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TESTING OF BIOMOLECULES AND NOVEL SURFACES FOR PERIODONTAL AND PERI-IMPLANT REGENERATION

Manuel Gómez Florit

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Dr. Marta Monjo Cabrer and Dr. Joana Maria Ramis Morey, of the Universitat de les Illes Balears.

WE DECLARE:

That the thesis titled TESTING OF BIOMOLECULES AND NOVEL SURFACES FOR PERIODONTAL AND PERI-IMPLANT REGENERATION, presented by Manuel Gómez Florit to obtain a doctoral degree, has been completed under our supervision and meets the requirements to opt for an European Doctorate.

For all intents and purposes, we hereby sign this document.

Signatures

Marta Monjo

Joana M. Ramis

Palma de Mallorca, 15th of May 2015

It always seems impossible until it's done

Nelson Mandela

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Manu Gómez

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Abbreviations

ACTA2	Alpha-smooth muscle actin
ALP	Alkaline phosphatase
AP-1	Activating protein-1
APTES	(3-Aminopropyl)triethoxysilane
ATP	Adenosine triphosphate
bFGF	Basic fibroblast growth factor
cDNA	Complementary DNA
CCN2	Connective tissue growth factor
COL12A1	Collagen type XII alpha 1
COL1A1	Collagen type I alpha 1
COL3A1	Collagen type III alpha 1
COL4A2	Collagen type IV alpha 2
COL5A1	Collagen type V alpha 1
COX2	Cyclooxigenase-2
Cp	Crossing point
DCFH-DA	2',7'-dichlorodihydrofluorescein diacetate
DCN	Decorin
ECM	Extracellular matrix
EDN1	Endothelin-1
ELISA	Enzyme-linked immunosorbent assay
EMD	Emdogain
FN1	Fibronectin-1
hGF	Human gingival fibroblasts
hMSCs	Human mesenchymal stem cells
IL-10	Interleukin-10
IL-1β	Interleukin-1 beta
IL-6	Interleukin-6
IL-8	Interleukin-8
ITGA2	Integrin alpha 2
ITGA8	Integrin alpha 8
ITGB3	Integrin beta 3
LDH	Lactate dehydrogenase
LPS	Bacterial lipopolysaccharide
MMP1	Matrix metalloproteinase-1
MMPs	Matrix metalloproteinases
modMA	Machined + acid etched surfaces
mRNA	Messenger RNA
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
OPG	Osteoprotegerin
PDGF	Platelet-derived growth factor
PGE2	Prostaglandin E2
PMNLs	Polymorphonuclear leukocytes

RANK	Receptor activator of NF-κB
RANKL	Receptor activator of NF-κB ligand
RGD	Arginylglycylaspartic acid
ROS	Reactive oxygen species
RT-PCR	Reverse transcription-polymerase chain reaction
S. epidermidis	*Staphylococcus epidermidis*
S. mutans	*Streptococcus mutans*
Sa	Surface roughness
SEM	Scanning electron microscopy
SPARC	Osteonectin
TGFB1	Transforming growth factor-beta 1
Ti	Titanium
TIMP1	Tissue inhibitors of matrix metalloproteinase-1
TIMPs	Tissue inhibitors of matrix metalloproteinases
TNF-α	Tumor necrosis factor-alpha
VCAN	Versican
VIM	Vimentin
Zr	Zirconium

Abstract

Periodontal and peri-implant diseases are related to an infection and inflammatory process around teeth or dental implants, respectively. The progressive loss of supporting tissues and the final loss of teeth or dental implants are the hallmarks of these pathological conditions. Unfortunately, these processes are frequent among populations worldwide and current therapeutic strategies have not achieved enough satisfactory results. Since inflammation and oxidative stress lie in the middle of these diseases, in this thesis five flavonoids (chrysin, diosmetin, galangin, quercitrin and taxifolin) and two pineal methoxyindoles (melatonin and 5-methoxytryptophol) with antioxidant and anti-inflammatory potential were screened *in vitro* with the aim to find new biomolecules to protect and regenerate the integrity of periodontal tissues.

The results obtained point to quercitrin, among the different biomolecules studied, as the most promising biomolecule to promote soft and hard tissue regeneration. Quercitrin showed anti-inflammatory effects, increased the expression of extracellular matrix related-markers and decreased soft tissue destruction in basal and inflammatory conditions in human gingival fibroblasts. Moreover, quercitrin increased the differentiation of human mesenchymal stem cells to bone-forming cells in basal, osteogenic and inflammatory conditions.

In parallel, the same principles apply to peri-implant healing, i.e. the intended regenerative process may favour soft and hard tissues integration, which guarantees dental implant success. It is known that physical and chemical properties of dental implant surfaces influence soft and hard tissue integration with the dental implant. In this thesis, different dental implant surfaces for soft tissue integration were tested. First, the response of human gingival fibroblasts to titanium (Ti) and titanium-zirconium (TiZr) surfaces with three different surface topographies (polished, microgrooved and rough) was compared. Ti and TiZr were biocompatible, i.e. allowed human gingival fibroblasts growth. Regarding the surface topography, microgrooved surfaces induced a less inflammatory and more regenerative cellular gene expression profile than polished and rough ones and guided cell alignment on the microgrooves of their surfaces. Then, quercitrin was used to biofunctionalise Ti surfaces and the effects on *Streptococcus mutans* and human gingival fibroblasts were analysed. Quercitrin-functionalised surfaces decreased bacterial adhesion while increased human gingival fibroblasts adhesion. Furthermore, these surfaces showed the same beneficial effects than quercitrin in solution on human gingival fibroblasts, i.e. anti-inflammatory effects, prevention of extracellular matrix destruction and promotion of tissue regeneration.

All in all, the results derived from this thesis suggest quercitrin as a potential treatment for periodontal and peri-implant diseases. Moreover, this molecule can be used to functionalise dental implants to improve both hard and soft tissue integration, increasing dental implant success.

Resumen

Las enfermedades periodontales y periimplantarias son condiciones patológicas relacionadas con un proceso de infección e inflamación alrededor del diente o de un implante dental, respectivamente. La pérdida progresiva de los tejidos que los rodean y la pérdida final de las piezas son las características principales de estas patologías. Desafortunadamente, estos procesos son frecuentes en la población y las opciones terapéuticas disponibles actualmente no han alcanzado resultados suficientemente satisfactorios. Debido a que la inflamación y el estrés oxidativo se encuentran entre los principales causantes de estas enfermedades, en esta tesis se han evaluado cinco flavonoides (crisina, diosmetina, galangina, quercitrina y taxifolina) y dos metoxiindoles pineales (melatonina y 5-methoxytryptophol) con potencial actividad antioxidante y antiinflamatoria mediante estudios *in vitro* con el objetivo de encontrar nuevas biomoléculas para proteger y regenerar la integridad de los tejidos periodontales.

Los resultados obtenidos apuntan a la quercitrina, entre las diferentes biomoléculas analizadas, como la biomolécula con más potencial para suscitar la regeneración de los tejidos periodontales. La quercitrina mostró efectos antiinflamatorios, incrementó la expresión de los marcadores relacionados con la matriz extracelular (ECM) y disminuyó la destrucción del tejido blando gingival en condiciones basales e inflamatorias, usando como modelo fibroblastos gingivales humanos (hGF). Además, la quercitrina incrementó la diferenciación de células madre humanas a células formadoras de hueso en condiciones basales, osteogénicas e inflamatorias.

En paralelo, los mismos principios se pueden aplicar al proceso de cicatrización de los tejidos periodontales alrededor de un implante dental después de una cirugía implantaria. El proceso regenerativo que se pretende conseguir podría favorecer la integración de los tejidos periodontales alrededor del implante dental, lo que garantizaría su supervivencia. Las propiedades fisicoquímicas de la superficie de los implantes influencian la integración de los tejidos periodontales al implante. En esta tesis se evaluaron diferentes superficies seleccionadas para incrementar la integración del tejido periodontal blando. Primero, se comparó la respuesta de los hGF a superficies de titanio (Ti) y titanio-zirconio (TiZr) con diferentes topografías (pulida, micro-estriada y rugosa). Los dos materiales fueron biocompatibles ya que permitieron el crecimiento normal de los hGF. En cuanto a la topografía, las superficies micro-estriadas produjeron un alineamiento de los hGF con las micro-estrías y produjeron una respuesta menos inflamatoria y más regenerativa que las superficies pulidas y rugosas, según el perfil de expresión de los marcadores génicos estudiados. A continuación, la quercitrina se usó para biofuncionalizar superficies de Ti y se analizaron los efectos de estas superficies en *Streptococcus mutans* y en hGF. Las superficies funcionalizadas con quercitrina disminuyeron la adhesión bacteriana e incrementaron la adhesión de hGF. Asimismo, estas superficies produjeron los mismos efectos beneficiosos que la quercitrina en solución en los hGF, es decir, efectos antiinflamatorios, prevención de la destrucción de la ECM y promoción de la regeneración tisular.

En conclusión, los resultados derivados de esta tesis sugieren a la quercitrina como tratamiento potencial para la enfermedad periodontal y periimplantitis. Además, la quercitrina podría usarse para funcionalizar implantes dentales y con ello mejorar su integración con los tejidos periodontales, mejorando la tasa de supervivencia de los implantes.

List of publications

This thesis is based on the following papers:

Paper I **Gómez-Florit, M.**, Ramis, J. M., & Monjo, M. Anti-fibrotic and anti-inflammatory properties of melatonin on human gingival fibroblasts in vitro. *Biochemical Pharmacology* (2013), 86(12), 1784–90. doi:10.1016/j.bcp.2013.10.009.

Impact Factor (2013) = 4.650 (Q1).

Paper II **Gomez-Florit, M.**, Monjo, M., & Ramis, J. M. Identification of Quercitrin as Potential Therapeutic Agent for Periodontal Applications. *Journal of Periodontology* (2014), 85(7), 966-974. doi: 10.1902/jop.2014.130438.

Impact Factor (2013) = 2.565 (Q1).

Paper III **Gomez-Florit, M.**, Monjo, M., & Ramis, J. M. Quercitrin for periodontal regeneration: effects on human gingival fibroblasts and human mesenchymal stem cells. *Manuscript* (2015).

Paper IV **Gomez-Florit, M.**, Ramis, J. M., Xing, R., Taxt-Lamolle, S., Haugen, H. J., Lyngstadaas, S. P., & Monjo, M. Differential response of human gingival fibroblasts to titanium- and titanium-zirconium-modified surfaces. *Journal of Periodontal Research* (2013), 49(4):425-436. doi:10.1111/jre.12121.

Impact Factor (2013) = 2.215 (Q1).

Paper V **Gomez-Florit, M.**, Pacha-Olivenza, M. A., Fernández-Calderón, M-C., Córdoba, A., González-Martín, M. L., Monjo, M., & Ramis J. M. Flavonoid-functionalised titanium surfaces favour gingival cells in the race for the surface against oral bacteria. *Manuscript* (2015).

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1 INTRODUCTION

1.1 Background of the study

Oral health is essential to general health and quality of life for individuals and the population as whole. In fact, millions of dental – oral – craniofacial procedures, ranging from tooth restorations to major reconstruction of hard and soft tissues are performed annually (Taba et al., 2005).

A key component of oral health is periodontal health. It includes tissues around natural teeth and around their biomaterial equivalents, dental implants, used to replace missing teeth (Tonetti et al., 2015a). Periodontal and peri-implant diseases are the result of an infection and inflammation characterised by the progressive loss of supporting tissues, and the loss of teeth or the dental implant are the final stage of these pathological conditions (Sculean et al., 2014).

Current strategies for periodontal and peri-implant diseases treatment are generally successful in eliminating active disease and some of them have achieved a certain degree of regeneration although the outcomes of such modalities are not always predictable. These strategies are based on the use of antibiotics, anti-inflammatory drugs and growth factors. However, antibiotics can induce the development of bacterial antimicrobial resistance and possible allergic reactions while anti-inflammatory drugs only achieved a decrease in the inflammatory response. On the other hand, growth factors are unstable and have short-lives. The overall goal is to develop less invasive, fast, safe and predictable therapy that re-establishes a healthy periodontal situation to maintain the teeth/implant (Han et al., 2013; Li and Jin, 2015; Ramseier et al., 2012; Shin et al., 2015).

Nature is often a source of inspiration for finding new bioactive molecules. Natural-derived products with multi-target actions, which promise higher therapeutic efficacy and safety (Koeberle and Werz, 2014), represent an alternative to pharmaceuticals and animal-derived compounds due to their low immunogenicity and toxicity (Friedman, 2007).

1.2 Periodontal tissues

Periodontal tissues are a functional unit of tissues that surround the teeth (periodontium). They are composed of hard (cementum and alveolar bone) and soft tissue (periodontal ligament and gingiva), which maintain teeth function and provide a protective barrier against bacterial infection (**Figure 1**) (Bartold et al., 2000; Hassell, 1993).

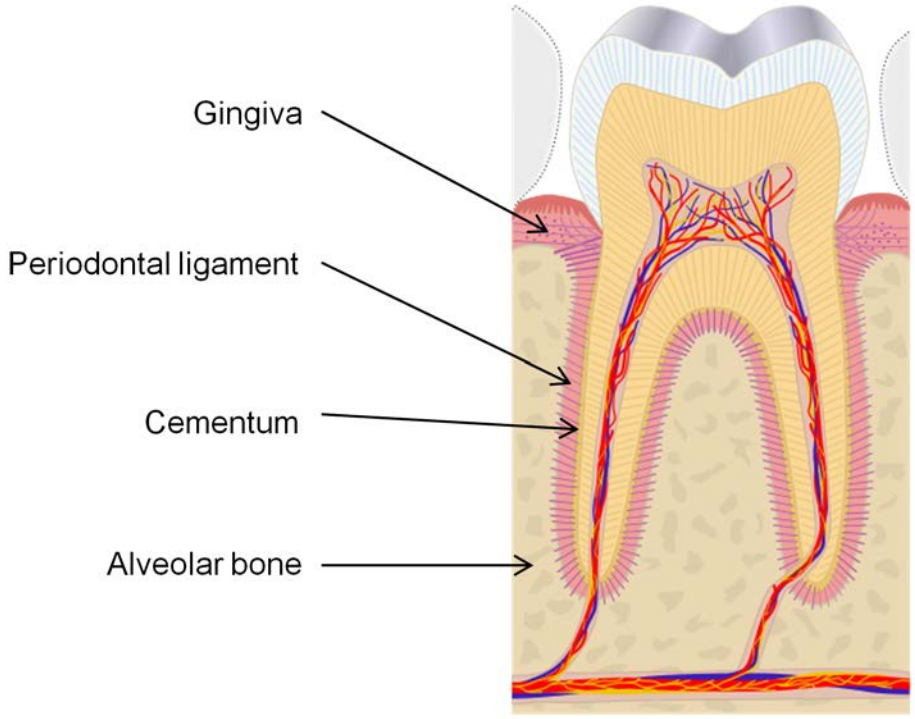

Figure 1. Graphic representation of the periodontium in cross section. Adapted from (Tek-en, 2015).

1.2.1 Cementum

The cementum is a thin calcified tissue that covers the surface of tooth roots in mammals. Periodontal ligament fibres are embedded into it, providing attachment between the periodontal ligament and the tooth. It also prevents root resorption during remodelling of the periodontium. It is less mineralised than other mineral tissues and it does not normally undergo remodelling. The cells responsible for its formation are cementoblasts, which are derived from fibroblast-like cells in the periodontal ligament (Hassell, 1993; Hughes, 2015).

1.2.2 Alveolar bone

The alveolar bone is a part of the mandibular and maxillary bone that forms the bony sockets to support teeth roots. It is characterised by a continuous and rapid tooth-dependent turnover, allowing tooth eruption and movement. However, this characteristic may be detrimental to the progression of periodontal disease (see page 4). The bone matrix is formed from a collagen scaffold filled with hydroxyapatite crystals. The cells present in alveolar bone are osteoblasts, osteocytes and osteoclasts. Osteoblasts are cells of mesenchymal origin, which, when fully differentiated, are primarily responsible for the production of bone matrix. After maturation, osteoblasts stop synthesizing matrix and become encased in it as osteocytes or remain on the bone surface as bone-lining cells. Osteoclasts are responsible for bone resorption through the secretion of acids and enzymes that dissolve bone matrix (Sodek and McKee, 2000).

1.2.3 Periodontal ligament

The periodontal ligament is a dense fibrous tissue that attaches teeth to alveolar bone and dissipates loads applied to teeth during function. Type I collagen forms the principal fibres of periodontal ligament that are inserted into both the alveolar bone and cementum. Periodontal ligament fibroblasts are the most abundant cells together with osteoblasts and cementoblasts, which are present adjacent to alveolar bone and cementum, respectively. Furthermore, periodontal ligament fibroblasts monitor mechanical stress and maintain periodontal ligament homeostasis (Hughes, 2015; McKnight et al., 2014).

1.2.4 Gingiva

Healthy gingiva normally covers the alveolar bone and tooth root to the cement-enamel junction, providing a tissue sealing around the cervical portion of teeth and covering the alveolar bone. It is composed of an overlying epithelial structure and an underlying connective tissue. The epithelium is predominantly cellular in nature while the connective tissue is less cellular and largely a fibrous network (Bartold et al., 2000).

1.2.4.1 Gingival connective tissue

Gingival connective tissue accounts for the major proportion of the gingiva. It is mainly composed of a collagenous (60-65%) extracellular matrix (ECM). Type I (65%) and type III (30%) collagens are the main collagen species although type IV, V and VI collagens are also present in gingival connective tissue. They are organised in different groups of fibre bundles arranged to bind the gingiva to the underlying tooth and bone. Proteoglycans are also ubiquitous constituents of gingival connective tissue. They act as matrix organizers and tissue space fillers although specific proteoglycans such as decorin and fibronectin interact with collagen fibres. The interaction of cells with their surrounding matrix is usually mediated by integrins. They are heterodimeric proteins composed of α- and β-subunits, which bind cells principally to matrix proteins such as fibronectin and collagen. Each subunit has an extracellular, a transmembrane, and a cytoplasmatic domain that attaches to the cytoskeleton (Bartold et al., 2000; Hassell, 1993; Hughes, 2015).

1.2.4.2 Gingival fibroblasts

Several cell types have been identified within gingival connective tissue, among these, fibroblasts account for most connective tissue cells and are responsible for the constant adaptation of the tissue. Fibroblasts are mesenchymal cells with many vital functions during development and in adult organisms. Although fibroblasts are among the most accessible mammalian cells to culture *in vitro*, they are poorly defined in molecular terms. In practice, fibroblasts are usually identified by their spindle-shaped morphology, ability to adhere to plastic culture surfaces, positive for vimentin immunostaining and the absence of markers for other cell lineages (Chang et al., 2002).

Fibroblasts are responsible for producing and maintaining the ECM components. Furthermore, fibroblasts are also involved in tissue homeostasis regulation, inflammation and wound healing repair and regeneration (**Figure 2**) (Bartold et al., 2000; Hassell, 1993). ECM undergoes a constant turnover of its components, in both

normal and diseased conditions, mainly through matrix metalloproteinases (MMPs) mediated collagen degradation (Bartold and Narayanan, 2006). The activity of MMPs is controlled by tissue inhibitors of matrix metalloproteinases (TIMPs) (Bartold and Narayanan, 2006; Sakagami et al., 2006).

