEGF, INFγ, TNFα,

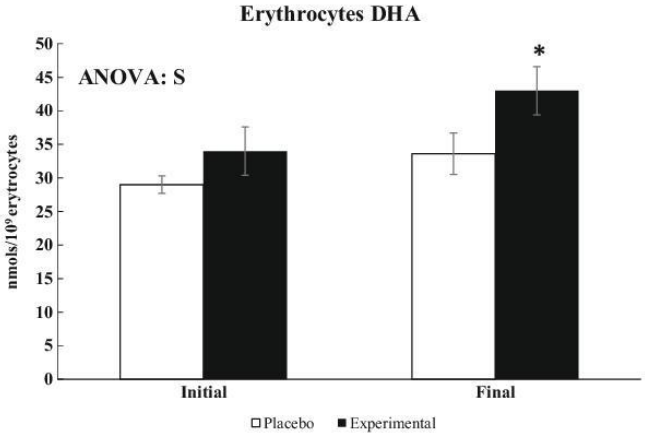

Fig. 2 Effects of DHA diet supplementation on erythrocyte composition

Table 1 Effects of acute exercise and DHA diet supplementation on PBMCs population

		Basal	Post-exercise	E	S	E×S
PBMC counts (10^3 cells/μL)	Placebo	3.34 ± 0.15	3.19 ± 0.16			
	Experimental	3.99 ± 0.35	3.13 ± 0.16			
Cell population						
Lymphocytes (%)	Placebo	74.1 ± 3.18	69.2 ± 1.48[b]	X		
	Experimental	71.2 ± 2.42	69.3 ± 1.89[b]			
Monocytes (%)	Placebo	15.0 ± 1.43	24.1 ± 1.77[b]	X		
	Experimental	13.3 ± 1.16	24.1 ± 1.53[b]			
Basophils (%)	Placebo	2.17 ± 0.36	1.32 ± 0.25[b]	X		
	Experimental	2.27 ± 0.38	1.61 ± 0.26[b]			
Eosinophils (%)	Placebo	8.70 ± 2.43	5.31 ± 0.26			
	Experimental	7.24 ± 2.14	4.91 ± 1.37			

Statistical analysis: two-way ANOVA, $P < 0.05$. One-way ANOVA, $P < 0.05$. Results are the mean ± SEM

E significant effect of exercise, *S* significant effect of supplementation, *E*×*S* significant interaction between both exercise and supplementation

[a] Significant differences between placebo and experimental groups

[b] Significant differences between basal and post-exercise

IL1β, and MCP1 productions by LPS-activated PBMCs after exercise were significantly lower in the experimental group than in the placebo. No differences were observed when the results were expressed per blood or per monocytes (results not shown). The different distribution of PBMC subtypes induced by acute exercise did not alter these pictures of exercise and DHA diet supplementation effects on the rate of cytokine production in response to LPS.

The rate of IL1α, MCP1, and EGF production significantly increased after acute exercise but only in the placebo group, while no effects of acute exercise were observed in the experimental group. IL8 production rate significantly increased after acute exercise in both experimental and placebo groups. The rate of IL2 production significantly decreased after acute exercise, but only in the placebo group. There is an interaction in the effect of acute exercise and DHA diet supplementation on the production rate of IL4, which significantly increased as a consequence of acute exercise in the placebo group, whereas it was maintained in the experimental group. No effects of acute exercise were observed in IL10 production rate, although the production rate was significantly lower in the placebo group in basal conditions. VEGF production rate significantly increased after acute exercise, but only in the placebo group. VEGF production rate in the experimental group was significantly lower than the placebo group both in basal and post-exercise conditions. INFγ production rate significantly increased after acute exercise, but this increase was only significant in the placebo group. INFγ production rate in basal conditions was significantly higher in the experimental group, but was significantly lower in the experimental group after acute exercise.

The effects of temperature and diet supplementation with DHA on basal PBMC production of cytokines and growth factors by PBMCs after activation with LPS are shown in Table 3. No effects of temperature or DHA supplementation were observed in EGF, IL1α, IL10, or IL4 production. Temperature significantly affected basal PBMC rate of production of IL2, IL6, IL8, IL1β, and MCP1. Diet supplementation with DHA did not alter the effects of temperature on cytokine production by LPS-activated PBMCs; however, it did affect the rate of VEGF production, as this was significantly lower in the experimental than placebo group at 37 °C. No differences were observed when the results were expressed per blood or per monocytes (results not shown). IL2 production rate by LPS-activated PBMCs in the placebo group significantly decreased with the rise in temperature, although the experimental group maintained a similar IL2 rate at 37 °C as at 39.5 °C. The rates of production of IL6, IL8, IL1β, and MCP1 by LPS-activated PBMCs were significantly higher at 39.5 °C than at 37 °C in both the placebo and experimental groups. IL8 production rate significantly increased in

Table 2 Effects of exercise and DHA diet supplementation on the rate of production of cytokines and growth factors in LPS-stimulated PBMCs and in TLR4 protein levels

		Basal	Post exercise	E	S	E×S
IL-2 (pg/h 10^6 cells)	Placebo	0.404 ± 0.011a	0.124 ± 0.053b	X	X	X
	Experimental	0.322 ± 0.048a	0.384 ± 0.050a			
IL-4 (pg/h 10^6 cells)	Placebo	1.10 ± 0.10a	1.34 ± 0.08b			X
	Experimental	1.17 ± 0.06ab	1.11 ± 0.05a			
IL-6 (pg/h 10^6 cells)	Placebo	81.0 ± 19.6b	198 ± 32c	X	X	X
	Experimental	34.7 ± 1.72a	80.8 ± 11.2b			
IL-8 (pg/h 10^6 cells)	Placebo	8559 ± 2301	13483 ± 4592	X	X	
	Experimental	7796 ± 1851	23344 ± 3285*#			
IL-10 (pg/h 10^6 cells)	Placebo	1.72 ± 0.44	1.12 ± 0.34		X	
	Experimental	0.601 ± 0.109*	0.485 ± 0.056			
VEGF (pg/h 10^6 cells)	Placebo	10.2 ± 0.92	13,8 ± 2.2#	X	X	
	Experimental	5,94 ± 0.73*	8.65 ± 1.23*			
INFγ (pg/h 10^6 cells)	Placebo	0.041 ± 0.012a	0.211 ± 0.020b	X	X	X
	Experimental	0.089 ± 0.004c	0.110 ± 0.009c			
TNFα (pg/h 10^6 cells)	Placebo	341 ± 88	1266 ± 134#	X	X	
	Experimental	239 ± 55	977 ± 108#*			
IL1α (pg/h 10^6 cells)	Placebo	0.411 ± 0.101	1.62 ± 0.16#	X		
	Experimental	0.882 ± 0.283	1.20 ± 0.26			
IL1β (pg/h 10^6 cells)	Placebo	3.19 ± 0.77a	16.3 ± 1.9b	X	X	X
	Experimental	2.36 ± 0.11a	7.31 ± 1.35c			
MCP1 (pg/h 10^6 cells)	Placebo	1.09 ± 0.08a	6.71 ± 1.06b	X	X	X
	Experimental	1.16 ± 0.09a	2.44 ± 0.30a			
EGF (pg/h 10^6 cells)	Placebo	21.0 ± 2.5	28.2 ± 3.7#	X	X	
	Experimental	17.0 ± 1.7	22.2 ± 1.4			
Protein levels						
TLR4 (AU)	Placebo	1.00 ± 2.33	2.03 ± 3.92#	X		
	Experimental	1.27 ± 17.3	1.71 ± 3.02			

Statistical analysis: two-way ANOVA, $P < 0.05$. One-way ANOVA, $P < 0.05$. When interaction $E \times S$ exists between supplementation and activation factors, different lowercase letters reveal significant differences. Results are the mean ± SEM

E significant effect of exercise, *S* significant effect of supplementation, $E \times S$ significant interaction between both exercise and supplementation

*Significant differences between placebo and experimental groups; #Significant differences between basal and post-exercise.

both placebo and experimental groups as a consequence of the temperature increase. No effects of DHA diet supplementation were observed.

Discussion

It was classically thought that exercise-induced increase in plasma cytokines was a consequence of an immune response due to local damage [49] and that immune cells are responsible for this increase, as occurs during sepsis [47]. However, it is well established that contracting skeletal muscle per se is the main source of circulating cytokines in response to exercise [56]. The main feature of the present results is that acute exercise affects PBMCs' capabilities to produce cytokines and growth factors in response to LPS stimulation. In accordance with our initial hypothesis, diet supplementation with omega-3 fatty acids reduced the rate of cytokine and growth factor production by activated PBMCs mainly after exercise. Body temperature also modulated PBMCs' response to LPS by increasing the rate of pro-inflammatory cytokine production.

Diet supplementation with a DHA-rich beverage increases the DHA content of erythrocyte membranes in accordance with other nutritional interventions with fish oil capsules [5] or foods enriched with omega 3 fatty acids [6, 20]. The fatty acid composition of erythrocytes

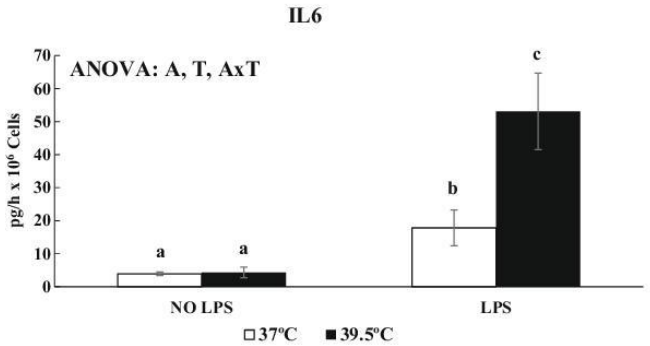

Fig. 3 Activation effects of LPS and temperature on the basal rate of IL6 production by PBMCs. Statistical analysis: two-way ANOVA, $P < 0.05$. *T* temperature diet supplementation effect, *A* LPS activation effect, $A \times T$ interaction between supplementation and activation effects. *Differences between stimulated and no-stimulated groups; #differences between 37.0 and 39.5 °C groups. When interaction $A \times T$ exists between supplementation and activation factors, different *lowercase letters* reveal significant differences. Results are the mean ± SEM

is suggested to reflect the fatty acid composition of other cells such as immune cells [69], and consequently, we can suppose that PBMCs from the experimental group have also increased their DHA content.

Table 3 Temperature and DHA diet supplementation effect on the basal rate of production of cytokines and growth factors LPS-stimulated PBMCs

Blood		37 °C	39.5 °C	T	S	T×S
IL-2 (pg/h 10^6 cells)	Placebo	0.404 ± 0.011	0.132 ± 0.057#	X		
	Experimental	0.332 ± 0.048	0.200 ± 0.115			
IL-4 (pg/h 10^6 cells)	Placebo	1.10 ± 0.10	1.36 ± 0.15			
	Experimental	1.17 ± 0.06	1.20 ± 0.20			
IL-6 (pg/h 10^6 cells)	Placebo	81.0 ± 19.6	179 ± 52#	X		
	Experimental	34.7 ± 1.7	144 ± 16#			
IL-8 (pg/h 10^6 cells)	Placebo	8559 ± 2301	23509 ± 3665#	X		
	Experimental	7796 ± 1851	21841 ± 5409#			
IL-10 (pg/h 10^6 cells)	Placebo	1.72 ± 0.44	0.861 ± 0.158			
	Experimental	0.601 ± 0.109	0.793 ± 0.038			
VEGF (pg/h 10^6 cells)	Placebo	10.2 ± 0.91	8.57 ± 1.68		X	
	Experimental	5.95 ± 0.73*	6.59 ± 1.92			
INFγ (pg/h 10^6 cells)	Placebo	0.041 ± 0.012	0.142 ± 0.040			
	Experimental	0.089 ± 0.004	0.100 ± 0.015			
IL1α (pg/h 10^6 cells)	Placebo	0.411 ± 0.102	5.69 ± 2.22			
	Experimental	0.882 ± 0.283	1.73 ± 0.067			
IL1β (pg/h 10^6 cells)	Placebo	3.19 ± 0.77	22.4 ± 6.1#	X		
	Experimental	2.36 ± 0.11	10.6 ± 0.38			
MCP1 (pg/h 10^6 cells)	Placebo	1.09 ± 0.08	3.47 ± 0.77#	X		
	Experimental	1.16 ± 0.09	3.61 ± 0.64#			
EGF (pg/h 10^6 cells)	Placebo	21.0 ± 2.5	23.8 ± 3.5			
	Experimental	17.0 ± 1.7	25.6 ± 2.3			

Statistical analysis: two-way ANOVA, $P < 0.05$. One-way ANOVA, $P < 0.05$. When interaction T×S exists between supplementation and activation factors, different lowercase letters reveal significant differences. Results are the mean ± SEM

T significant effect of temperature, *S* significant effect of supplementation, *T*×*S* significant interaction between both factors

*Significant differences between placebo and experimental groups; #Significant differences between 37 and 39.5 °C

Effects of LPS stimulation on PBMC IL6 production

LPS is considered a PAMP that binds to TLR4 leading to an inflammatory response [31, 58]. The recognition of LPS by TLR4 triggers the activation of intracellular signaling pathways such as the one mediated by NFκβ, which results in the production of anti-microbial molecules and pro-inflammatory cytokines [19]. In accordance, we stimulated PBMCs with LPS to induce an inflammatory response in order to evaluate the effects of acute exercise, temperature, and DHA diet supplementation on this PBMC response. LPS stimulates PBMCs to produce IL6; the rate of IL6 production is increased at 39.5 °C, a body temperature attainable during acute exercise, with respect to the 37.0 °C basal body temperature [43]. Body temperature could enhance the host defense program by production of inflammatory cytokines induced by PAMPs or at least by PAMPs interacting with TLR4.

Effects of acute exercise and DHA diet supplementation on the number and class of circulating PBMCs

Acute exercise, but not DHA diet supplementation, influenced the number and class of circulating PBMCs. Lymphopenia and monocytosis were present 2 h after acute exercise in a similar way to other studies [62, 66]. This change in the cellular proportion after exercise with a major contribution of monocytes could be responsible for the major cellular response to LPS. Acute exercise enhances PBMC capabilities to produce cytokines and growth factors in response to LPS, which is in accordance with higher protein levels of TLR4. However, a decrease in monocyte cell-surface expression of TLRs after training and both acute aerobic and chronic resistance to exercise has been reported [27, 36]. These contradictory results could be due to the fact that acute exercise increases TLR4 levels in the whole set of PBMCs although TLR4 can decrease after exercise on the surface of CD4 monocytes [27, 36]. This would be in accordance with the evidence that 2 h after a marathon, the number of monocytes that respond to stimulation with LPS is increased [63]. Our results also point out that acute exercise increases TLR4 cellular levels because TLR4 increased in a higher degree than monocytes after exercise; these cellular TLR4 increased levels could be responsible for a greater PBMC response to LPS. There is also additional evidence indicating that acute exercise stimulates T cell subset activation in vivo and in response to mitogen and antigen stimulation [71]. However, this increased post-exercise cellular sensitivity to LPS is not reflected in an elevation of plasma IL6 or TNFα levels after prolonged running [63]. The role of blood in exercise adaptation is generally neglected [50], but we evidence that PBMCs are primed to synthesize cytokines and growth factors after acute exercise, probably mediated by an increase in TLR4 levels in the PBMC cell pool.

Effects of acute exercise and DHA diet supplementation on TLR4 protein levels

Diet supplementation with DHA attenuates the effects of acute exercise on TLR4 levels in PBMCs and the production rate of some cytokines and growth factors. In accordance, it has been reported that the synthesis of IL1β, IL1α, and TNFα can be suppressed by dietary supplementation with long-chain n-3 fatty acids [21]. A strong relationship has been described between IL6 production and the presence of TLR4 on the surface of CD4 monocytes [64]. These attenuating effects of DHA diet supplementation are parallel to the attenuated induction of TLR4 protein levels by exercise in the DHA-supplemented group. The anti-inflammatory effect of DHA could be partially explained by this attenuating response to exercise of TLR4 in PBMCs. Alternatively, the decreased cytokine production rates in the experimental group could also be attributable to an action subsequent to the interaction of LPS with TLR4 receptor; it could be related to alterations in the activation of NFκβ. The capability of omega-3 fatty acids to reduce inflammation is related to the capability to inhibit the activation of NFκβ [29]. NFκβ transcription complexes are present in a latent, inactive state in the cytoplasm where they are bound to an inhibitor (IκB). Many stimuli can rapidly activate these transcription complexes by freeing them from their inhibitor and enabling them to translocate to the nucleus; they can bind to kB sites inside the nucleus enabling the expression of more than 150 genes [52]. The gene expression of COX2, IL8, and TNFα are mediated by the activation and internalization into the nucleus of NFκβ [52]; thus, DHA diet supplementation could interact with the NFκβ pathway activation and translocation into the nucleus or affect the binding to κB sites of DNA. In fact, products of DHA oxidation such as resolvins generated during the resolution phase of inflammation actively promote the termination of the process [4, 26, 61]. Moreover, it has been

pointed out that DHA activates cytosolic phospholipase A_2 to produce prostaglandin E_2, which plays an anti-inflammatory role in macrophages by inhibiting the NFκβ pathway [39].

Effects of acute exercise and DHA diet supplementation on cytokine production by LPS-stimulated PBMCs

LPS activates PBMCs and triggers the production of various pro-inflammatory Th1-type cytokines such as IFNγ, TNFα, and IL8 and Th2-type cytokines such as IL4 [45]. We determined the influence of acute exercise and DHA diet supplementation on the rates of Th1 and Th2 cytokine production by PBMCs stimulated with LPS. Both acute exercise and DHA diet supplementation altered the basal inflammatory response of PBMCs to LPS: exercise enhanced the stimulatory effects of LPS on the synthesis of the Th1 and Th2 cytokines, whereas DHA diet supplementation attenuated this activation by exercise. Exercise significantly increased the rate of INFγ and IL4 production by PBMCs, cytokine activators of Th1 and Th2 cells, respectively [46]. Th1 cells are characterized by their ability to produce IFNγ, but not IL4, whereas Th2 cells produce IL4, but not IFNγ [46]. It was evidenced that exercise primes PBMCs to respond to LPS stimulation by increasing both Th1 and Th2 cytokine production; however, the response of Th2 cells is lower than that of Th1 cells because INFγ production rate is increased five times after exercise, whereas IL4 only increases 1.2 times. Th1 cells play critical roles in the cell-mediated immune response, whereas Th2 cells are involved in humoral immunity. The number and percentage of CD8+ T cells expressing IL2, IFNγ, TNFα, IL6, IL4, and IL10 increase at 1 h after cycling at 95 % maximal steady state exercise [37]. Exercise may prime late-differentiated blood CD8+ T cells to initiate effector functions in preparation for their extravasation into tissues in accordance with the rates of cytokine production measured in the present experience.

Cytokine production is influenced by the presence of other cytokines [3, 21, 56]. LPS has been described to induce a marked and quick increase in circulating TNFα, which is followed by an increase in IL6 [56]. TNFα pro-inflammatory properties lead to the recruitment and activation of inflammatory cells to the site of injury where the production of various cytokines including IL1, IL6, IL8, and MCP1 are induced [3]. Acute exercise enhances the production of TNFα by PBMCs whereas DHA diet supplementation attenuates it. It has been also described that the synthesis of IL1β, IL1α, and TNFα by PBMCs stimulated in vitro with LPS can be suppressed by dietary supplementation with long-chain n-3 fatty acids [21].

IL6 has been described to influence the synthesis of IL10 [56]. Changes in the rate of production of IL6 by LPS-stimulated PBMCs induced by exercise and DHA diet supplementation also influence the rates of production of IL10. IL10 suppresses the production of inflammatory cytokines and the anti-inflammatory agent IL1rα; in addition, the suppressive effects of IL10 result in the inhibition of the functions mediated by T cells, monocytes/macrophages, and natural killer (NK) cells [14, 32, 44]. IL10 produced from both Th1 and Th2 subpopulations inhibits the production of IL12, as well as other Th1 cytokines including IFNγ and IL2 [13, 70]. DHA diet supplementation reduces the rate of TNFα, IL6, and IL10 production by LPS-activated PBMCs, in accordance with the stimulatory effects of TNFα on IL6 and the stimulatory effect of IL6 on IL10 production.

A rapid increase in IL8 in response to LPS which is enhanced after exercise, but is not influenced by DHA diet supplementation, was detected. The high rates of IL8 production could indicate that IL8 is produced in the first response to LPS stimulation, although it has been pointed out that IL8 is produced as a result of TNFα stimulation [3]. IL8 is a chemokine that primarily attracts neutrophils but also acts as an angiogenic factor [3]. There is considerable evidence suggesting that angiogenesis and chronic inflammation are co-dependent in inflammatory diseases such as rheumatoid arthritis [67]. We evidenced that the PBMCs activated with LPS increase the basal production of growth factors such as VEGF and EGF in response to exercise and that diet supplementation with DHA attenuated this effect.

IL2 shows a particular pattern of change to acute exercise, different from the other cytokines analyzed: the rate of IL2 production decreased after exercise, and DHA diet supplementation prevented this effect. A decrease in lymphocyte IL2, TNFα, and IL10 production was evidenced after a marathon race which was not influenced by 60-day fish oil supplementation [60]. However, decreased IL2, TNFα, and IL10 production in concanavalin A-stimulated lymphocytes was reported after the race compared with cells from the control group [60]. The decrease in IL2 was speculated to result in less reactive cells to antigenic stimuli, although further research is needed [15].

Effects of temperature and diet supplementation on PBMC cytokine production

Body temperature is a factor that influences the inflammatory response to infection and also the rates of cytokine production by PBMCs [35]. The heat production induced by physical activity could influence the cytokine production associated to acute exercise [43, 54]. The greater rise in rectal temperature during exercise in an ambient temperature of 32 °C increases the concentrations of serum IL8, IL10, IL1rα, TNFα, and plasma myeloperoxidase [53]. We analyzed a possible effect of body temperature to mimic the ability of exercise to prime PBMCs to produce cytokines and growth factors in response to LPS. We determined the rate of cytokine and growth factor production by LPS-stimulated PBMCs at 39.5 °C, which is the core body temperature attained when sportsmen perform exercise at 75–80 % VO_2max in a hot environment of 32 °C at 75–78 % humidity [43]. The incubation of LPS-activated cells at 39.5 °C selectively altered the production of IL2, IL6, IL1β, and MCP1, whereas the other cytokines and growth factors maintained the same production rates as at 37 °C. Then, the increase of central body temperature as a result of exercise probably alters the capabilities of cytokine production by PBMCs. Body temperature increased by exercise may prime PBMCs to enhance their effector functions, probably in preparation for their extravasation into tissues after exercise. Acute exercise and temperature differently alter the capabilities of cytokine production by PBMCs; this fact rules out a key participation of body temperature in cytokine production by PBMCs after exercise and reinforces the idea that PBMCs do not contribute to plasma cytokine levels after exercise. Accordingly, it has been described that circulating monocytes are not the source of elevations in plasma IL6 and TNFα levels after prolonged running [63]. The lack of influence of DHA supplementation on temperature effects points out that temperature and acute exercise act through different mechanisms.

In summary, both acute exercise and temperature enhance cytokine and growth factor production by PBMCs in response to LPS activation. PBMCs are primed to synthesize cytokines and growth factors, probably mediated by increased TLR4 levels after acute exercise. Temperature and acute exercise differ in responsiveness of individual cytokine production induced by LPS, indicating the operation of different mechanisms of cytokine induction. DHA diet supplementation attenuates the enhanced effects of acute exercise on cytokine production by LPS-stimulated PBMCs but does not affect the enhanced effects of temperature on cytokine production. Acute exercise primed PBMCs to respond to LPS which was modulated by DHA diet supplementation, whereas temperature also primed PBMCs to respond to LPS in a different way to acute exercise but without any influence by DHA diet supplementation.

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Conflict of interest The authors declare that they have no conflict of interest.