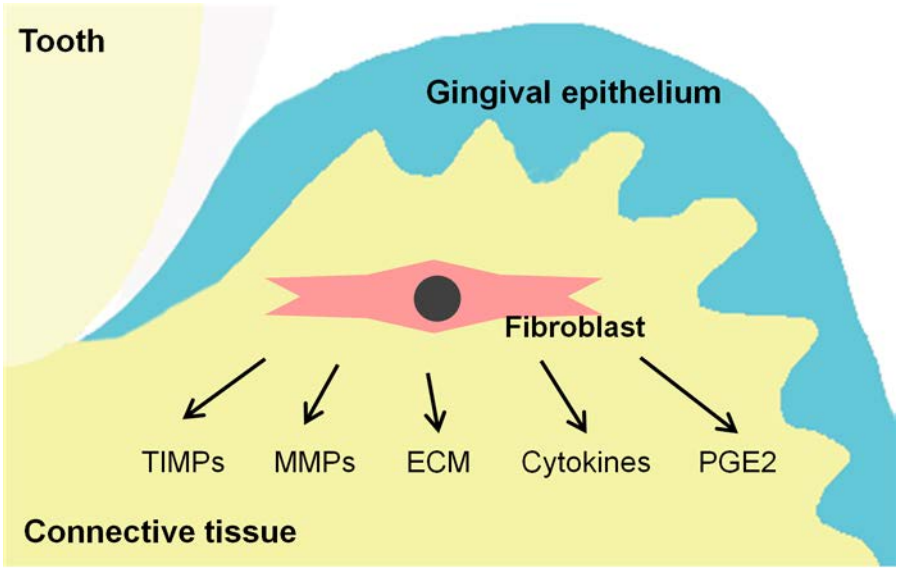

Figure 2. Central role of fibroblasts in gingival connective tissue. Adapted from (Bartold et al., 2000).

During wound healing, fibroblasts migrate into the wound site, where they synthesize and remodel a new ECM (Gurtner et al., 2008). The differentiation of fibroblasts into myofibroblasts, which express highly contractile proteins including α-smooth muscle actin, is necessary to close the wound (Hinz et al., 2007). These processes can produce either a scarred (repaired) tissue or a regenerated tissue, depending on the local signals. Scarring, which has been associated with an excessive inflammatory response and myofibroblast differentiation, results in an architecturally and functionally non-restored tissue. Contrary, a regeneration process, associated with a decreased inflammatory response and myofibroblast differentiation, produces a new tissue architecturally and functionally identical to the original tissue (Satish and Kathju, 2010).

1.3 Periodontal disease

Periodontal disease is the result of a complex interaction between the microbial challenge induced by dental plaque biofilm and the host inflammatory response around periodontal tissues. The conditions range from gingivitis, an inflammation of the gingiva in which the soft tissue attachment to the tooth remains at its original level, to periodontitis, an inflammation of the supporting tissues of the teeth with progressive attachment loss and bone destruction (**Figure 3**). Gingivitis and periodontitis are best viewed as a continuum of a chronic inflammatory disease (Kinane and Attström, 2005; Tonetti et al., 2015b). Gingivitis may persist for many years and with good oral hygiene is completely reversible. If left untreated, it may lead to periodontitis, the non-reversible

destructive stage that affects hard tissues and ends in tooth loss. Periodontal disease is characterized by strong release of inflammatory mediators and reactive oxygen species (ROS) in response to oral pathogens, which might end in tissue destruction, whereas periodontal health is characterized by the opposite (Dentino et al., 2013).

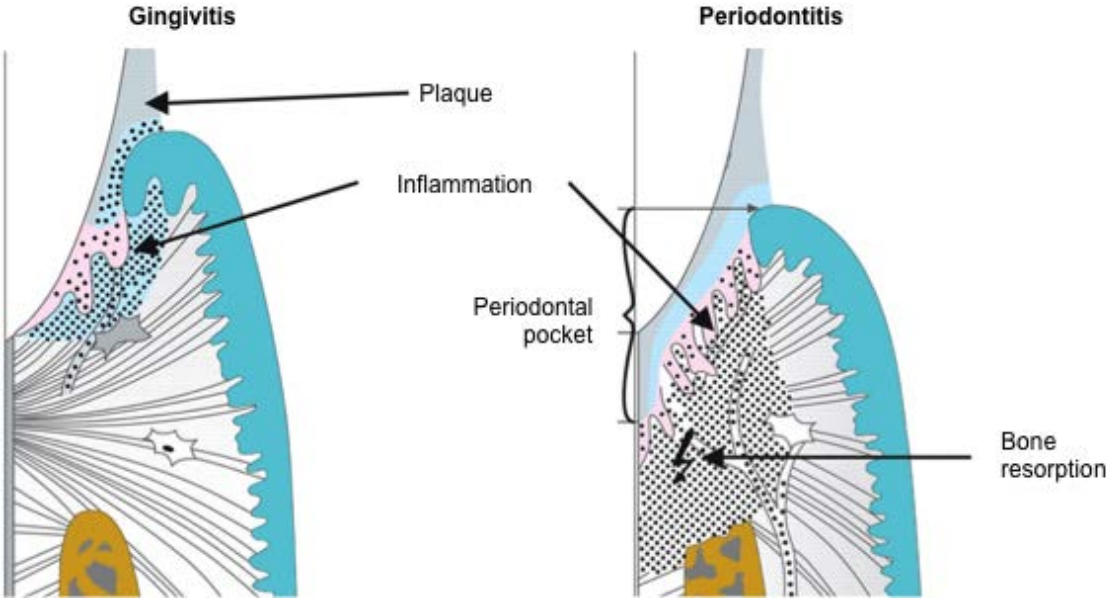

Figure 3. Periodontal disease. While gingivitis affects the marginal soft tissue (gingiva), periodontitis affects deeper periodontal tissues (soft tissue attachment and bone). Adapted from (Sanz et al., 2010).

Both gingivitis and periodontitis are very common in populations worldwide. The reported worldwide prevalence among adults is 50-90% for gingivitis and 5-20% for severe forms of periodontitis (Dye, 2012). In general, the rate of progression is slow but there are a number of risk factors associated with periodontal disease progression, including genetic, lifestyle-related (e.g. smoking, alcohol and stress), and other diseases (e.g. diabetes mellitus, obesity, metabolic syndrome, osteoporosis, and low dietary calcium and vitamin D) (Genco and Borgnakke, 2013). In fact, severe periodontitis is the sixth most prevalent human disease, according to the 2010 global burden of diseases study, with a standardized prevalence of 11.2% (Kassebaum et al., 2014).

1.3.1 Pathogenesis of periodontal disease

Periodontal disease can be outlined in three stages: (1) accumulation of bacterial biofilm in the gingival sulcus (colonization) and penetration of epithelium and connective tissue (invasion), (2) stimulation of a host response that involves activation of the innate and acquired immunity (inflammation), and (3) destruction of connective tissue attachment to the tooth surface (gingivitis) and bone that is irreversible (periodontitis) (Graves et al., 2011).

Bacterial adhesion and colonization of the teeth surface results in biofilm formation. Highly potent first-line antimicrobial defence systems can sense and destroy invaders and attract immune cells. However, some bacteria invade tissues, which trigger the

inflammatory response. Although bacteria are necessary for disease initiation, they are not sufficient to cause disease progression unless there is an associated inflammatory response within a susceptible host (Cekici et al., 2014). The host releases several proteolytic enzymes, a cascade of inflammatory mediators and ROS in the gingiva (Cekici et al., 2014). More specifically, a loss of homeostatic balance between proteolytic enzymes and their inhibitors, and ROS and the antioxidant defence systems that protect and repair tissues is believed to be responsible (Chapple and Matthews, 2007). The host response against bacterial plaque is considered as a "two-edged sword" (Preshaw, 2008). That is, the response is protective in nature to control bacterial infection. However, in individuals prone to periodontitis the inflammatory process results in excessive production and activation of proinflammatory mediators and destructive enzymes, causing soft and hard tissue damage (Kirkwood et al., 2007).

1.3.1.1 Colonization and invasion

Unlike many infectious diseases, periodontal disease appears to be mediated by the overgrowth of commensal organisms, rather than by the acquisition of an exogenous pathogen. A large proportion of the initial colonizers are streptococci although there are about 700 bacterial species in the oral cavity (Paster et al., 2006). Plaque accumulation leads to gingivitis, but host factors together with the overgrowth of Gram-negative species and anaerobic bacteria has been related to the shift to periodontitis due to the ability of these organisms to penetrate the gingival epithelium and to release endotoxins, cytotoxic enzymes and toxic molecules (Sbordone and Bortolaia, 2003).

Biofilm formation on teeth surfaces, also called dental plaque, is typically divided in four steps (**Figure 4**): (1) **primary attachment** is the reversible cell-to-surface attachment of the primary colonizers after formation of the conditioning film on the tooth enamel; (2) in **secondary attachment**, the initial colonizers irreversibly attach to the tooth and cell-to-cell attachment with mid- and late-colonizers occurs; (3) during biofilm **maturation** bacteria proliferate and produce ECM; (4) the final step is the detachment of bacteria from the biofilm and their **dispersion**, which contribute to their expansion (Crawford et al., 2012; Davey and O'toole, 2000).

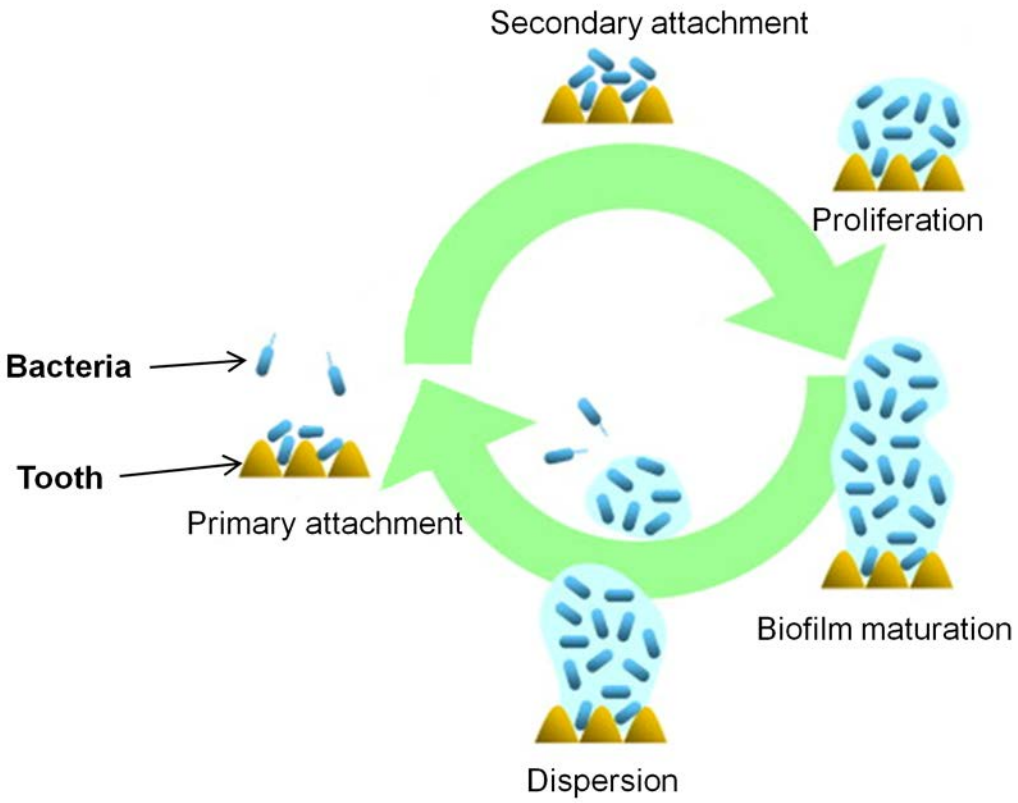

Figure 4. Biofilm formation and its life cycle. Adapted from (Crawford et al., 2012).

1.3.1.2 Inflammation

The bacterial assault triggers the activation of the innate immunity by the binding of various bacterial components to toll-like receptors and the activation of both activating protein-1 (AP-1) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathways, which promote the release of inflammatory mediators (Mahanonda and Pichyangkul, 2007) (**Figure 5**). Mast cells, within the connective tissue, release vasoactive amines, which increase vascular permeability, together with **tumor necrosis factor-alpha** (TNF-α) and **interleukin-8** (IL-8) that activate the inflammatory response. Polymorphonuclear leukocytes (PMNLs) migrate to the site of injury where they release antimicrobial factors, including defensins and lysosomal enzymes. PMNLs also generate an **oxidative burst** to facilitate phagocytosis and killing of bacteria although ROS do not discriminate between host and bacteria and contribute to tissue degradation (Chapple and Matthews, 2007; Yucel-Lindberg and Båge, 2013). Resident fibroblasts within the gingival connective tissue are also stimulated to secrete **MMPs** and inflammatory mediators. **Interleukin-1 beta** (IL-1β), **interleukin-6** (IL-6), TNF-α and prostaglandins (e.g. **prostaglandin E2** (PGE2)) are key elements of the inflammatory response. In response to these signals, monocytes mature into macrophages, which produce more proinflammatory mediators (Cekici et al., 2014). At this point, 60–70% of the collagen in the gingival connective tissue is degraded, but the bone is still intact (Page and Schroeder, 1976; Yucel-Lindberg and Båge, 2013).

At the same time, the elevated levels of proinflammatory mediators are counter-balanced by a protective response in the host with increased levels of anti-inflammatory cytokines, such as **interleukin-10** (IL-10), and **TIMPs**. However, if inflammation prevails, cells of the adaptive immune system appear. Antigen presenting cells process bacterial antigens to present them to naïve CD4 T-cells. Naïve T-cells differentiate into various T-cell subsets that produce proinflammatory or immunosuppressive cytokines, to increase or inhibit the production of inflammatory mediators (Graves, 2008; Yucel-Lindberg and Båge, 2013). If the inflammation continues, osteoclasts are derived from macrophages, which degrade alveolar bone. (Graves et al., 2011; Hernández et al., 2011).

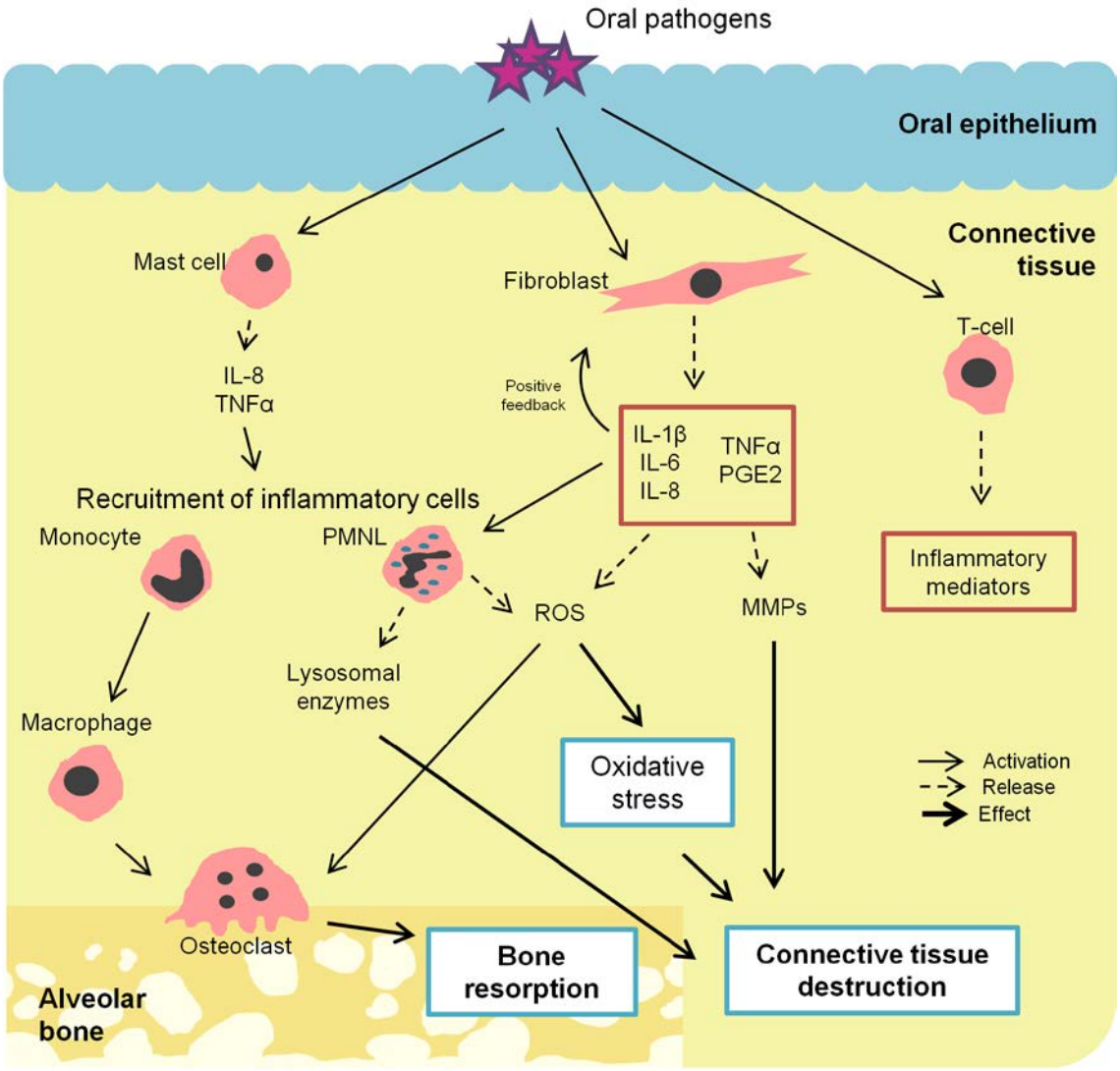

Figure 5. Outline of the pathogenesis of periodontal disease.

1.3.1.3 Tissue loss: mechanisms of tissue damage

In periodontal disease, bacterial virulence factors are able to cause direct tissue damage, although the major mechanisms of soft and hard tissue damage are host-derived.

MMPs/TIMPs ratio

Gingival connective tissue undergoes through a constant turnover of its components, in both normal and diseased conditions, mainly mediated through MMP-mediated collagen degradation (Bartold and Narayanan, 2006). The activity of MMPs is controlled at a number of levels: at gene transcriptional level; at post-transcriptional level (through proteolytic activation); and at inhibitory level (through TIMPs inhibition). During inflammation, proinflammatory cytokines such as IL-1β and TNF-α induce MMP production and activation while TIMPs are downregulated. This produces an imbalance of MMPs over their inhibitors, TIMPs, which triggers ECM degradation (60-70% collagen is degraded) (Birkedal-Hansen, 1993; Soell et al., 2002). In addition, the proinflammatory cytokine network induces fibroblasts to attempt tissue repair that ends in tissue scarring instead of a structural and functional regenerative response (Bartold and Narayanan, 2006).