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Manuscript VII

Effects of docosahexaenoic supplementation and in vitro vitamin C on the oxidative and inflammatory neutrophil response to activation

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Research Article

Effects of Docosahexaenoic Supplementation and *In Vitro* Vitamin C on the Oxidative and Inflammatory Neutrophil Response to Activation

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We studied the effects of diet supplementation with docosahexaenoic (DHA) and *in vitro* vitamin C (VitC) at physiological concentrations on oxidative and inflammatory neutrophil response to phorbol myristate acetate (PMA). Fifteen male footballers ingested a beverage enriched with DHA or a placebo for 8 weeks in a randomized double-blind study. Neutrophils were isolated from blood samples collected in basal conditions at the end of nutritional intervention. Neutrophils were cultured for 2 hours at 37°C in (a) control media, (b) media with PMA, and (c) media with PMA + VitC. PMA induces neutrophil degranulation with increased extracellular myeloperoxidase and catalase activities, nitric oxide production, expression of the inflammatory genes cyclooxygenase-2, nuclear factor $\kappa\beta$, interleukin 8 and tumor necrosis factor α, and interleukin 6 production. DHA diet supplementation boosts the exit of CAT from neutrophils but moderates the degranulation of myeloperoxidase granules induced by PMA. VitC facilitates azurophilic degranulation of neutrophils and increases gene expression of myeloperoxidase induced by PMA. VitC and DHA diet supplementation prevent PMA effects on inflammatory gene expression, although together they do not produce additional effects. DHA diet supplementation enhances antioxidant defences and anti-inflammatory neutrophil response to *in vitro* PMA activation. VitC facilitates neutrophil degranulation but prevents an inflammatory response to PMA.

1. Introduction

Neutrophils, along with other granulocytes, play an important role in inflammation both for clearing pathogens and for immune regulation [1] and also participate in remodelling damaged tissue. Neutrophils are recruited to the site of injury or infection where they can trigger an inflammatory response by producing cytokines or other chemical mediators and antimicrobial agents to deal with possible infection or contribute to tissue repair [2]. The phagocytic neutrophil function involves an oxidative burst carried out by the enzyme NADPH-oxidase and the secretion of lysosomal enzymes such as proteases, phospholipases, and glycosidases among others [3].

Exercise produces an acute phase immune response characterised by neutrophilia and lymphopenia [4, 5], thus altering the lymphocyte and neutrophil oxidative balance [6]. Acute exercise primes neutrophils to oxidative burst [7], and it significantly increases neutrophil ROS production after stimulation with zymosan or phorbol myristate acetate (PMA) [8]. The priming of neutrophils induced by acute exercise is in parallel with neutrophil secretion of CAT [7]. Extracellular CAT regulates extracellular hydrogen peroxide levels, probably to minimize oxidative damage in plasma and

also to end the signalling action of the hydrogen peroxide [8]. Low levels of antioxidant enzymes in neutrophils after exercise enhance the importance of low molecular weight antioxidant molecules in order to prevent oxidative damage. Ascorbate is found in normal circulating human neutrophils in millimolar concentration [6, 9] and its levels duplicate in neutrophils after prolonged acute exercise [6]. The contribution of ascorbate is a key element to neutrophil protection against oxidative imbalance after acute exercise [6, 10]. However, the influence of ascorbate on neutrophil activation and on its immune function has been little studied [11].

Ascorbate influences some neutrophil functions, including augmented chemotaxis, increased particulate ingestion, enhanced lysozyme-mediated nonoxidative killing, and protection against the toxic effects of superoxide anion radical [11]. Some studies on humans have shown that high doses of ascorbate (20 mM) can inhibit production of IL6 and TNFα in monocytes without affecting IL1 or IL8 levels, but the same ascorbate concentrations have also been found to inhibit IL2 production without affecting TNFα or IFNγ levels in lymphocytes [12]. It has been indicated that the physiological concentration of ascorbate in neutrophils is about 1–3 mM depending on exercise status [5]. These physiological ascorbate levels are lower than ascorbate concentrations assayed in other studies [12, 13]. The effects of physiological doses of ascorbate after vigorous exercise on neutrophil inflammatory and immune capabilities have not been studied, even though there is evidence that a millimolar physiological concentration of ascorbate could inhibit NF$\kappa\beta$ activation in endothelial cells [13].

PMA is a molecular mimetic of diacylglycerol [14] used to activate neutrophils in order to mimic neutrophil response to acute exercise [8]. PMA activates several isoforms of protein kinase C, particularly PKCδ and ε, which in turn activate the NF$\kappa\beta$ signalling pathway [15–17] in neutrophils [18, 19]. PMA induces neutrophil degranulation and the assembly and activation of lysosomal NADPH-oxidase [20, 21]. Moreover, PMA increases reactive oxygen species [22] production and antioxidant enzyme gene expression in neutrophils [18] and in HL60 cells [8]. The production of ROS, induced by PMA, is attributed to NADPH-oxidase operation in the cell or lysosomal membranes [23], but it is also attributed to the mitochondrial respiratory chain function [8, 24]. Activated neutrophils produce an oxidative burst that in turn can cause oxidative damage in proteins, lipids, or nucleic acid [25]. Similarly, neutrophils may modify the pattern of nitric oxide (NO) availability after intense exercise [10]. Nitric oxide (NO) regulates several important neutrophil functions, including chemotaxis, adhesion, aggregation, and PMN-mediated bacterial killing or tissue damage [26, 27]. NO is synthesized in neutrophils by constitutive and inducible nitric oxide synthase isoforms (iNOS). iNOS in combination with NAD(P)H-oxidase play a role in peroxynitrite production acting as a microbiocide together with other products such as hypochlorous acid, produced by myeloperoxidase [28–30].

The neutrophil response to PMA is affected by omega-3-PUFAs in isolated neutrophils in rats [31]. Omega-3-PUFAs increase phagocytic and antifungal activity without altering nitric oxide production. They increase production of hydrogen peroxide and superoxide anion radical in the presence of PMA but do not modify superoxide anion production in the presence of zymosan [31]. In goats, DHA increases neutrophil defensive functions by upregulating phagocytosis activity and downregulating ROS production, thus reducing excessive tissue damage [32]. In humans, no significant effects on neutrophil chemotaxis or superoxide anion radical production induced by DHA have been reported [33]. Although DHA diet supplementation has little influence on neutrophil capabilities to produce ROS after exercise, it reduces the time at which maximal neutrophil ROS production after stimulation with opsonized zymosan is attained; DHA supplementation induces a faster response to zymosan after exercise in neutrophils [8]. The "*in vitro*" generation of inflammatory precursors by human neutrophils does not seem to be modulated by DHA added in the cell medium [34]. In a similar way, DHA diet supplementation hardly modifies the antioxidant adaptive response of neutrophils to training, and no effects have been reported on the production of reactive oxygen and nitrogen species (ROS and RNS) by neutrophils stimulated with PMA after acute exercise [35], probably attributable to a lack of neutrophil activation after exercise. However, the effects of DHA diet supplementation on neutrophil response against a molecular mimetic of DAG, such as PMA, in trained sportsmen are not known.

The aim of this study was to assess the potential role of dietary supplementation with DHA for 8 weeks in highly trained footballers in the oxidative and inflammatory neutrophil response against their activation "*in vitro*" with PMA and the role of physiological neutrophil ascorbate levels in these immune responses of activated neutrophils.

Table 1: Anthropometric and physical activity characteristics of subjects.

	Placebo	Experimental
Age (years)	19.3 ± 0.4	20.4 ± 0.5
Weight (kg)	76.5 ± 1.8	76.4 ± 3.5
Height (cm)	179 ± 2	180 ± 3
Body mass index (BMI, kg/m^2)	24.0 ± 0.6	23.5 ± 0.5
Waist-hip ratio (WHR)	0.805 ± 0.012	0.814 ± 0.012
Fat mass (Yuhasz, %)	7.53 ± 0.24	7.21 ± 0.25
Fat-free mass (%)	92.5 ± 0.2	92.8 ± 0.3
Intense physical activity time (min/day)	96.4 ± 57.9	50.4 ± 13.1
Moderate physical activity time (min/day)	68.6 ± 17.1	63.2 ± 14.6
VO_2max	60.4 ± 18	62.0 ± 0.9
Neutrophils (10^6 cells/mL blood)	3.47 ± 0.53	2.80 ± 0.12

Statistical analysis: Student's t-test for unpaired data, $p < 0.05$.

2. Material and Methods

2.1. Subjects and Study Design. Neutrophils were obtained from fifteen male professional and federated football players who volunteered to take part in this study (Table 1). The subjects and study design was the same as described previously [35, 36]. It was a double-blind study of eight weeks

of nutritional intervention with DHA diet supplementation which was registered at ClinicalTrial.gov (NCT02177383). The participants were 22 football players randomly allocated either to the supplemented ($n = 11$) or to the placebo group ($n = 11$). During the nutritional intervention 2 football players of the experimental group left to train with Mallorca B team and they abandoned the study, and 5 football players of the placebo group dropped out of trial for different reasons, one of them broke the anterior cruciate ligament of knee, 2 football players left the team, and the other two were promoted to Mallorca A team. Each group consumed one litre of DHA-enriched or placebo (DHA-nonsupplemented) drinks five times a week. All subjects were informed of the purpose and demands of the study before giving their written consent to participate. The study protocol was in accordance with the Declaration of Helsinki for research on human subjects and was approved by the Ethical Committee of Clinical Investigation of the Autonomous Community of the Balearic Islands (Palma de Mallorca, Balearic Islands, Spain). Participants in the study were 19.7 ± 0.4 years old, 76.5 ± 2.5 kg in weight, and 179.5 ± 2.5 cm tall. Waist circumference was 78.4 ± 0.9 cm; hip circumference was 97.4 ± 1.2 cm; and waist-hip ratio was 0.81 ± 0.01 WHR. Body mass index was 23.7 ± 0.55 BMI, kg/m^2. The football players had 92.6 ± 0.2% fat-free mass. VO_2max, determined following the test of Leger-Boucher [37], was 61.4 ± 1.35 mL/kg min. The study was performed at the beginning of the competitive season. DHA supplementation was administered to the footballers for eight weeks, using an almond-based experimental drink enriched with DHA. In order to avoid beverage influence, DHA-nonsupplemented drink was given to the placebo group. Both experimental and placebo drinks were enriched with the same amount of α-tocopherol-acetate. There were no differences between anthropometric characteristics or physical activity capabilities, such as age, body weight, body mass index, waist-hip ratio, percentage of fat mass, percentage of fat-free mass, daily physical activity time, or their performance status measured as the VO_2max between placebo and experimental groups. Neither were there any significant differences found between the number of circulating neutrophils in the experimental and placebo group [35].

2.2. Drinks Composition and Diet Supplementation. Both the placebo and the experimental drinks contained 3.0% almond, 0.8% sucrose, and 0.8% different lipids, depending on the type of drink: traces of lemon and cinnamon flavours and 40 mg/1L of vitamin E (α-tocopherol-acetate). The lipid content in the placebo drink was 0.8% refined olive oil whereas in the experimental drink it was 0.6% of the same refined olive oil and 0.2% DHA-S Market (Martek Biosciences Corporation, Columbia, EEUU). The two almond drinks were produced by Liquats Vegetals S.A. (Girona, Spain) following standardized procedures [35, 36]. The placebo and experimental beverages were identical in both taste and appearance.

The experimental drink had significantly higher concentrations of the fatty acids C20:3 (21 μM), C22:0 (76 μM), C22:5 (1715 μM), and C22:6n3 (3457 μM) than the placebo drink in which they were undetected. The daily intake of 1 litre of experimental beverage for five days a week during eight weeks represented a mean daily supplementation intake of 1.14 g of DHA to which the intake of omega-3 in the diet must be added. DHA intake of the placebo group was only from the diet. Nutrient intake by the diet was determined beforehand using a previously published seven-day questionnaire [35].

2.3. Assessment of Nutritional Intervention. The impact of DHA diet supplementation was measured by determining fatty acid composition of erythrocyte membranes before and after nutritional intervention. Erythrocytes were obtained from the blood samples taken at the beginning and at the end of the nutritional intervention as indicated above. Fatty acids were extracted [38] and analysed following a previously described procedure [35].

2.4. Neutrophil Purification. Venous blood samples were obtained from the antecubital vein of sportsmen in suitable vacutainers with EDTA as anticoagulant. Blood samples were obtained after eight weeks of nutritional intervention at 08:00 on a training day after 12 h overnight fasting. Neutrophil fraction was purified following an adaptation of the method described by Bøyum [39]. Blood was carefully introduced on Ficoll in a proportion of 1.5 : 1 and was then centrifuged at 900 g, at 4°C for 30 min. The precipitate containing the erythrocytes and neutrophils was incubated at 4°C with 0.15 M ammonium chloride to haemolyse erythrocytes. The suspension was centrifuged at 750 g, at 4°C for 15 min, and the supernatant was then discarded. The neutrophil phase at the bottom was washed first with ammonium chloride and then with phosphate buffer saline (PBS), pH 7.4.

2.5. Cell Culture. Purified neutrophils from experimental and placebo groups were divided into three aliquots, each of which was cultured with RPMI 1640 culture medium containing 2 mM L-glutamine, but three different treatments were applied: control group: neutrophils treated only with culture medium (RPMI 1640); PMA group: neutrophils treated with culture medium (RPMI 1640) in addition to PMA (PMA 5 μg/mL); PMA and vitamin C group: neutrophils treated with culture medium (RPMI 1640) in addition to PMA (PMA 5 μg/mL) and ascorbate (3 mM). All neutrophil groups were incubated in polypropylene tubes at 37°C for 2 hours. Subsequently, the cells were pelleted by centrifugation (900 ×g, 5 min, 4°C) and cell-free supernatants were stored at −80°C until biochemical determinations; the determinations made in the cell-free supernatants will be considered as determinations in the extracellular media. Neutrophils were resuspended with 2 mL of PBS and divided into two aliquots of 1 mL. One aliquot was centrifuged 900 ×g, 5 min, 4°C, and the precipitate containing the neutrophils was lysed with distilled water and stored at −80°C; determinations performed in the neutrophils lysates will be considered as determinations in the intracellular media. A second aliquot was centrifuged 900 ×g, 5 min, 4°C, and the neutrophil phase

Table 2: Primer sequences and conditions.

Gene	Primer	Conditions	
18S	Fw: 5′-ATG TGA AGT CAC TGT GCC AG-3′	95°C	10 s
	Rv: 5′-GTG TAA TCC GTC TCC ACA GA-3′	60°C	10 s
		72°C	12 s
NFκβ	Fw: 5′-AAACACTGTGAGGATGGGATCTG-3′	95°C	10 s
	Rv: 5′-CGAAGCCGACCACCATGT-3′	60°C	10 s
		72°C	15 s
TNFα	Fw: 5′-CCCAGGCAGTCAGATCATCTTCTCGGAA-3′	95°C	10 s
	Rv: 5′-CTGGTTATCTCTCAGCTCCACGCCATT-3′	63°C	10 s
		72°C	15 s
IL8	Fw: 5′-GCTCTGTGTGAAGGTGCAGTTTTGCCAA-3′	94°C	10 s
	Rv: 5′-GGCGCAGTGTGGTCCACTCTCAAT-3′	63°C	10 s
		72°C	15 s
MPO	Fw: 5′-TGAACATGGGGAGTGTTTCA-3′	95°C	5 s
	Rv: 5′-CCAGCTCTGCTAACCAGGAC-3′	60°C	7 s
		72°C	10 s
COX2	Fw: 5′-TTGCCTGGCAGGGTTGCTGGTGGTA-3′	95°C	10 s
	Rv: 5′-CATCTGCCTGCTCTGGTCAATGGAA-3′	63°C	10 s
		72°C	15 s

at the bottom was used to obtain RNA by adding 1 mL of Tripure and storing at −80°C until further processing.

2.6. Enzymatic Determinations. The activities of CAT and MPO were determined both in the lysed neutrophil solution (intracellular media) and in the cell-free culture supernatant (extracellular media). Both enzyme activities were determined with a Shimadzu UV-2100 spectrophotometer at 37°C. MPO activity was measured by guaiacol oxidation [40]. The reaction mixture contained sodium phosphate buffer pH 7 and 13.5 mM guaiacol. The reaction was initiated by adding 300 mM H_2O_2, and changes at 470 nm were monitored. CAT activity was measured by the spectrophotometric method of Aebi [41] based on following the decomposition of H_2O_2 at 240 nm.

2.7. Cytokine Determination. Cytokine (IL6 and TNFα) determinations were performed in neutrophil-free supernatant using individual ELISA kits (Diaclone, lit for GENPROBE) following the manufacturer's instructions for use. The overall intra-assay coefficient of variation was calculated to be 3.3% for TNFα and 4.4% for IL6; the calculated overall interassay coefficient of variation was 9.0% for TNFα and 9.1% for IL6.

2.8. Nitrite and Nitrate Determination. We centrifuged the neutrophil lysates at 900 ×g at 4°C for 10 min to eliminate cellular debris. The resulting supernatant and the cell-free supernatants were used to measure nitrite and nitrate levels by its transformation into NO which was detected by gas-phase chemiluminescence reaction with ozone using a nitric oxide analyzer (NOA) 280i (Sievers). Nitrite levels were determined following an adaptation of the method described by Castegnaro et al. [42]. Briefly, the purge vessel was loaded with 50 mM KCI in glacial acetic acid and 400 μL of antifoam. A nitrite standard (0.5–10 μM) was used to calculate nitrite concentration. 100 μL of sample or standard was injected in the purge vessel and the area under the curve of NO peaks was recorded and processed using Liquid software. Nitrate levels were determined following an adaptation of the method described by Braman and Hendrix [43]. The purge vessel was loaded with a saturated VCl_3 solution in 1 M HCl and tempered to 90°C with a current of hot water. To prevent damage to the NOA from the hydrochloric acid vapour, a gas bubbler filled with 1 M NaOH was installed between the purge vessel and the NOA. A nitrate standard (5–200 μM) was used to calculate nitrate concentration. 10 μL of sample or standard was injected in the purge vessel and the area under the curve of NO peaks was recorded and processed using Liquid software. NO_x was calculated by adding the extracellular and intracellular concentrations of nitrates and nitrites. To calculate intracellular nitrate concentration it was considered a neutrophil volume of 30×10^{-8} μL/neutrophil [44].

2.9. Neutrophil RNA Extraction and Relative Quantitative RT-PCR Assay. Total RNA was isolated from neutrophils by Tripure extraction (Roche Diagnostics, Germany). RNA (1 μg) from each sample was reverse-transcribed using 50 U of Expand Reverse Transcriptase (Roche Diagnostics, Germany) and 20 pmol oligo [45] for 60 min at 37°C in a 10 μL final volume. The resulting cDNA (2.5 μL) was amplified using the LightCycler FastStart DNA MasterPLUS SYBR Green I kit (Roche Diagnostics, Germany). COX2 TNFα, IL8 NFκβ, and MPO mRNA expression were determined by multiplex real time rtPCR using human 18S rRNA as invariant reference. The primers and amplification conditions used are listed in Table 2. Relative quantification was performed by standard calculations considering $2^{(\Delta\Delta Ct)}$. mRNA levels from the control were arbitrarily referred to as 1.

2.10. Statistical Analysis. Statistical analysis was carried out using the Statistical Package for Social Sciences (SPSS) v.18.0

Table 3: Effects of PMA activation, *in vitro* vitamin C, and DHA diet supplementation on the distribution of catalase (CAT) and myeloperoxidase (MPO) activities between intracellular and extracellular neutrophil compartments.

		Control	PMA	PMA + VitC	S	A	S × A
Extracellular activity							
CAT (K/10^9 cells)	Placebo	16.2 ± 1.9	39.4 ± 8.1$^{\#}$	46.1 ± 7.3$^{\#}$	X	X	
	Experimental	10.3 ± 3.2	112 ± 31$^{\#*}$	90.6 ± 17.8$^{\#}$			
MPO (nkat/10^9 cells)	Placebo	9.18 ± 0.86	34.3 ± 3.8$^{\#}$	135 ± 25$^{\#\$}$		X	
	Experimental	6.94 ± 1.07	40.3 ± 4.3$^{\#}$	84.5 ± 33.6$^{\#\$}$			
Intracellular activity							
CAT (K/10^9 cells)	Placebo	62.1 ± 12.9	28.2 ± 6.0$^{\#}$	77.2 ± 7.29$^{\$}$		X	
	Experimental	67.2 ± 6.26	21.6 ± 3.0$^{\#}$	68.8 ± 11.9$^{\$}$			
MPO (nkat/10^9 cells)	Placebo	31.5 ± 11.8^{a}	38.1 ± 8.93^{a}	1.19 ± 0.06^{c}		X	X
	Experimental	16.9 ± 2.4^{ac}	83.6 ± 18.5^{b}	1.23 ± 0.06^{c}			

Statistical analysis: two-way ANOVA, $p < 0.05$. S: supplementation effect; A: activation effect; S × A: interaction between supplementation and activation effects. X indicates significant effect of the statistical factor S, A, or S × A. *Differences between placebo and experimental groups; $^{\#}$difference with respect to the control group; $^{\$}$differences between PMA and PMA + VitC group. When interaction S × A exists between supplementation and activation factors, different letters reveal significant differences. Results are the mean ± SEM.

for Windows. Results are expressed as mean ± SEM. And $p < 0.05$ was considered statistically significant. A Kolmogorov-Smirnov test was applied to assess the normal distribution of the data. The statistical significance of the data was assessed by two-way analysis of variance (ANOVA). The statistical factors analysed were beverage supplementation (S) and neutrophil treatment (T). The sets of data in which there was a significant SxE interaction were tested by one-way ANOVA. When significant effects of S or E factor were found, Student's *t*-test for unpaired data was used to determine the differences between the groups involved.

3. Results

3.1. Assessment of Nutritional Intervention by Fatty Acid Erythrocyte Composition. DHA supplementation with the experimental beverage significantly increased the levels of this fatty acid in the erythrocyte membranes. At the beginning of the nutritional intervention there were no differences in the fatty acid composition of erythrocyte membranes between the placebo (DHA concentration is 29.0 ± 1.3 nmol/10^9 erythrocytes) and experimental groups (DHA concentration is 34.0 ± 3.6 nmol/ 10^9 erythrocytes). After eight weeks of nutritional intervention with DHA-enriched or non-DHA-enriched drinks the DHA concentration in erythrocytes of the placebo group was 33.6 ± 3.16 nmol/10^9 erythrocytes and 43.0 ± 3.66 nmol/10^9 erythrocytes in the supplemented group. Nutritional intervention increased DHA levels 26.5% with respect to initial values in erythrocytes from the experimental group, whereas its levels were unchanged in the placebo group. No effects of either beverage were reported on the amounts of saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), or PUFAs on erythrocyte fatty acid composition.

3.2. Influence of PMA, Vitamin C, and DHA Diet Supplementation on Neutrophil Oxidative Response. We have studied the influence of PMA, vitamin C, and DHA diet supplementation on neutrophil CAT and MPO activities in lysed neutrophils solution (intracellular media) and in free-cells culture supernatants (extracellular media).

PMA activation significantly increased total CAT activity in neutrophils (1.8 times) with respect to unstimulated control cells in the experimental group, whereas no differences were reported in the placebo group (Figure 1(a)). These differential responses to PMA resulted in significantly higher total CAT activity in the experimental group when compared to the placebo group. The addition of vitamin C combined with PMA significantly increased total CAT activity both in the placebo and in the experimental groups, without differences between them. Neutrophil activation with PMA, DHA diet supplementation, and vitamin C addition influence extra/intracellular CAT activity distribution, with a significant interaction between these factors on the percentage of neutrophil extracellular CAT activity (Figure 1(b)). The percentage of CAT activity in the extracellular medium significantly increased after neutrophil activation with PMA both in the placebo (1.71 times) and in the experimental (5.71 times) groups. PMA activation produced a significantly higher percentage of CAT activity in the extracellular medium (1.4 times) in the experimental rather than the placebo group. The addition of vitamin C attenuated the effect of PMA activation on the percentage of extracellular enzyme activity, mainly in the placebo group, which returned to control level, whereas the experimental group continued with higher values than the control level. The intra/extracellular distribution of neutrophil CAT activity was significantly altered by PMA activation in both groups (Table 3). Neutrophil CAT release into the extracellular media was reinforced by DHA with a significant increase in CAT activity, about 10 times, in the experimental group, but only about 2.4 times in the placebo group, after PMA stimulation. In fact, extracellular CAT activity in the experimental group was 2.8 times higher than the placebo group after PMA activation. Vitamin C did not modify the CAT activity measured in the extracellular

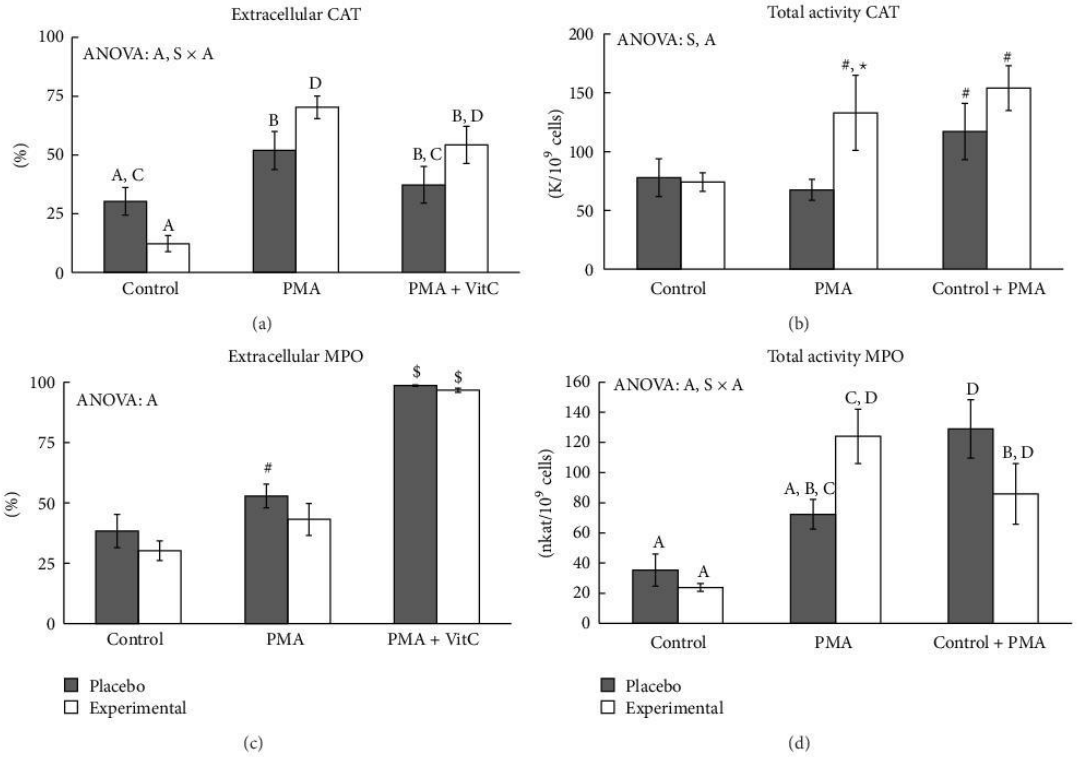

FIGURE 1: Effects of *in vitro* PMA activation, vitamin C, and DHA diet supplementation on total CAT and MPO enzyme activities and on their distribution in the extracellular compartment of neutrophils. Statistical analysis: two-way ANOVA, $p < 0.05$. S: significant effect of DHA dietary supplementation; A: significant effect of PMA activation; S × A: significant interaction between DHA dietary supplementation and PMA activation effects. * indicates differences between placebo and experimental groups; # indicates difference with respect to the control group; $ indicates differences between PMA and PMA + VitC group. When interaction S × A exists between supplementation and activation factors, different lowercase letters reveal significant differences. Results are the mean ± SEM.

media in PMA-activated neutrophils. These pictures are complementary to those present in the intracellular CAT activity of neutrophils with a decrease about 2.5 after PMA activation, both in the placebo and in the experimental groups. However, the addition of vitamin C together with PMA maintained intracellular CAT activity at control levels.

The possible effect of vitamin C present in the culture medium on the determination of catalase activity was assessed. The presence of vitamin C in the medium used to determine CAT activity could contribute to consumption of hydrogen peroxide, reducing its availability for CAT. The 3 mM concentration of vitamin C added in the neutrophil incubation medium resulted in a concentration of 40 μM in the medium used to determine CAT activity. There was no evidence that the addition of 3 mM vitamin C to the neutrophil incubation medium influenced CAT activity measurement at this concentration of vitamin C.

Neutrophil activation with PMA significantly increased total MPO activity in the experimental group but not in the placebo group, whereas the combined addition of PMA and vitamin C increased total MPO activity in both groups with respect to the control values (Figure 1(c)). The addition of vitamin C to PMA-activated neutrophils significantly increased total MPO activity in the placebo (1.8 times higher than PMA-activated neutrophils) group, while the vitamin C addition significantly decreased the total MPO activity (1.4 times) in the experimental group. Neutrophil activation with PMA significantly increased the percentage of extracellular MPO activity in the placebo group but to a lesser extent than in the experimental group (Figure 1(d)). The addition of vitamin C combined with PMA significantly increased the percentage of extracellular MPO activity in both groups until practically reaching 100% MPO. The distribution of MPO activity between the intra- and extracellular neutrophil medium was strongly influenced by the addition of vitamin C and PMA (Table 3). Extracellular MPO activity increased after neutrophil activation with PMA, both in the placebo and in the experimental groups, and the addition of vitamin C to PMA-activated neutrophils significantly reinforced this response. Intracellular MPO activity significantly increased

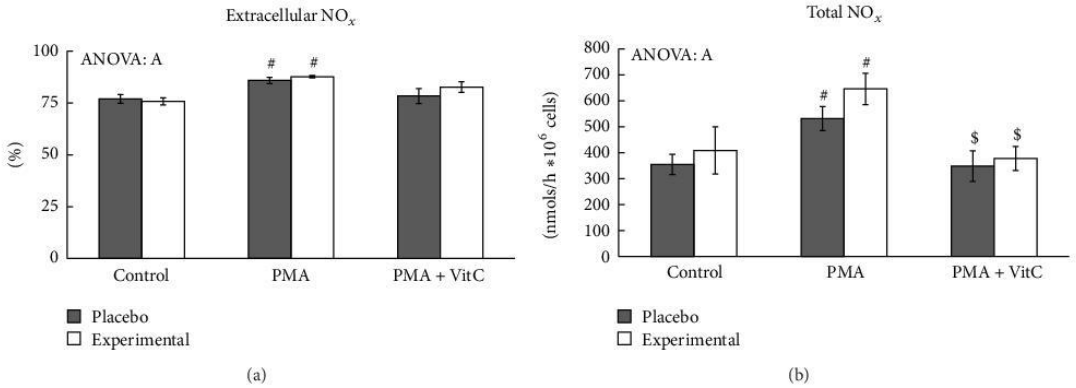

FIGURE 2: Effects of PMA activation, *in vitro* vitamin C, and DHA diet supplementation on the percentage of extracellular NO_x and the total NO_x production. Statistical analysis: two-way ANOVA, $p < 0.05$. S: DHA diet supplementation effect; A: PMA activation effect; S × A: interaction between supplementation and activation effects. # indicates difference with respect to the control group; $ indicates differences between PMA and PMA + VitC group. When interaction S × A exists between supplementation and activation factors, different lowercase letters reveal significant differences. Results are the mean ± SEM.

TABLE 4: Effects of PMA activation, *in vitro* vitamin C, and DHA diet supplementation on nitrite and nitrate distribution between extracellular and intracellular neutrophil compartments.

		Control	PMA	PMA + VitC	ANOVA		
					S	A	S × A
Extracellular							
Nitrate (mM)	Placebo	1.07 ± 0.05	1.23 ± 0.04$^{\#}$	1.03 ± 0.07$^{\#\$}$	X	X	
	Experimental	1.16 ± 0.10	1.41 ± 0.06$^{*\#}$	1.10 ± 0.07$^{\#\$}$			
Nitrite (nM)	Placebo	458 ± 54	648 ± 48$^{\#}$	422 ± 68$^{\#\$}$	X	X	
	Experimental	545 ± 104	798 ± 65$^{*\#}$	495 ± 69$^{\#\$}$			
Intracellular							
Nitrate (mM)	Placebo	572 ± 60	447 ± 45	441 ± 65		X	
	Experimental	715 ± 119	494 ± 78$^{\#}$	437 ± 60$^{\#}$			
Intracellular/extracellular relationships							
Nitrate ratio	Placebo	1366 ± 213	712 ± 77$^{\#}$	1305 ± 284$^{\$}$		X	
	Experimental	1363 ± 130	646 ± 87$^{\#}$	954 ± 152$^{\#}$			

Statistical analysis: two-way ANOVA, $p < 0.05$. S: supplementation effect; A: activation effect; S × A: interaction between supplementation and activation effects. X indicates significant effect of the statistical factor S, A, or S × A. *Differences between placebo and experimental groups; $^{\#}$difference with respect to the control group; $^{\$}$differences between PMA and PMA + VitC group. When interaction S × A exists between supplementation and activation factors, different letters reveal significant differences. Results are the mean ± SEM.

4.9 times after neutrophil activation with PMA in the experimental group, whereas the placebo group maintained control values. Vitamin C addition to PMA-activated neutrophils emptied them of MPO, both in the placebo and in the experimental groups.

3.3. Influence of PMA, Vitamin C, and DHA Diet Supplementation on Neutrophil Inflammatory Response. Nitrite and nitrate production by immune cells was measured as indicative of NO production related to neutrophil immune toxicity and inflammation activation. We have analysed nitrite and nitrate levels in lysed neutrophils solution (intracellular media) and in free-cells culture supernatants (extracellular media).

PMA stimulation significantly increased total NO_x production in both placebo and experimental groups (Figure 2(a)). Vitamin C addition to PMA-activated neutrophils resulted in total NO_x production similar to control nonactivated neutrophil values. NO_x distribution between intracellular and extracellular media was influenced by PMA neutrophil stimulation increasing the percentage in the extracellular media, whereas vitamin C addition to PMA-stimulated neutrophils resulted in NO_x distribution similar to the control situation. No significant effects were observed in NO_x production due to DHA diet supplementation. Extracellular nitrate and nitrite concentration demonstrated a similar pattern of change induced by PMA stimulation, vitamin C, and DHA diet supplementation (Table 4). PMA stimulation

Table 5: Effects of PMA activation, *in vitro* vitamin C, and DHA diet supplementation on inflammatory gene expression.

		Control	PMA	PMA + VitC	ANOVA S	ANOVA A	ANOVA S × A
Gene expression							
COX2	Placebo	1.00 ± 0.12	4.37 ± 1.65$^{\#}$	1.21 ± 0.31$^{\$}$		X	
	Experimental	1.14 ± 0.25	2.32 ± 0.81	1.54 ± 0.33			
NF$\kappa\beta$	Placebo	1.0 ± 0.14^{a}	1.91 ± 0.34^{b}	0.88 ± 0.09^{a}	X	X	X
	Experimental	0.90 ± 0.08^{a}	0.98 ± 0.14^{a}	0.84 ± 0.06^{a}			
IL8	Placebo	1.00 ± 0.15^{a}	3.19 ± 1.19^{b}	1.01 ± 0.36^{a}			X
	Experimental	0.89 ± 0.13^{a}	0.89 ± 0.12^{a}	1.09 ± 0.18^{a}			
TNFα	Placebo	1.00 ± 0.12	2.30 ± 0.51$^{\#}$	0.99 ± 0.14		X	
	Experimental	1.03 ± 0.15	1.24 ± 0.26	0.97 ± 0.12$^{\$}$			
MPO	Placebo	1.00 ± 0.16	1.32 ± 0.27	1.73 ± 0.59	X	X	
	Experimental	2.09 ± 0.61	1.86 ± 0.66	4.72 ± 1.23$^{\#\$}$			

Statistical analysis: two-way ANOVA, $p < 0.05$. S: supplementation effect; A: activation effect; S × A: interaction between supplementation and activation effects. X indicates significant effect of the statistical factor S, A, or S × A. $^{\#}$Difference with respect to the control group; $^{\$}$differences between PMA and PMA + VitC group. When interaction S × A exists between supplementation and activation factors, different letters reveal significant differences. Results are the mean ± SEM.

significantly increased extracellular nitrate and nitrite concentrations in both the placebo and the experimental groups, whereas vitamin C addition to PMA stimulated neutrophils returned to control values. Intracellular nitrate concentration reflected a complementary picture to extracellular nitrate concentration.

The effects of PMA activation, vitamin C, and DHA diet supplementation on the expression of inflammatory genes and cytokine production in neutrophils are shown in Table 5. COX2 expression was significantly increased when neutrophils were stimulated with PMA in the placebo group, and vitamin C addition prevented this increase. An interaction between PMA activation and DHA diet supplementation factors on NF$\kappa\beta$ expression was observed. NF$\kappa\beta$ expression maintained control levels in all treatments in the experimental group, whereas it increased in the placebo group after stimulation with PMA but not after the addition of vitamin C. The pattern of IL8 expression was very similar to NF$\kappa\beta$ expression, with an interaction between neutrophil activation and DHA-diet supplementation factors. IL8 expression significantly increased after neutrophil stimulation with PMA only in the placebo group. TNFα expression was affected by PMA activation with a significant increase in the expression only in the placebo group. The addition of vitamin C together with PMA maintained TNFα expression at control level both in the placebo and in the experimental groups. There were no significant changes in the expression of MPO when neutrophils were stimulated with PMA. However, MPO expression increased significantly (more than 4 times) in the experimental group, but not in the placebo group, after the addition of vitamin C in combination with PMA.

We have analysed the production rate of IL6 and TNFα (Figure 3). The rate of IL6 production in the extracellular medium increased significantly when neutrophils were stimulated with PMA but was only significant in the placebo group. The addition of vitamin C enhanced the response to PMA activation. No significant differences in the rate of neutrophil TNFα production in the extracellular medium were observed.

4. Discussion

4.1. Assessment of Nutritional Intervention. Diet supplementation for 8 weeks with a DHA-enriched beverage increases DHA content in erythrocytes in accordance with similar nutritional interventions performed in humans [46, 47]. The membrane composition of the placebo and experimental groups' neutrophils should be different in line with the greater DHA content in experimental group's erythrocytes. In fact, similar changes in the fatty acid composition of erythrocyte and leukocyte membranes have been pointed out in child patients with alterative-exudative and allergic inflammation [48], in the same way as diet supplementation with fish oil is known to significantly increase the DHA content of lymphocyte and monocytes membranes [47, 49]. The enrichment of DHA in cellular membranes could modulate several signalling responses to different stimuli [50, 51]. Following fish oil supplementation, omega-3 fatty acids are incorporated into cellular membranes, which can affect lipid-protein interactions and, therefore, the function of embedded proteins [52].

4.2. PMA, DHA, and Vitamin C Effects on Oxidative Neutrophil Response. PMA activates the protein kinase signalling pathways in neutrophils [18, 19, 23] and induces neutrophil degranulation [18]. PMA induces higher MPO and CAT activities in extracellular neutrophil media, in accordance with the degranulation effects of PMA [18]. Neutrophils contain several types of exocytosable organelles which hold a battery of molecules that contribute to the precise execution of many neutrophil functions [53]. First, secretory vesicles are mobilized, while tertiary granules, specific granules, and MPO containing azurophilic granules are sequentially mobilized in response to increasingly strong stimuli [53]. The exocytosable CAT is detected in neutrophil organelles [7] but the specific type of organelle is not known. CAT and MPO exit from neutrophils in response to PMA are influenced differently by DHA-diet supplementation and vitamin C addition. DHA diet supplementation enhances exocytosable

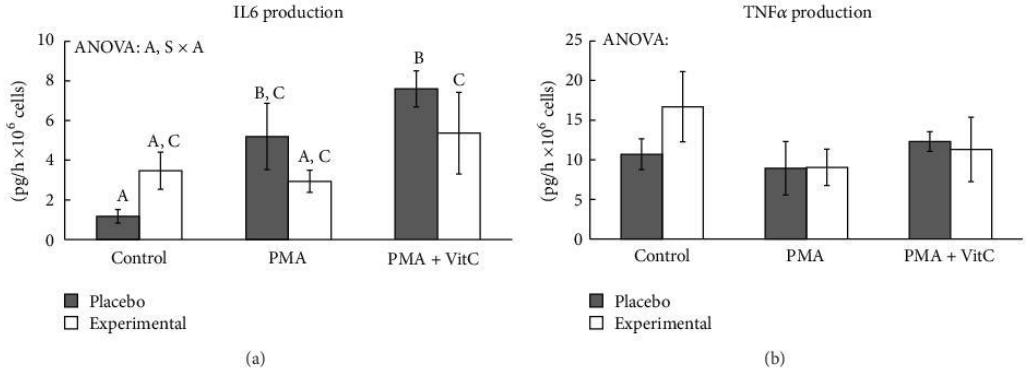

FIGURE 3: Effects of PMA activation, *in vitro* vitamin C, and DHA diet supplementation on IL6 and TNFα production rate. Statistical analysis: two-way ANOVA, $p < 0.05$. S: DHA diet supplementation effect; A: PMA activation effect; S × A: interaction between supplementation and activation effects. When interaction S × A exists between supplementation and activation factors, different lowercase letters reveal significant differences. Results are the mean ± SEM.

catalase and also moderates the degranulation of azurophilic MPO containing granules induced by PMA. Vitamin C addition enhances MPO exit but practically prevents (in placebo) or reduces (in the DHA-supplemented group) CAT exit from neutrophils activated by PMA. This may indicate the presence of these two enzymes in different neutrophil organelles. Despite the fact that DHA diet supplementation enhances CAT exit and moderates neutrophil degranulation of azurophilic MPO containing granules induced by PMA, total neutrophil MPO and CAT activities increase in response to PMA in the DHA-supplemented group. DHA supplementation enhances neutrophil oxidative burst by increasing the activity of NAD(P)H-oxidase [54]. The parallelism in the effects of DHA on NAD(P)H-oxidase and CAT activities of PMA-activated neutrophils could be the result of a need to reduce, or to remove, the large amount of ROS produced during the oxidative burst induced by PMA. In addition, MPO gene expression is enhanced by PMA only when a physiological concentration of vitamin C is present, mainly in the DHA-supplemented group. The presence of vitamin C is required to allow complete PMA-induced neutrophil azurophilic granule degranulation until neutrophils are emptied of MPO in response to PMA, whereas DHA diet supplementation enhances PMA-induced CAT exit from neutrophils.

Intense physical activity brings about similar changes in neutrophil CAT and MPO activities as those induced by PMA: decreasing CAT, superoxide dismutase, and glutathione peroxidase activities in neutrophils increasing CAT exit [6, 7] and MPO neutrophil degranulation since increased levels of plasma MPO activity were reported after exercise [50]. Moreover, vitamin C diet supplementation facilitates greater neutrophil activation and antioxidant enzyme secretion to extracellular media induced by intense exercise [4]. We found evidence that vitamin C plays a necessary role in facilitating neutrophil degranulation induced by PMA, in a similar way to that observed in physiological situations such as intense exercise [4, 6, 7, 55].

4.3. PMA, DHA, and Vitamin C Effects on Inflammatory Neutrophil Response. Neutrophil capability of producing nitric oxide is stimulated by PMA since total NO_x levels, markers of NO synthesis, increased after neutrophil activation with PMA. Similarly, it has been pointed out that PMA increases iNOS gene expression in HL60 cells [8], probably as a result of the previous ROS production induced by PMA [8, 24, 56] or due to the presence of inflammatory cytokines such as TNFα or IL1 [57]. Vitamin C and DHA diet supplementation influence nitric oxide production and release in response to neutrophil stimulation with PMA. The DHA diet supplemented group had greater intracellular and extracellular nitrate and nitrite levels than placebo group, but the response to PMA stimulation and vitamin C addition was similar to the placebo group. PMA increases the rate of NO production by neutrophils and the extracellular markers of NO synthesis. Neutrophil activation via PMA is driven by protein kinase C isoforms by activating NFκβ [15] which regulates the transcription of many acute phase proteins and a large variety of stress response genes, such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX2). It also regulates the expression of inflammatory cytokines, chemokines, immunoreceptors, and cell adhesion molecules [58]. The addition of vitamin C at physiological concentration avoids the stimulatory effects of PMA on NO production and NO_x distribution, maintaining these parameters at non-PMA-stimulated neutrophil level. In this way it has been pointed out that millimolar concentrations of ascorbate inhibit NFκβ activation in endothelial cells [13]. The vitamin C effects could indicate the participation of ROS produced by neutrophil activation with PMA in NO production by neutrophils. In fact, it has been pointed out that ROS mediates the induction of iNOS gene expression [56, 59]. However, DHA diet supplementation does not influence the neutrophil response to PMA on NO synthesis.

PMA activates an inflammatory response in neutrophils reflected by its effects on neutrophil degranulation, inflammatory gene expression, and IL6 production. This

inflammatory response by neutrophils is mediated by both DHA diet supplementation and vitamin C, both of which prevent the inductive effects of PMA on IL6 production and inflammatory gene expression. It reinforces the idea that both ROS and NF$\kappa\beta$ participate in the signalling pathway activated by PMA in neutrophils. Several studies have demonstrated the anti-inflammatory effects of omega-3 fatty acids [50, 60, 61]. DHA diet supplementation prevents the enhanced expression of IL8, TNFα, COX2, and NF$\kappa\beta$ genes induced by PMA. This attenuating neutrophil inflammatory response could be due to direct DHA prevention of NF$\kappa\beta$ activation and translocation into the nucleus. DHA has been described as preventing I-$\kappa\beta$ phosphorylation and NF$\kappa\beta$ activation, thereby also preventing NF$\kappa\beta$ translocation into the nucleus [62], which is required to activate gene expression. Therefore, the DHA attenuating response of neutrophil stimulation with PMA on the gene expression of IL8, TNFα, and COX2 could be mediated by DHA interference with NF$\kappa\beta$ signalling [58]. COX2 [63], IL8, and TNFα gene expression [64] are also mediated by activation and internalization into the nucleus of the NF$\kappa\beta$ pathway which also enables the expression of more than 150 genes [58].

Vitamin C influences the expression of inflammatory genes induced by PMA-activated neutrophils because vitamin C attenuates increased expression of COX2, IL8, TNFα, and NF$\kappa\beta$ in response to PMA. This reinforces the idea that PMA-induced ROS production mediates the activation and translocation of NF$\kappa\beta$ into the nucleus and the induction of inflammatory genes into neutrophils [13, 64]. The presence of both vitamin C and DHA diet supplementation hinders PMA effects on inflammatory gene expression, but these two factors together do not produce additional effects on the expression of COX2, NF$\kappa\beta$, IL8, and TNFα. However, MPO gene expression follows a different pattern of change in response to PMA, DHA diet supplementation, and vitamin C. The presence of vitamin C at a physiological concentration only induces increased expression of the MPO gene in the DHA-supplemented group, but not in the placebo group. This different pattern of response is in accordance with the role of vitamin C in the neutrophil degranulation of azurophilic granules indicated above. It suggests the participation of different signalling pathways regulating PMA-induced neutrophil degranulation.

In conclusion, DHA diet supplementation enhances neutrophil antioxidant capability and attenuates inflammatory response after PMA activation. DHA enhances CAT and MPO activities, releases CAT, and moderates degranulation of azurophilic granules containing MPO after PMA activation of neutrophils. Vitamin C facilitates the azurophilic degranulation in PMA-activated neutrophils. Vitamin C and DHA diet supplementation prevent PMA effects on inflammatory gene expression in neutrophils, but these two factors together do not produce additional effects on the expression of inflammatory genes.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Manuscript VIII

Composición farmacéutica que comprende la 5-dodecanolida, su preparación y su uso

COMPOSICIÓN FARMACEUTICA QUE COMPRENDE 5-DODECANOLIDA, SU PREPARACIÓN Y SU USO

CAMPO DE LA INVENCIÓN

La presente invención pertenece al campo de las composiciones farmacéuticas con actividad anti-inflamatoria. Más particularmente se refiere a una composición farmacéutica que comprende como principio activo la 5-dodecanolida. Este principio activo ha sido aislado de la manteca del cerdo a través un tratamiento específico. Así pues la presente invención también se refiere a un procedimiento para obtener la composición de la invención así como al uso de la misma con fines terapéuticos y más particularmente con el fin de tratar procesos inflamatorios.