ROS

Another mechanism of tissue damage is the imbalance between oxidants and antioxidants in favour of the oxidants, termed "oxidative stress" (Sies, 1997). Oxidative stress has been implicated in a number of human diseases, including periodontal disease (Chapple and Matthews, 2007). Damage occurs either as a direct result of excess ROS activity/antioxidant deficiency or indirectly as a result of the activation of redox-sensitive transcription factors and the creation of a proinflammatory state (Chapple and Matthews, 2007). The mechanisms by which ROS induce tissue damage include oxidation of cellular (lipids, carbohydrates, proteins and DNA) and extracellular targets (extracellular matrix components, collagens and structural proteins) (Chapple and Matthews, 2007; Waddington et al., 2000) in addition of being proinflammatory mediators (Naik and Dixit, 2011). Furthermore, the imbalance of MMPs over TIMPs could be the result of direct ROS damage to TIMPs or ROS-induced alterations in MMPs and TIMPs expression (Chapple and Matthews, 2007).

RANKL/OPG ratio

Bone formation and resorption occur continuously in healthy alveolar bone. The receptor activator of NF-κB ligand (RANKL) binds to the receptor activator of NF-κB (RANK) that is expressed by pre-osteoclasts, inducing the formation of multinucleated osteoclasts. In addition, the decoy receptor osteoprotegerin (OPG) acts as a competitive inhibitor of RANKL. Bone resorption is the result of an imbalance between RANKL and OPG. In periodontal disease, RANKL expression is markedly elevated in osteoblasts, T-cells and to a lesser extend in gingival fibroblasts, in response to proinflammatory mediators, while low levels of OPG have been reported (Kajiya et al., 2010; Mogi et al., 2004). Thus, a high ratio of RANKL/OPG creates proresorptive conditions while a low ratio is antiresorptive (Graves et al., 2011). In addition, certain ROS activate osteoclasts and promote osteoclastogenesis, contributing to bone resorption (Garrett et al., 1990).

1.4 Current strategies for periodontal regeneration

Regeneration refers to the restoration of a lost or injured part, in contrast to repair, which describes healing of a wound by tissue that does not fully restore the architecture or the function of the part (Wang et al., 2005). The ultimate goal of periodontal therapy is the regeneration of both hard and soft tissues destroyed by the disease. Periodontal regeneration requires the restoration of alveolar bone height, the formation of cementum attached to root surfaces and periodontal ligament fibres and the re-establishment of gingival tissues.

Current conventional therapy in periodontal disease is based on the **control of bacterial infection** through mechanical dental cleaning, subgingival scaling and root planning (Dentino et al., 2013). Furthermore, **local and systemic antimicrobials** are used as adjuncts to the mechanical treatment (Preshaw, 2008). The use of local antibiotics and antiseptics has shown some benefits over the manual debridement alone although the effects were modest and mostly temporary. On the other hand, the use of systemic antibiotics, despite of the benefits to the clinical progression of periodontal disease, can induce the development of bacterial antimicrobial resistance and possible allergic reactions (Heitz-Mayfield and Lang, 2013). Furthermore, in severe cases, **periodontal surgery** is required to provide access for the debridement of residual dental plaque, reduce the depth of periodontal pockets, and stimulate the regeneration of lost tissues by grafting with biomaterials (Heitz-Mayfield and Lang, 2013). These strategies alone are insufficient since periodontal disease is the result of destructive inflammation; thus, if successful, treatment frequently results in a process of gingival fibrosis and limited bone remodelling, rather than in true regeneration of the periodontal tissues (Han et al., 2013; Hughes, 2015).

1.4.1 Host-modulation therapy

Research in periodontal disease treatment has targeted the host component of the disease (Kirkwood et al., 2007; Preshaw, 2008; Salvi and Lang, 2005) since the concept of host-modulation therapy was introduced in the early 1990s (L. M. Golub et al., 1992). It aims at damping down the host response to either decrease the excess production proinflammatory mediators and destructive enzymes in order to reduce damage to the periodontal tissues, or to stimulate the regenerative process, allowing for the restoration of soft tissue attachment and bone formation. To date, MMP inhibitors, non-steroidal anti-inflammatory drugs and bisphosphonates, have shown advantages in the management of periodontal disease (Preshaw, 2008; Salvi and Lang, 2005). However, the ultimate goal of periodontal treatment is not only to cease and prevent further periodontal tissue destruction, but also to regenerate the periodontal apparatus (Chantarawaratit et al., 2014).

1.4.2 Tissue engineering

Tissue engineering, which involves the use of appropriate cells, signals and engineering materials, has emerged as an alternative approach to further facilitate and optimize periodontal regeneration (Han et al., 2013). Bone substitutes (autografts, allografts, xenografts and alloplasts) and guided tissue regeneration are the two techniques with histological documentation of periodontal regeneration. However, the results of these

existing therapeutic methods overall remain unpredictable (Li and Jin, 2015; Ramseier et al., 2012). On the other hand, the use of gene therapy or growth factors has also been explored but its use has shortcomings such as the instability and short half-life of growth factors and the undesired host immune reactions or potential tumorigenesis of gene therapy (Anusaksathien and Giannobile, 2002; Li and Jin, 2015; Shin et al., 2015). However, there is a considerable body of clinical and pre-clinical data that demonstrates the regenerative properties of platelet-derived growth factor (PDGF) and Emdogain (EMD) (Institut Straumann, Basel, Switzerland), a purified acid extract of proteins from pig enamel matrix extracted from developing porcine teeth (Darby and Morris, 2012; Gestrelius et al., 2000; Kaigler et al., 2011; Lyngstadaas et al., 2001; Sculean et al., 2007; Weinreb and Nemcovsky, 2015). As with the use of bone substitutes and guided tissue regeneration, a systematic Cochrane review point to a high degree of heterogeneity among the included trials when using Emdogain (Esposito et al., 2009).

1.5 Antioxidants

Antioxidants are any molecule that delays, prevents or removes the oxidation of other molecules. Their role is the inhibition of free radical mediated oxidation of biological molecules. In addition to these direct antioxidant effects, many antioxidants enhance the expression of antioxidant and detoxifying enzymes or other pathways. Antioxidants can be synthesized by the body (endogenous) or obtained through the diet (exogenous) (Sen and Packer, 1996).

1.5.1 Pineal methoxyindoles

Melatonin is synthesized from serotonin in the pineal gland and in a variety of other tissues. Melatonin regulates the biological rhythms and the reproductive behaviour of several animal species, including humans (Brzezinski, 1997). However, many of its effects differ to its primary neurohormonal functions due to its properties as anti-inflammatory, effective free radical scavenger and stimulator of antioxidant enzymes (Allegra et al., 2003; Reiter et al., 2000; Rodriguez et al., 2004; Tan et al., 2003). Melatonin has been shown to protect the oral cavity from oxidative stress-related tissue damage (Blasiak et al., 2011; Cutando et al., 2007; Gómez-Moreno et al., 2010). Moreover, melatonin levels correlate with the periodontal health status indicating that it may have a protective role against periodontal disease (Almughrabi et al., 2013; Gómez-Moreno et al., 2007).

5-Methoxytryptophol is also synthesized in the pineal gland from serotonin and is involved in numerous functions, including the biological rhythms and the reproductive behaviour (Ouzir et al., 2013). In addition, 5-methoxytryptophol shows immunomodulatory, antioxidant and anticancer properties (García et al., 2000; Lissoni et al., 2000, 1997, 1996; Rodriguez-Naranjo et al., 2012).

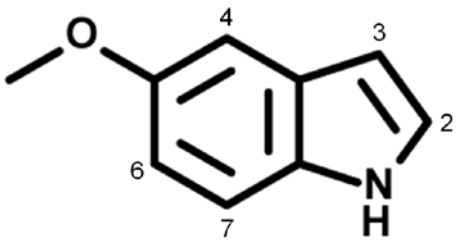

Figure 6. Basic structure of a pineal methoxyindole. Melatonin and 5-methoxyindole differ in the C3 substitute.

1.5.2 Flavonoids

Natural products have been used in traditional medicine for a long time and represent the most abundant antioxidants in the human diet. Polyphenolic compounds are ubiquitous, abundant and offer a range of properties and an array of functions such as antioxidant, anti-inflammatory and antimicrobial capacity (An et al., 2011; Cushnie and Lamb, 2011; Izzi et al., 2012; Middleton et al., 2000; Pietta, 2000), among others (Friedman, 2007). Flavonoids are a class of polyphenols characterized by the presence of three rings that are formed in plants from phenylalanine, tyrosine and malonate (**Figure 7**). Variations on the basic structure of flavonoids yield different classes. Most common flavonoids include flavones, flavonols and flavanones (Bravo, 1998; Manach et al., 2004; Pietta, 2000). They are widespread constituents of plants, especially common in leaves, flowering tissues, stems and barks, and they are important in the plant for normal growth development and defence against infection and injury (Kähkönen et al., 1999; Manach et al., 2004).

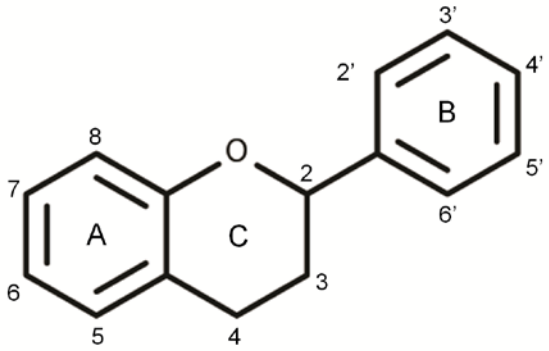

Figure 7. Basic flavonoid structure.

The increasing interest in polyphenols is due to their antioxidant properties and multi-target biological actions (Daglia, 2012; Koeberle and Werz, 2014; Spatafora and Tringali, 2012). Epidemiological, clinical, and animal studies support a role of polyphenols in the prevention of various chronic diseases, including cardiovascular, inflammatory, metabolic and neurodegenerative diseases, and some cancers (Middleton et al., 2000). Furthermore, consistent studies have shown that polyphenols could have a therapeutic effect against periodontal disease (Al-Shaher et al., 2004; Ara et al., 2010; Feghali et al., 2012; Gutiérrez-Venegas and Contreras-Sánchez, 2013; Gutiérrez-Venegas et al., 2014, 2007; Hosokawa et al., 2011; Palaska et al., 2013; Petti and Scully, 2009; Zhao et al., 2013).

Quercitrin (quercetin 3-*O*-rhamnoside) is a pigment found in many plants and foods (Alvarez et al., 2012; Fabjan et al., 2003; Hamoudová et al., 2004; Qiao et al., 2014;

Slimestad et al., 2007). It was originally isolated from oaks (from Latin *Quercus*), and used as a brilliant yellow dye (from *citron*, lemon for its colour). Beyond its antioxidant and anti-inflammatory properties (Babujanarthanam et al., 2011; Dai et al., 2013; Ding et al., 2010; Luo et al., 2015; Totilo, 2007; Yin et al., 2013), quercitrin has shown antiproliferative and apoptotic effects on cancer cells (Cincin et al., 2014; Ham et al., 2012) and antiviral activity (Alvarez et al., 2012; X. Chen et al., 2011).

Taxifolin (dihydroquercetin) is a flavonoid commonly found in onions, milk thistle and pine bark (Rohdewald, 2002; Slimestad et al., 2007; Wallace et al., 2005). It is used in different complex preparations such as silymarin, approved for the prevention of recurrent hepatitis C by the European Medicaments Agency (European Medicines Agency, 2011). Furthermore, it shows certain anti-cancer mechanisms and antiviral activity due to its antioxidant capacity (Weidmann, 2012).

1.6 Dental implants

Dental implants have been widely used to replace missing teeth since Brånemark introduced them (Brånemark et al., 1969). A single-tooth implant is usually composed of a crown, an abutment and an implant screw (**Figure 8**). The integration of the implant screw with the hard tissue, a process termed osseointegration, provides mechanical support to the dental implant. Furthermore, peri-implant soft tissue integration to the abutment is also essential in order to protect bone and implant from bacterial penetration.

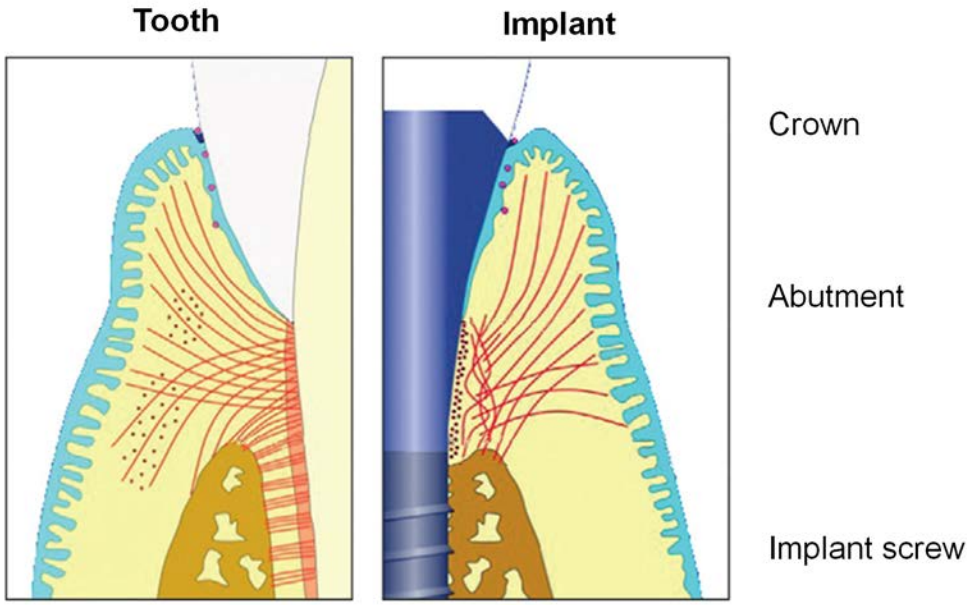

Figure 8. Comparison of a natural tooth and a dental implant. Adapted from (Gruber and Bosshardt, 2015).

The attachment of a tooth and a dental implant to the surrounding bone is completely different, as the implant surface directly contacts the bone and lacks a periodontal ligament that connects teeth and bone. The biomaterials capability to allow bone growth on their surface is termed “osteoconductivity” (Gruber and Bosshardt, 2015; Sculean et al., 2014).

1.6.1 Soft tissue integration to dental implants

Soft tissue integration is fundamentally the result of wound healing around the dental implant, which should establish a biological seal between the oral environment and the bone-surrounded implant (**Figure 9**). The soft tissue around a dental implant is composed of a barrier epithelium that resembles the junctional epithelium around the tooth, and the connective tissue. Connective tissue around the implants differs from that around teeth in the higher amount of collagen fibres, which are not attached to cementum, and in the lower number of fibroblasts and blood vessels. Furthermore, most studies revealed a collagen fibre orientation parallel to the implant surface, although a perpendicular direction may also occur (**Figure 9**) (Berglundh et al., 1991; Gruber and Bosshardt, 2015; Sculean et al., 2014).

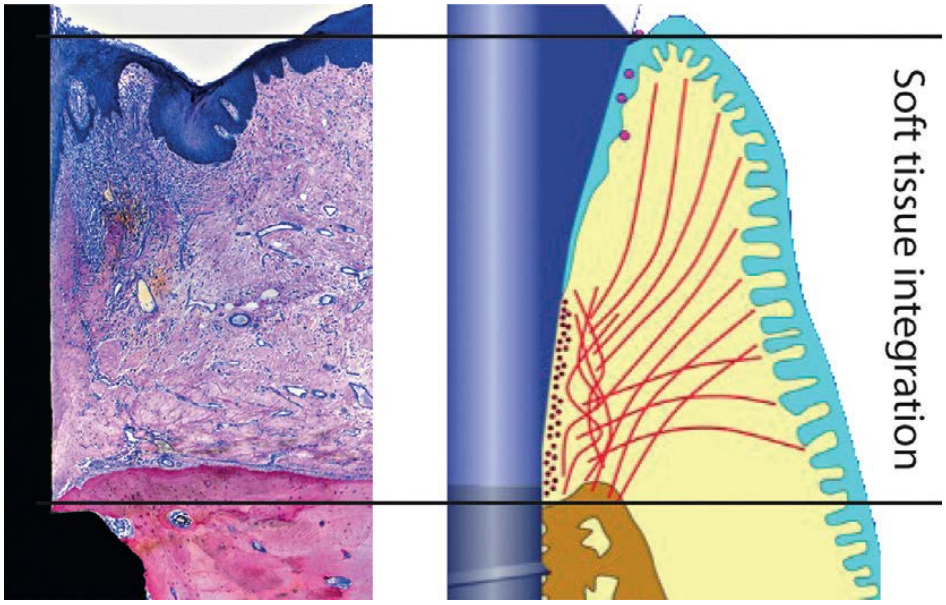

Figure 9. The soft tissue integration protects bone and implant from the microbial challenges in the oral cavity. Image adapted from (Gruber and Bosshardt, 2015)**.**

1.6.1.1 Wound healing around a dental implant

Peri-implant soft tissue healing follows the four overlapping phases of wound healing, i.e., haemostatic, inflammatory, new tissue formation and remodelling phases. After an implant placement a blood coagulum is formed which provides a provisional fibrin-network matrix for cell migration. Inflammatory cells migrate, phagocytise bacteria and necrotic tissue and release inflammatory mediators to either finalise the inflammation or to amplify it. Fibroblasts then invade the fibrin network and produce collagen-rich connective tissue in contact to the implant surface. Some fibroblasts undergo

transformation into myofibroblasts and express α-smooth muscle actin to contract the wound. The final phase involves collagen remodelling which can produce either a scarred (repaired) tissue or a structural and functional regenerated tissue, depending on the microenvironmental signals. During this process, the adsorption of saliva to the implant surface induces bacterial accumulation (dental plaque formation), which triggers the inflammatory process (Sculean et al., 2014; Villar et al., 2011).

Inflammation is necessary for the effective defence against pathogens and to set in motion tissue repair following injury (Gurtner et al., 2008). However, excessive inflammation has been shown to delay healing and to result in increased scarring, compromising tissue regeneration (Eming et al., 2007).

1.6.2 Peri-implant diseases

Infection and inflammation on the implant surface and peri-implant tissues cause soft tissue destruction and bone loss, which result in implant failure (Jung et al., 2008; Sculean et al., 2014), in a process similar to that observed in periodontal disease (Bullon et al., 2004; Carcuac and Berglundh, 2014; Tonetti et al., 2015a). There are two types of peri-implant diseases, which are infectious in nature. Peri-implant mucositis describes an inflammatory lesion that resides in the gingiva (similar to gingivitis). In some cases, this situation can progress to peri-implantitis, in which the supporting bone around osseotintegrated implants is also affected (similar to periodontitis) (Lindhe and Meyle, 2008; Tonetti et al., 2015a). Recent meta-analyses have estimated that the weighted mean prevalence of peri-implant mucositis and peri-implantitis is 43% and 22%, respectively (Derks and Tomasi, 2015). These inflammatory processes are one of the largest concerns jeopardizing the long-term efficacy of implants.

The sequence of microbial colonization on dental implants and biofilm formation is similar to that of teeth (Tanner et al., 1997). Surface roughness plays a crucial role in this process. Increased surface roughness facilitates biofilm formation at the implant-soft tissue interface (Subramani et al., 2009), although changes in surface roughness below 200 nm do not influence it (Teughels et al., 2006).

1.6.3 Abutment modification for improvement of soft tissue integration

1.6.3.1 Abutment materials

Commonly used abutment materials include titanium, gold, porcelain, aluminium and zirconium (Rompen et al., 2006; Sculean et al., 2014). Only titanium, zirconium and aluminium have shown biocompatibility and allowed the formation of a soft tissue on long-term studies while dental porcelain or gold are less biocompatible (Abrahamsson and Cardaropoli, 2007; Abrahamsson et al., 1998; Rompen, 2012). Apart from gold abutments, which showed increased bacterial colonization than other abutment materials (Kim et al., 2012), the mechanical properties and biocompatibility are the most important factors for selecting dental abutment materials.

Titanium (Ti) is the most widely used material in implantology because of its mechanical strength, resistance to corrosion and excellent biocompatibility (Steinemann, 1998). Furthermore, Ti is covered with an oxide layer, spontaneously

formed in presence of oxygen molecules, a process known as passivation. This titanium dioxide (TiO_2) surface layer is chemically stable in the body and protects Ti from corrosion (Liu et al., 2004). However, Ti mechanical properties are limited in the case of small diameter implants when placed in a narrow bone space (e.g. maxilla front) (Saulacic et al., 2012). To enhance its strength, Ti can be alloyed with other elements, such as zirconium (Zr), niobium, tantalum, palladium, indium and vanadium. Among the Ti alloys, a titanium-aluminum-vanadium alloy, also known as Ti-6Al-4V, is one of the most popular alloys although the release of toxic vanadium and aluminium ions poses safety risks (Grandin et al., 2012).

Titanium-Zirconium alloys have shown improved tensile and fatigue strength and corrosion resistance than Ti (Ho et al., 2008; Khan et al., 1999; Kobayashi et al., 1995; Thoma et al., 2011) with improved biocompatibility and similar osseointegration properties (Grandin et al., 2012; Linkevicius and Apse, 2008; Pae et al., 2009). Therefore, TiZr has been selected to develop small diameter implant applications.

1.6.3.2 Abutment topography

The surface properties of implants influence adhesion and differentiation of cells surrounding implants, including gingival fibroblasts (Rompen et al., 2006). Fibroblasts prefer smooth than rough surfaces (Ponsonnet et al., 2002; Wieland et al., 2002), although finely grooved surfaces perform better than smooth ones (Chou et al., 1995; Den Braber et al., 1998; Guillem-Marti et al., 2013; Kokubu et al., 2009; Wieland et al., 2002). In other studies, fibroblast proliferation increased on micro-grooved rough surfaces compared with smooth surfaces (Guida et al., 2013; Kim et al., 2009; Lee et al., 2009). *In vivo* studies indicate that a certain surface roughness is required for the formation of a stable soft tissue seal around the abutments (Brunette and Chehroudi, 1999; Schwarz et al., 2007a, 2007b). This disagreement on the effect of roughness could be due to the interference of surface texture, which also influences fibroblast growth. The rough surfaces that improve fibroblast growth more than smooth ones have a microgrooved texture. Microgrooved surfaces have been shown to enhance fibroblast apposition compared to smooth ones (Den Braber et al., 1998; Guillem-Marti et al., 2013; Kokubu et al., 2009; Wieland et al., 2002). The positive effect of microgrooved surfaces on fibroblast growth may be related to the contact guidance by the grooved texture (Brunette et al., 1983; Dunn and Brown, 1986; Kearns et al., 2013; Manwaring et al., 2004). Furthermore, micro-textured surfaces perpendicularly oriented to the migration direction of epithelial tissue impede its downgrowth (Chehroudi et al., 1990).

1.6.3.3 Abutment biofunctionalization

Research efforts have focused the modification of implant surfaces to enhance osseointegration. There are a limited number of studies that focus surface functionalization for enhanced soft tissue integration, which have been summarized in Table 1.

Table 1. Strategies for surface functionalization to enhance soft tissue integration.

Coating	Cell culture model	Cell response	References
Biomimetic strategies			
Collagen	Primary human fibroblasts	Increased cell adhesion and activation	(Marín-Pareja et al., 2014; Nagai et al., 2002)
Laminin	Primary human fibroblasts and *in vivo*	Increased cell adhesion	(Dean et al., 1995)
Fibronectin	Primary human fibroblasts and hamster fibroblasts	Slightly increased cell adhesion	(Cannas et al., 1988; Dean et al., 1995)
RGD-sequences	Primary human fibroblasts	Increased cell adhesion and viability	(Zhao et al., 2006)
Antimicrobial peptide (GL13K)	Primary human fibroblasts	Biocompatible. Increased cell adhesion and viability.	(Holmberg et al., 2013; Zhou et al., 2015)
Growth factors			
PDGF, EMD	*In vivo*	Increased speed and quantity of soft tissue healing	(Bates et al., 2013)
CNN2	Primary human fibroblasts	Increased cell adhesion and viability	(Wei et al., 2012)
RGD-sequences + bFGF	Primary human fibroblasts	Increased cell adhesion and viability	(Jin et al., 2012)
Small molecules			
H	Primary human fibroblasts	Increased cell adhesion and cell function	(Gomez-Florit et al., 2014)
Ca, Mg	Fibroblastic NIH3T3 mouse cells	Increased cell adhesion and expression of adhesion proteins	(Okawachi et al., 2012)
TiN, ZrN	Primary human fibroblasts	Increased cell adhesion	(Grössner-Schreiber et al., 2006)
Antibacterial coatings			
Ag	Fibroblast-like HT1080 cells	No effect	(Mei et al., 2014)
ZnO_2	Primary human fibroblasts	Decreased cell compatibility	(Chang et al., 2012)
Polyethylene glycol-like	Fibroblasts	No effect	(Buxadera-Palomero et al., 2015)

A number of modified surfaces with integrated antibiotics, growth factors, biomimetic peptides or complex formulations of artificial extracellular matrix components have been developed in order to improve soft tissue integration to dental implants (**Table 1**). However, biocompatibility of most anti-bacterial surfaces is still uncertain while the lack of stability of growth factors after implantation are the main reasons for the absence of such a product from the current implant market (Bhatavadekar, 2012; Busscher et al., 2012; Campoccia et al., 2013). On the other hand, surface modification with proteins, hydrogen, calcium, magnesium, TiN or ZrN promoted fibroblast adhesion but no other beneficial effects have been described (**Table 1**). Therefore, there is a need to develop a new generation of bioactive dental implant surfaces to improve soft and hard tissue integration (Gruber and Bosshardt, 2015).

2 OBJECTIVES

Taking into account that the etiology and clinical manifestations of periodontal and peri-implant diseases have many features in common, it stands to a reason that those molecules that induce the regeneration of periodontal and peri-implant tissues while controlling bacterial infection and inflammation would be useful for the management of both, periodontal and and peri-implant diseases. In addition, functionalization of dental implant abutments with such molecules may improve soft tissue integration to the implant abutment, mimicking natural gingiva around tooth and preventing the penetration of oral bacteria and therefore avoiding peri-implant disease.

In this thesis, the following specific aims were defined, which are outlined in Figure 10:

1. **To select potential antioxidant biomolecules to improve periodontal/peri-implant regeneration.**

First we screened *in vitro* among different natural biomolecules with antioxidant and anti-inflammatory activity for potential beneficial effects on diseased periodontal and peri-implant tissues.

The effects of two groups of biomolecules on human gingival fibroblasts and bacterial growth were evaluated: pineal methoxyindoles (melatonin and 5-methoxytryptophol) [**Paper I**] and flavonoids (chrysin, diosmetin, galangin, quercitrin and taxifolin) [**Paper II**]. The results derived from this screening pointed to melatonin and quercitrin as candidates to improve periodontal regeneration. In a second stage, an inflammatory model was set to mimic the environment of periodontal and peri-implant diseases *in vitro*. For these studies, quercitrin was selected to evaluate its effects on human gingival fibroblasts and on human mesenchymal stem cells [**Paper III**].

2. **To evaluate metal implant surfaces to increase soft tissue integration.**

To achieve the second aim, the response of human gingival fibroblasts to two materials and three topographies was firstly evaluated [**Paper IV**]. Then, from the outcome of the first objective, quercitrin was used to biofunctionalise titanium surfaces and the behaviour of human gingival fibroblasts and bacteria on them was analysed [**Paper V**].

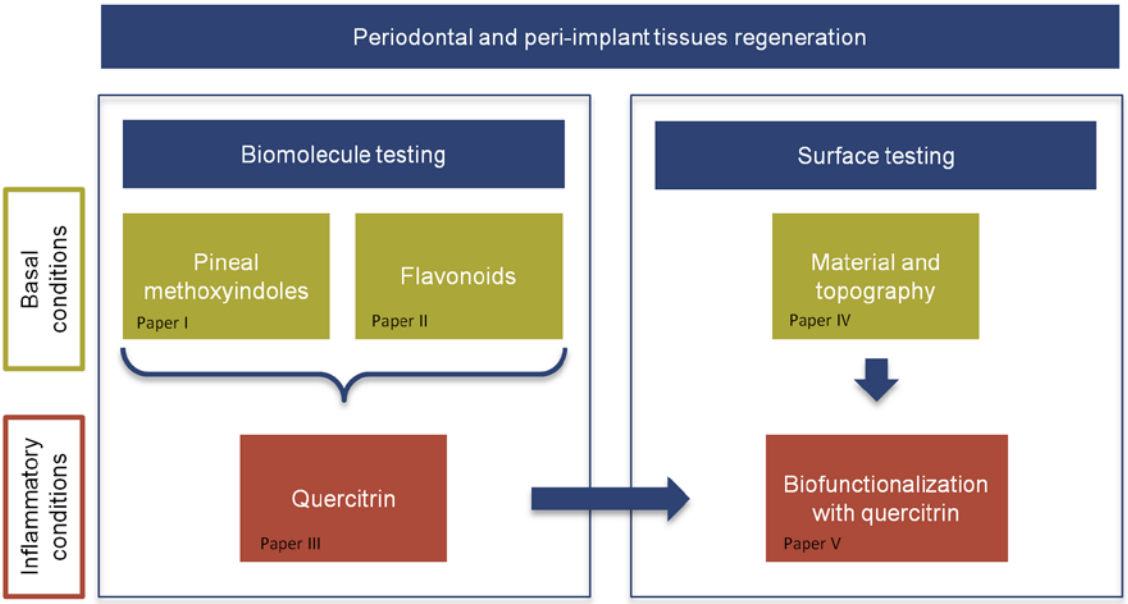

Figure 10. Outline of the experimental design followed in this thesis.

3 METHODOLOGICAL CONSIDERATIONS

This chapter intends to discuss the methods used during this thesis by providing the advantages or disadvantages of the selected methods with regards to the aim of research. For a detailed description of the equipment, materials and specific methods used, see the "Material and Methods" section of each individual paper [Appendix 1].

3.1 Biomolecule selection

Emerging data from clinical studies show that antioxidant supplementation has beneficial effects on periodontal disease status (Hirasawa et al., 2002; Hrishi et al., 2015; Iwasaki et al., 2013, 2012; Maruyama et al., 2011; Mathur et al., 2013; Staudte et al., 2005; Tomofuji et al., 2009). Here, the effects of different antioxidants were screened for their potential in periodontal applications, specially focused in periodontal and peri-implant tissue regeneration.

3.1.1 Flavonoids

Structural diversity in each flavonoid family arises from the various hydroxylation, methoxylation, and glycosylation patterns of ring substitution. Flavonoids usually occur as glycosides in plants because the effect of glycosylation renders the flavonoid less reactive and more water soluble, permitting its storage in the cell vacuole. The activity of flavonoids is closely linked to their structure. Flavonoid structure-activity studies point to the *ortho*-dihydroxy (3',4'-diOH, i.e. catechol) functionality in the B-ring, the C2-C3 double bond (in conjugation with the 4-oxo group) and both 3-OH and 5-OH groups as responsible of high antioxidant capacity, inhibitory activity of prooxidant enzymes and anti-inflammatory activity (Amić et al., 2007; Kim et al., 2004; Rice-Evans et al., 1996). In this thesis, two flavones (chrysin and diosmetin), two flavonols (galangin and quercitrin), a flavanone (taxifolin), representing the most common flavonoids, were selected to evaluate its potential in periodontal applications (**Figure 11**).

3.1.2 Pineal methoxyindoles

Melatonin and 5-methoxytryptophol, which are synthesized in the pineal gland, are involved in numerous biological actions (Brzezinski, 1997; Ouzir et al., 2013). Both have been shown as free radical scavengers or antioxidants and immunomodulatory agents due to their similar molecular structures (Allegra et al., 2003; García et al., 2000; Lissoni et al., 1997; Reiter et al., 2000). However, they show variable effectiveness and potency depending on the different substituents and position (**Figure 12**).

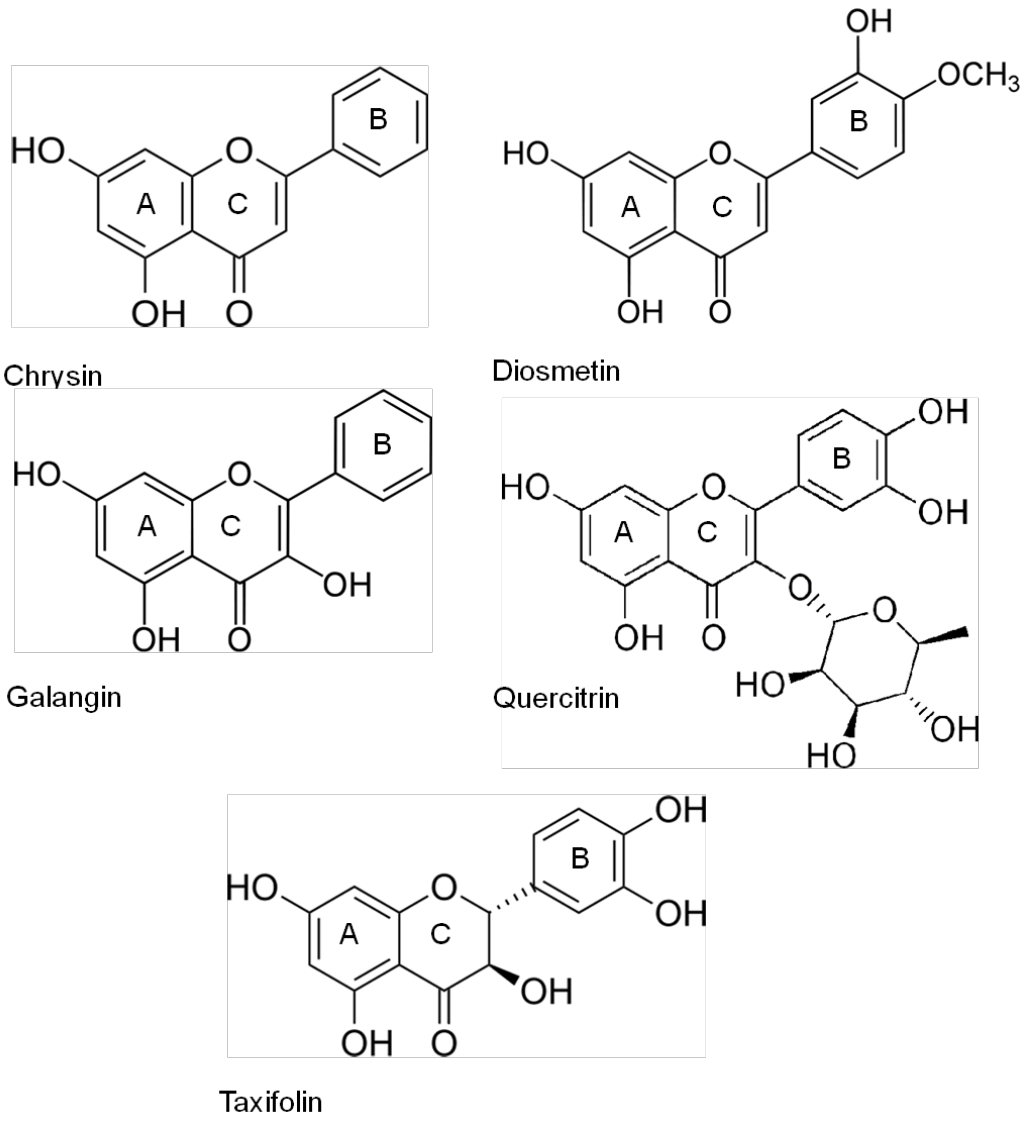

Figure 11. Structure of the flavonoids used in the study. Adapted from paper II.

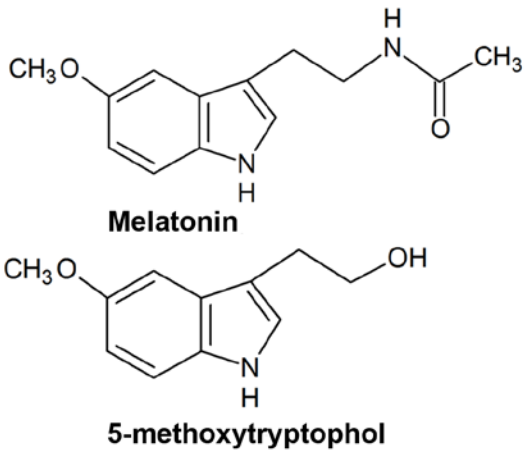

Figure 12. Structure of melatonin and 5-methoxytryptophol.

3.2 Biological characterization

3.2.1 Selection of in vitro models

Many *in vivo* models aim at simulating, as closely as possible, human exposure to a certain treatment (Clift et al., 2011). However, the ethical issues regarding animal experimentation, the time required to obtain results and the costs associated to this research, limits it for a first screening and characterization of response to a certain stimuli (Habibovic et al., 2007). *In vitro* assays have been developed as a simplified reflection of the *in vivo* situation in controlled and well-defined conditions, in order to reduce the need for animal experimentation, giving results at a lower cost, more quickly and being more reproducible (Clift et al., 2011). Obviously, *in vitro* models also have limitations: they assay narrow aspects of complex processes, ignoring the holistic nature of life. Despite these shortcomings, the establishment of an *in vitro* model is attractive and useful for a first characterization of the biological effects of a specific biomolecule or a new biomaterial.

Primary cell culture refers to cells that are placed in culture directly from the tissue of origin. On the other hand, cell lines are derived from primary cell cultures that have been immortalised and, therefore, they escape the normal control of cell cycle. Although primary cell cultures have a limited life span compared to cell lines, they provide data of much greater value than cell lines (Stacey et al., 2009). It is widely accepted that multiple donors need to be investigated to consider donor-to-donor variability. Pooling the cells from various donors might result in an average of very different populations and the result might describe a population that does not exist, losing a lot of valuable information. On the other hand, performing an experiment with similar responding donors may bias the data towards one population and exclude donor populations (Stoddart et al., 2012). In this thesis, commercial human primary cells from different donors have been used for testing the biomolecules and novel implant surfaces, as detailed below.

Human gingival fibroblasts

Fibroblasts are mesenchymal cells with many vital functions during development and in adult organisms. They synthesize much of ECM components in connective tissues, play key roles in wound healing and are involved in the maintenance of tissue homeostasis. Within gingival connective tissue, gingival fibroblasts account for most connective tissue cells and are responsible for maintenance of tissue homeostasis as well as for wound healing and are also involved in different regulatory processes, including inflammation (Bartold et al., 2000). Furthermore, after installation of a dental implant, fibroblasts from the oral connective tissue are the preferred cells to form a collagen-rich connective tissue to repopulate the wound (Palaiologou et al., 2001).