ANTECEDENTES DE LA INVENCIÓN

La inflamación es un proceso tisular constituido por una serie de fenómenos moleculares, celulares y vasculares de finalidad defensiva frente a agresiones físicas, químicas o biológicas. Los aspectos básicos que se destacan en el proceso inflamatorio son en primer lugar, la focalización de la respuesta, que tiende a circunscribir la zona de lucha contra el agente agresor. En segundo lugar, la respuesta inflamatoria es inmediata y preponderantemente inespecífica, aunque puede favorecer el desarrollo posterior de una respuesta específica. En tercer lugar, el foco inflamatorio atrae a las células inmunitarias de los tejidos cercanos. La inflamación provoca una gran dilatación de los vasos sanguíneos junto con una apertura de sus poros, incrementando su permeabilidad y permitiendo el paso de líquido, sustancias y células desde la sangre a los tejidos, por lo que éstos aumentan de volumen y temperatura. La finalización de la inflamación no es un proceso pasivo, sino que puede estar mediado por la síntesis de diversas moléculas derivadas de la oxidación de ácidos grasos poliinsaturados como el eicosapentaenoico y el docosahexaenoico. Entre estas moléculas encontramos las resolvinas y los hidroxi-derivados de la oxidación de ácidos grasos omega-3 de cadena larga (18, 20, 22 carbonos) (Patente US

2009/0180961 A1), o derivados sintéticos de éstos (Patente MX 2011010827). Su composición permite que se puedan administrar en forma de partículas y nanopartículas para finalizar la inflamación (WO2012135032 (A2)).

La inflamación está descrita ya en papiros egipcios 3000 años a.C. Celsus (escritor romano), durante el primer siglo d.C, expone sus signos cardinales: Rubor, Tumor, Calor y Dolor. Seguidamente, Virchow expuso el quinto signo: limitación funcional. En 1793, el cirujano escocés John Hunter planteó que "no es una enfermedad sino una respuesta no específica que tiene un efecto saludable sobre el huésped". En 1839-1884, Julius Cohnheim describió los primeros hallazgos microscópicos de esta repuesta de defensa: aumento de la permeabilidad vascular y migración leucocitaria. Posteriormente, Elie Metchnikoff concluyó que el proceso de la inflamación genera leucocitos y anticuerpos para la defensa contra los microorganismos, estableciéndose desde entonces la relación INFLAMACIÓN + INFECCIÓN; es decir, cuando las bacterias entran en el organismo producen una infección y el cuerpo en su defensa genera una inflamación. Más aún, Sir Thomas Lewis, quien sobre las bases de experimentos simples de la respuesta inflamatoria realizados en piel, estableció que "sustancias químicas, tales como la histamina, localmente inducidas por el daño, median los cambios vasculares de la inflamación", este concepto fundamenta el descubrimiento importante de mediadores químicos de la inflamación y el uso potencial de agentes anti-inflamatorios.

De forma general podemos dividir la inflamación en cinco etapas:

1-Liberación de mediadores: Son moléculas, la mayor parte de ellas, de estructura elemental que son liberadas o sintetizadas bajo la actuación de determinados estímulos. Los tejidos al lesionarse van a liberar mediadores de la inflamación.

Entre los mediadores destacan aminas como la histamina o la serotonina, enzimas proteolíticas, el óxido nítrico, citoquinas proinflamatorias (como el factor quimiotáctico de eosinófilos y el factor quimio táctico de neutrófilos) y heparina.

Otras sustancias de carácter lipídico constituyen un segundo grupo importante de mediadores de la inflamación. Éstas son sustancias sintetizadas *de novo* y derivadas del ácido araquidónico a través de dos vías metabólicas, la de la enzima ciclooxigenasa (COX) que determina la producción de prostaglandinas (PGs) y tromboxanos y la de la lipooxigenasa (LOX) que conduce a la formación de leucotrienos (LTs).

2- Efecto de los mediadores: Una vez liberadas, estas moléculas producen alteraciones vasculares y efectos quimiotácticos que favorecen la llegada de moléculas y células inmunitarias al foco inflamatorio. Se produce un aumento de la permeabilidad vascular favoreciendo la llegada a la zona afectada, desde la sangre, de moléculas y células del sistema inmunitario y se estimulan las terminaciones nerviosas del dolor.

3-Llegada de moléculas y células inmunes al foco inflamatorio: Desde el punto de vista cronológico, los mediadores de la inflamación van a producir básicamente dos efectos. En una primera fase inicial, alteraciones vasculares que facilitan el trasvase de moléculas desde la sangre al foco inflamatorio, así como la producción de edema. En una segunda fase, más tardía, las propias alteraciones vasculares, así como la liberación en el foco de factores quimiotácticos, determinan la llegada de células inmunes (basófilos, neutrófilos, macrófagos, linfocitos y eosinófilos) procedentes de la sangre y de los tejidos circundantes.

4-Regulación del proceso inflamatorio: Como la mayor parte de las respuestas inmunes, el fenómeno inflamatorio también integra una serie de mecanismos inhibidores tendentes a finalizar o equilibrar el proceso. Algunos de los mediadores que producen activación, al variar su concentración o actuar sobre distintos receptores, van a producir inhibición, consiguiendo, de esta forma, un equilibrio o modulación de la respuesta inflamatoria. La síntesis de moléculas, como las resolvinas a partir de los ácidos grasos poliinsaturados omega 3 de los fosfolípidos de membrana, entra en este proceso de finalización de la inflamación.

5-Reparación: Fase constituida por fenómenos que van a determinar la reparación total o parcial de los tejidos dañados por el agente agresor o por la propia respuesta inflamatoria. Estos procesos integran la llegada a la zona de fibroblastos que van a proliferar y sintetizar colágeno, proliferación de células epiteliales y proliferación de vasos dentro de la herida.

Para reducir el proceso inflamatorio se usan básicamente productos anti-inflamatorios no esteroideos (AINEs). Su funcionamiento se basa en la inhibición de la enzima COX reduciendo la síntesis de PGs. Los AINEs constituyen el pilar básico del tratamiento de las enfermedades inflamatorias crónicas, como las tan extendidas artrosis y artritis, que al no tener cura requieren del uso de fármacos para el tratamiento de los síntomas (tratamiento sintomático). Esto significa que disminuyen la rigidez y el dolor por su efecto anti-inflamatorio y analgésico, pero no curan ni modifican la enfermedad. Se han identificado otras moléculas, mediadores lipídicos que pueden contribuir a la finalización de la inflamación y cuya función, utilización, síntesis y forma de aplicación han sido previamente patentados (Patente US 2009/0180961 A1; Patente MX 2011010827; WO2012135032 (A2) — 2012-10-04).

Las formas de aplicación tópicas son preparaciones semisólidas y deformables para extender sobre la piel o mucosas. Están formadas por una base o vehículo y el principio activo o medicamento. Las bases o vehículos contienen el principio activo y dan la consistencia de la presentación, su adecuada elección depende del tipo de piel donde se va a aplicar y de las propiedades físico-químicas del medicamento para facilitar su liberación. La finalidad de estos preparados es la penetración de las sustancias activas a través del estrato córneo de la piel con función aislante. La penetración consta de dos fases, absorción, que es el paso del medicamento a través de la piel hasta llegar a nivel de las células. La segunda fase es la difusión, es decir, la propagación a través de los tejidos.

En el tratamiento por vía tópica se deben cumplir los siguientes requisitos: piel intacta, inflamación de localización superficial y focal, y además, la aplicación debe ser continua. Su uso está indicado en traumatismos, esquinces, contusiones, tendinitis, etc.

Dentro de la cultura tradicional, el sebo de cerdo se ha utilizado para el tratamiento de golpes y procesos inflamatorios agudos pero sin que se sepa los componentes que pueden ser responsables de estos efectos anti-inflamatorios.

Por otro lado hay descritos en la literatura ejemplos donde la grasa de cerdo se ha usado en la elaboración de composiciones con actividad anti-inflamatoria.
Por ejemplo, el documento WO2009077635 se refiere a una composición a base de aceite de oliva, manteca de cerdo y miel de abeja en proporciones 1:1:1 y a su uso como anti-inflamatorio, anti-edematoso, anti-eritematoso y regenerador de tejidos.

El documento JP2009149599 también describe una composición de un aceite medicinal a base de grasas y aceites de cerdo negro alimentado con una dieta suplementada con α-tocoferol útil en el tratamiento cosmético y alimenticio de dermatitis atópica, inflamación, pérdida de cabello y eczema.

Por su parte el documento ES2087036 tiene por objeto una pomada de acción rápida contra las varices, que se constituye a base de una mezcla de alcanfor, manteca de cerdo y bicarbonato.

US2014377370A1 tiene por objeto una composición homeopática que comprende una grasa animal, entre ellas la manteca de cerdo, cebolla y romero para el tratamiento de articulaciones con rigidez, dolor, inflamación, calambres, lesiones, gota o rango limitado de movimiento.
Sin embargo, ninguno de los documentos anteriormente descritos hace referencia a por medio de que componentes la grasa de cerdo ejerce un efecto anti-inflamatorio.
Los autores de la presente invención han conseguido a partir de un determinado tratamiento de la grasa del cerdo identificar una

serie de compuestos con actividad anti-inflamatoria. Pero de manera más sorprendente han podido identificar que mediante dicho tratamiento aparece un compuesto un compuesto que no se encuentra en la grasa de cerdo sin tratar y que muestra un efecto anti-inflamatorio muy potente. Este compuesto identificado por los inventores es la 5-dodecanolida que es la base de las composiciones de la presente invención.

BREVE DESCRIPCIÓN DE LAS FIGURAS

Figura 1: Efectos del ungüento sobre la inflamación inducida con zymosan sobre el diámetro de las patas de las ratas.

Figura 2: Efectos del ungüento sobre la inflamación inducida con zymosan a las 4 horas sobre el peso de la pata.

DESCRIPCIÓN DETALLADA DE LA INVENCIÓN

Un primer objeto de la invención es una composición farmacéutica que comprende 5-dodecanolida como principio activo y uno o más excipientes o vehículos farmacéuticamente aceptables.

La composición de la invención se puede preparar a partir del compuesto obtenido comercialmente o por síntesis o a partir de un proceso de tratamiento y purificación de la manteca del cerdo que es a partir de donde los inventores han conseguido aislarlo e identificar su potente actividad anti-inflamatoria.

Cuando la 5-dodecanolida se obtiene a partir del tratamiento de la manteca del cerdo que se describirá más adelante se observa que además de la 5-dodecanolida la manteca tratada posee otra serie de compuestos que sirven para potenciar la actividad anti-inflamatoria.

Así pues, en una realización particular la composición de la invención comprende además de 5-dodecanolida, uno o más de los compuestos seleccionados entre octadecenamida, resolvina D1, ácido hexadecanoico, ácido 9-octadecenoico, ácido 6-

octadecenoico, ácido 9,12-octadecadienoico, ácido octadecanoico, ácido 5,8,11,14-eicosatetraenoico, ácido 7-hexadecenoico, ácido 11,14-octadecadienoico, hexadecanamida.

La 5-dodecanolida puede estar presente en la composición de la invención en un rango de entre 2-6% del peso total de la composición, preferiblemente entre el 3-5%.

En una realización particular la composición de la invención comprende octadecenamida en un porcentaje de 2-8% del peso total de la composición.

En otra realización particular la composición de la invención comprende resolvina D1 en un porcentaje de 1-4% del peso total de la composición.

Otra realización particular la composición de la invención comprende ácido hexadecanoico en un porcentaje de 0,01-0,7% del peso total de la composición.

Otra realización particular la composición de la invención comprende ácido 9-octadecenoico, ácido 6-octadecenoico o ambos en un porcentaje de 56-65% del peso total de la composición.

Otra realización particular la composición de la invención comprende ácido 9,12-octadecadienoico en un porcentaje de 12-15% del peso total de la composición.

Otra realización particular la composición de la invención comprende ácido octadecanoico en un porcentaje de 0,1-0,5% del peso total de la composición.

Otra realización particular la composición de la invención comprende ácido 5,8,11,14-eicosatetraenoico en un porcentaje de 5,1-6,3% del peso total de la composición.

Otra realización particular la composición de la invención comprende ácido 7-hexadecenoico en un porcentaje de 1,2-3% del peso total de la composición.

Otra realización particular la composición de la invención comprende ácido 11,14-octadecadienoico en un porcentaje de 5,5-6,1% del peso total de la composición.

Otra realización particular la composición de la invención comprende hexadecanamida en un porcentaje de 0,7-1,4% del peso total de la composición.

Una realización particular viene representada por una composición que comprende en porcentaje en peso con respecto al total de la composición:

a) 4,34-4,63% de 5-dodecanolida
b) 0,05-0,7% de ácido hexadecanoico
c) 59,4-63,7% de ácido 9-octadecenoico o ácido 6-octadecenoico
d) 12,5-14% de ácido 9,12-octadecadienoico
e) 0,13-0,47% de ácido octadecanoico
f) 5,32-6,21% de ácido 5,8,11,14-eicosatetraenoico
g) 1,33—2,83% de ácido 7-hexadecenoico
h) 5,67-6,00% de ácido 11,14-octadecadienoico
i) 0,89-1,28% de hexadecanamida
j) 3,19-6,84% de octadecenamida
k) 1,93-3,02% de resolvina D1.

La composición de la invención además comprende uno o más excipientes o vehículos farmacéuticamente aceptables. Dependiendo de la vía de administración se pueden usar unos u otros excipientes o vehículos farmacéuticos que son ampliamente conocidos para un experto en la materia como por ejemplo materiales de soporte, lubricantes, cargas, disolventes, diluyentes, colorantes, acondicionadores del aroma y el sabor tales como azúcares, antioxidantes, ceras o ésteres de ácidos grasos, conservantes,

emulsionantes y/o aglutinantes. La selección de estas aditivos auxiliares o vehículos y las cantidades que van a usarse dependerán de la forma de administración de la composición farmacéutica.

La forma de administración preferida en por vía tópica y en este sentido la forma preferida de la composición de la invención incluye ungüentos, pomadas, geles, cremas, lociones, suspensiones o emulsiones. La realización preferida de la invención implica la composición en forma de ungüento.

Un segundo objeto de la invención es un procedimiento para obtener una composición de acuerdo con la invención que comprende:

a) Extracción de la manteca de cerdo
b) Trituración de la manteca
c) Cocción de la manteca triturada
d) Separación por tamizado de la manteca líquida de los chicharrones
e) Opcionalmente, extracción hidroalcohólica de la manteca líquida

Antes de llevar a cabo la primera etapa del procedimiento de se lleva a cabo el despiece del cerdo.

La primera etapa comprende la extracción de la manteca del cerdo. La manteca de cerdo se obtiene de la panceta y del tejido adiposo de la región del epiplón. Se separa la parte magra del tocino blanco y la grasa adherida al epiplón. La alimentación del cerdo tiene importancia determinando la cantidad y composición de grasa acumulada por el animal.

En un segundo paso la manteca de cerdo formada por el tocino blanco y la grasa del epiplón se trituran por medios mecánicos como, por ejemplo, en una trituradora de carne con un disco de picado de orificios grandes (diámetro de unos 2 cm). Una vez triturados, el tocino y la grasa del epiplón se mezclan hasta conseguir una masa homogénea, aunque no demasiado triturada. Aproximadamente se obtienen unos 20 Kg. de masa por animal.

A continuación la manteca triturada se somete a cocción. Par ello la manteca se introduce preferiblemente en una caldera de acero inoxidable para su cocción a fuego fuerte. La grasa se remueve constantemente durante todo el proceso de cocción con una cuchara de madera, para evitar que la grasa se pegue en el fondo de la caldera y se queme. Durante el proceso de cocción la grasa se licua y posteriormente alcanza la ebullición (60-80°C). El tiempo promedio de cocción para unos 20 Kg de grasa es de unos 50 minutos a fuego fuerte, hasta que los chicharrones estén cocidos y adquieran un color dorado intenso, lo que puede pasar tras 90 minutos de cocción. Durante esta cocción se producen y liberan los principios anti-inflamatorios. La producción de los componentes con carácter anti-inflamatorio puede incrementarse, incrementando la proporción de proteína y utilizando grasa procedente de cerdos con una alimentación suplementada con ácidos grasos poliinsaturados omega 3.

Una vez la manteca está cocida, se saca del fuego y se deja reposar en la misma cazuela. Cuando ha enfriado lo suficiente se pasa por un tamiz con el fin de separar los chicharrones de la manteca líquida. Posteriormente, estando aun la manteca líquida se puede o bien utilizar en el paso siguiente del procedimiento o almacenarse en recipientes de cristal donde termina de enfriarse y se solidifica para su uso opcional en la etapa de extracción hidroalcohólica.

La extracción hidroalcohólica y la purificación de los principios activos es opcional aunque recomendable ya que permite una mayor concentración de los compuestos con actividad anti-inflamatoria. Para obtener el extracto hidroalcohólico del sebo de cerdo se funde y se añade una mezcla de alcohol:agua (65:35, v:v), preferiblemente metanol:agua (65:35, v:v) y un ácido concentrado preferiblemente HCl 1M. Se agita vigorosamente con ayuda mecánica y se separan las fases por centrifugación. Seguidamente se purifican los principios anti-inflamatorios mediante una extracción en fase sólida en la que los principios activos anti-inflamatorios se adsorben a un material hidrofóbico que se lava

con agua, hexano y finalmente se desadsorben y liberan con metilformiato.
El extracto hidroalcohólico obtenido por este procedimiento posee además de 5-dodecanolida los siguientes compuestos: octadecenamida, resolvina D1, ácido hexadecanoico, ácido 9-octadecenoico, ácido 6-octadecenoico, ácido 9,12-octadecadienoico, ácido octadecanoico, ácido 5,8,11,14-eicosatetraenoico, ácido 7-hexadecenoico, ácido 11,14-octadecadienoico, hexadecanamida.
El extracto obtenido se puede utilizar directamente como ungüento para aplicación tópica o puede procesarse para su formulación como composición farmacéutica adaptado a cualquier vía de administración tal y como se ha explicado antes.

Por otro lado, hay que señalar que tanto el sebo de cerdo tratado, como el extracto hidroalcohólico contienen principios anti-inflamatorios que pueden utilizarse directamente o bien introducirse en diversos vehículos de aplicación sobre las zonas inflamadas. Estos vehículos de aplicación pueden ser ungüentos, pomadas, disoluciones, pulverizaciones, etc.

Alternativamente se puede preparar la composición de la invención utilizando 5-dodecanolida sintetizada o purificada de otras fuentes diferentes del sebo de cerdo o una mezcla de esta con uno o más de los siguientes compuestos: octadecenamida, resolvina D1, ácido hexadecanoico, ácido 9-octadecenoico, ácido 6-octadecenoico, ácido 9,12-octadecadienoico, ácido octadecanoico, ácido 5,8,11,14-eicosatetraenoico, ácido 7-hexadecenoico, ácido 11,14-octadecadienoico, hexadecanamida.

Un último objeto de la presente invención viene representado por el uso de la composición de la invención.

De manera general la composición de la invención tiene su aplicación en el tratamiento de la inflamación.

De manera más específica la composición de la invención puede usarse para el tratamiento de la inflamación asociada a

enfermedades articulares como la artritis o artrosis. Así también y, dado que se ha demostrado eficaz en la reducción del proceso inflamatorio iniciado por fragmentos de las paredes celulares de microorganismos como *E. coli*, también puede usarse para el tratamiento de la inflamación asociada a enfermedades infecciosas como la que se produce en la mastitis que obstruye algún conducto galactóforo causado por infecciones bacterianas con capacidad de hacer biofilms durante la lactancia. Así mismo, puede disminuir la inflamación asociada al acné que también es consecuencia de la actividad bacteriana.

En cualquier caso la composición de la invención se usa de manera preferente para el tratamiento de la inflamación por vía tópica ya sea en forma de ungüento, pomada, gel, crema, loción, suspensión o emulsión, y por tanto, la composición de la invención se hace especialmente útil en el tratamiento inflamaciones a nivel superficial como traumatismos, esguinces, contusiones o tendinitis.

EJEMPLOS

La presente invención se ilustra mejor mediante los siguientes ejemplos, que no pretenden ser limitadores de su alcance. Así, por ejemplo, las concentraciones concretas y la naturaleza de los ingredientes y aditivos descrita en los ejemplos pueden extenderse a otros y a otras concentraciones. La raza de cerdo utilizada para la obtención del producto así como su proceso de engorde pueden variar y no son limitantes de la invención. La maquinaria e instrumentos que se utilizan en los ejemplos pueden variar y no son limitantes de la invención.

EJEMPLO 1

En este ejemplo se ilustra una realización de cómo se obtiene tanto el sebo de cerdo tratado así como el extracto hidroalcohólico a partir de este extracto.

En primer lugar se llevó a cabo el despiece del cerdo.

A continuación se obtuvo la manteca de la panceta y del tejido adiposo de la región del epiplón. Se separa la parte magra del tocino blanco y la grasa adherida al epiplón.

La manteca de cerdo formada por el tocino blanco y la grasa del epiplón se trituraron en una trituradora de carne con un disco de picado de orificios grandes (diámetro de unos 2 cm). Una vez triturados, el tocino y la grasa del epiplón se mezclaron hasta conseguir una masa homogénea, aunque no demasiado triturada. Aproximadamente se obtuvieron unos 20 Kg. de masa por animal.

A continuación la manteca triturada se sometió a cocción en una caldera de acero inoxidable para su cocción a fuego fuerte. La grasa se removió constantemente durante todo el proceso de cocción con una cuchara de madera, para evitar que la grasa se pegara en el fondo de la caldera y se quemara. Durante el proceso de cocción, la grasa se licuó y posteriormente alcanzó la ebullición (60-80°C). La manteca se coció durante 90 minutos momento en el cual los chicharrones quedaron cocidos y adquirieron un color dorado intenso.

Una vez la manteca estuvo cocida, se sacó del fuego y se dejó reposar en la misma cazuela. Cuando se enfrió lo suficiente se pasó por un tamiz con el fin de separar los chicharrones de la manteca líquida. Estando aun la manteca líquida se almacenó en recipientes de cristal donde terminó de enfriarse y se solidifico. Parte de esta manteca se usó después en el resto de los ejemplos y otra parte se sometió a la extracción hidroalcohólica.

Para realizar esta extracción, se procedió como se describe a continuación. 1 g de sebo de cerdo se fundió a 50-60°C y,

posteriormente, se añadieron 5 mL de metanol:agua (65:35, v:v) y 50 µL de HCl 1M. Se agitó vigorosamente con ayuda de un vórtex durante 1 minuto y, a continuación, se separaron las fases por centrifugación a 3.000 rpm durante 5 min. Se pasó la fase acuosa por una columna tipo C-18(Sep-Pak® Vac 12cc (2g)), y se limpió la columna con 10 mL de agua, 6 mL de hexano y finalmente se eluyó con 8 mL de metilformiato. El eluido se secó bajo corriente de nitrógeno a 55°C y se obtuvo un extracto hidroalcohólico del sebo de cerdo en estado sólido.

EJEMPLO 2

En este ejemplo se desarrolló un experimento que demuestra las propiedades anti-inflamatorias del ungüento (sebo de cerdo). Para ello se usó un modelo animal en el que se indujo una inflamación aguda, y tras la cual se testó el producto anti-inflamatorio.

Procedimiento experimental. Para llevar a cabo el experimento de utilizaron ratas Sprague de 350-400 gramos de peso. Las ratas se dividieron en dos grupos (n=6): un primer grupo control que fue sometido a inflamación, y un segundo grupo en el que tras la inflamación fue tratado con el ungüento anti-inflamatorio.