In order to overcome the shortcomings of monolayer cultures, different high-order *in vitro* models have been suggested. Complex interactive co-cultures of gingival keratinocytes and gingival fibroblasts have been used to test biomolecules for periodontal regeneration and dental biomaterials (Moharamzadeh et al., 2007; Schulz et al., 2012). Also, three-dimensional models with fibroblasts or keratinocytes embedded or cultured on matrices have also been used. However, these systems have been used to a very limited extent (Weinreb and Nemcovsky, 2015). Moreover, co-cultures or three-

dimensional systems are not the optimal test system to perform screening analysis. For these reasons, in this thesis, experiments were performed with monolayer cultures of primary human gingival fibroblasts.

Human gingival fibroblasts were initially [paper I, II, IV] cultured with commercial culture media supplemented with foetal bovine serum, as recommended by the supplier. When studying the expression of collagens, some treatments increased their messenger RNA (mRNA) levels while protein quantification did not change (see section 3.2.8). This discrepancy can be explained by the lack of ascorbic acid in culture media. Ascorbic acid or vitamin C is essential for the synthesis of hydroxyproline, which is necessary to stabilise the collagen triple-stranded helix (Alberts et al., 2002). Therefore, lack of ascorbic acid leads to the accumulation of unprocessed procollagen in the cell culture medium, which is discarded with every media change (Chen and Raghunath, 2009). To enhance the deposition of secreted collagen, ascorbic acid was added to cell culture media in papers III and V.

Human mesenchymal stem cells

Mesenchymal stem cells (MSCs) are multipotent cells that can differentiate along a variety of cell lineages, such as chondrocytes, osteoblasts and adipocytes (Pittenger et al., 1999) when receiving the appropriate signals. Different studies have shown that bone-marrow MSCs have the capacity to promote the regeneration of alveolar bone, cementum and periodontal ligament (Kawaguchi et al., 2004; Li and Jin, 2015). Periodontal ligament derived MSCs are an obvious alternative for periodontal regeneration studies. However, the difficulty of collecting enough periodontal ligament MSCs from the same donor limits its use for the treatment of periodontal disease (Li and Jin, 2015). Furthermore, periodontal ligament MSCs are comparable to bone marrow MSCs since they have a similar differentiation capacity and ability to regenerate periodontal bone (F. M. Chen et al., 2011; Monsarrat et al., 2014). Actually, bone marrow MSCs showed periodontal ligament-like cell features when cocultured with the periodontal ligament *in vivo* (Kramer et al., 2004). In this thesis, human bone marrow derived MSCs were selected as *in vitro* model to test the osteogenic potential of quercitrin [paper III].

To create the osteogenic media, we used ascorbic acid, β-glycerophosphate and dexamethasone, as previously described (Jaiswal et al., 1997). As explained before, ascorbic acid is essential for collagen synthesis. β-glycerophosphate is a source of organic phosphate used by alkaline phosphatase needed for the mineralization process. Finally, the precise mechanism by which dexamethasone induces osteogenesis is unknown although it prevents cell apoptosis and promotes hMSCs proliferation (Langenbach and Handschel, 2013).

Inflammatory model

We used the inflammatory mediator IL-1β to mimic the inflammatory process in periodontal disease [paper III] and after an implant placement [paper V]. IL-1β is an important mediator in inflammatory responses, broadly used to induce experimental inflammation and to enhance the proinflammatory response (Furuhashi et al., 2005; Kida et al., 2005; Ono et al., 2011), imitating the inflammatory pathways activated in response to oral pathogens (Yucel-Lindberg and Båge, 2013). The concentrations used

are in the range with the IL-1β levels usually found in patients with periodontitis (Nokhbehsaim et al., 2011). The major disadvantages when using IL-1β are that the stimulation that induces may be, in some cases, too potent due to positive feedback mechanisms (Ghosh and Hayden, 2008) and that the only addition of IL-1β does not reflect the entire inflammatory process in periodontal disease.

Bacterial lipopolysaccharide (LPS) is a component of gram-negative bacterial cell wall that is also widely used to induce experimental inflammation by stimulation of toll-like receptors. However, it is a thermostable and UV-resistant molecule that can induce inflammatory reactions in very low concentrations (Lieder et al., 2013). Therefore, it could resist conventional cleaning and sterilization methods, contaminate a cell culture cabin and induce undesired responses in other on-going experiments.

3.2.2 Cell toxicity

Lactate dehydrogenase (LDH) is a stable cytoplasmatic enzyme present in all cells that catalyses the conversion of lactate to pyruvate. LDH is rapidly released into the cell supernatant upon damage of the plasma membrane, so it can be used to quantify cytotoxicity in cell cultures. By performing this assay, it is possible to measure the adverse effects induced by different treatments and/or by a biomaterial in contact with the cells. In this thesis, LDH release was measured as an index of biomolecules toxicity [paper I, II, II] but also as an index of biocompatibility of the tested biomaterials [paper IV, V].

Alternatively, the cytotoxicity of treatments and biomaterials can be measured through assessment of changes in membrane integrity by trypan blue exclusion or by nucleic acid staining using propidium iodide or through assessment of the metabolic activity by the tetrazodium salt assay or by resazurin dye. However, many of them are destructive assays while the advantage of non-destructive assays (LDH and resazurin) is that cell culture can continue after collecting the culture media for the assay.

3.2.3 Cell visualization

Fluorescence imaging is one of the most basic tools used in biological sciences for the visualization of cells and tissues. In this thesis, conventional fluorescence microscopy [paper V] and confocal microscopy [paper IV, V] were used. The most important feature of a confocal microscope is the capability of isolating and collecting only light from a focal plane within a sample, allowing image capture at higher resolution without out-of-focus signal compared to conventional fluorescent microscopy. This allows observing the fine detail in the samples, for example, when immunostaining protocols are used [paper IV, V]. On the other hand, the advantage of conventional fluorescence is that the entire thickness of the sample is excited at the same time allowing a complete and faster visualization of the specimen, necessary when larger areas are processed [paper V].

As described above, another alternative to visualize cell morphology on biomaterials is using scanning electron microscopy. This technique is useful when evaluating cell coverage of the surface and cell orientation although it does not allow distinguishing cell parts.

3.2.4 Cell quantification

By quantification of the DNA content it is possible to get an estimation of the number of cells. Among the different methods for DNA quantification, the specific DNA dye Hoechst was used [paper IV]. This method is an accurate, sensitive and specific method for quantifying DNA in which fluorescence units are correlated with the cell number using a linear standard curve (Rao and Otto, 1992). This quantitative method was supplemented with cell imaging. However, when using these two methods a lot of samples are required. For this reason, in paper V, cells on the disks were stained and visualized through fluorescence microscopy. Two pictures from each sample were taken, covering a large area of the sample, and nuclei were counted using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Using this technique, cell morphology visualization and cell number quantification can be performed at the same time although one shortcoming is that cell counting is more time-consuming than DNA quantification.

Alternatively, the number of cells can be related to DNA absorbance measurement at 260 nm although interfering molecules can introduce high variability and error in the method. Also, the number of cells can be related to the metabolic activity, as described previously.

3.2.5 Wound healing assay

Wound healing assay is a classic and commonly used method for studying cell migration and the biology underlying it (Lampugnani, 1999). To perform a wound healing assay, a cell monolayer is scratched using an object such as a pipette tip or syringe needle. The monolayers recover and heal the wound in a process that can be observed overtime (**Figure 13**) (Yarrow et al., 2004). There are a number of limitations of this scratch assay compared to other available methods, such as Boyden chamber assays (where cells move from one compartment to another). The scratch assay takes a relatively longer time to perform, a relatively large amount of cells and chemicals are required and open-area quantification is manual and highly subjective (Liang et al., 2007). Despite these limitations, the scratch assay was used in this thesis because it is easy to set up, does not require any specialized equipment and all materials required for the assay are available in any laboratory that performs cell culture. In this thesis, the effect of biomolecules [paper I, II] and surfaces [paper IV] on wound healing of human gingival fibroblasts was evaluated. To overcome the subjective open-area quantification, an automated software for the analysis of wound healing assays was used (Gebäck et al., 2009). For this, cells were followed taking live pictures of the same area over time. However, for the experiments with surfaces, a qualitative end-point analysis was performed since cells on surfaces could not be followed live.

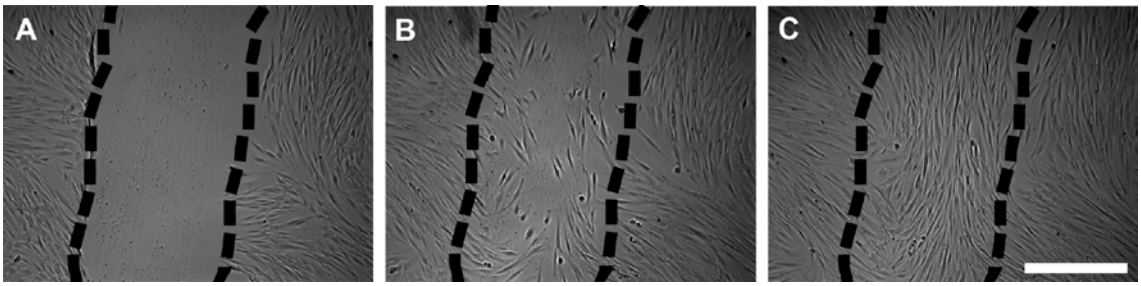

Figure 13. Wound healing assay with human gingival fibroblasts on plastic multiwell plates. (A) Initial scratch; (B) 24 h after scratching; (C) 48 h after scratching. Dotted line indicates scratch mark. Scale bar = 500 μm.

3.2.6 Real-time RT-PCR

Real-time reverse transcription-polymerase chain reaction (real-time RT-PCR) provides a quantitative, sensitive and fast technique for detection of specific mRNA sequences. It detects mRNA sequences from very tiny amounts of sample (nanogram level), reduces the probabilities of variability and contamination, and does not need post-reaction analyses (Ginzinger, 2002). Before performing a gene expression measurement by real-time RT-PCR, total RNA is first isolated by using the guanidine thiocyanate method (Chomczynski and Sacchi, 1987). After RNA isolation, the same amount of total RNA is retro-transcripted to its complementary DNA (cDNA). The cDNA synthesized is then used as template for real-time PCR amplification of reference and target genes. The amount of product formed is monitored during the course of the reaction using fluorescent dyes introduced into the reaction. The increase in fluorescence signal is proportional to the amount of DNA synthesized during each amplification cycle. Each reaction is characterized by the cycle fraction at which fluorescence first arise a statistically significant increase in fluorescence, a parameter known as the crossing point (Cp), which correlates with the initial amount of cDNA target, i.e. low Cp values are correlated to high levels of cDNA template. In this thesis, SYBR Green dye was used to monitor the amount product formed. It binds specifically to double-stranded DNA and it exhibits little fluorescence when unbounded. During each elongation cycle the number of double-stranded DNA formed is increased and, consequently, the amount of SYBR Green that binds to it also increases. SYBR Green detection is the simplest method to detect real-time PCR products. However, SYBR Green may bind to any double-stranded DNA, including primer-dimer and other non-specific reaction products although the specificity of the reaction was assessed through melting curve analysis and agarose gels to determine the product size (Bustin, 2000).

To allow relative quantification after real-time PCR, reaction efficiencies are calculated from standard curves constructed using Cp values of cDNA serial dilutions. Then, Cp values for each of the unknown samples are used to calculate the expression levels using the ΔCp method (Pfaffl, 2001). Samples were normalized by the geometric mean of the expression levels of reference genes, to minimize differences in extraction yield, and amplification efficacy, thus enabling comparisons across different samples (Vandesompele et al., 2002).

By definition, a reference gene is a constitutive gene that is required for the maintenance of basic cellular functions and expressed in all cells of the organism under normal and pathological conditions. These genes should have constant mRNA levels in all samples and they should not vary by the experimental treatment. Stability of reference genes was calculated using a statistical tool (BestKeeper, Technical University of Munich, Weihenstephan, Germany) (Pfaffl et al., 2004). The target genes analysed in this thesis were related to ECM organization and turnover, cell adhesion and structure, inflammation and wound healing (**Table 2**). The function and importance of the proteins encoded by the genes analysed through the different studies are described below.

Table 2. Function and importance of the different target genes studied in this thesis.

	Protein (Gene)	Function	References
ECM organization and turnover	Collagens (COL1A1, COL3A1, COL4A2, COL5A1, COL12A1)	The gingival connective tissue consists of a dense network of collagen fibrils bundles that provide firmness to the gingiva and attach it to the tooth and alveolar bone. Type I, III and V are fibrillar collagens; type XII is a fibril-associated collagen; and type IV is a network forming collagen. Collagen type I account for 90% of body collagen and type III is associated with scarless responses.	(Alberts et al., 2002; Bartold et al., 2000; Satish and Kathju, 2010)
	Decorin (DCN)	A small dermatan sulfate proteoglycan that binds to type I collagen fibres and transforming growth factor-β. Lack of decorin inhibits collagen fibril formation and organization.	(Alberts et al., 2002; Häkkinen et al., 2000a)
	Matrix metalloproteinase-1 (MMP1)	MMPs degrade matrix proteins such as collagen and fibronectin in normal physiological processes. However, MMPs are upregulated during inflammation, which leads to an accelerated ECM destruction. Specifically, MMP-1 is a secreted MMP that breaks down collagen type I and III.	(Birkedal-Hansen, 1993)
	Tissue inhibitor of MMP-1 (TIMP1)	MMPs are inhibited by TIMPs. TIMP-1 binds to secreted MMPs, like MMP-1.	(Brew et al., 2000)

Table 2. Continuation.

Cell adhesion and structure	Versican (VCAN)	It is a large ECM proteoglycan. It participates in cell adhesion, migration and proliferation.	(Zimmermann et al., 1994)
	Vimentin (VIM)	VIM is an intermediate filament that is constitutively expressed in cells. As an intermediate filament, VIM is a major component of the cytoskeleton. Its downregulation may reveal impaired cell growth.	(Alberts et al., 2002)
	Osteonectin (SPARC)	SPARC is a glycoprotein related to adhesion, proliferation and ECM mineralization. It is able to mediate integrin-dependent cell adhesion.	(Häkkinen et al., 2000b)
	Fibronectin-1 (FN1)	Is an ECM protein involved in cell adhesion to the matrix and in cell guidance. Integrins on the cell surface are receptors for fibronectin.	(Alberts et al., 2002; Chou et al., 1995)
	Integrins (ITGA2, ITGA8, ITGB3)	The α2 integrin subunit is collagen receptor while α8 subunit is a fibronectin receptor. Integrin β3 subunit is a vitronectin receptor, a molecule involved in focal contacts whose upregulation enhanced gingival fibroblasts attachment.	(Hormia and Könönen, 1994; Palaiologou et al., 2001)
Inflammation	Interleukin-6 and interleukin-8 (IL6, IL8)	IL-6 and IL-8 mediate important signals in the proinflammatory cytokine network, including in periodontal disease.	(Bartold and Narayanan, 2006; Cekici et al., 2014)
	Cyclooxigenase-2 (COX2)	COX2 is an inducible enzyme that converts free arachidonic acid into prostaglandins, such as prostaglandin E2 (proinflammatory mediators). COX2 is upregulated during inflammation.	(Noguchi and Ishikawa, 2007)
	Tumor necrosis factor-alpha (TNFα)	It is a multifunctional proinflammatory cytokine involved in periodontal disease.	(Bartold and Narayanan, 2006; Cekici et al., 2014)
	Interleukin-10 (IL10)	IL10 plays a major role in suppressing immune responses. It plays a protective role in tissue destruction and upregulates TIMPs expression.	(Cekici et al., 2014)

Table 2. Continuation.

Wound healing	Alpha-smooth muscle actin (ACTA2)	It is a highly contractile protein that is the hallmark of activated fibroblasts or myofibroblasts. Myofibroblasts are contractile cells that, over time, bring the edges of a wound together. Its expression is necessary for wound healing although excessive fibroblast activation is typical of fibrosis and scarring.	(Hinz et al., 2007, 2003)
	Transforming growth factor-beta 1 (TGFB1)	It is a multifunctional cytokine that regulates multiple processes. In fibroblats, TGF-β induces the expression of ACTA2 and prompt the conversion to the myofibroblast phenotype.	(Hinz et al., 2007, 2003)
	Endothelin-1 (EDN1)	Its accumulation initiates the differentiation of fibroblasts into myofibroblasts.	(Hinz et al., 2007, 2003)

3.2.7 Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) is a widely used sensitive immunoassay that uses an enzyme linked to an antibody or antigen as a marker for the detection of a specific immunogen (Lequin, 2005). To allow quantification, a standard curve with known amounts of the immunogen of interest is run in every assay. It is an accurate and quantitative method, but it requires a proper setting up of the assay (depending on the range of detection and sensibility) to get a successful detection. The limitation of the ELISA lies in the specificity of the primary antibody. ELISA was used to detect MMP-1, TIMP-1 [papers I, II, IV] and PGE2 [papers III, V].

3.2.8 Collagen quantification

Collagens constitute the main component of ECM (Alberts et al., 2002), therefore it was of interest to quantify collagen production. Collagen can be found soluble in the culture medium or mostly entrapped in the ECM (Chen and Raghunath, 2009). The Sirius Red dye method for collagen quantification is based on the selective binding of Sirius Red F3BA to collagen. Subsequent elution with sodium hydroxide and read-out at 540 nm can be done in cuvettes, in microplates with collagen extract adsorbed to the plastic and using microplate readers (Chen and Raghunath, 2009; Walsh et al., 1992). This method was used in this thesis in papers I and II. However, it requires many replicates and a lot of sample, which was not available in the studies performed with the surfaces due to the small size of the implants. Furthermore, even under acid ascorbic supplementation, fibroblasts secrete minimal amounts of collagen that is discarded with every medium change (Chen and Raghunath, 2009). For this reasons, the analysis of collagen mRNA levels was used to study collagen production in papers III, IV and V.

There are different methods to study total and specific collagens. Before using the Sirius Red dye method, quantification of collagen from culture media was tested using ELISA, although the sensibility of the kit was not enough to detect it.

3.2.9 Oxidative stress assay

As more extensively explained in previous sections, oxidative stress lies at the heart of periodontal and peri-implant diseases. In paper II, the antioxidant capacity of quercitrin and taxifolin, after exposure of human gingival fibroblasts to oxygen peroxide, was assayed. The cell-permeant 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), a chemically reduced form of fluorescein, was used as an indicator for ROS. The theory behind using DCFH-DA is that oxidative non-fluorescent fluorescein derivatives will emit fluorescence after being oxidized by hydrogen peroxide. The emitted fluorescence is directly proportional to the concentration of hydrogen peroxide. When applied to intact cells, DCFH-DA crosses cell membranes and is hydrolyzed enzymatically by intracellular esterases to nonfluorescent DCFH. In the presence of ROS, DCFH is oxidized to highly fluorescent DCF. One shortcoming of the method is that DCFH can be photooxidised to DCF. To overcome this problem, a fluorescent microplate reader with fast light excitation and measurement was used. Therefore, the intracellular DCF fluorescence is a direct measure of overall oxidative stress in cells (Wang and Joseph, 1999). Alternative methods to assess oxidative damage include the measurement of lipid peroxidation products and DNA adducts although they do not measure oxidative stress directly.

3.2.10 Alkaline phosphatase activity and calcium quantification

In paper III, the effect of quercitrin on the osteogenic differentiation of hMSCs was evaluated. For this purpose, alkaline phosphatase (ALP) activity, an enzyme present in the cell membrane necessary for initiating bone mineralization (E. E. Golub et al., 1992), was determined, and calcium, the hallmark of osteoblast differentiation, was quantified.

Determination of ALP activity is based on a colorimetric method that follows the hydrolization of p-Nitrophenyl phosphate with the formation of a yellow end product that can be measured spectrophotometrically. To determine ALP activity a previous step of cell membrane disruption with Triton X-100 (a surfactant) and freeze/thawing cycles was conducted.

Calcium was determined by inductively coupled plasma atomic emission spectroscopy (ICP-AES). The major advantage of this method over commercial kits is that it is a highly sensitive and precise analytical technique for detection of the concentration of a wide range of elements present in a solution, calcium among others, based in the emission of electromagnetic radiation at characteristic wavelengths of each particular element from excited atoms by inductively coupled plasma. The concentration of calcium was calculated correlating the emission intensity of each sample to standard curves. Special attention was taken to avoid contamination of the samples with calcium from other sources (autoclaved material and glass labware) since it is a very sensitive method.

3.3 Biomaterials

The biomaterials tested in this thesis were coin-shaped Ti and TiZr samples. For *in vitro* studies, coin-shaped samples had a perfect fit in the multiwell plastic dishes used. With this arrangement, cells could not be followed with the available conventional inverted-light microscopes used for cell culture, which allow live cell visualization. Instead of this, fluorescence, confocal and scanning electron microscopy was used to visualize cells cultured onto the biomaterials, as described above. Moreover, cells seeded onto tissue culture plastic were used to monitor each cell experiment with the inverted-light microscope.

3.3.1 Polished, machined and acid-etched surfaces

For paper IV, coin-shaped samples (diameter 4.39 mm, thickness 2 mm) made of grade IV titanium and titanium-zirconium alloy, containing 13-17% zirconium, were kindly provided by Institut Straumann AG (Basel, Switzerland) as machined and machined + acid etched (modMA) surfaces. The modMA surfaces were produced from machined surfaces acid etched in HCl/H_2SO_4 at 125-130 °C for 5 min, rinsed in NaCl under N_2 protection and stored in 0.9% NaCl solution (Rupp et al., 2006). Mechanical wet polishing was used to fabricate the polished surfaces from M surfaces. Polishing was performed using silicon carbide abrasives from coarse to fine ones. The final step was carried out on a neoprene cloth using suspension of colloidal silica with particle around 30-100 nm. Polished samples were cleaned by ultrasonic baths with 40% NaOH and 50% HNO_3 to remove silicon carbide and silica (Lamolle et al., 2009). Samples were dipped in phosphate buffered saline before cell seeding.

3.3.2 Functionalization of Ti surfaces with flavonoids

For paper V, machined coin-shaped samples (diameter 6.2 mm, thickness 2 mm) made of grade IV Ti were purchased from Implantmedia (Lloseta, Spain). Polished surfaces were fabricated using wet mechanical polishing. Surfaces were cleaned as described above prior to functionalization with flavonoids.

First, Ti surfaces were passivated in order to remove the superficial carbon layer (contamination) and create a layer of hydroxyls (Liu et al., 2004; Martin et al., 2007). The functionalization of the surfaces consisted in binding covalently quercitrin to titanium using an aminosilane as linker agent by reaction of the carbonyl group of the flavonoid with the terminal amino group of the aminosilane, forming a imine bond (Córdoba et al., 2015b). However, in aqueous dissolutions, imine bonds can be hydrolysed. For this reason, the coating was also treated with the reducing agent $NaCNBH_3$ to irreversibly reduce the imine bond (-C=N-) to an amine (-C–N-) in order to decrease bond reactivity and increase the stability over time of the coating (**Figure 14**) (Córdoba et al., 2015a).

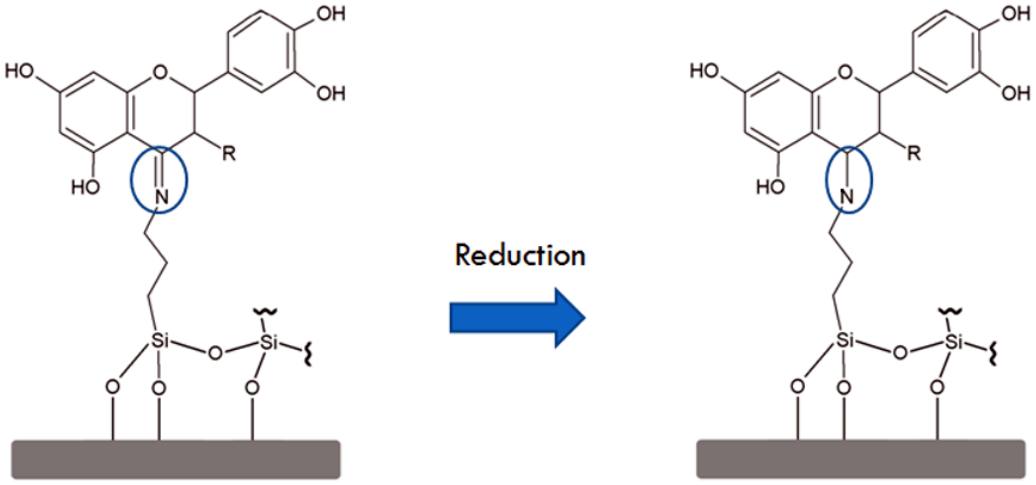

Figure 14. (Left) Quercitrin-coated surfaces. (Right) Quercitrin-coated surfaces after reduction of the imine bond to an amine. Courtesy of Alba Córdoba.

Silanes are widely used as coupling agents for surface modification (Treccani et al., 2013). (3-Aminopropyl)triethoxysilane (APTES) was chosen as crosslinker since it forms biocompatible self-assembled monolayers on hydroxylated surfaces, such as TiO_2 (Bauer et al., 2013; Toworfe et al., 2009) and its amino terminal group allows its bonding with carboxylic groups.

3.4 Surface characterization

3.4.1 Scanning electron microscopy

Scanning electron microscopy (SEM) is a fast qualitative method that allows visualizing relatively large surface areas at high magnification. Materials of different electron density appear in different grey values; the higher the electron density the lighter the material appears on the image. SEM was used in this thesis for imaging surfaces [paper IV] and cell/bacteria morphology and distribution on the surfaces [paper IV, V]. Samples containing cells/bacteria need to be fixed and dried to preserve its structure under the vacuum conditions of the specimen chamber.

3.4.2 Optical profilometry

Optical profilometry is a rapid and reliable technique to examine surface topography and to give information on material roughness at high resolution. Surface roughness (Sa) is defined as the absolute average height deviation from the mean plane. It is a widely used parameter to describe surface topography (**Figure 15**). In this thesis, surface roughness was measured in paper IV.

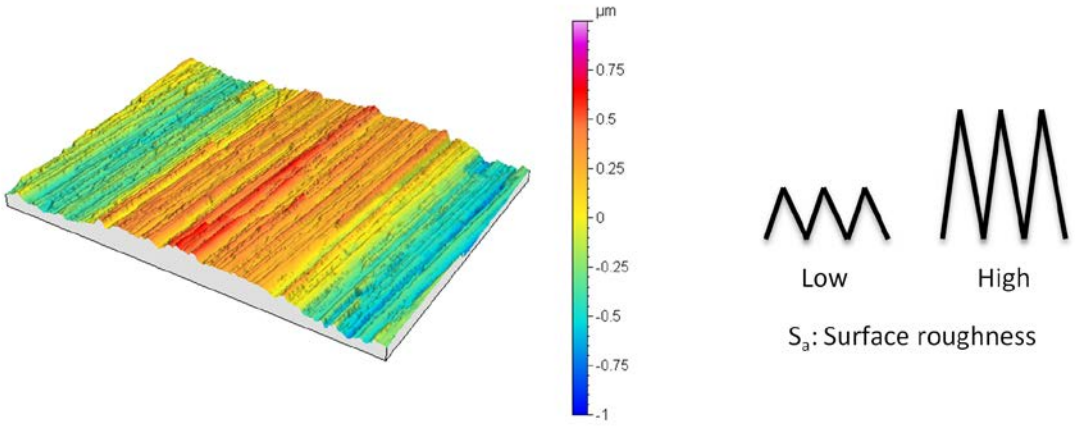

Figure 15. (Left) Topographical image of machined Ti surfaces from profilometry (250 x 190 µm). (Right) Schematic illustration of surface roughness assessed from topographical images. Profilometry image is a courtesy of Dr. Rui Xing.

3.5 Bacterial experiments

In this thesis two bacterial strains were used, *Staphylococcus epidermidis* and *Streptococcus mutans*. These two species are among the initial colonizers during the multistage dental plaque formation process (Sbordone and Bortolaia, 2003), preparing a favourable environment for late colonizers. Moreover, both are recognized biofilm forming bacteria (Ha et al., 2005; Lévesque et al., 2005; Mack et al., 2006). *S. epidermidis* plays a major role in infections of implants (Nablo et al., 2005) while *S. mutans* is considered the primary causative agent of dental caries (Lévesque et al., 2005). Furthermore, both bacterial strains are innocuous compared to other bacterial strains used for the same purpose. Therefore, they represent a suitable and safe model to investigate the anti-bacterial effect and the biofilm formation.

The methods used include the evaluation of *S. epidermidis* growth in the presence of different biomolecules [paper I, II] and the evaluation of *S. mutans* adhesion and biofilm formation on different surfaces [paper V].

3.5.1 Bacterial growth

There are different methods to measure bacterial growth such as absorbance measurement, colony count or adenosine triphosphate (ATP) measurement. To evaluate bacterial growth [paper I, II] in this thesis, bacterial suspensions were incubated with the different biomolecules in 96-well flat-bottomed plates for 24 h at 37˚C under shaking. At regular time intervals, the optical density was measured at 600 nm to determine bacterial proliferation. When absorbance is measured, increased bacterial number decreases transmission of light. The major advantages of this method include that it does not require special equipment (but the spectrophotometer), reagents or manual colony counting.

3.5.2 Bacterial adhesion

Bacterial adhesion onto different biomaterial surfaces was carried out with artificial saliva (Gal et al., 2001) to mimic the physiologic conditions of *S. mutans* growth [paper V]. To evaluate bacterial adhesion, a flow system was used. Briefly, it was a modified Robbins device where disks were fixed to removable ports that allow contact between the surfaces under study and a flow of suspended bacteria in laminar conditions. Before each experiment, the whole system was filled with artificial saliva and preconditioned at 37˚C. Afterwards, the bacterial suspension was allowed to flow through the system at a flow rate of 2 ml/min, corresponding to a shear rate at the wall of the flow chamber of 0.97 s^{-1} (unpublished results). This shear rate is such that the specific interactions that could occur between the cells and the different substrata dominate over the drag force of the fluid flow. Bacteria adhered to the different surfaces were counted using a Live/Dead staining method in which live bacteria, with intact bacterial membranes, fluorescence green, while dead bacteria, with damaged membranes, fluorescence red. However, it has been shown that not all red-stained bacteria correspond to dead cells, as some of them are able to survive in a nutrient rich media. An alternative to this method would be colony counting although it requires removing bacteria from the surfaces, inoculating fresh media plates, incubating the plates and colony counting.

3.5.3 Biofilm formation

Bacterial suspension was cultured on the surfaces at 37˚C and gentle shaking (20 rpm). Biofilm formation was measured using a commercial kit based on ATP quantification [paper V]. A linear relationship has been described between the detected amount of bacterial ATP and the number of viable cells in the biofilm (Fan and Wood, 2007). Then, the biofilm formed on the different surfaces was observed with the SEM. Alternatively, safranin staining is also used to measure biofilm formation but it requires several washing steps and dye liberation, which introduce variability in the assay.

4 DISCUSSION

Oxidative stress and inflammation lie in the middle of many diseases, including periodontal and peri-implant diseases. The biomolecules studied in this thesis were five flavonoids (chrysin, diosmetin, galangin, quercitrin and taxifolin) and two pineal methoxyindoles (melatonin and 5-methoxytryptophol), which were selected for their antioxidant and anti-inflammatory potential (Allegra et al., 2003; García-Lafuente et al., 2009; Pietta, 2000; Reiter et al., 2000; Rodriguez et al., 2004; Rodriguez-Naranjo et al., 2012; Tan et al., 2003; Williams et al., 2004).

Periodontal and peri-implant diseases are the result of an excessive inflammatory process initiated by bacterial accumulation, which end in the destruction of periodontal and peri-implant tissues. Even though some differences exist between tissues supporting natural teeth and dental implants, the ideal periodontal/peri-implant treatment should decrease bacterial infection, damp down the host inflammatory response against bacteria, decrease the excessive production of proinflammatory mediators, destructive enzymes and free radicals and it should stimulate soft and hard tissue regeneration (Giannobile, 2008; Kinane et al., 2011; Preshaw, 2008). Furthermore, the same principles apply to peri-implant healing, i.e. the intended regenerative process may favour the soft and hard tissues integration, which guarantees dental implant success (Kantarci et al., 2015).

4.1 Periodontal and peri-implant tissue regeneration

The first step to achieve periodontal and peri-implant regeneration is to curb bacterial infection. Among the different biomolecules studied in this thesis, only quercitrin showed a moderate anti-bacterial effect on *S. epidermidis* [paper II, Figure 1]. However, previous studies showed that melatonin and different flavonoids have antibacterial effects against different gram-positive and gram-negative bacteria (Srinivasan et al., 2012; Tekbas et al., 2008), indicating that further analysis should be done to confirm or discard the effects of the biomolecules on microbial growth. In a second stage, quercitrin was selected for the biofunctionalization of dental implant abutments. Quercitrin-functionalised surfaces may help to win the “race for the surface” between bacteria and host cells (Gristina, 1987) since they decreased *S. mutans* adhesion while increasing human gingival fibroblast attachment [paper V, Figures 1-2]. It is interesting to highlight that in a sterile implant surgery, the total number of bacteria that infect a wound/surface reaches up to 270 bacteria/cm^2 (Busscher et al., 2012). Therefore, the effect of decreasing bacterial adhesion while increasing cell adhesion may guarantee implant success.

To shift from disease conditions to periodontal and peri-implant tissues regeneration it is required to shorten or minimize the inflammation caused by the continuous presence of pathogens in the oral cavity. Among the different biomolecules screened in a first stage, quercitrin and melatonin showed the strongest anti-inflammatory effects. As

periodontal and peri-implant diseases occur in an inflammatory environment, in a second stage, the effects of quercitrin in inflammatory conditions were evaluated. Using this approach, quercitrin decreased the release of the pro-inflammatory PGE2 when added to the cell culture media [paper III, Figure 2] and when grafted to Ti surfaces [paper V, Figure 4], suggesting its potential use as anti-inflammatory agent for periodontal/peri-implant diseases management (Kats et al., 2013; Tipton et al., 2003). This result is supported by previous studies where quercitrin had anti-inflammatory effects on LPS-stimulated macrophages (Fang et al., 2008) and *in vivo* in different animal models under inflammation (Comalada et al., 2005; Totilo, 2007; Yin et al., 2013). Both quercitrin and commonly used non-steroidal anti-inflammatory drugs inhibit COX2, an enzyme necessary for PGE2 synthesis. Therefore, the anti-inflammatory effects of quercitrin could be compared with those of non-steroidal anti-inflammatory drugs, which have shown beneficial effects in the conventional treatment of periodontal disease in different clinical trials (Kirkwood et al., 2007; Noguchi and Ishikawa, 2007; Salvi and Lang, 2005). However, these treatments only focus on decreasing inflammation while the ultimate goal of periodontal treatment is not only to cease it, but also to regenerate the soft and hard periodontal tissues (Chantarawaratit et al., 2014).

Another objective for periodontal and peri-implant regeneration is to prevent soft tissue breakdown and to promote new tissue formation. The results from this thesis suggest that melatonin, 5-methoxytryptophol, quercitrin and taxifolin may promote soft tissue formation since they increased different ECM related-markers [paper I, II]. In addition, melatonin and quercitrin decreased the levels of pro-fibrotic markers, suggesting that they may produce a structural and functional regenerated tissue rather than a scarred (fibrotic) tissue. Another interesting finding is that melatonin and quercitrin decreased MMP/TIMP ratio, which is related to a reduced MMP-mediated collagen degradation (Soell et al., 2002). Furthermore, quercitrin also showed potential beneficial effects on soft tissue re-arrangement in inflammatory conditions, both, when added to the cell culture media [paper III, Figure 1] and when grafted to Ti surfaces [paper V, Figure 3]. The effects of quercitrin, inhibiting MMP, may be compared to those of doxycycline, a MMP inhibitor that reduced periodontal disease and inhibited early stage bone resorption in different clinical trials. However, prolonged use of doxycycline, even at sub-antimicrobial doses, can lead to the development of antibiotic resistance (Preshaw, 2008; Preshaw et al., 2004). Therefore, quercitrin may represent a safer option for periodontal and peri-implant tissue regeneration.

Quercitrin's anti-inflammatory effect may also promote soft tissue regeneration since excessive inflammation delays regeneration and wound healing processes, resulting in increased scarring and compromising tissue regeneration (Eming et al., 2007; Sculean et al., 2014). Much research efforts in soft tissue regeneration have focused on the direct delivery of growth factors or through gene therapy with promising results. However, the unpredictability of the clinical outcomes probably due to the lack of stability of growth factors has awaked the necessity for alternative options (Anusaksathien and Giannobile, 2002; Li and Jin, 2015; Ramseier et al., 2012; Shin et al., 2015). In this context, quercitrin may represent a promising option to improve soft tissue regeneration.

Further progression of gingivitis and peri-implant mucositis leads to periodontitis and peri-implantitis, which is characterised by alveolar bone resorption. For this reason, it is also important to promote hard tissue formation to achieve a satisfactory periodontal

regeneration treatment. The anabolic action of melatonin on bone metabolism has been proved both *in vitro* and *in vivo*, as it promotes osteoblast proliferation and differentiation, decreases osteoclast-related bone resorption and accelerates osseointegration of dental implants (Cutando et al., 2008; Liu et al., 2013). Since the effects of melatonin on bone formation seem very clear, the potential of quercitrin to enhance bone regeneration was studied in this thesis. Quercitrin enhanced the osteogenic differentiation and increased the mineralization of hMSCs in basal, osteogenic and inflammatory conditions [paper III, Figure 3]. This is in agreement with previous reports where different flavonoids promoted osteoblast differentiation (Choi, 2011; Jeong et al., 2011; Jia et al., 2003; Lee et al., 2011). Quercitrin has also been shown to decrease osteoclast formation *in vitro* (Satué et al., 2013); and to promote the differentiation of MSCs when grafted to Ti surfaces (Córdoba et al., 2015b), suggesting that quercitrin could enhance the osseointegration process of dental implants. These results together with the findings presented in this thesis, point to quercitrin as a potential biomolecule to enhance bone regeneration in periodontitis and peri-implantitis patients.