Los animales fueron anestesiados con pentobarbital sódico. La inflamación fue inducida mediante una inyección subplantar una de las patas posteriores con zymosan (0,1 ml en suero salino de una concentración 10 mg/ml). El zymosan está formado por fragmentos de pared celular de levadura (*Saccharomices cerevisiae*) y su aplicación genera una inflamación aguda, generando un modelo para el estudio de sustancias anti-inflamatorias. La otra pata actuó como control negativo y en ella se inyectó 0,1 ml de suero salino. Mediante este tratamiento se obtiene una inflamación manifiesta en la región plantar con un máximo de edema a la hora de la inyección.

Una hora después de la inyección, a los animales del primer grupo se les extiende una capa de 0,5 g de vaselina que actúa como control, mientras que al segundo grupo se le extiende una capa de

0,5 g del ungüento. Ambos productos de aplican de forma uniforme de manera que quede una capa recubriendo toda la zona inflamada.

Se mide el diámetro de las patas en los tiempos 0 (antes inducir la inflamación), tiempo 1 hora (antes de poner el ungüento o la vaselina), y tiempo 2, 3 y 4 horas (ver figura 1).

El diámetro de las patas se ve influido por el tratamiento al que es sometido. El diámetro a tiempo 0 es similar en todos los animales. Una hora después de la inyección se observa una respuesta diferencial entre los grupos salino (el diámetro prácticamente no se ve modificado) y los grupos de zymosan (donde el incremento en el diámetro por la inflamación es evidente). A los tiempos 3 y 4 horas se observa como el grupo tratado con el ungüento se recupera de la inflamación de forma significativa más rápidamente que el grupo tratado únicamente con vaselina. El ungüento actúa de forma eficaz reduciendo los síntomas externos de la inflamación.

EJEMPLO 3

Este ejemplo ilustra cómo la aplicación del ungüento en la zona inflamada reduce algunos de los mediadores bioquímicos implicados en el proceso inflamatorio.

El procedimiento experimental es el mismo que el llevado a cabo en el experimento 2. A las 4 horas de haber generado la inflamación los animales son sacrificados por decapitación extrayendo el tejido subplantar que se pesó (ver figura 2) y luego se proceso y recogiendo muestras de sangre.

El tejido fue homogeneizado en tampón fosfato 50 mM, pH 7,0 que contenía 0,5% de hexadeciltrimetilamonio bromide. Los homogenados fueron centrifugados a 8.000 g a 4°C y se recogió el sobrenadante. En el sobrenadante se determinaron las actividades enzimáticas de enzimas antioxidantes (catalasa, y glutatión peroxidasa) y la actividad mieloperoxidasa (enzima que se expresa de forma exclusiva en neutrófilos y es indicativo de inflamación). También se determinaron los niveles de nitrito (indicador indirecto de la

producción de óxido nítrico) y niveles de malondialdehido (marcador de daño en lípidos) y de grupos carbonilo en proteínas (marcador de daño en proteínas).

A partir de la sangre se purificaron los neutrófilos. 5 ml de sangre se mezclaron con 5 ml de PBS pH 7,4 y se introdujeron sobre 4 ml de Ficoll. Se centrifugaron las muestras a 900 g durante 30 minutos a 18°C. Se retiró el sobrenadante y se añadieron a los precipitados 10 ml de dextrano al 5%. Se dejaron reposar las muestras 30 minutos a temperatura ambiente y finalmente se centrifugaron a 750 g, 10 minutos a 4°C obteniendo un precipitado con los neutrófilos. En los neutrófilos de determinaron las actividades de los enzimas antioxidantes y de la mieloperoxidasa.

Determinaciones enzimáticas. Las determinaciones se realizaron en un espectrofotómetro Shimadzu UV-2100 a 37°C.

La actividad catalasa fue determinada mediante un método basado en la descomposición del H_2O_2. Esta descomposición se monitoriza espectrofotométricamente a 240 nm. La concentración final de H_2O_2 era de 10 mM. Se introducía el volumen adecuado de muestra, y se ajustaba con tampón fosfato a 2 ml. La reacción se iniciaba añadiendo 1 ml de H_2O_2. El volumen final del ensayo era de 3 ml.

La actividad glutatión peroxidasa (GPx) requiere H_2O_2 como substrato y la reacción se seguía acoplando la reacción enzimática catalizada por la glutatión reductasa. Las concentraciones finales de glutatión reductasa, NADPH y H_2O_2 eran respectivamente de 0,24 U/ml, 200 µM y 265 µM. El volumen total de la reacción se ajustaba a 1 ml con tampón fosfato 200 mM con EDTA 1 mM y pH 7. Se seguía la absorbancia a 339 nm, durante 3 minutos.

La actividad mieloperoxidasa (MPO) monitoriza la oxidación del guaiacol. La mezcla de la reacción contenía tampón fosfato pH 7 y 13,5 mM de guaiacol. La reacción se iniciaba con la adición de H_2O_2 300 µM, monitorizando los cambios a 470 nm.

Determinación de los niveles de nitrito. Los niveles de nitrito fueron determinados como marcador indirecto del óxido nítrico, mediante un método colorimétrico en microplaca de 96 pocillos.

Las determinaciones se realizaron en tejido plantar y neutrófilos. Las muestras fueron desproteinizadas añadiendo 1,5 volúmenes de acetona, y se dejaron toda la noche a –20°C. Se centrifugaron durante 10 minutos a 15.000 g y 4°C, y se recogieron los sobrenadantes. Para el ensayo, en cada pocillo se introdujeron 100 µl de muestra. Posteriormente se añadieron 50 µl de una disolución de sulfanilamida al 2 % peso/volumen en HCl 5 % y, seguidamente, se añadieron 50 µl de una disolución de N-(1-Naphthyl) etilendiamina al 0,1 % peso/volumen en agua. Se agitaron las placas y se leyeron a 540 nm, tras 30 minutos de incubación. Las determinaciones se realizaron por duplicado. Para los cálculos se realizó una recta patrón con nitrito sódico.

Determinación de los niveles de MDA. La concentración de MDA se determinó mediante el uso de un kit colorimétrico (Calbiochem) siguiendo las instrucciones del fabricante.

Determinación de los grupos carbonilo. Para realizar las determinaciones se precipitaron las muestras con ácido tricloroacético (Metcalf) al 30%. Los precipitados eran resuspendidos con 500 µl de una solución 10 mM de 2,4-dinitrofenilhidrazina (DNPH), durante 60 minutos a 37°C. Seguidamente las muestras fueron precipitadas con 500 µl de TCA al 20%, centrifugadas durante 10 minutos a 1.000 g a 4°C, y el sobrenadante fue descartado. El precipitado obtenido era lavado dos veces con 1 ml de etanol-acetato de etilo (1:1; v/v) para remover el DNPH que permanecía libre, y centrifugado 10 minutos a 1.000 g a 4°C. El precipitado final era resuspendido en 1 ml de guanidina 6 M en tampón fosfato 2 mM y pH 2,3. Las muestras fueron incubadas durante 40 minutos a 37°C. Finalmente, las muestras eran centrifugadas durante 5 minutos a 3.000 g a 4°C para clarificar el sobrenadante. La concentración de grupos carbonilo se determinó a 360 nm, donde el DNPH presenta el máximo de absorción.

Todos los resultados obtenidos en el tejido plantar se corrigieron por los niveles de proteína determinados usando un kit comercial (Biorad), mientras que los obtenidos en neutrófilos se expresan corregidos por los niveles de DNA determinados de forma fluorimétrica.

Los resultados relativos al efecto del ungüento sobre la inflamación inducida por zymosan sobre el peso de la pata se muestran en la figura 2.

Tras 4 horas después de inducir la inflamación el peso de la pata presenta valores similares entre los dos grupos de salino. El tratamiento con el ungüento reduce de forma significativa la inflamación como se evidencia con un menor peso de la pata.

En la tabla 1 se muestran los resultados relativos a las actividades enzimáticas, los marcadores de daño oxidativo y los niveles de nitrito en el tejido plantar.

Tabla 1. Actividades enzimáticas, marcadores de daño oxidativo y niveles de nitrito en el tejido plantar.

	Salino	Salino ungüento	Zymosan	Zymosan ungüento
Actividades enzimáticas				
MPO (nKat/mg prot)	2,79 ± 0,30	3,02 ± 0,28	21,8 ± 2,4[a]	17,8 ± 3,0[a]
GPx (nKat/mg prot)	0,29 ± 0,02	0,32 ± 0,04	0,38 ± 0,02	0,36 ± 0,06
Catalasa (mK/mg prot)	11,3 ± 2,8	12,9 ± 1,2	9,17 ± 1,0	10,7 ± 2,2
Daño oxidativo				
MDA (nmol/g prot)	523 ± 79	542 ± 86	648 ± 48	591 ± 79
Carbonilos (µmol/mg prot)	15,0 ± 1,1	16,7 ± 1,7	23,9 ± 0,9[a]	24,2 ± 1,1[a]
Nitrito (µmol/mg prot)	5,81 ± 1,06	6,04 ± 0,54	14,1 ± 1,0[a]	11,6 ± 0,7[b]

Letras distintas indican diferencias significativas entre los tratamientos (p<0,05, ANOVA de un factor).

La actividad de la MPO se usa como indicador de inflamación al ser indicativo de la infiltración de neutrófilos en el tejido estudiado, al ser un enzima que se expresa exclusivamente en este

tipo celular. El tratamiento con zymosan produce un aumento acusado de la actividad MPO. El ungüento no reduce la actividad MPO respecto al grupo con vaselina, posiblemente porque las señales quimiotácticas para los neutrófilos se liberarían al inicio del proceso de inflamación, antes de añadir el ungüento.

No se observan diferencias significativas entre los diferentes tratamientos en las actividades de los enzimas antioxidantes.

La existencia de daño oxidativo inducido por el zymosan sólo es evidente en el caso de los grupos carbonilo, sin diferencias entre el grupo tratado con el ungüento y el grupo tratado con vaselina.

El nitrito se usa como marcador de la producción de óxido nítrico. El óxido nítrico es un mediador importante en el proceso inflamatorio. El tratamiento con zymosan incrementa de forma significativa los niveles de nitrito, los cuales se ven reducidos por la acción del ungüento. Esta reducción participaría en una más rápida recuperación del proceso inflamatorio.

En la tabla 2 se muestran los resultados de la actividad enzimática en neutrófilos circulantes.

Tabla 2. Actividades enzimáticas en neutrófilos circulantes.

	Salino	**Salino + ungüento**	**Zymosan**	**Zymosan + ungüento**
MPO (nKat/ug DNA)	$4{,}47 \pm 0{,}82^{a}$	$4{,}74 \pm 0{,}44^{a}$	$7{,}73 \pm 1{,}03^{b}$	$4{,}44 \pm 0{,}67^{a}$
GPx (nKat/ug DNA)	$0{,}80 \pm 0{,}06$	$0{,}79 \pm 0{,}06$	$0{,}87 \pm 0{,}0$	$0{,}64 \pm 0{,}04$
Catalasa (K/mg DNA)	$0{,}15 \pm 0{,}02^{a}$	$0{,}14 \pm 0{,}01^{a}$	$0{,}27 \pm 0{,}06^{b}$	$0{,}16 \pm 0{,}03^{a}$

Letras distintas indican diferencias significativas entre los tratamientos ($p<0{,}05$, ANOVA de un factor).

El grupo tratado con zymosan y vaselina presenta unas actividades MPO y catalasa significativamente más elevadas que el grupo tratado con zymosan y el ungüento. De hecho, el grupo tratado con el ungüento presenta unas actividades similares a los dos grupos tratados con salino. La aplicación del ungüento reduce el grado de activación de los neutrófilos circulantes y, por lo tanto,

reduciría el riesgo de sufrir daño oxidativo inducido por la acción de los propios neutrófilos.

EJEMPLO 4

En este ejemplo se desarrolla un experimento que demuestra las propiedades anti-inflamatorias del extracto hidroalcohólico del sebo de cerdo. Para ello se usó un modelo celular (PBMCs, células mononucleares de sangre periférica humana y neutrófilos humanos) en el que se simuló un proceso de inflamación aguda mediante el contacto de las células con un componente de las paredes bacterianas de naturaleza lipopolisacarídica (LPS) como el de *Escherichia coli*. La instauración del proceso inflamatorio se testó mediante la cuantificación de la producción de citoquinas inflamatorias como el TNF-α, y tras la cual se testó el efecto del extracto hidroalcohólico como producto anti-inflamatorio.

Las células se aislaron a partir de sangre de la vena antecubital de 8 individuos mediante punción con vacutainers con EDTA como anticoagulante. Se purificaron las fracciones de PBMCs y de neutrófilos utilizando un procedimiento previamente descrito (Boyum 1964; Sureda et al. 2004a). Brevemente, la sangre se introduce en Ficoll en una proporción 1,5:1 y se centrifuga a 900 x g, a 4°C durante 30 minutos. La fracción de PBMCs se extrae cuidadosamente, se conservan el sedimento, que contiene neutrófilos y eritrocitos, y la fase de plasma, descartándose la fase intermedia de Ficoll. La fracción de PBMCs se lava dos veces con tampón fosfato (PBS), pH 7,4, y se centrifuga a 1.000 g, a 4°C durante 10 minutos, consiguiendo finalmente el sedimento de PBMCs.

El sedimento que contiene eritrocitos y neutrófilos, se incuba a 4°C con cloruro de amonio 0,15 M para hemolizar los eritrocitos. La suspensión se centrifuga a 750 g, a 4°C durante 15 minutos y se descarta el sobrenadante. El sedimento de neutrófilos se lava primero con cloruro de amonio 0,15 M y posteriormente con PBS. El último lavado con PBS, tanto del sedimento de PBMCS como del de neutrófilos, se realiza con un volumen de 10 mL. Se reparte

equitativamente este volumen en 5 alícuotas de 2 mL en cinco tubos de manera que en cada tubo esté el mismo número de células. Los tubos con las alícuotas de 2mL se centrifugan a 1.000 g a 4°C durante 10 minutos, se descarta el sobrenadante y se obtiene un precipitado de células que son la que se utilizan para evidenciar los efectos inflamatorios del LPS y los efectos anti-inflamatorios del extracto hidroalcohólico del sebo de cerdo.

Las PBMCs y los neutrófilos se resuspenden en 2 mL de medio de cultivo RPMI-1640 (Sigma-Aldrich, España) solo (control) o con medio de cultivo RPMI-1640 que contiene los diferentes aditivos para realizar la experimentación. Los diferentes cultivos celulares se incuban a 37°C durante 2 horas. Al acabar la incubación se centrifugan a 1.000 g, a 4°C durante 10 minutos, se recogen los sobrenadantes y se guardan a -80°C para el posterior análisis de marcadores de inflamación como el TNF-α.

En este experimento se ensayaron los siguientes medios/aditivos repetido 8 veces con células procedentes de 8 donantes en un volumen final de 2 mL:

- Cultivo Control: contiene el medio de cultivo y las células.

- Grupo LPS: contiene el medio de cultivo con LPS 1 µg/mL como aditivo y las células (lipopolysaccharides from *Escherichia coli* 0127:B8; Sigma-Aldrich, España).

- Grupo Extracto: contiene el medio de cultivo con un extracto hidroalcohólico de sebo de cerdo a una concentración de 1 mg/mL y las células.

- Grupo Extracto + LPS: contiene el medio de cultivo con un extracto hidroalcohólico de sebo de cerdo a una concentración de 1 mg/mL y las células al que se le añade LPS (1 µg/mL) al cabo de 30 minutos de incubación.

- Grupo LPS + Extracto: contiene el medio de cultivo con LPS 1 µg/mL y las células al que se le añade un extracto hidroalcohólico

de sebo de cerdo a una concentración de 1 mg/mL al cabo de 30 minutos de incubación.

En los sobrenadantes de los medios de incubación se determinan los siguientes compuestos: TNF-α, IL-6, IL-8.

Los análisis que se realizaron mediante los kits ELISA siguientes:

- TNF-α (Diaclone, Francia, coeficiente de variación intra-ensayo 3,3%, inter-ensayo 9,0%)

- IL-6 (Diaclone, Francia, coeficiente de variación intra-ensayo 4,4%, inter-ensayo 9,1%)

- IL-8 (RayBio, coeficiente de variación intra-ensayo 10%, inter-ensayo 12%)

Las diferencias entre los resultados se analizaron estadísticamente mediante análisis de la varianza de un factor y un post hoc DMS, $p<0,05$, utilizando el programa estadístico SPSS, versión 21.0.

Los resultados (ver tabla 3) se expresan como tasa de producción de los diferentes parámetros referidos a las células que los producen (cantidad/10^3 cel x mL/h).

Tabla 3. Tasa de producción.

PBMCs					
	Control	**LPS**	**Extracto**	**Extracto + LPS**	**LPS+ Extracto**
TNF-α (pg/10^3 cel x mL x h)	148 ± 12^{a}	390 ± 32^{b}	$74,5 \pm 26,7^{c}$	$106 \pm 8^{a,c}$	$133 \pm 18^{a,c}$
IL-6 (pg/10^3 Cel x mL x h)	$28,1 \pm 3,2^{a}$	$39,2 \pm 5,4^{b}$	$2,71 \pm 1,19^{c}$	$21,1 \pm 5,9^{a}$	$2,93 \pm 0,57^{c}$
IL-8 (pg/10^3 cel x mL x h)	857 ± 82^{a}	1296 ± 162^{b}	243 ± 16^{c}	433 ± 66^{c}	186 ± 71^{c}
PGE-1(pg/10^3 cel x mL x h)	$62,6 \pm 20,5^{a}$	$41,6 \pm 6,0^{a}$	1646 ± 307^{b}	2007 ± 385^{b}	2005 ± 394^{b}
Neutrófilos					
IL-6 (pg 10^3 cel x mL x h)	$5,70 \pm 0,69^{a}$	$9,66 \pm 2,45^{b}$	$4,24 \pm 0,56^{a}$	$3,89 \pm 0,49^{a}$	$4,91 \pm 0,78^{a}$

Letras distintas indican diferencias significativas entre los tratamientos (p<0,05, ANOVA de un factor).

La presencia de LPS incrementa la tasa de producción de TNF-α, IL-6, IL-8 por parte de las PBMCs, y la tasa de producción de IL-6 en el caso de los neutrófilos. Se pone de manifiesto que el LPS produce un efecto inflamatorio en PBMCs y en neutrófilos, probablemente a través de la activación y translocación al núcleo del NFκB que permite la expresión de genes inflamatorios como el TNF-α, IL-6 y IL-8. La adición del extracto hidroalcohólico de sebo de cerdo ya reduce la tasa de producción de TNF-α, IL-6 y IL-8 del cultivo de PBMCs control y reduce mucho más la tasa de producción de estos factores inflamatorios por parte de las PBMCs activadas con LPS, poniendo de manifiesto un potente efecto anti-inflamatorio del extracto hidroalcohólico del sebo de cerdo. El orden en el que se introduce el extracto hidroalcohólico de cerdo, antes o después del LPS parece que no influye en la respuesta anti-inflamatoria del extracto hidroalcohólico. Estos resultados se diferencian así del mecanismo anti-inflamatorio que presentan algunos productos de oxidación de fosfolípidos naturales, que tan sólo actúan si previamente a la adición del estímulo inflamatorio ya están presentes en el medio.

Así mismo, el extracto hidroalcohólico del sebo de cerdo incrementa significativamente la producción de PGE_1 por parte de las PBMCs sin que la presencia de LPS modifique la tasa de producción. Se ha indicado que la PGE-1 tiene efectos anti-inflamatorios. Los resultados obtenidos ponen de manifiesto que o bien el extracto hidroalcohólico presenta unos niveles elevados de PGE_1 o bien de un precursor de su síntesis como el ácido graso 9,12-octadecadienoico.

EJEMPLO 5

Este ejemplo ilustra la diferente composición del sebo de cerdo y del extracto hidroalcohólico del sebo de cerdo respecto de la composición de la materia de partida para la elaboración del sebo de cerdo siguiendo el procedimiento descrito en la presente invención. Los componentes con actividad anti-inflamatoria están presentes en el sebo de cerdo ya tratado y en el extracto hidroalcohólico, pero no lo están en la materia de partida de la

elaboración del sebo o están en concentraciones inferiores, aunque algunos componentes precursores de compuestos anti-inflamatorios están presentes en menor concentración en la materia de partida.

Para determinar la composición del sebo de cerdo de la panceta de partida se realizó una extracción del contenido total de lípidos con disolventes orgánicos, se derivatizaron por trans-esterificación/esterificación a los metil ésteres de los ácidos grasos, se separaron y cuantificaron estos metil ésteres y otros productos no metilados por cromatografía de gases. Así mismo, se cuantificó por procedimientos inmunológicos el contenido de resolvina en el extracto orgánico seco del sebo y de la panceta. También se identificaron y cuantificaron los componentes del extracto hidroalcohólico del sebo de cerdo y de la panceta. Los componentes del extracto hidroalcohólico se trans-esterificaron/esterificaron y se separaron por cromatografía de gases acoplada a un espectrofotómetro de masas. La identificación de los diferentes picos cromatográficos se realiza por comparación con los espectros de masas de substancias puras y por el tiempo de retención de los picos cromatográficos. En algunos casos se utilizaron patrones de los productos puros para confirmar su identificación.

Los procedimientos seguidos se resumen a continuación. El contenido lipídico del sebo y de la panceta previamente triturada (5 g) se extrae siguiendo una modificación del método de Folch (Folch et al., 1957) mediante cloroformo/metanol (2:1, v:v) con 0,01% hidroxibutilanisol (BHA) como antioxidante y 2 µL de ácido n-heptadecanoico (15 mM) como patrón interno. La fase orgánica resultante se evapora bajo corriente de nitrógeno a 55°C. El residuo seco se disuelve en 100 µL de hexano y, posteriormente, se añaden 25 µL de reactivo de derivatización (Meth-Prep™ II) dejando reaccionar durante 30 minutos a temperatura ambiente. Una alícuota de 1 µL se inyecta al cromatógrafo de gases usando helio como fase móvil con un flujo de 2,17 mL/min, medido a 150°C en cabeza de columna. El cromatógrafo de gases es un Agilent modelo 5890 (Agilent Technologies, Santa Clara, CA, EEUU) con un detector de ionización de llama (FID) y una columna Supelcowax® 10

Capillary GC column, 30 m x 0,53 mm, df 0,50 µm (Supelco, Bellefonte, PA, EEUU). La rampa de temperatura empieza a 150°C con un gradiente de temperatura de 4°C/min hasta 260°C y después una temperatura isoterma mantenida durante 15 minutos. El inyector se encuentra a 280°C y el FID a 300°C.

El residuo seco de los extractos hidoralcohólicos del sebo de cerdo o de la panceta de cerdo obtenidos según el procedimiento descrito procedente de 10g se disuelve en 100 µL de reactivo de derivatización (Meth-Prep™ II) dejando reaccionar durante 30 minutos a temperatura ambiente. Una alícuota de 5 µL se inyecta al cromatógrafo de gases con helio como fase móvil con un flujo de 0,5 mL/min, medido a 150°C en cabeza de columna. El cromatógrafo de gases Agilent modelo 6890 (Agilent Technologies, Santa Clara, CA, EEUU) está acoplado a un detector de masas de impacto electrónico Agilent modelo 5975 (Agilent Technologies, Santa Clara, CA, EEUU). La columna cromatográfica es una columna Supelcowax® 10 Capillary GC column, 30 m x 0,53 mm, df 0,50 µm (Supelco, Bellefonte, PA, EEUU). La rampa de temperatura empieza a 150°C con un gradiente de temperatura de 4°C/min hasta 260 °C y después una temperatura isoterma mantenida durante 15 minutos. El inyector se encuentra a 280°C.

La determinación de la resolvina D1 se realiza del extracto hidroalcohólico de 1 g de sebo cerdo o de panceta, mediante el procedimiento descrito anteriormente, utilizando un kit de ELISA para la determinación de resolvina D1 (Cayman, EEUU, coeficient of variation intra-assay 11,4%), siguiendo las instrucciones y con las interferencias que allí se describen.

Los resultados referidos a la composición del sebo de cerdo y de la panceta de cerdo, teniendo en cuenta tanto los componentes determinados en el extracto orgánico como los determinados en el extracto hidroalcohólico como los determinados con el análisis inmunológico se detallan a continuación:

Tabla 4. Composición del sebo tratado y de la panceta de partida.

Molécula (TR, min)	Sebo (%)	Panceta (%)
C12:0 (6,213)	0,937 ± 0,064	0,674 ± 0,119
C14:0 (9,826)	0,380 ± 0,065	0,465 ± 0,210
C16:0 (13,785)	10,2 ± 1,27	4,30 ± 0,09
C16:1 (14,390)	3,18 ± 0,09	2,70 ± 0,94
C18:0 (18,048)	13,9 ± 1,1	5,43 ± 1,19
C18:1n9 (18,540)	15,6 ± 2,5	8,84 ± 0,51
C18:2n6 (19,577)	3,99 ± 0,429	4,09 ± 0,47
C18:3n6 (20,254)	0,784 ± 0,158	0,283 ± 0,070
C18:3n3 (20,982)	1,67 ± 0,34	1,61 ± 0,08
C20:0 (22,306)	0,372 ± 0,044	0,204 ± 0,057
C20:1n9 (22,789)	2,81 ± 0,24	10,4 ± 3,1
C20:2 (23,826)	0,784 ± 0,185	0,920 ± 0,153
C20:3 (24,409)	0,150 ± 0,033	0,385 ± 0,071
C20:4n6 (24,942)	4,92 ± 2,26	2,18 ± 0,63
C20:5n3 (25,212)	0,414 ± 0,128	0,211 ± 0,017
C22:0 (26,398)	2,78 ± 0,79	6,38 ± 0,31
C22:1n9 (26,897)	0,634 ± 0,145	1,39 ± 0,09
C22:2 (27,918)	1,97 ± 0,27	0,565 ± 0,028
C24:0 (30,603)	4,75 ± 1,00	6,24 ± 0,31
C24:1n9 (31,244)	2,45 ± 0,46	4,40 ± 0,57
C22:6n3 (31,437)	6,80 ± 1,63	11,4 ± 0,2
SFA	33,3 ± 0,8	23,7 ± 1,3
MUFA	24,6 ± 2,5	27,7 ± 1,5
PUFA	21,5 ± 3,1	21,7 ± 1,5
5-Dodecanolida	0,00543 ± 0,00018	ND
Hexadecanamida	0,00129 ± 0,00025	ND
Octadecenamida	0,00607 ± 0,00060	ND
Resolvina D_1 (pg/g de sebo)	2360 ± 234	509 ± 20
H_2O (%)	0,657 ± 0,078	
Área identificada	79,4 ± 2,5	73,04 ± 1,5
Área no identificada	20,6 ± 2,1	26,96 ± 1,05

ND no detectado. C20:3 = C20:3n6 + C20:3n3. SFA ácidos grasos saturados (saturated fatty acids), MUFA ácidos grasos monoinsaturados (monounsaturated fatty acids), PUFA ácidos grasos poliinsaturados (polyunsaturated fatty acids).

El perfil de ácidos grasos del sebo de cerdo y de la panceta de cerdo es diferente dando a entender los efectos de la elaboración del sebo sobre la composición de ácidos grasos. Es destacable que el proceso de elaboración del sebo produce nuevos componentes como 5-dodecanolida, hexadecanamida y octadecenamida, así como la liberación de ácidos grasos como el ácido hexadecanoico, ácido 9-octadecenoico o el ácido 6-Octadecenoico, el ácido 9,12-octadecadienoico, el ácido octadecanoico, el ácido 5,8,11,14-eicosatetraenoico, el ácido 7-hexadecenoico, el ácido 11,14-octadecadienoico que aparecen en la composición del extracto

hidroalcohólico y que en la composición del sebo y panceta de cerdo están incluidos en los respectivos valores globales de cada uno de estos ácidos grasos.

La composición del extracto hidroalcohólico del sebo de cerdo se indica en la siguiente tabla 5. En el extracto hidroalcohólico de la panceta no se detectaron estos componentes a excepción de la resolvina D1 que se detectó tanto en el extracto hidroalcohólico del sebo (2360 ± 234 pg/g sebo) como en el de la panceta (509 ± 20 pg/g panceta).

Tabla 5. CG-masas composición extracto hidroalcohólico del sebo de cerdo

Molécula (Certeza, %)	Fórmula molecular	TR (min)	Porcentaje (%)	Min-Max (%)
Hexadecanoico, metil éster (93%)	$C_{17}H_{34}O_2$	4,788	0,603 ± 0,096	0,505 – 0,701
9-Octadecenoico, metil éster (99%) **6-Octadecenoico, metil éster** (99%)	$C_{19}H_{36}O_2$	5,632	61,6 ± 2,2	59,4 – 63,7
9,12-Octadecadienoico, metil éster (99%)	$C_{19}H_{34}O_2$	6,367	13,3 ± 0,8	12,5 – 14,0
Octadecanoico, metil éster (85%)	$C_{19}H_{38}O_2$	7,107	0,307 ± 0,166	0,136 – 0,478
5,8,11,14-eicosatetraenoico, etil éster (90%)	$C_{22}H_{36}O_2$	10,929	5,77 ± 0,42	5,32 – 6,21
5-Dodecanolida (81%)	$C_{12}H_{22}O_2$	12,935	4,49 ± 0,15	4,34 – 4,63
7-hexadecenoico, metil éster (89%)	$C_{17}H_{32}O_2$	13,176	2,08 ± 0,72	1,33 – 2,83
11,14-octadecadienoico, metil éster (93%)	$C_{19}H_{34}O_2$	16,956	5,84 ± 0,15	5,67 – 6,00
Hexadecanamida (92%)	$C_{16}H_{33}NO$	18,513	1,07 ± 0,21	0,890 – 1,28
Octadecenamida (82%)	$C_{18}H_{35}NO$	22,261	5,02 ± 0,50	3,19 – 6,84
Resolvina D_1 (µg/g de extracto)	$C_{22}H_{32}O_5$		2,86 ± 0,28	1,93-3,02

La identificación de los componentes del extracto hidroalcohólico se realizó por espectrometría de masas, teniendo en cuenta el tiempo de retención del compuesto. En la tabla se indican los derivados metilados de los compuestos del extracto hidroalcohólico que son los que se separan por cromatografía de gases. Así mismo, hay compuestos presentes que no se han metilado y que se separan en su forma nativa del extracto hidroalcohólico. Entre paréntesis se indica el porcentaje de similitud del espectro de masas del pico cromatográfico correspondiente a cada tiempo de retención con el del compuesto puro de referencia. En el caso de la 5-dodecanolida y de la oleamida, se utilizaron compuestos patrón para comprobar su elución en el mismo tiempo de retención y su

espectro de masas. Los resultados de los espectros de masas se muestran a continuación: los picos cromatográficos en los tiempos de retención de 12,935 minutos y 22,261 minutos separados del extracto hidroalcohólico tienen un espectro de masas que incluye los espectros de masas correspondientes a la 5-dodecanolida y a la oleamida; a su vez, estos productos puros tienen los mismos tiempos de retención.

Algunos de estos componentes de los extractos hidroalcohólicos del sebo de cerdo tienen actividad anti-inflamatoria.

EJEMPLO 6

En este ejemplo se desarrolla un experimento que demuestra las propiedades anti-inflamatorias de algunos de los componentes del extracto hidroalcohólico del sebo de cerdo. Se utilizaron componentes puros del extracto hidroalcohólico del sebo de cerdo obtenidos de casas comerciales de productos químicos. Se usó un modelo celular (neutrófilos humanos) en el que se simuló un proceso de inflamación aguda mediante el contacto de las células con un componente de las paredes bacterianas de naturaleza lipopolisacarídica (LPS) de *Escherichia coli*. La instauración del proceso inflamatorio se testó mediante la cuantificación de la liberación de catalasa y de mieloperoxidasa al medio de cultivo como indicadores de la desgranulación de neutrófilos activados por LPS. Se testó el efecto de 5-dodecanolida, resolvina D1, octadecenamida y el extracto hidroalcohólico del sebo de cerdo. Los productos puros como la resolvina D1, oleamida y 5-dodecanolida proceden de Quimigen S.L., España. Los neutrófilos se obtuvieron a partir de sangre venosa de seis donantes, siguiendo el protocolo descrito en el ejemplo 4. El extracto hidroalcohólico del sebo de cerdo se obtuvo siguiendo el procedimiento descrito en esta patente. Las actividades enzimáticas catalasa y mieloperoxidasa se determinaron siguiendo los procedimientos descritos en el ejemplo 2 en los sobrenadantes de la centrifugación de los cultivos de neutrófilos incubados según las condiciones descritas en el ejemplo 3. Las concentraciones de los diferentes componentes del extracto

hidroalcohólico ensayadas corresponden con las que proporciona el extracto hidroalcohólico en el medio de cultivo.

Los neutrófilos de cada participante se distribuyeron entre los siguientes tipos de cultivos:

- Grupo Control: medio de cultivo RPMI-1640 (Sigma-Aldrich, España).

- Grupo LPS: medio de cultivo con LPS de *Escherichia coli* 0127:B8 (Sigma-Aldrich, España) a una concentración final de 1 µg/mL.

- Grupo LPS con resolvina: medio de cultivo con LPS (1 µg/mL) y Resolvina D1 (1 ng/mL).

- Grupo LPS con oleamida: medio de cultivo con LPS (1 µg/mL) y Oleamida (0,06 mg/mL). - Grupo LPS con 5-dodecanolida: medio de cultivo con LPS (1 µg/mL) y 5-dodecanolida (0,04 mg/mL).

- Grupo LPS con Extracto hidroalcohólico: medio de cultivo con LPS 1 µg/mL y extracto hidroalcohólico (1 mg/mL).

Tabla 6:Actividad catalasa (CAT) y mieloperoxidasa (MPO) en los sobrenadantes de las incubaciones de neutrófilos en presencia de diversos componentes del extracto hidroalcohólico del sebo de cerdo.

	CAT ($K/10^6$ neutrófilos)	**CAT** (%)	**MPO** ($nKat/10^6$ neutrófilos)	**MPO** (%)
Control	$53.7 \pm 7.2^{a,c}$	$100 \pm 13.3^{a,c}$	69.1 ± 39.8^{a}	100 ± 57.6^{a}
LPS	116 ± 19^{b}	215 ± 35.7^{b}	177 ± 62.0^{b}	197 ± 86.3^{b}
LPS+ Resolvina	$75.2 \pm 2.2^{b,c}$	$139 \pm 4.04^{b,c}$	7.38 ± 2.03^{c}	10.7 ± 2.93^{c}
LPS + Oleamida	$34.3 \pm 13.8^{a,c}$	$63.7 \pm 25.7^{a,c}$	10.9 ± 3.01^{c}	15.8 ± 4.35^{c}
LPS + 5-Dodecanolida	$51.3 \pm 14.2^{a,c}$	$95.5 \pm 26.5^{a,c}$	11.7 ± 1.95^{c}	16.9 ± 2.81^{c}
LPS + Extracto sebo	20.8 ± 7.4^{a}	38.7 ± 13.8^{a}	8.67 ± 1.92^{c}	12.5 ± 2.77^{c}

Análisis estadístico: Letras distintas indican diferencias significativas entre los tratamientos, ANOVA de un factor, posthoc DMS, p<0,05.

La presencia del LPS induce una respuesta inflamatoria en los neutrófilos propiciando la desgranulación y un incremento significativo de la actividad catalasa y mieloperoxidasa en el

medio extracelular. El extracto hidroalcohólico del sebo reduce significativamente el proceso inflamatorio inducido por el LPS, a valores incluso inferiores que el propio control sin activar con LPS. La adición de resolvina D1, de 5-dodecanolida y de oleamida reduce al nivel control la actividad catalasa extracelular y la actividad mieloperoxidasa extracelular al nivel del extracto hidroalcohólico del sebo de los cultivos de neutrófilos activados con LPS.

EJEMPLO 7

En este ejemplo se desarrolla un experimento que demuestra las propiedades anti-inflamatorias de algunos de los componentes del extracto hidroalcohólico del sebo de cerdo. Se utiliza uno de los componentes del extracto hidroalcohólico del sebo de cerdo, la 5-dodecanolida procedente de Quimigen S. L., España. Se usó un modelo celular (neutrófilos humanos) en el que se simuló un proceso de inflamación aguda mediante el contacto de las células con un componente de las paredes bacterianas de naturaleza lipopolisacarídica (LPS) de *Escherichia coli*. La instauración del proceso inflamatorio se testó mediante la cuantificación de la producción de la citoquina pro-inflamatoria, el factor necrótico tumoral alfa (TNF-α, y tras la cual se testó el efecto de 5-dodecanolida, uno de los componentes del extracto hidroalcohólico del sebo de cerdo, a diferentes concentraciones. Los neutrófilos se obtuvieron a partir de sangre venosa de nueve donantes, siguiendo el protocolo descrito en el ejemplo 4. Se determina los niveles de TNF-α en los sobrenadantes de la centrifugación de los cultivos de neutrófilos incubados según los procedimientos y condiciones descritas en el ejemplo 3.

Tabla 7: Niveles de TNF-α en los sobrenadantes de las incubaciones de neutrófilos en presencia de LPS y de diferentes concentraciones de 5-dodecanolida

	TNF-α (pg /10^6 neutrófilos·mLx h)
Control	15,4 ± 3,05[a,c]
LPS	79,1 ± 11,0[b]
LPS + 5-dodecanolida (0,1 mg/mL)	9,15 ± 1,53[a]
LPS + 5-dodecanolida (0,06 mg/mL)	13,5 ± 2,15[a,c]
LPS + 5-dodecanolida (0,01 mg/mL)	25,9 ± 3,78[c]
5-dodecanolida (0,1 mg/mL)	7,08 ± 0,73[a]
5-dodecanolida (0,06 mg/mL)	6,87 ± 0,75[a]
5-dodecanolida (0,01 mg/mL)	8,2 ± 1,60[a]

Análisis estadístico: Letras distintas indican diferencias significativas entre los tratamientos, ANOVA 1 factor, posthoc DMS, p<0,05.

El LPS desencadena una respuesta infamatoria activando significativamente la síntesis de TNF-α que incrementa unas cinco veces. La 5-dodecanolida no altera la producción de TNF-α control; sin embargo, elimina totalmente el efecto pro-inflamatorio del LPS de una manera dependiente de la concentración de 5-Dodecanolida. El efecto anti-inflamatorio de la 5-dodecanolida, medido como la menor capacidad de la producción de TNF-α por parte de neutrófilos activados con LPS, es unas 3 veces mayor si la concentración de 5-dodecanolida es de 0,1mg/mL que si es 0,01mg/mL, aunque todas las concentraciones ensayadas reducen a nivel control la tasa de producción de TNF-α por parte de los neutrófilos activados con LPS.

REIVINDICACIONES

1. Composición farmacéutica que comprende 5-dodecanolida como principio activo y uno o más excipientes o vehículos farmacéuticamente aceptables.

2. Composición de acuerdo con la reivindicación 1 que adicionalmente comprende uno o más de los compuestos seleccionados entre octadecenamida, resolvina D1, ácido hexadecanoico, ácido 9-octadecenoico, ácido 6-octadecenoico, ácido 9,12-octadecadienoico, ácido octadecanoico, ácido 5,8,11,14-eicosatetraenoico, ácido 7-hexadecenoico, ácido 11,14-octadecadienoico, hexadecanamida.

3. Composición de acuerdo con cualquiera de las reivindicaciones anteriores donde el 5-dodecanolida se encuentra en un porcentaje de entre 2-6% del total del peso de la composición.

4. Composición de acuerdo con cualquiera de las reivindicaciones anteriores que comprende en porcentaje en peso con respecto al total de la composición:

a) 4,34-4,63% de 5-dodecanolida
b) 0,05-0,7% de ácido hexadecanoico
c) 59,4-63,7% de ácido 9-octadecenoico o ácido 6-octadecenoico
d) 12,5-14% de ácido 9,12-octadecadienoico
e) 0,13-0,47% de ácido octadecanoico
f) 5,32-6,21% de ácido 5,8,11,14-eicosatetraenoico
g) 1,33—2,83% de ácido 7-hexadecenoico
h) 5,67-6,00% de ácido 11,14-octadecadienoico
i) 0,89-1,28% de hexadecanamida
j) 3,19-6,84% de octadecenamida

k) 1,93-3,02% de resolvina D1.

5. Composición de acuerdo con cualquiera de las reivindicaciones anteriores en forma de ungüento para aplicación tópica.

6. Procedimiento para obtener una composición de acuerdo con cualquiera de las reivindicaciones anteriores que comprende:

a) Extracción de la manteca de cerdo
b) Trituración de la manteca
c) Cocción de la manteca triturada
d) Separación por tamizado de la manteca liquida de los chicharrones
e) Opcionalmente, extracción hidroalcohólica de la manteca líquida

7. Procedimiento de acuerdo con la reivindicación 6 donde la manteca se extrae a partir de la panceta y del tejido adiposo del epiplón.

8. Procedimiento de acuerdo con cualquiera de las reivindicaciones 6 o 7 donde la trituración se hace por medios mecánicos hasta alcanzar una masa homogénea.

9. Procedimiento de acuerdo con cualquiera de las reivindicaciones 6 a 8 donde la cocción implica llevar a ebullición de la grasas de la manteca durante un tiempo de entre 50 a 90 minutos.

10. Procedimiento de acuerdo con cualquiera de las reivindicaciones 6 a 9 donde la extracción hidroalcohólica se hace en una solución alcohol:agua 65:35 (v:v),

preferiblemente metanol:agua y opcionalmente en presencia de un ácido, preferiblemente HCl.

11. Composición de acuerdo con cualquiera de las reivindicaciones 1 a 5 para su uso en el tratamiento de la inflamación.

12. Composición para uso de acuerdo con la reivindicación 11 donde la inflamación tratada es la inflamación asociada a enfermedades articulares como la artritis o artrosis.

13. Composición para uso de acuerdo con la reivindicación 11 donde la inflamación tratada es la inflamación asociada a enfermedades infecciosas como la mastitis o el acné.

14. Composición para uso de acuerdo con la reivindicación 11 donde el tratamiento de la inflamación se hace por vía tópica.

15. Composición para uso de acuerdo con la reivindicación 13 donde el tratamiento por vía tópica permite tratar inflamaciones a nivel superficial como traumatismos, esguinces, contusiones, tendinitis, la mastitis o el acné.

Figuras

Figura 1: Efectos del ungüento sobre la inflamación inducida con zymosan sobre el diámetro de las patas de las ratas.

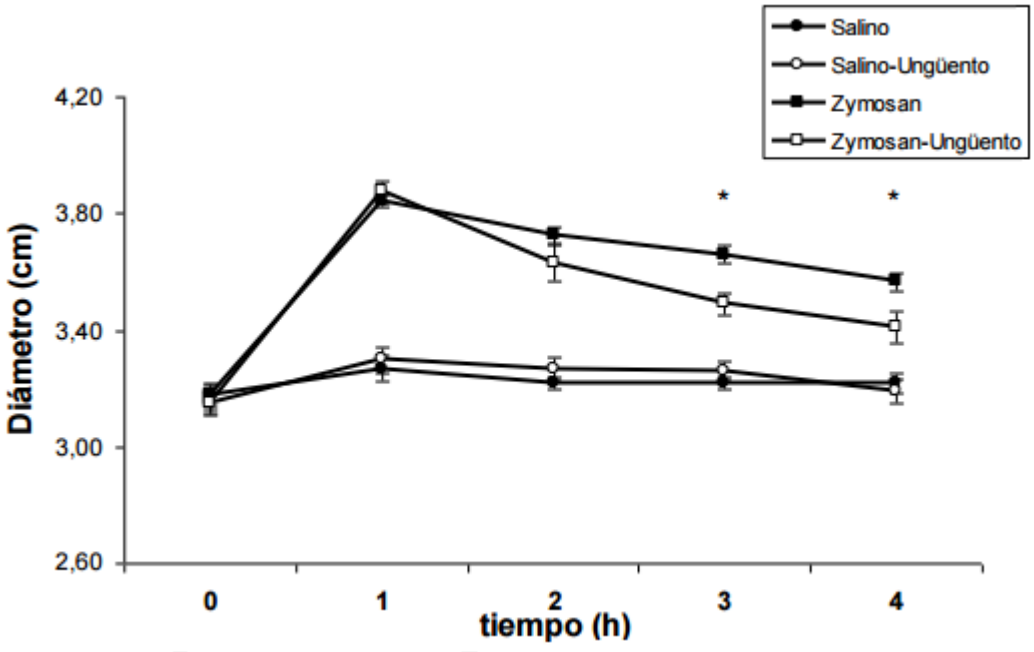
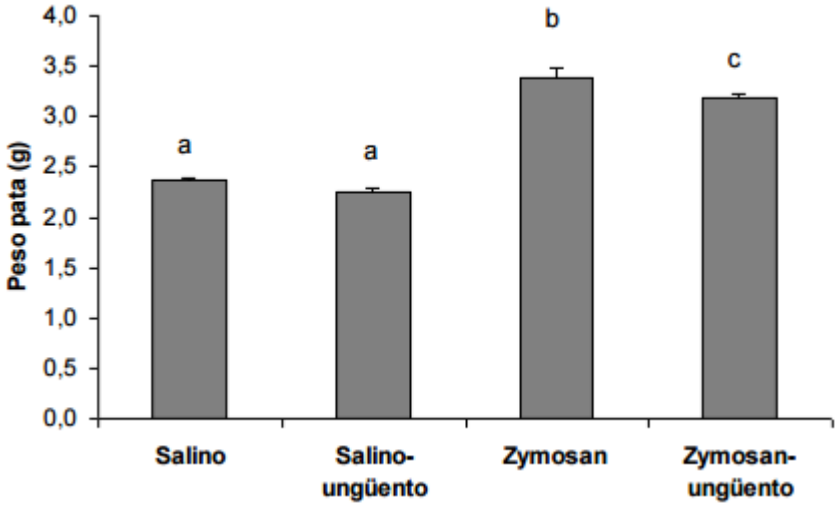


Figura 2: Efectos del ungüento sobre la inflamación inducida con zymosan a las 4 horas sobre el peso de la pata.



IV. RECAPITULACIÓN

Efectos de la suplementación con DHA y el ejercicio sobre el estrés oxidativo

Se ha postulado que un aumento de la ingesta de ácidos grasos omega 3 puede incrementar el daño oxidativo debido a que dichos ácidos grasos son más susceptibles a la oxidación por la presencia en su estructura de numerosos dobles enlaces (Richard et al. 2008). Hemos observado que la suplementación de la dieta durante 8 semanas con una bebida funcional enriquecida con DHA incrementa de forma significativa los niveles de dicho ácido graso en las membranas eritrocitarias, hecho que también podría producirse en las células inmunitarias incrementándose la disponibilidad de DHA en dichas células (Toft et al. 2000). Una mayor disponibilidad de DHA puede modular la fluidez de las membranas celulares, alterar vías de señalización intracelular, modificar receptores de membrana (como los TLR) o nucleares (como el PPARγ), estimular la producción de eicosanoides o alterar la producción de RONS (Martins de Lima et al. 2007). No se han observado efectos de la suplementación de la dieta con DHA sobre marcadores de daño oxidativo como los niveles de MDA, nitrotirosinas o carbonilos en PBMCs; sin embargo hemos podido observar un incremento significativo en los niveles proteicos de UCP-3 en PBMCs atribuible a la suplementación de la dieta con DHA. Un incremento en los niveles de UCP-3 supone una mayor protección antioxidante a nivel mitocondrial y coincide con los resultados obtenidos en un estudio precedente realizado en ratones (Cha et al. 2001). Además, este hecho podría estar relacionado con la menor producción de ROS a nivel mitocondrial observada en las PBMCs del grupo experimental al ser activadas con LPS o PMA después de la realización de un ejercicio agudo.