Biological activity of flavonoids is related to its antioxidant activity, which depends on the substitution pattern of the rings (Amić et al., 2007; Rice-Evans et al., 1996). In fact, quercitrin possesses structural characteristics that have been related to high antioxidant and anti-inflammatory activity. Quercitrin's B-ring catechol group is capable of readily donating hydrogen (electron) to stabilise ROS. Furthermore, its C2-C3 double bound and the 4-oxo group are also important for its antioxidant function. On the other hand, the catechol group together with the 3-OH can chelate transition metal ions such as copper and iron. In this thesis, quercitrin decreased oxidative stress in human gingival fibroblasts [paper II, Figure 4]. Furthermore, flavonoids can also exert its antioxidant activity via the activation and/or upregulation of antioxidant detoxifying enzymes. Actually, quercitrin has been shown to increase enzymatic antioxidant defence systems in different *in vitro* and *in vivo* models (Babujanarthanam et al., 2011; Kim et al., 2015; Yin et al., 2013). However, flavonoids antioxidant activity is unlikely to be the sole explanation for their cellular effects (Williams et al., 2004). Accumulating evidence suggests that their effects are mediated by interactions with signalling pathways (Kim et al., 2004; Trzeciakiewicz et al., 2009). In particular, quercitrin has been shown to have an inhibitory effect on AP-1 and NF-κB pathways, which have central roles regulating cell differentiation and inflammation, among other downstream targets (Dai et al., 2013; Ding et al., 2010). This may explain the positive effects of quercitrin on periodontal tissues regeneration.

4.2 Considerations on soft tissue integration to dental implants

Soft tissue integration can prevent the penetration of oral pathogens towards the bone, protecting bone and implant from biomaterial-related infections and increasing dental implant long-term success. In this thesis, two strategies were used to modify the surface properties of dental implant abutments to enhance soft tissue integration. First, the behaviour of human gingival fibroblasts on Ti and TiZr with different topographies was

compared. In a second stage, taking into account benefits of quercitrin on soft and hard tissue cells, it was used to biofunctionalise Ti surfaces.

When comparing, the response of human gingival fibroblasts to Ti and TiZr surfaces with different topographies, cells preferred polished and machined smooth surfaces that modMA rough surfaces [paper IV, Figure 3-5], in agreement with previous studies (Ponsonnet et al., 2002; Rompen et al., 2006; Wieland et al., 2002). Furthermore, machined surfaces performed better than polished ones probably due to the cell alignment and the less inflammatory and more regenerative profile induced by machined surfaces. Hence, abutment surface with grooved structures may be more promising than polished surfaces in terms of soft tissue regeneration. Moreover, cells aligned to a specific pattern also produce aligned collagen fibre bundles (Kearns et al., 2013). Regarding the material, both Ti and TiZr showed similar biocompatibility, in agreement with previous reports (Linkevicius and Apse, 2008; Pae et al., 2009). Although TiZr showed slightly higher cell adhesion, it also increased MMP/TIMP ratio [paper IV, Figure 7], which is related to collagen breakdown. For this reason and since the availability of TiZr was limited, Ti was used for further studies. Nevertheless, in the case of small diameter implants, TiZr achieves good osseointegration and high success rates both in animal and in clinical studies (Grandin et al., 2012).

Among the different biomolecules studied in this thesis, quercitrin showed the most promising activities in periodontal and peri-implant tissue regeneration. Therefore, the second strategy to improve soft tissue integration consisted in evaluating quercitrin grafted to Ti surfaces. As detailed above, the results suggest that quercitrin-fuctionalised surfaces may decrease bacterial attachment while increasing human gingival fibroblast adhesion. Furthermore, quercitrin-fuctionalised surfaces may also prevent tissue destruction and inflammation while promoting new collagen synthesis. Compared with often-reported monofunctional surface coatings on which bacterial adhesion and biofilm formation is discouraged or they promote host tissue integration, but not both at the same time (Busscher et al., 2012; He et al., 2015), quercitrin-functionalised surfaces have shown multifunctional activities. Furthermore, quercitrin-functionalised surfaces may overcome many shortcomings related to the biocompatibility of some antibiotic-coated surfaces or the lack of stability of growth factors (Anusaksathien and Giannobile, 2002; Zhao et al., 2009).

4.3 Future perspectives and potential applications

The translation of *in vitro* studies to human use requires its confirmation following well-established stages. First, the pre-clinical development should be completed with *in vivo* studies, which should confirm the results of this thesis. A single animal model that represents all aspects of human anatomy and physiology does not exist (Kantarci et al., 2015). Different animal models have been developed to test different aspects of periodontal diseases and wound healing around implants. For example, mouse, rat and rabbit represent cheap and well-defined systems to study inflammation on soft and hard tissues. On the other hand, miniature pigs and dogs are more suitable for biomaterial testing since periodontal tissues and the size of teeth are, in general, similar to those in humans (Kantarci et al., 2015; Schwarz et al., 2007b). Finally, the clinical development

of a product consists in human studies and efficacy evaluation that must accomplish all required criteria for its approval and launch (Trofin et al., 2013).

Inflammation and oxidative stress are behind periodontal disease (Chapple and Matthews, 2007; Waddington et al., 2000). The beneficial effects of antioxidant supplementation (Hirasawa et al., 2002; Hrishi et al., 2015; Iwasaki et al., 2013, 2012; Maruyama et al., 2011; Mathur et al., 2013; Staudte et al., 2005; Tomofuji et al., 2009) and treatment with anti-inflammatory drugs (Kirkwood et al., 2007; Noguchi and Ishikawa, 2007; Salvi and Lang, 2005) on periodontal disease status have been demonstrated in different clinical trials. Unlike these options, however, quercitrin may have a broad range of actions rather than single antioxidant and/or anti-inflammatory activity. Taking into account the results presented in this thesis, it seems possible that quercitrin could promote periodontal and peri-implant tissues regeneration. Different options could be considered for the administration of quercitrin such as toothpastes, mouthrinses or topical application. In fact, antioxidants added to oral hygiene products improve periodontal disease indexes (Battino et al., 2005; Palaska et al., 2013). Furthermore, quercitrin could be used in combination with other biomaterials, i.e. gels, or scaffolds required in severe cases of periodontal and peri-implant diseases.

Taken all together, the results derived from this thesis suggest that antioxidants in general and quercitrin in particular may have a clinical use for periodontal and peri-implant regeneration (**Figure 16**).

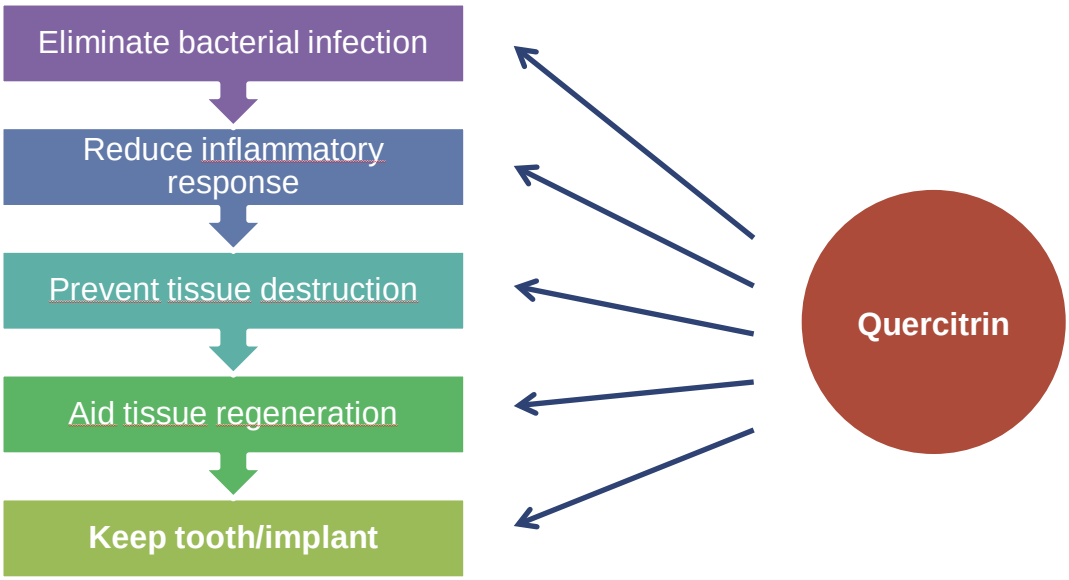

Figure 16. Ideal sequence to achieve periodontal/peri-implant regeneration and potential effects of quercitrin.

CONCLUSIONS

I. Melatonin and quercitrin decrease the inflammatory response and show beneficial effects in extracellular matrix formation and regeneration in human gingival fibroblasts. In addition, quercitrin causes the same positive effects in an inflammatory environment.

II. Quercitrin increases the differentiation of human mesenchymal stem cells in basal, osteogenic and inflammatory conditions.

III. Titanium and titanium-zirconium surfaces are biocompatible with human gingival fibroblasts. Regarding the surface texture, human gingival fibroblasts prefer microgrooved surfaces than polished and rough surfaces, inducing cell alignment and a less inflammatory and more regenerative profile.

IV. Biofunctionalization of titanium with quercitrin decreases *Streptococcus mutans* adhesion while increases human gingival fibroblasts adhesion. Quercitrin-functionalised surfaces also show anti-inflammatory effects and induce extracellular matrix formation and remodelling.

V. All in all, these findings suggest quercitrin as a potential bioactive molecule to enhance both soft and hard periodontal tissue regeneration.

REFERENCES

Abrahamsson, I., Berglundh, T., Glantz, P.O., Lindhe, J., 1998. The mucosal attachment at different abutments. An experimental study in dogs. *J. Clin. Periodontol.* 25, 721–727. doi:10.1111/j.1600-051X.1998.tb02513.x

Abrahamsson, I., Cardaropoli, G., 2007. Peri-implant hard and soft tissue integration to dental implants made of titanium and gold. *Clin. Oral Implants Res.* 18, 269–74. doi:10.1111/j.1600-0501.2006.01326.x

Al-Shaher, A., Wallace, J., Agarwal, S., Bretz, W., Baugh, D., 2004. Effect of propolis on human fibroblasts from the pulp and periodontal ligament. *J. Endod.* 30, 359–61. doi:10.1097/00004770-200405000-00012

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., Walter, P., 2002. Molecular Biology of the Cell. Garland Science, New York, USA.

Allegra, M., Reiter, R.J., Tan, D.-X., Gentile, C., Tesoriere, L., Livrea, M.A., 2003. The chemistry of melatonin's interaction with reactive species. *J. Pineal Res.* 34, 1–10. doi:10.1034/j.1600-079X.2003.02112.x

Almughrabi, O.M., Marzouk, K.M., Hasanato, R.M., Shafik, S.S., 2013. Melatonin levels in periodontal health and disease. *J. Periodontal Res.* 48, 315–21. doi:10.1111/jre.12010

Alvarez, A.L., Melón, S., Dalton, K.P., Nicieza, I., Roque, A., Suárez, B., Parra, F., 2012. Apple pomace, a by-product from the asturian cider industry, inhibits herpes simplex virus types 1 and 2 in vitro replication: study of its mechanisms of action. *J. Med. Food* 15, 581–7. doi:10.1089/jmf.2011.0308

Amić, D., Davidović-Amić, D., Beslo, D., Rastija, V., Lucić, B., Trinajstić, N., 2007. SAR and QSAR of the antioxidant activity of flavonoids. *Curr. Med. Chem.* 14, 827–45. doi:10.2174/092986707780090954

An, J., Zuo, G.Y., Hao, X.Y., Wang, G.C., Li, Z.S., 2011. Antibacterial and synergy of a flavanonol rhamnoside with antibiotics against clinical isolates of methicillin-resistant Staphylococcus aureus (MRSA). *Phytomedicine* 18, 990–993. doi:10.1016/j.phymed.2011.02.013

Anusaksathien, O., Giannobile, W. V, 2002. Growth factor delivery to re-engineer periodontal tissues. *Curr. Pharm. Biotechnol.* 3, 129–139. doi:10.2174/1389201023378391

Ara, T., Honjo, K.-I., Fujinami, Y., Hattori, T., Imamura, Y., Wang, P.-L., 2010. Preventive effects of a kampo medicine, orento on inflammatory responses in lipopolysaccharide treated human gingival fibroblasts. *Biol. Pharm. Bull.* 33, 611–616. doi:10.1248/bpb.33.611

Babujanarthanam, R., Kavitha, P., Mahadeva Rao, U.S., Pandian, M.R., 2011. Quercitrin a bioflavonoid improves the antioxidant status in streptozotocin: induced diabetic rat tissues. *Mol. Cell. Biochem.* 358, 121–9. doi:10.1007/s11010-011-0927-x

Bartold, P.M., Narayanan, a S., 2006. Molecular and cell biology of healthy and diseased periodontal tissues. *Periodontol. 2000* 40, 29–49. doi:10.1111/j.1600-0757.2005.00140.x

Bartold, P.M., Walsh, L.J., Narayanan, a S., 2000. Molecular and cell biology of the gingiva. *Periodontol. 2000* 24, 28–55. doi:10.1034/j.1600-0757.2000.2240103.x

Bates, C., Marino, V., Fazzalari, N.L., Bartold, P.M., 2013. Soft Tissue Attachment to Titanium Implants Coated with Growth Factors. *Clin. Implant Dent. Relat. Res.* 15, 53–63. doi:10.1111/j.1708-8208.2010.00327.x

Battino, M., Ferreiro, M.S., Armeni, T., Politi, a, Bompadre, S., Massoli, a, Bullon, P., 2005. In vitro antioxidant activities of antioxidant-enriched toothpastes. *Free Radic. Res.* 39, 343–50. doi:10.1080/10715760400023853

Bauer, S., Schmuki, P., von der Mark, K., Park, J., 2013. Engineering biocompatible implant surfaces. *Prog. Mater. Sci.* 58, 261–326. doi:10.1016/j.pmatsci.2012.09.001

Berglundh, T., Lindhe, J., Ericsson, I., Marinello, C.P., Liljenberg, B., Thornsen, P., 1991. The soft tissue barrier at implants and teeth. *Clin. Oral Implants Res.* 2, 81–90. doi:10.1034/j.1600-0501.1991.020206.x

Bhatavadekar, N., 2012. Peri-implant soft tissue management: Where are we? *J. Indian Soc. Periodontol.* 16, 623–7. doi:10.4103/0972-124X.106938

Birkedal-Hansen, H., 1993. Role of matrix metalloproteinases in human periodontal diseases. *J. Periodontol.* 64, 474–84. doi:10.1902/jop.1993.64.5s.474

Blasiak, J., Kasznicki, J., Drzewoski, J., Pawlowska, E., Szczepanska, J., Reiter, R.J., 2011. Perspectives on the use of melatonin to reduce cytotoxic and genotoxic effects of methacrylate-based dental materials. *J. Pineal Res.* 51, 157–62. doi:10.1111/j.1600-079X.2011.00877.x

Brånemark, P.I., Adell, R., Breine, U., Hansson, B.O., Lindström, J., Ohlsson, A., 1969. Intra-osseous anchorage of dental prostheses. I. Experimental studies. *Scand. J. Plast. Reconstr. Surg.* 3, 81–100. doi:10.1097/00006534-197107000-00067

Bravo, L., 1998. Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. *Nutr. Rev.* 56, 317–333. doi:10.1111/j.1753-4887.1998.tb01670.x

Brew, K., Dinakarpandian, D., Nagase, H., 2000. Tissue inhibitors of metalloproteinases: evolution, structure and function. *Biochim. Biophys. Acta - Protein Struct. Mol. Enzymol.* 1477, 267–283. doi:10.1016/S0167-4838(99)00279-4

Brunette, D.M., Chehroudi, B., 1999. The effects of the surface topography of micromachined titanium substrata on cell behavior in vitro and in vivo. *J. Biomech. Eng.* 121, 49–57. doi:10.1115/1.2798042

Brunette, D.M., Kenner, G.S., Gould, T.R., 1983. Grooved titanium surfaces orient growth and migration of cells from human gingival explants. *J. Dent. Res.* 62, 1045–1048. doi:10.1177/00220345830620100701

Brzezinski, A., 1997. Melatonin in humans. *N. Engl. J. Med.* 336, 186–195. doi:10.1056/NEJM199701163360306

Bullon, P., Fioroni, M., Goteri, G., Rubini, C., Battino, M., 2004. Immunohistochemical analysis of soft tissues in implants with healthy and peri-implantitis condition, and aggressive periodontitis. *Clin. Oral Implants Res.* 15, 553–559. doi:10.1111/j.1600-0501.2004.01072.x

Busscher, H.J., van der Mei, H.C., Subbiahdoss, G., Jutte, P.C., van den Dungen, J.J. a. M., Zaat, S. a. J., Schultz, M.J., Grainger, D.W., 2012. Biomaterial-Associated Infection: Locating the Finish Line in the Race for the Surface. *Sci. Transl. Med.* 4, 153rv10–153rv10. doi:10.1126/scitranslmed.3004528

Bustin, S., 2000. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J. Mol. Endocrinol.* 25, 169–193. doi:10.1677/jme.0.0250169

Buxadera-Palomero, J., Canal, C., Torrent-Camarero, S., Garrido, B., Javier Gil, F., Rodríguez, D., 2015. Antifouling coatings for dental implants: Polyethylene glycol-like coatings on titanium by plasma polymerization. *Biointerphases* 10, 029505. doi:10.1116/1.4913376

Campoccia, D., Montanaro, L., Arciola, C.R., 2013. A review of the biomaterials technologies for infection-resistant surfaces. *Biomaterials* 34, 8533–8554. doi:10.1016/j.biomaterials.2013.07.089

Cannas, M., Denicolai, F., Webb, L.X., Gristina, A.G., 1988. Bioimplant surfaces: binding of fibronectin and fibroblast adhesion. *J. Orthop. Res.* 6, 58–62. doi:10.1002/jor.1100060108

Carcuac, O., Berglundh, T., 2014. Composition of human peri-implantitis and periodontitis lesions. *J. Dent. Res.* 93, 1083–8. doi:10.1177/0022034514551754

Cekici, A., Kantarci, A., Hasturk, H., Van Dyke, T.E., 2014. Inflammatory and immune pathways in the pathogenesis of periodontal disease. *Periodontol. 2000* 64, 57–80. doi:10.1111/prd.12002

Chang, H.Y., Chi, J.-T., Dudoit, S., Bondre, C., van de Rijn, M., Botstein, D., Brown, P.O., 2002. Diversity, topographic differentiation, and positional memory in human fibroblasts. *Proc. Natl. Acad. Sci. U. S. A.* 99, 12877–82. doi:10.1073/pnas.162488599

Chang, Y.-Y., Lai, C.-H., Hsu, J.-T., Tang, C.-H., Liao, W.-C., Huang, H.-L., 2012. Antibacterial properties and human gingival fibroblast cell compatibility of TiO2/Ag compound coatings and ZnO films on titanium-based material. *Clin. Oral Investig.* 16, 95–100. doi:10.1007/s00784-010-0504-9

Chantarawaratit, P., Sangvanich, P., Banlunara, W., Soontornvipart, K., Thunyakitpisal, P., 2014. Acemannan sponges stimulate alveolar bone, cementum and periodontal ligament regeneration in a canine class II furcation defect model. *J. Periodontal Res.* 49, 164–178. doi:10.1111/jre.12090

Chapple, I.L.C., Matthews, J.B., 2007. The role of reactive oxygen and antioxidant species in periodontal tissue destruction. *Periodontol. 2000* 43, 160–232. doi:10.1111/j.1600-0757.2006.00178.x

Chehroudi, B., Gould, T.R.L., Brunette, D.M., 1990. Titanium-coated micromachined grooves of different dimensions affect epithelial and connective-tissue cells differentlyin vivo. *J. Biomed. Mater. Res.* 24, 1203–1219. doi:10.1002/jbm.820240906

Chen, C.Z., Raghunath, M., 2009. Focus on collagen: in vitro systems to study fibrogenesis and antifibrosis _ state of the art. *Fibrogenes. Tissue Repair* 2, 7. doi:10.1186/1755-1536-2-7

Chen, F.M., Wu, L.A., Zhang, M., Zhang, R., Sun, H.H., 2011. Homing of endogenous stem/progenitor cells for in situ tissue regeneration: Promises, strategies, and translational perspectives. *Biomaterials* 32, 3189–3209. doi:10.1016/j.biomaterials.2010.12.032

Chen, X., Wang, Z., Yang, Z., Wang, J., Xu, Y., Tan, R.-X., Li, E., 2011. Houttuynia cordata blocks HSV infection through inhibition of NF-κB activation. *Antiviral Res.* 92, 341–5. doi:10.1016/j.antiviral.2011.09.005

Choi, E.M., 2011. Honokiol isolated from Magnolia officinalis stimulates osteoblast function and inhibits the release of bone-resorbing mediators. *Int. Immunopharmacol.* 11, 1541–1545. doi:10.1016/j.intimp.2011.05.011

Chomczynski, P., Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162, 156–159. doi:10.1016/0003-2697(87)90021-2

Chou, L., Firth, J.D., Uitto, V.J., Brunette, D.M., 1995. Substratum surface topography alters cell shape and regulates fibronectin mRNA level, mRNA stability, secretion and assembly in human fibroblasts. *J. Cell Sci.* 108, 1563–1573.