Se han analizado los efectos de una bebida funcional elaborada con almendras, y enriquecida con aceite de oliva, vitamina E y DHA, sobre marcadores de estrés oxidativo en plasma y eritrocitos. Dicha bebida funcional ha sido administrada en dos grupos de deportistas (jóvenes y sénior) durante 6 semanas. La suplementación de la dieta con la bebida funcional reduce de forma significativa los niveles de MDA plasmáticos después de la realización de un ejercicio agudo. El hecho que los niveles de MDA plasmáticos se vean reducidos al consumir la bebida funcional pero no por la suplementación con DHA, podría atribuirse a la presencia de otros componentes en la bebida con propiedades antioxidantes como los polifenoles y la vitamina E. En

este sentido, el descenso en los niveles de MDA plasmático coincidirían con los resultados obtenidos con una bebida similar o con zumos enriquecidos con polifenoles (Martorell et al. 2014, Rangel-Huerta et al. 2015). No se han observado efectos de la suplementación con la bebida funcional sobre los marcadores de daño oxidativo en proteínas, ni en plasma ni en eritrocitos, ni en deportistas jóvenes ni en deportistas sénior. La suplementación con la bebida funcional puede incrementar ligeramente la expresión de ciertos enzimas antioxidantes como la GPx y la GRd en PBMCs de atletas jóvenes, pudiendo incrementar así la protección frente al estrés oxidativo. En resumen, la suplementación de la dieta con una bebida funcional elaborada a base de almendra, aceite de oliva y, enriquecida con DHA y vitamina E reduce el nivel de estrés oxidativo en plasma asociado a la realización de un ejercicio extenuante, a la vez que induce una respuesta antioxidante en PBMCs. Además, estos efectos son dependientes de la edad puesto que se observan principalmente en atletas jóvenes.

La realización de un ejercicio agudo incrementa la demanda y consumo de oxígeno y consecuentemente incrementa la producción de ROS (Alessio 1993, Ji 1999). Este aumento en la producción de ROS, si deriva de un ejercicio de elevada intensidad, se manifiesta con un aumento en los niveles de marcadores de daño oxidativo como el MDA, nitrotirosinas y en el daño en el ADN (*comet assay*) en PBMCs. Estos efectos pro-oxidantes del ejercicio agudo no se vieron alterados por la suplementación de la dieta con DHA. Asimismo, como consecuencia de la realización de un ejercicio agudo las PBMCs incrementan de forma significativa sus mecanismos de defensa antioxidante, con un aumento de las actividades enzimáticas –CAT, GPx y GRd-, y de los niveles proteicos –CAT, GPx, Cu/Zn-SOD, UCP-2 y tiorredoxina reductasa (TrxR1)-. Es importante señalar que la suplementación de la dieta con DHA incrementa adicionalmente los niveles proteicos de Cu/Zn-SOD en PBMCs, potenciando la capacidad antioxidante endógena. Los aumentos en las actividades y niveles proteicos de las enzimas antioxidantes no se vieron reflejados en un incremento en la expresión de dichos genes, este hecho podría atribuirse a regulaciones post-traduccionales de estas enzimas antioxidantes como ya se ha descrito en linfocitos y eritrocitos (Tauler et al. 2005, Mestre-Alfaro et al. 2011). En resumen la realización de un ejercicio agudo provoca un incremento en los

marcadores de daño oxidativo en lípidos, proteínas y ADN a la vez que induce una respuesta antioxidante en las PBMCs. La activación de las defensas antioxidantes en PBMCs parece ser paralela a la generación de daño oxidativo, poniendo de manifiesto que las mismas causas que inician el daño oxidativo pueden ser responsables de la activación de las defensas antioxidantes. En todo caso, las defensas antioxidantes no son suficientes para evitar el daño oxidativo asociado a la realización de un ejercicio agudo, a pesar de que existe una respuesta adaptativa antioxidante en las PBMCs frente al ejercicio.

La edad es una de las variables que influyen en la situación de estrés oxidativo, observándose un aumento progresivo de los marcadores de daño oxidativo con el envejecimiento (Bailey et al. 2010). Algunos estudios han demostrado que existe una correlación positiva entre la edad y el daño oxidativo en proteínas (Gil et al. 2006). Se han estudiado los patrones de daño oxidativo y de estrés oxidativo inducidos por la actividad física y la suplementación de la dieta con antioxidantes y DHA en atletas jóvenes y en atletas sénior. Los atletas sénior son más susceptibles a padecer daño oxidativo en las proteínas del plasma y de eritrocitos que los atletas jóvenes después de realizar una prueba de esfuerzo, aunque el daño oxidativo en lípidos no se ve alterado por la edad. El menor daño oxidativo en proteínas evidenciado en los atletas jóvenes es coincidente con sus defensas antioxidantes más eficientes o más potentes. Los atletas jóvenes presentan una mayor expresión de Cu/Zn-SOD después de la realización de un ejercicio agudo en comparación a los atletas sénior. Por tanto, podemos decir que los atletas jóvenes están más protegidos y presentan menores tasas de daño oxidativo que los atletas sénior frente a la realización de un ejercicio agudo.

La realización de un ejercicio agudo induce una respuesta adaptativa antioxidante y daño oxidativo en PBMCs. Una sucesión de repeticiones de ejercicios agudos, lo que constituye un entrenamiento, también puede inducir una respuesta acumulativa antioxidante y de daño oxidativo. Tras ocho semanas de entrenamiento los niveles de peroxidación lipídica se reducen mientras que incrementan los niveles de carbonilación y de nitración de proteínas, se reduce la actividad CAT y se incrementa la actividad GPx y los niveles de UCP-2 y UCP-3 en PBMCs.

La realización de ejercicio de forma regular modifica la manifestación de daño oxidativo y la estrategia de defensa antioxidante en PBMCs. Mientras que el entrenamiento regular reduce el grado de peroxidación lipídica, puesto de manifiesto también en otras experiencias de entrenamiento de futbolistas (Ferrer et al. 2009), a la vez incrementa el daño oxidativo y nitrosativo en proteínas. Paralelamente, el entrenamiento provoca un cambio en la estrategia de las PBMCs para eliminar las ROS pasándose de un sistema basado en la CAT a un sistema de eliminación de ROS basado en la actividad GPx, presentando esta segunda una mayor capacidad para eliminar peróxido de hidrógeno en concentraciones bajas (Ferrer et al. 2009). Adicionalmente, el entrenamiento incrementa los niveles de UCP-2 y UCP-3, dos proteínas que se han asociado a una menor tasa de producción mitocondrial de ROS (Cannon et al. 2006, Sahlin et al. 2010). Las adaptaciones al entrenamiento manifestadas en las PBMCs podrían dar lugar a una disminución de la síntesis de ROS a nivel mitocondrial y una adaptación del patrón antioxidante hacia la utilización de vías de eliminación de ROS más eficientes a bajas concentraciones. A pesar de estas adaptaciones de las PBMCs al entrenamiento, se acumula daño oxidativo en proteínas, probablemente como consecuencia de la reacción de carbonilación entre proteínas y MDA y de la reacción de nitración entre proteínas y peroxinitrito, este último producto de la reacción entre el anión superóxido y el óxido nítrico.

En resumen, podemos decir que la suplementación de la dieta con DHA, o con una bebida funcional elaborada con almendras, aceite de oliva y enriquecida con DHA y vitamina E no incrementa los niveles de daño oxidativo sino al contrario, lo reducen en algunos casos y potencian las defensas antioxidantes endógenas, principalmente a nivel mitocondrial en PBMCs. El ejercicio agudo induce una respuesta antioxidante aunque dicha respuesta no es suficiente para reducir el daño oxidativo en lípidos, proteínas y ADN. La realización de ejercicio de forma regular, el entrenamiento, modifica la manifestación de daño oxidativo y la estrategia de defensa antioxidante, potencia las defensas antioxidantes y acumula daño oxidativo y nitrosativo en proteínas.

Efectos de la suplementación con DHA, el ejercicio agudo y el entrenamiento sobre el estado inflamatorio

El ejercicio agudo induce una respuesta inflamatoria como consecuencia del daño muscular (Pedersen et al. 1997, Nieman and Pedersen 1999). Este proceso inflamatorio se ve reflejado en un incremento de toda una serie de citoquinas, cuya secreción depende de la intensidad del ejercicio realizado (Ostrowski et al. 1998, Pedersen 2000). Esta respuesta se caracteriza principalmente por un incremento en los niveles plasmáticos de IL-6, pudiendo incrementar también la concentración en plasma de otras citoquinas proinflamatorias como el TNF-α y IL-1β, aunque éstas se incrementan únicamente tras ejercicios extenuantes (Cannon and Kluger 1983, Steensberg et al. 2002). Quimioquinas como la IL-8, MIP-1, GM-CSF y G-CSF también ven aumentados sus niveles después de la realización de un ejercicio prolongado como una maratón (Ostrowski et al. 1998, Pedersen 2000). Se ha detectado únicamente un incremento significativo en los niveles plasmáticos de IL-6 tras la realización de una sesión planificada de entrenamiento de 2 horas de duración al 70% de la VO_2max durante el 50% del entrenamiento. Las otras citoquinas analizadas (IL-5, TNF-α, GM-CSF, MIP-1α, IL-1β, IL-2, IL-4, IL-8, IL-10, VEGF, INF-γ EGF, MCP-1, TNF-α) no alteran sus niveles basales a las dos horas de acabar dicha sesión de entrenamiento. Factores como la intensidad, duración, edad, nivel de entrenamiento, tipo de ejercicio y daño muscular influyen en la respuesta de las citoquinas plasmáticas como marcadores del estado de inflamación (Cannon and Kluger 1983, Ostrowski et al. 1998, Pedersen 2000, Steensberg et al. 2002). Incluso se ha puesto en evidencia que la fuente de IL-6 circulante, que incrementa sus niveles tras ejercicios prolongados, no puede atribuirse a las células inmunitarias sino que es fruto de la secreción muscular (Starkie et al. 2001, Steensberg et al. 2002, Pedersen and Febbraio 2008). Se ha postulado también que la IL-6 secretada como consecuencia de la contracción muscular induce la secreción de citoquinas con un perfil anti-inflamatorio como la IL-10 y la IL-1rα, participando de esta manera en la respuesta anti-inflamatoria asociada a la práctica regular de actividad física (Pedersen 2013). La respuesta de las citoquinas plasmáticas a las dos horas de acabar un ejercicio agudo estaría parcialmente en consonancia con esta ausencia de actividad secretora por parte de las células inmunitarias. Sin embargo, también

hemos puesto de manifiesto, en un experimento con actividad física extenuante y controlada en atletas jóvenes y atletas sénior, que se incrementan algunos marcadores plasmáticos de activación de las células inmunitarias como la molécula de adhesión soluble L-selectina (sL-selectina) y la molécula de adhesión intercelular 3 soluble (sICAM3)(del Pozo et al. 1994, Akimoto et al. 2002). Así mismo, se ha evidenciado que la realización de una actividad física extenuante en estos atletas y la realización de una sesión de entrenamiento en futbolistas federados incrementan los niveles de NFκB activado en PBMCs. El NFκB activado penetra en el núcleo donde modula la expresión de multitud de genes, tanto inflamatorios como antioxidantes (Pahl 1999). Entre los genes que facilita su expresión están genes antioxidantes (Hemooxigenasa 1, Mn-SOD, UCP-2), genes pro-inflamatorios (iNOS, COX-2, TNF-α, IL-1β), y otros genes como factores de crecimiento y moléculas de adhesión. Estos resultados ponen de manifiesto que el ejercicio agudo prepara las PBMCs para dar respuestas inflamatorias y antioxidantes, aunque no se aprecia un perfil pro-inflamatorio en las citoquinas plasmáticas. Similarmente, el ejercicio agudo extenuante en atletas jóvenes y sénior o una sesión de entrenamiento de futbolistas no alteran los niveles plasmáticos de PGE_1 aunque sí incrementan los niveles de PGE_2 y disminuyen los de lipoxina. Esta diferente respuesta al ejercicio de los niveles plasmáticos de PGE_1 y de PGE_2 puede atribuirse en parte a diferencias en sus vías de síntesis. La PGE_2 se sintetiza a partir del AA, mientras que la PGE_1 se sintetiza a partir del ácido dihomo-γ-linoleico (DGLA), un precursor del AA. Estos dos precursores de las prostaglandinas podrían estar diferentemente disponibles frente a la realización de un ejercicio agudo. De hecho la disponibilidad de DHA influye en la respuesta al ejercicio agudo de los niveles plasmáticos de PGE_2 dado que tan sólo se incrementan tras la suplementación de la dieta con DHA.

Las lipoxinas son mediadores lipídicos sintetizados por parte de las LOXs, a partir del AA y juegan un papel muy importante en la resolución de la inflamación (Serhan et al. 2011, Chandrasekharan and Sharma-Walia 2015). Presentan potentes efectos quimiotácticos pero sin activar vías inflamatorias, además, son capaces de inhibir la infiltración de los neutrófilos y de estimular la fagocitosis de los neutrófilos apoptóticos (Serhan 2007). Se ha observado que los niveles de lipoxina A_4 descendieron de forma significativa después de la realización de un ejercicio agudo

principalmente en los atletas sénior, dicho descenso podría ser debido a que se ha observado que después de la realización de un ejercicio agudo se produce una eliminación efectiva de lipoxina A_4 por la orina (Gangemi et al. 2003). Los efectos del ejercicio agudo sobre los niveles circulantes de mediadores lipídicos como la PGE_2 y la lipoxina A_4 pueden considerarse como parte del perfil anti-inflamatorio inducido por la actividad física.

La práctica de ejercicio regular o el entrenamiento tienen efectos anti-inflamatorios a nivel sistémico (Petersen and Pedersen 2005, Mathur and Pedersen 2008, Sallam and Laher 2016). A pesar de ello, no hemos detectado variaciones en las concentraciones plasmáticas basales de citoquinas después de 8 semanas de entrenamiento regular. Se ha descrito que el ejercicio regular ayuda a reducir los niveles circulantes de citoquinas pro-inflamatorias en patologías asociadas a una inflamación crónica como la obesidad y diabetes en las que es habitual detectar elevados niveles plasmáticos de dichas citoquinas (Petersen and Pedersen 2005, Oberbach et al. 2006, Pedersen and Saltin 2006), mientras que no se han observado efectos en los niveles plasmáticos basales de citoquinas en atletas. Sin embargo, hemos detectado un incremento significativo de los niveles de PGE_1 en plasma durante este mismo periodo de entrenamiento. La PGE_1 tiene potentes efectos vasodilatadores, con capacidad de reducir la agregación plaquetaria y de las células inmunitarias, además, se ha observado que reduce la formación de edemas (Frassdorf et al. 2006) y tiene propiedades antinflamatorias (Frassdorf et al. 2006, Uchida et al. 2009). Incluso se administra PGE_1 como tratamiento en casos de enfermedades relacionadas con procesos isquémicos y mala circulación (Frassdorf et al. 2006). Se ha descrito que el ejercicio regular puede inducir cambios en la actividad de algunos enzimas que participan en el metabolismo de los eicosanoides (Nikolaidis and Mougios 2004). Se ha observado que la Δ5-desaturasa presenta una menor actividad en ratones entrenados (Helge et al. 1999) por lo que debido a esta menor actividad la mayor parte de DGLA es insertado en las membranas plasmáticas, incrementándose así la disponibilidad de DGLA y facilitando la síntesis de PGE_1 (Rubin and Laposata 1991). El incremento en los niveles plasmáticos basales de PGE_1 producido tras un periodo de 8 semanas de entrenamiento

contribuiría a los efectos anti-inflamatorios asociados a la práctica regular de ejercicios.

Los efectos de los ácidos grasos omega 3 sobre la producción de citoquinas y sobre el estado inflamatorio asociado a algunas enfermedades están ampliamente descritos (Calder 2006, Gonzalez-Periz et al. 2009, Hassan et al. 2010). La suplementación de la dieta con DHA durante 8 semanas no ha mostrado efecto alguno sobre los niveles basales ni los niveles post-ejercicio de las citoquinas analizadas. Tan sólo los niveles post-ejercicio de MIP-1α son ligeramente más elevados en el grupo suplementado con DHA que en el grupo placebo tras 8 semanas de entrenamiento. Estas ligeras diferencias pueden atribuirse a que los sujetos del grupo experimental presentaron ya inicialmente unos niveles basales de MIP-1α en plasma ligeramente superiores que los sujetos del grupo placebo, manteniéndose estas diferencias tras ocho semanas de entrenamiento. De forma similar se ha descrito que la suplementación de la dieta con cápsulas de ácidos grasos omega 3 no altera el perfil de citoquinas circulantes tras la realización de un ejercicio extenuante como es una maratón. Los ácidos grasos omega 3 pueden generar vía COX y LOX una amplia gama de mediadores lipídicos (Serhan et al. 2002, Serhan et al. 2015), que podrían contribuir a los efectos anti-inflamatorios de los ácidos grasos omega-3. De hecho, hemos observado un incremento en los niveles de COX-2 después de 8 semanas de suplementación con DHA, hecho que podría estar relacionado con los mayores niveles de PGE_2 observados en el grupo suplementado después de la realización de un ejercicio agudo en el grupo experimental.

También se han analizado los efectos de la bebida elaborada con almendra, aceite de oliva y enriquecida con vitamina E y DHA sobre el estado inflamatorio en atletas jóvenes y sénior tras la práctica de un ejercicio agudo. En este caso se ha observado un efecto modulador del estado inflamatorio ya que por una parte la bebida disminuye de forma significativa los niveles plasmáticos de sICAM-3 y sL-Selectina pero por otra incrementa de forma significativa las concentraciones circulantes de TNF-α y su expresión génica en PBMCs, de forma más evidente en los atletas sénior. Hemos evidenciado que el ejercicio agudo prepara a las PBMCs para dar una respuesta pro-inflamatoria y antioxidante; la suplementación de la dieta con la bebida funcional disminuye el nivel de activación de las PBMCs atendiendo a la

disminución de los niveles de las moléculas de adhesión. Además no altera el nivel de activación de NFkB y no evita un cierto perfil pro-inflamatorio dado que incrementa los niveles plasmáticos de TNF-α y su expresión génica en PBMCs, principalmente después de un ejercicio extenuante en atletas sénior. Esta potenciación del carácter pro-inflamatorio de las PBMCs post-ejercicio inducido por el consumo de la bebida funcional rica en polifenoles, DHA y vitamina E podría atribuirse en parte a la aportación de otros ácidos grasos con la bebida como el ácido palmítico. En este sentido, se ha descrito que el ácido palmítico induce efectos pro-inflamatorios (Wu et al. 2014).

Para concluir, ocho semanas de entrenamiento regular o la realización de un ejercicio intenso y prolongado provoca pocos cambios en los niveles de citoquinas plasmáticas en futbolistas sanos y entrenados. Sin embargo, el ejercicio intenso y prolongado incrementa los niveles de PGE_2, además una sesión de ejercicio extenuante incrementa el grado de activación del NFκB en PBMCs e incrementa los niveles plasmáticos de sICAM-3 y sL-Selectina, indicando que el ejercicio puede preparar a las PBMCs para tener una respuesta pro-inflamatoria post-ejercicio. La suplementación de la dieta con DHA, no afecta los niveles plasmáticos de citoquinas, sin embargo incrementa los niveles de PGE_2 después de un ejercicio intenso y prolongado en futbolistas entrenados y después de un ejercicio extenuante en atletas jóvenes y sénior. El consumo de una bebida de almendra enriquecida con DHA, vitamina E y aceite de oliva atenúa el incremento de los niveles plasmáticos de sICAM-3 y sL-Selectina provocados por un ejercicio extenuante.

Efectos de la suplementación con DHA y el ejercicio sobre la capacidad de producción de citoquinas de las PBMCs

Durante y después de la realización de un ejercicio agudo las PBMCs se movilizan debido a la liberación de hormonas de estrés (Gleeson et al. 2006, Dimitrov et al. 2010, Mestre-Alfaro et al. 2012, Reihmane et al. 2012), a la producción de citoquinas asociada al ejercicio (Walsh et al. 2011, Del Giacco and Scorcu 2014) y a factores como el calor producido durante la contracción muscular (Gleeson et al. 2006, Mestre-Alfaro et al. 2012). Hemos puesto de manifiesto que la realización de un ejercicio agudo prepara las PBMCs para una respuesta inflamatoria potenciando su capacidad para producir citoquinas y factores de crecimiento en respuesta a estímulos inmunológicos como el LPS. Hemos relacionado esta preparación pro-inflamatoria con los mayores niveles de TLR-4 y de NFkB activado en PBMCs después de la realización de un ejercicio intenso y prolongado como una sesión de entrenamiento y tras un ejercicio extenuante, aunque no hemos observado cambios en los niveles proteicos de COX-1 o COX-2, enzimas clave de la síntesis de mediadores lipídicos pro- y anti-inflamatorios. La respuesta de los niveles de TLR-4 en PBMCs al ejercicio agudo parece no ser coincidente con los resultados de algunos estudios preexistentes (Lancaster et al. 2005, Gleeson et al. 2006) en los que se aprecia una reducción de la expresión de los TLR-4 en monocitos tanto tras la realización de un ejercicio agudo como consecuencia de la práctica de ejercicio de forma regular. Probablemente, estas discrepancias sean atribuibles al distinto tipo celular analizado en estos estudios (PBMCs o monocitos) y a la metodología de análisis usada (western-blot o citometría de flujo). En este sentido, debemos tener en cuenta que como consecuencia de un ejercicio agudo se cambian las proporciones de las subpoblaciones de PBMCs, se reduce el número de linfocitos y se incrementa el número de monocitos circulantes (Cases et al. 2005, Tauler et al. 2006, Okutsu et al. 2008), por lo tanto el incremento en los niveles de TLR-4 observados podría ser debido al cambio en la proporción linfocitos/monocitos que se produce tras la realización de un ejercicio agudo. Sin embargo, las diferencias en el número de monocitos y de linfocitos que hemos observado tras el ejercicio intenso y prolongado no pueden explicar la magnitud del cambio en los niveles de TLR-4. Se refuerza la idea de que las PBMCs presentan una mayor capacidad de respuesta frente a estímulos como el LPS tras la realización de una actividad física intensa y

prolongada. Hemos observado que el ejercicio intenso y prolongado modifica significativamente la respuesta de las PBMCs frente a un estímulo externo como el LPS, puesto que se detecta un incremento en la producción de IL-6, IL-8, VEGF, MCP-1, EGF, INF-γ, TNF-α, IL-1α e IL-1β, a la vez que disminuyen los niveles de IL-2, se ha postulado que un descenso en los niveles de IL-2 puede provocar una respuesta inmunitaria disminuida (Gaffen and Liu 2004, Del Giacco and Scorcu 2014). Por este motivo podemos concluir que la realización de un ejercicio intenso y prolongado prepara o activa a las PBMCs incrementando la presencia de los TLR-4 que promueven una potenciación de la secreción de citoquinas frente al estímulo con LPS. Las PBMCs además de alterar el patrón de secreción de citoquinas, también incrementan su capacidad de síntesis de mediadores lipídicos como la RvD_1 como consecuencia de la realización de un ejercicio intenso y prolongado. Aumentos en los mediadores lipídicos inducidos por un ejercicio han sido descritos previamente por otros autores (Markworth et al. 2013), lo que podría relacionarse en parte con los efectos anti-inflamatorios asociados a la realización de ejercicio. La realización de un ejercicio intenso y prolongado no altera los niveles de COX-1 ni COX-2 en PBMCs, sin embargo, incrementa la capacidad de las PBMCs de producir PGE_1 y PGE_2 en respuesta al LPS, lo que cuestiona que las PBMCs sean las responsables de los cambios observados en los niveles circulantes de estas PGs en respuesta al ejercicio.

La suplementación de la dieta con ácidos grasos omega 3 puede modificar la composición en ácidos grasos de las células inmunes (Toft et al. 2000). Estos cambios pueden alterar la función de las células inmunitarias, incrementando la actividad citotóxica de las *NK,* alterar la producción de citoquinas, modificar la secreción de mediadores lipídicos (Calder 1998, Paschoal et al. 2013, Santos et al. 2013, Liu et al. 2014, Da Boit et al. 2015). Los efectos anti-inflamatorios de los ácidos grasos omega 3 están ampliamente descritos (Calder 2006, Gonzalez-Periz et al. 2009, Hassan et al. 2010). Hemos evidenciado que la suplementación de la dieta con DHA reduce la producción de TNF-α, IL-6, IL-1β, IL-1α, y MCP-1 por parte de PBMCs estimulados con LPS reduciendo así el potencial inflamatorio de las células inmunes. Además, la suplementación de la dieta con DHA provoca un incremento en los niveles de COX-2, a la vez que atenúa de forma notable la capacidad de producción de PGE_1 y PGE_2 después de la realización de un ejercicio intenso y prolongado,

mostrándose una discordancia entre los niveles de COX-2 y la capacidad de síntesis de PGs por parte de las PBMCs, hecho descrito previamente en otros estudios (Carroll et al. 2013).

La temperatura corporal es un factor que influencia la respuesta inflamatoria frente a una infección y además puede alterar las tasas de producción de citoquinas por parte de la PBMCs (Kluger et al. 1998). Es sabido que la realización de un ejercicio agudo incrementa la temperatura corporal y los niveles plasmáticos de IL-6 (Pearson et al. 2011, Mestre-Alfaro et al. 2012), por lo que el incremento de la temperatura corporal asociado al ejercicio podría influir en la capacidad de las PBMCs de producir citoquinas. La incubación de PBMCs estimuladas con LPS a 39.5ºC incrementa la tasa de producción de IL-2, IL-6, IL-1β y MCP-1 mientras que la tasa de producción de las otras citoquinas no se ve alterada por el incremento en la temperatura de incubación. Como ya se ha comentado el ejercicio intenso y prolongado incrementa la capacidad de producción de citoquinas por parte de las PBMCs estimuladas con LPS, sin embargo, las citoquinas que ven potenciada su tasa de producción son diferentes a las que ven potenciada su tasa de producción por el calor. Mientras que el ejercicio físico altera la producción de IL-2, IL-6, IL-8, VEGF, EGF, MCP-1, TNF-α, IL-1β e IFN-γ, el incremento de temperatura sólo provoca variaciones en los niveles de IL-2, IL-6, IL-1β y MCP-1.

En este sentido, el incremento en la temperatura corporal asociada a la realización de un ejercicio agudo altera las capacidades de las PBMCs para producir citoquinas. El hecho que el patrón de las PBMCs de síntesis de citoquinas provocado por el ejercicio agudo y por la temperatura sea diferente, permite pensar que el ejercicio agudo y la temperatura afectan la producción de citoquinas por dos mecanismos diferentes. Esta idea se ve reforzada por el hecho de que la suplementación de la dieta con DHA no afecta de ninguna manera la producción de citoquinas cuando las PBMCs se incubaron a 39.5ºC, mientras que sí altera la producción de citoquinas por parte de las PBMCs después de la realización de un ejercicio agudo.

En resumen, el ejercicio intenso y prolongado incrementa la capacidad de síntesis de citoquinas por parte de las PBMCs, dicho incremento estaría relacionado con una mayor cantidad de TLR-4 en las PBMCs después de la realización de un ejercicio

intenso y prolongado, además el ejercicio agudo también incrementa de forma significativa la capacidad de producción de mediadores lipídicos como la RvD_1, PGE_1 y PGE_2. Un aumento en la temperatura corporal también incrementa la capacidad de producción de citoquinas por parte de las PBMCs estimuladas con LPS pero el patrón de citoquinas secretado es diferente al causado por la realización de un ejercicio intenso y prolongado. La suplementación de la dieta con DHA atenúa de forma notable el incremento en la tasa de producción de citoquinas y PGs causado por la realización de un ejercicio intenso y prolongado, sin embargo no altera el incremento en la tasa de producción de citoquinas causado por la temperatura.

Efectos de la suplementación con DHA y Vitamina C sobre el estado oxidativo e inflamatorio en neutrófilos

El *Phorbol Myrstate Acetate* es una molécula que mimetiza los efectos del diacilglicerol (DAG) y que se ha usado para simular la respuesta de los neutrófilos a la actividad física (Uauy-Dagach et al. 1994, Ferrer et al. 2010). Se sabe que el PMA activa la proteína quinasa C que a la vez provoca una activación de la vía del NFκB en neutrófilos (Niwa et al. 1996, Giembycz and Lindsay 1999), además es capaz de

incrementar la producción de ROS y activar la expresión de genes antioxidantes en neutrófilos y células HL60 (Niwa et al. 1996, Ferrer et al. 2010). Hemos observado que el tratamiento con PMA incrementa las actividades CAT y MPO en el medio extracelular de cultivos de neutrófilos. Este hecho puede ser debido a la activación del proceso de degranulación de los neutrófilos después de ser tratados con PMA (Niwa et al. 1996). La liberación de estas dos enzimas al medio extracelular está modulada por la suplementación de la dieta con DHA y por la adición de vitamina C al medio de cultivo. La suplementación de la dieta con DHA provoca que lo neutrófilos respondan al PMA potenciando la liberación de CAT a la vez que reducen la liberación de MPO al medio extracelular. Por su parte, la adición de vitamina C al medio de cultivo de los neutrófilos potencia la liberación de MPO a la vez que permite recuperar la actividad CAT intracelular a pesar de ser estimulados con PMA. Estos efectos de la adición de vitamina C al medio de cultivo se producen tanto en los neutrófilos del grupo placebo como en los del grupo suplementado con DHA aunque la reducción es mayor en el grupo placebo donde prácticamente inhibe la liberación de la CAT. Esta pauta de influencia de la vitamina C y del DHA sobre las actividades CAT y MPO extracelulares pondría de manifiesto la presencia de estos enzimas en dos tipos diferentes de vesículas de secreción en el interior de los neutrófilos.

La estimulación de los neutrófilos con PMA incrementa su capacidad para producir óxido nítrico, puesto que hemos detectado un incremento en los niveles de NOx (nitrato + nitrito) productos de la metabolización del NO. La síntesis de óxido nítrico se produce por acción de la iNOS a partir de L-arginina, siendo la isoforma de los neutrófilos inducible (Ratajczak-Wrona et al. 2013). El incremento en la tasa de producción de derivados del NO podría atribuirse a cambios en la actividad o niveles de este enzima, de hecho se han descrito incrementos en la expresión de la iNOS en células HL60 después de su estimulación con PMA. (Ferrer et al. 2010). Hemos observado que la suplementación de la dieta con DHA y la adición de vitamina C al medio de cultivo modulan la liberación de nitratos y nitritos al medio extracelular. La suplementación con DHA incrementa los niveles de nitrito y nitrato en el medio extracelular en respuesta al PMA. Por su parte, la adición de vitamina C inhibe la producción de NOx inducida por el PMA, manteniéndose los niveles a una

concentración muy similar a las del grupo control no activado con PMA, sin presencia de vitamina C.

La estimulación de los neutrófilos con PMA provoca un incremento en los niveles de IL-6, además de incrementar la expresión de varios genes que participan en la respuesta inflamatoria como el TNF-α, la IL-8, el NFκB y la COX-2. Esta respuesta inflamatoria inducida por PMA está modulada por la suplementación con DHA y por la vitamina C, ya que ambos factores reducen la producción de IL-6 y la expresión de genes pro-inflamatorios inducida por el PMA. La suplementación de la dieta con DHA evita de forma notable el incremento en la expresión IL-8, TNF-α, COX-2, y NFκB inducido por el PMA. Esta reducción en la respuesta inflamatoria podría ser debida en parte a los efectos del DHA sobre la activación del NFκB, puesto que algunos estudios han demostrado que puede inhibir la fosforilación de I-κB, evitando así su translocación hacia el núcleo (Adkins and Kelley 2010). La vitamina C también modula la respuesta inflamatoria provocada por el PMA, ya que se observa una reducción en la expresión de COX-2, IL-8, TNF-α y NFκB. La vitamina C podría neutralizar gran parte de las ROS producidos por la estimulación con el PMA y, al reducirse los niveles de ROS, se reduciría también la activación de NFκB, limitando así la respuesta inflamatoria.

Resumiendo, la suplementación de la dieta con DHA potencia las capacidades antioxidantes de los neutrófilos a la vez que atenúa la respuesta inflamatoria después de la activación con PMA. Además la suplementación de la dieta con DHA potencia las actividades CAT y MPO y modera la desgranulación de los gránulos azurofílicos después de estimular a los neutrófilos con PMA. La vitamina C facilita la desgranulación de los gránulos de azurofílicos. La combinación del DHA y la vitamina C previenen la expresión de genes pro-inflamatorios.

Efectos de la suplementación con una bebida funcional sobre el rendimiento deportivo

Está ampliamente descrito que el rendimiento deportivo está influenciado por la alimentación y la hidratación (Williams 2002). De hecho, es una práctica muy extendida entre los deportistas el consumo de suplementos nutricionales ricos en hidratos de carbono, proteínas, vitaminas y minerales, además de otros nutrientes

capaces de reducir la inflamación y la oxidación (Colls Garrido et al. 2015) con el objetivo de mejorar el rendimiento deportivo. De todos modos en la mayoría de casos no se ha podido demostrar efectos ergogénicos de dichos suplementos.

Se ha descrito que las medidas de la temperatura corporal y de la piel así como la percepción de la fatiga medida con el índice de Borg en respuesta a una actividad física extenuante pueden ser parámetros útiles a la hora de valorar el nivel de rendimiento deportivo (Borg 1970, Mee et al. 2015). Se ha planificado una prueba de esfuerzo máximo siguiendo la evolución de la temperatura corporal y de la piel así como los niveles de lactato en sangre y la percepción de fatiga para valorar el nivel de rendimiento deportivo de atletas jóvenes y sénior, antes y después de un periodo de suplementación de la dieta con una bebida funcional de almendra y aceite de oliva enriquecida con vitamina E y DHA. Durante la prueba de esfuerzo incrementa de forma significativa la temperatura corporal y la de la piel, los niveles sanguíneos de lactato y la percepción de fatiga mediante el índice de Borg, a la vez desciende el grado de almacenamiento de calor (*heat storge*). Este perfil de cambio y los valores de estos parámetros durante la prueba de esfuerzo máximo inicial no se modifican en la prueba de esfuerzo máximo tras el consumo de la bebida funcional durante ocho semanas. Podemos afirmar que la suplementación de la dieta con una bebida funcional de almendras y aceite de oliva, y enriquecida con DHA, vitamina E no influye en el rendimiento deportivo de atletas jóvenes ni de atletas sénior. Son muchos los estudios que han intentado demostrar efectos ergogénicos del DHA, vitaminas o polifenoles sobre el rendimiento deportivo. Algunos de estos estudios han mostrado pequeñas mejorías en el rendimiento como consecuencia de la suplementación con DHA (Esteve 2002 , Lewis et al. 2015), vitaminas (Nikolaidis et al. 2012, Bentley et al. 2015, Paschalis et al. 2016) y polifenoles (Davis et al. 2010, Richards et al. 2010), aunque la mayoría no han evidenciado efectos que potencien el rendimiento o bien mejoras en la VO_2máx (Guezennec et al. 1989, Oostenbrug et al. 1997, Gomez-Cabrera et al. 2008, Dean et al. 2009, Bigelman et al. 2010, Yfanti et al. 2010, Roberts et al. 2011) . Cabe destacar que la percepción del grado de fatiga es menor en los atletas sénior que en los atletas jóvenes, resultados coincidentes con la bibliografía preexistente (Schlader et al. 2010, Flouris and Schlader 2015).

Compuestos y propiedades antiinflamatorias del sebo de cerdo

Se ha descrito que algunos productos de la oxidación enzimática y no enzimática de los ácidos grasos presentan la capacidad de regular la respuesta inflamatoria (Bochkov and Leitinger 2003). El 1-palmitoil-2-araquidonoil-sn-glicero-3-fosforilcolina, un derivado del AA, tiene potentes efectos anti-inflamatorios pudiendo inhibir la producción de TNF-α en células estimuladas con LPS (Bochkov et al. 2002). El sebo de cerdo, un alimento producto de la cocción prolongada del tejido adiposo blanco del animal, se ha utilizado en la cultura tradicional para tratar golpes y procesos inflamatorios, pero sin saber que componentes pueden ser los

responsables de dichos efectos. Hemos puesto de manifiesto que el tratamiento vía tópica con sebo de cerdo es capaz de disminuir la respuesta inflamatoria provocada por la inyección subplantar de zymosan en ratas Sprague-Dawley. El diámetro de la zona inflamada de la pata de la rata proporciona el nivel de inflamación y se observa, que los animales tratados con el sebo de cerdo en forma de ungüento presentan un menor diámetro de la zona inflamada de la pata. Además muestran una recuperación más rápida que los animales control tratados con un ungüento de vaselina. El proceso de inflamación también se sigue mediante las determinaciones de las actividades de enzimas antioxidantes (CAT y GPx), marcadores de daño oxidativo (MDA y carbonilos), de enzimas inflamatorios (MPO) y de marcadores de inflamación como los niveles de nitrito en homogenado de tejido subplantar a las 4 horas de haber inducido la inflamación. La inyección subplantar de zymosan incrementa significativamente la actividad MPO, los niveles de nitrito y los de carbonilos. El sebo de cerdo a modo de ungüento no modula los efectos del zymosan sobre los carbonilos ni sobre la actividad MPO, sin embargo, reduce la concentración de nitritos retrayendo o evitando el nivel de vasodilatación asociada a la inflamación de la pata. Los niveles de nitrito son un indicador de la producción de óxido nítrico que es un importante mediador de la respuesta inflamatoria (Hierholzer et al. 1998). El hecho de que los niveles de nitrito sean significativamente más bajos en el grupo tratado con sebo de cerdo que en el grupo control pone de manifiesto que el sebo de cerdo es capaz de reducir la respuesta inflamatoria inducida por el zymosan. Los efectos anti-inflamatorios del sebo de cerdo también se evidencian en los neutrófilos purificados de sangre extraída de las ratas inyectadas con zymosan tratadas con ungüento de sebo de cerdo o de vaselina. El zymosan incrementa de forma significativa las actividades MPO y CAT en neutrófilos como parte de la respuesta inflamatoria pero dicho incremento se revierte por la utilización del sebo de cerdo. Para poder confirmar las propiedades antinflamatorias del sebo de cerdo y averiguar los componentes responsables de dichas propiedades se hace un extracto hidroalcohólico del sebo de cerdo y se testan sus efectos en PBMCs y neutrófilos estimulados con LPS, con lo que se simula un proceso inflamatorio. El LPS incrementa significativamente la capacidad de producción de TNF-α, IL-6 e IL-8 y dicho incremento se revierte en su totalidad por la adición del extracto hidroalcohólico al medio de cultivo de PBMCs. Se evidencian así los potentes efectos

anti-inflamatorios del sebo de cerdo y del extracto hidroalcohólico obtenido. Estos resultados coinciden con lo observado en neutrófilos donde la adición del extracto hidroalcohólico del sebo de cerdo elimina el incremento en la producción de IL-6 provocado por el tratamiento con LPS. Adicionalmente, la adición del extracto hidroalcohólico también incrementa la capacidad de producción de PGE_1 por parte de PBMCs activadas con LPS, de la cual se han descrito efectos anti-inflamatorios (Frassdorf et al. 2006).

Con el objetivo de dilucidar el componente o componentes anti-inflamatorios del sebo de cerdo se analiza el perfil de ácidos grasos y derivados por cromatografía de gases/espectrometría de masas en el extracto hidroalcohólico del sebo de cerdo y en el mismo extracto hidroalcohólico del tejido adiposo del cerdo a partir del cual se obtiene en sebo. El perfil de ácidos grasos y derivados del tejido adiposo y del sebo de cerdo es diferente, hecho que es atribuible al proceso de cocción que se lleva a cabo durante la elaboración del sebo. En el sebo de cerdo aparecen nuevos compuestos que no están presentes en el tejido adiposo y que por tanto podrían ser los responsables de su carácter anti-inflamatorio. Aparecen compuestos como la 5-dodecanolida, la oleamida, ácido hexadecanoico, ácido 9-octadecenoico, ácido 6-octadecenoico, ácido 9,12-octadecadienoico, ácido octadecanoico, ácido 5,8,11,14-eicosatetraenoico, ácido 7-hexadecenoico, ácido 11,14-octadecadienoico, hexadecanamida como consecuencia del proceso de elaboración del sebo; además, algunos de los componentes del tejido adiposo incrementan su concentración en el sebo de cerdo como es el caso de la resolvina D_1, compuesto que se sabe que presenta potentes efectos de resolución de la inflamación (Serhan et al. 2002) y del EPA, ácido graso omega 3 con propiedades anti-inflamatorias (Lee et al. 1985, Calder 2006, Davidson et al. 2013). Con este motivo se testan los posibles efectos anti-inflamatorios de la resolvina D_1, la 5-dodecanolida, la oleamida, y del extracto hidroalcohólico en neutrófilos en cultivo estimulados con LPS. Las concentraciones ensayadas fueron equivalentes a las que contiene el extracto hidroalcohólico del sebo de cerdo. Los neutrófilos suelen ser las primeras células movilizadas frente al daño tisular o infección provocando una repuesta inflamatoria, que se caracteriza por la liberación del contenido de sus gránulos al medio externo y en un estallido oxidativo consistente en la producción de grandes cantidades de peróxido de

hidrogeno e hipoclorito con alto consumo de oxígeno (*Oxdative burst*) (Verhoef et al. 1993, Borregaard 2010). El tratamiento de neutrófilos con LPS incrementa de forma significativa las actividades CAT y MPO en el medio de cultivo; tanto el extracto hidroalcohólico como la oleamida y la 5-dodecanolida reducen de forma significativa la actividad CAT en el medio extracelular, aunque cabe destacar que el mayor descenso se observa en el tratamiento con el extracto hidroalcohólico. La actividad MPO en el medio extracelular disminuye de forma significativa con la adición de los tres compuesto puros probados, mostrando los tres unos efectos de la misma magnitud que los observados por el extracto hidroalcohólico. Finalmente, se analizan los efectos de diferentes concentraciones de 5-dodecanolida sobre la capacidad de producción de TNF-α de neutrófilos estimulados con LPS, se observa como el LPS incrementa de forma significativa la producción de TNF-α por parte de los neutrófilos y que dicho incremento se minimiza por la presencia de 5-dodecanolida en el medio de cultivo. Los efectos de inhibitorios sobre la producción de TNF-α de la 5-dodecanolida son dependientes de la dosis observándose una mayor inhibición en las concentraciones más elevadas de 5-dodecanolida.

En resumen, el sebo de cerdo tiene un potente efecto anti-inflamatorio, dicho efecto es atribuible en gran medida a la 5-dodecanolida, aunque los efectos de dicho compuesto por sí solo no alcanzan la magnitud observada por el extracto hidroalcohólico, con lo cual podemos deducir que la presencia en el extracto hidroalcohólico de otros compuestos como, la oleamida, ácido hexadecanoico, ácido 9-octadecenoico, ácido 6-octadecenoico, ácido 9,12-octadecadienoico, ácido octadecanoico, ácido 5,8,11,14-eicosatetraenoico, ácido 7-hexadecenoico, ácido 11,14-octadecadienoico, hexadecanamida y la resolvina D_1, incrementa el potencial anti-inflamatorio de la 5-dodecanolida.

V. CONCLUSIONES

1- El entrenamiento físico regular provoca un cambio en la estrategia de eliminación de especies reactivas de oxígeno en PBMCs, pasándose de un sistema basado en la acción de la catalasa a un sistema basado en la acción de la glutatión peroxidasa disminuyendo los marcadores de peroxidación lipídica. El ejercicio regular incrementa los niveles de UCP-2 y UCP-3 potenciando las defensas contra la producción de las especies reactivas de oxigeno mitocondriales.

2- Un ejercicio físico intenso y prolongado incrementa el nivel de estrés oxidativo, hecho que provoca una adaptación de los mecanismos antioxidantes reduciendo el daño oxidativo en PBMCs, aunque estas adaptaciones no son suficientes para evitarlo en su totalidad. La suplementación de la dieta con DHA durante ocho semanas incrementa las capacidades antioxidantes y reduce la producción de ROS en PBMCs activadas.

3- La suplementación de la dieta con una bebida funcional de almendra y aceite de oliva enriquecida con DHA y vitamina E durante un mes reduce los niveles de peroxidación lipídica en plasma después de la realización de un ejercicio extenuante. El ejercicio físico extenuante y la suplementación con la bebida funcional potencia la expresión de enzimas antioxidantes en PBMCs aunque únicamente en atletas jóvenes.

4- La práctica de ejercicio físico de forma regular durante 8 semanas no modifica los niveles plasmáticos basales de citoquinas en futbolistas bien entrenados. La realización de un ejercicio físico intenso y prolongado incrementa de forma significativa los niveles plasmáticos de IL-6 y mantiene inalterados los niveles de otras citoquinas pro-inflamatorias y anti-inflamatorias y de factores de crecimiento. Los niveles de citoquinas plasmáticas no se ven afectados como consecuencia de la suplementación de la dieta enriquecida con DHA.

5- La práctica de ejercicio físico de forma regular produce efectos anti-inflamatorios a nivel sistémico probablemente inducidos por el incremento de los niveles de basales de PGE1 y por la acción sinérgica del ejercicio físico y de la suplementación

de la dieta enriquecida con DHA que producen un incremento de los niveles plasmáticos de PGE_2.

6- El ejercicio físico extenuante incrementa la activación de NFκB en PBMCs y los niveles plasmáticos de sICAM3 y sL-Selectina, marcadores de una mayor activación de las células inmunitarias mononucleares y de los neutrófilos. La suplementación de la dieta durante un mes con la bebida funcional de almendra y aceite de oliva enriquecida con vitamina E y DHA atenúa el grado de activación de las células inmunes; sin embargo provoca un cierto grado pro-inflamatorio en respuesta a un ejercicio físico extenuante al incrementar los niveles plasmáticos de TNF-α y la expresión de esta en PBMCs, más evidente en atletas sénior.

7- El ejercicio físico intenso y prolongado y la temperatura incrementan la capacidad de producción de citoquinas proinflamatorias en PBMCs estimuladas *in vitro* con LPS. La suplementación de la dieta enriquecida con DHA atenúa la producción de citoquinas en respuesta al LPS únicamente en el caso del ejercicio, sugiriendo que el incremento en la capacidad de producción de citoquinas inducido por el aumento de temperatura es resultado de una vía de activación distinta a la que provoca el ejercicio.

8- El ejercicio físico intenso y prolongado potencia la capacidad de producción de PGE_1, PGE_2 y RvD_1 de las PBMCs activadas *in vitro* con LPS, creando así un ambiente anti-inflamatorio post-ejercicio.

9- La suplementación de la dieta con DHA potencia las capacidades antioxidantes de los neutrófilos y atenúa la respuesta inflamatoria después de la estimulación *in vitro* con PMA. La combinación de vitamina C con la suplementación dietética con DHA evita la expresión de genes pro-inflamatorios en neutrófilos estimulados *in vitro* con PMA.

10- La suplementación de la dieta con una bebida funcional de almendra y aceite de oliva enriquecida con DHA y vitamina E durante un mes no incrementa el rendimiento deportivo en atletas bien entrenados.

11- El sebo de cerdo vía tópica reduce la inflamación inducida mediante inyección plantar de zymosan en ratas. Un extracto hidroalcohólico del sebo reduce la producción de citoquinas proinflamatorias de las PBMCs estimuladas con LPS. Dichos efectos anti-inflamatorios son atribuidos a la 5-dodecanolida; sin embargo, la oleamida, resolvina $D_{1,}$ ácido hexadecanoico, ácido 9-octadecenoico, ácido 6-octadecenoico, ácido 9,12-octadecadienoico, ácido octadecanoico, ácido 5,8,11,14-eicosatetraenoico, ácido 7-hexadecenoico, ácido 11,14-octadecadienoico, hexadecanamida, todos ellos presentes en extracto hidroalcohólico del sebo, forman una mezcla que potencia los efectos anti-inflamatorios de la 5-dodecanolida.

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