Cincin, Z.B., Unlu, M., Kiran, B., Bireller, E.S., Baran, Y., Cakmakoglu, B., 2014. Molecular mechanisms of quercitrin-induced apoptosis in non-small cell lung cancer. *Arch. Med. Res.* 45, 445–54. doi:10.1016/j.arcmed.2014.08.002

Clift, M.J.D., Gehr, P., Rothen-Rutishauser, B., 2011. Nanotoxicology: a perspective and discussion of whether or not in vitro testing is a valid alternative. *Arch. Toxicol.* 85, 723–31. doi:10.1007/s00204-010-0560-6

Comalada, M., Camuesco, D., Sierra, S., Ballester, I., Xaus, J., Gálvez, J., Zarzuelo, A., 2005. In vivo quercitrin anti-inflammatory effect involves release of quercetin, which inhibits inflammation through down-regulation of the NF-κB pathway. *Eur. J. Immunol.* 35, 584–592. doi:10.1002/eji.200425778

Córdoba, A., Monjo, M., Hierro-Oliva, M., González-Martín, M.L., Ramis, J.M., 2015a. Bioinspired quercitrin nanocoatings enhance stem cell adhesion and osteoblast mineralization. *ACS Appl Mater Interfaces*. doi: 10.1021/acsami.5b05044

Córdoba, A., Satué, M., Gómez-Florit, M., Hierro-Oliva, M., Petzold, C., Lyngstadaas, S.P., González-Martín, M.L., Monjo, M., Ramis, J.M., 2015b. Flavonoid-Modified Surfaces: Multifunctional Bioactive Biomaterials with Osteopromotive, Anti-Inflammatory, and Anti-Fibrotic Potential. *Adv. Healthc. Mater.* 4, 540–549. doi:10.1002/adhm.201400587

Crawford, R.J., Webb, H.K., Truong, V.K., Hasan, J., Ivanova, E.P., 2012. Surface topographical factors influencing bacterial attachment. *Adv. Colloid Interface Sci.* 179-182, 142–149. doi:10.1016/j.cis.2012.06.015

Cushnie, T.P.T., Lamb, A.J., 2011. Recent advances in understanding the antibacterial properties of flavonoids. *Int. J. Antimicrob. Agents* 38, 99–107. doi:10.1016/j.ijantimicag.2011.02.014

Cutando, A., Gómez-Moreno, G., Arana, C., Acuña-Castroviejo, D., Reiter, R.J., 2007. Melatonin: potential functions in the oral cavity. *J. Periodontol.* 78, 1094–102. doi:10.1902/jop.2007.060396

Cutando, A., Gómez-Moreno, G., Arana, C., Muñoz, F., Lopez-Peña, M., Stephenson, J., Reiter, R.J., 2008. Melatonin stimulates osteointegration of dental implants. *J. Pineal Res.* 45, 174–9. doi:10.1111/j.1600-079X.2008.00573.x

Daglia, M., 2012. Polyphenols as antimicrobial agents. *Curr. Opin. Biotechnol.* 23, 174–81. doi:10.1016/j.copbio.2011.08.007

Dai, X., Ding, Y., Zhang, Z., Cai, X., Li, Y., 2013. Quercetin and quercitrin protect against cytokine-induced injuries in RINm5F β-cells via the mitochondrial pathway and NF-κB signaling. *Int. J. Mol. Med.* 31, 265–271. doi:10.3892/ijmm.2012.1177

Darby, I.B., Morris, K.H., 2012. A Systematic Review of the Use of Growth Factors in Human Periodontal Regeneration. *J. Periodontol.* 84, 1–14. doi:10.1902/jop.2012.120145

Davey, M.E., O'toole, G. a, 2000. Microbial biofilms: from ecology to molecular genetics. *Microbiol. Mol. Biol. Rev.* 64, 847–867. doi:10.1128/MMBR.64.4.847-867.2000

Dean, J.W., Culbertson, K.C., D'Angelo, A.M., 1995. Fibronectin and laminin enhance gingival cell attachment to dental implant surfaces in vitro. *Int. J. Oral Maxillofac. Implants* 10, 721–728.

Den Braber, E.T., Jansen, H. V., De Boer, M.J., Croes, H.J.E., Elwenspoek, M., Ginsel, L. a, Jansen, J. a, 1998. Scanning electron microscopic, transmission electron microscopic, and confocal laser scanning microscopic observation of fibroblasts cultured on microgrooved surfaces of bulk titanium substrata. *J. Biomed. Mater. Res.* 40, 425–433. doi:10.1002/(SICI)1097-4636(19980605)40:3<425::AID-JBM13>3.0.CO;2-I

Dentino, A., Lee, S., Mailhot, J., Hefti, A.F., 2013. Principles of periodontology. *Periodontol. 2000* 61, 16–53. doi:10.1111/j.1600-0757.2011.00397.x

Derks, J., Tomasi, C., 2015. Peri-implant health and disease. A systematic review of current epidemiology. *J. Clin. Periodontol.* 42, S158–S171. doi:10.1111/jcpe.12334

Ding, M., Zhao, J., Bowman, L., Lu, Y., Shi, X., 2010. Inhibition of AP-1 and MAPK signaling and activation of Nrf2/ARE pathway by quercitrin. *Int. J. Oncol.* 36, 59–67. doi:10.3892/ijo-00000475

Dunn, G.A., Brown, A.F., 1986. Alignment of fibroblasts on grooved surfaces described by a simple geometric transformation. *J. Cell Sci.* 83, 313–340.

Dye, B.A., 2012. Global periodontal disease epidemiology. *Periodontol. 2000* 58, 10–25. doi:10.1111/j.1600-0757.2011.00413.x

Eming, S. a, Krieg, T., Davidson, J.M., 2007. Inflammation in wound repair: molecular and cellular mechanisms. *J. Invest. Dermatol.* 127, 514–525. doi:10.1038/sj.jid.5700701

Esposito, M., Grusovin, M.G., Papanikolaou, N., Coulthard, P., Worthington, H. V, 2009. Enamel matrix derivative (Emdogain(R)) for periodontal tissue regeneration in intrabony defects. *Cochrane database Syst. Rev.* CD003875. doi:10.1002/14651858.CD003875.pub3

European Medicines Agency, 2011. Public summary of opinion on orphan designation. Silibinin-C-2′,3-dihydrogensuccinate, disodium salt for the prevention of recurrent hepatitis C in liver transplant recipients. [WWW Document]. *EMEA*. URL http://www.ema.europa.eu/ema/index.jsp?curl=pages/medicines/human/orphans/2011/01/human_orphan_000877.jsp&mid=WC0b01ac058001d12b&murl=menus/medicines/medicines.jsp (accessed 3.7.15).

Fabjan, N., Rode, J., Kosir, I.J., Wang, Z., Zhang, Z., Kreft, I., 2003. Tartary buckwheat (Fagopyrum tataricum Gaertn.) as a source of dietary rutin and quercitrin. *J. Agric. Food Chem.* 51, 6452–5. doi:10.1021/jf034543e

Fan, F., Wood, K. V, 2007. Bioluminescent assays for high-throughput screening. *Assay Drug Dev. Technol.* 5, 127–136. doi:10.1089/adt.2006.053

Fang, S.-H., Rao, Y.K., Tzeng, Y.-M., 2008. Anti-oxidant and inflammatory mediator's growth inhibitory effects of compounds isolated from Phyllanthus urinaria. *J. Ethnopharmacol.* 116, 333–40. doi:10.1016/j.jep.2007.11.040

Feghali, K., Feldman, M., La, V.D., Santos, J., Grenier, D., 2012. Cranberry proanthocyanidins: natural weapons against periodontal diseases. *J. Agric. Food Chem.* 60, 5728–35. doi:10.1021/jf203304v

Friedman, M., 2007. Overview of antibacterial, antitoxin, antiviral, and antifungal activities of tea flavonoids and teas. *Mol. Nutr. Food Res.* 51, 116–34. doi:10.1002/mnfr.200600173

Furuhashi, I., Iwata, S., Shibata, S., Sato, T., Inoue, H., 2005. Inhibition by licochalcone A, a novel flavonoid isolated from liquorice root, of IL-1beta-induced PGE2 production in human skin fibroblasts. *J. Pharm. Pharmacol.* 57, 1661–6. doi:10.1211/jpp.57.12.0017

Gal, J.Y., Fovet, Y., Adib-Yadzi, M., 2001. About a synthetic saliva for in vitro studies. *Talanta* 53, 1103–1115. doi:10.1016/S0039-9140(00)00618-4

García, J.J., Reiter, R.J., Cabrera, J.J., Pié, J., Mayo, J.C., Sáinz, R.M., Tan, D.X., Qi, W., Acuña-Castroviejo, D., 2000. 5-Methoxytryptophol preserves hepatic microsomal membrane fluidity during oxidative stress. *J. Cell. Biochem.* 76, 651–657. doi:10.1002/(SICI)1097-4644(20000315)76:4<651::AID-JCB13>3.0.CO;2-H

García-Lafuente, A., Guillamón, E., Villares, A., Rostagno, M. a, Martínez, J.A., 2009. Flavonoids as anti-inflammatory agents: Implications in cancer and cardiovascular disease. *Inflamm. Res.* doi:10.1007/s00011-009-0037-3

Garrett, I.R., Boyce, B.F., Oreffo, R.O., Bonewald, L., Poser, J., Mundy, G.R., 1990. Oxygen-derived free radicals stimulate osteoclastic bone resorption in rodent bone in vitro and in vivo. *J. Clin. Invest.* 85, 632–9. doi:10.1172/JCI114485

Gebäck, T., Schulz, M.M.P., Koumoutsakos, P., Detmar, M., Geback, T., 2009. TScratch: a novel and simple software tool for automated analysis of monolayer wound healing assays. *Biotechniques* 46, 265–274. doi:10.2144/000113083

Genco, R.J., Borgnakke, W.S., 2013. Risk factors for periodontal disease. *Periodontol. 2000* 62, 59–94. doi:10.1111/j.1600-0757.2012.00457.x

Gestrelius, S., Lyngstadaas, S.P., Hammarström, L., 2000. Emdogain - periodontal regeneration based on biomimicry. *Clin. Oral Investig.* 4, 120–125. doi:10.1007/s007840050127

Ghosh, S., Hayden, M.S., 2008. New regulators of NF-kappaB in inflammation. *Nat. Rev. Immunol.* 8, 837–48. doi:10.1038/nri2423

Giannobile, W. V, 2008. Host-response therapeutics for periodontal diseases. *J. Periodontol.* 79, 1592–600. doi:10.1902/jop.2008.080174

Ginzinger, D.G., 2002. Gene quantification using real-time quantitative PCR. *Exp. Hematol.* 30, 503–512. doi:10.1016/S0301-472X(02)00806-8

Golub, E.E., Harrison, G., Taylor, A.G., Camper, S., Shapiro, I.M., 1992. The role of alkaline phosphatase in cartilage mineralization. *Bone Miner.* 17, 273–278. doi:10.1016/0169-6009(92)90750-8

Golub, L.M., Suomalainen, K., Sorsa, T., 1992. Host modulation with tetracyclines and their chemically modified analogues. *Curr. Opin. Dent.* 2, 80–90.

Gomez-Florit, M., Xing, R., Ramis, J.M., Taxt-Lamolle, S., Haugen, H.J., Lyngstadaas, S.P., Monjo, M., 2014. Human gingival fibroblasts function is stimulated on machined hydrided titanium zirconium dental implants. *J. Dent.* 42, 30–8. doi:10.1016/j.jdent.2013.11.003

Gómez-Moreno, G., Cutando-Soriano, a, Arana, C., Galindo, P., Bolaños, J., Acuña-Castroviejo, D., Wang, H.-L., 2007. Melatonin expression in periodontal disease. *J. Periodontal Res.* 42, 536–40. doi:10.1111/j.1600-0765.2007.00978.x

Gómez-Moreno, G., Guardia, J., Ferrera, M.J., Cutando, a, Reiter, R.J., 2010. Melatonin in diseases of the oral cavity. *Oral Dis.* 16, 242–7. doi:10.1111/j.1601-0825.2009.01610.x

Grandin, H.M., Berner, S., Dard, M., 2012. A Review of Titanium Zirconium (TiZr) Alloys for Use in Endosseous Dental Implants. *Materials (Basel).* 5, 1348–1360. doi:10.3390/ma5081348

Graves, D., 2008. Cytokines that promote periodontal tissue destruction. *J. Periodontol.* 79, 1585–91. doi:10.1902/jop.2008.080183

Graves, D.T., Oates, T., Garlet, G.P., 2011. Review of osteoimmunology and the host response in endodontic and periodontal lesions. *J. Oral Microbiol.* 3, 1–15. doi:10.3402/jom.v3i0.5304

Gristina, A., 1987. Biomaterial-centered infection: microbial adhesion versus tissue integration. *Science (80-.).* 237, 1588–1595. doi:10.1126/science.3629258

Grössner-Schreiber, B., Herzog, M., Hedderich, J., Dück, A., Hannig, M., Griepentrog, M., 2006. Focal adhesion contact formation by fibroblasts cultured on surface-modified dental implants: an in vitro study. *Clin. Oral Implants Res.* 17, 736–45. doi:10.1111/j.1600-0501.2006.01277.x

Gruber, R., Bosshardt, D.D., 2015. Dental Implantology and Implants - Tissue Interface, in: Stem Cell Biology and Tissue Engineering in Dental Sciences. Elsevier Inc., pp. 735–747. doi:10.1016/B978-0-12-397157-9.00078-3

Guida, L., Oliva, A., Basile, M.A., Giordano, M., Nastri, L., Annunziata, M., 2013. Human gingival fibroblast functions are stimulated by oxidized nano-structured titanium surfaces. *J. Dent.* 41, 900–7. doi:10.1016/j.jdent.2013.07.009

Guillem-Marti, J., Delgado, L., Godoy-Gallardo, M., Pegueroles, M., Herrero, M., Gil, F.J., 2013. Fibroblast adhesion and activation onto micro-machined titanium surfaces. *Clin. Oral Implants Res.* 24, 770–80. doi:10.1111/j.1600-0501.2012.02451.x

Gurtner, G.C., Werner, S., Barrandon, Y., Longaker, M.T., 2008. Wound repair and regeneration. *Nature* 453, 314–321. doi:10.1038/nature07039

Gutiérrez-Venegas, G., Contreras-Sánchez, A., 2013. Luteolin and fisetin inhibit the effects of lipopolysaccharide obtained from Porphyromonas gingivalis in human gingival fibroblasts. *Mol. Biol. Rep.* 40, 477–85. doi:10.1007/s11033-012-2083-0

Gutiérrez-Venegas, G., Jiménez-Estrada, M., Maldonado, S., 2007. The effect of flavonoids on transduction mechanisms in lipopolysaccharide-treated human gingival fibroblasts. *Int. Immunopharmacol.* 7, 1199–210. doi:10.1016/j.intimp.2007.05.004

Gutiérrez-Venegas, G., Luna, O.A., Arreguín-Cano, J.A., Hernández-Bermúdez, C., 2014. Myricetin blocks lipoteichoic acid-induced COX-2 expression in human gingival fibroblasts. *Cell. Mol. Biol. Lett.* 19, 126–39. doi:10.2478/s11658-014-0186-4

Ha, K.-Y., Chung, Y.-G., Ryoo, S.-J., 2005. Adherence and biofilm formation of Staphylococcus epidermidis and Mycobacterium tuberculosis on various spinal implants. *Spine (Phila. Pa. 1976).* 30, 38–43. doi:00007632-200501010-00008 [pii]

Habibovic, P., Woodfield, T., Groot, K. De, 2007. Tissue Engineering, Advances in Experimental Medicine and Biology. Springer US, Boston, MA. doi:10.1007/978-0-387-34133-0

Häkkinen, L., Strassburger, S., Kähäri, V.M., Scott, P.G., Eichstetter, I., Lozzo, R. V, Larjava, H., 2000a. A role for decorin in the structural organization of periodontal ligament. *Lab. Invest.* 80, 1869–1880. doi:10.1038/labinvest.3780197

Häkkinen, L., Uitto, V.J., Larjava, H., 2000b. Cell biology of gingival wound healing. *Periodontol. 2000* 24, 127–152. doi:10.1034/j.1600-0757.2000.2240107.x

Ham, Y.-M., Yoon, W.-J., Park, S.-Y., Song, G.-P., Jung, Y.-H., Jeon, Y.-J., Kang, S.-M., Kim, K.-N., 2012. Quercitrin protects against oxidative stress-induced injury in lung fibroblast cells via up-regulation of Bcl-xL. *J. Funct. Foods* 4, 253–262. doi:10.1016/j.jff.2011.12.001

Hamoudová, R., Urbánek, M., Pospíšilová, M., Polášek, M., 2004. Assay of phenolic compounds in red wine by on-line combination of capillary isotachophoresis with capillary zone electrophoresis. *J. Chromatogr. A* 1032, 281–287. doi:10.1016/j.chroma.2004.01.014

Han, J., Menicanin, D., Gronthos, S., Bartold, P., 2013. Stem cells, tissue engineering and periodontal regeneration. *Aust. Dent. J.* 1–14. doi:10.1111/adj.12100

Hassell, T.M., 1993. Tissues and cells of the periodontium. *Periodontol. 2000* 3, 9–38. doi:10.1111/j.1600-0757.1993.tb00230.x

He, T., Zhang, Y., Lai, A.C.K., Chan, V., 2015. Engineering bio-adhesive functions in an antimicrobial polymer multilayer. *Biomed. Mater.* 10, 015015. doi:10.1088/1748-6041/10/1/015015

Heitz-Mayfield, L.J. a, Lang, N.P., 2013. Surgical and nonsurgical periodontal therapy. Learned and unlearned concepts. *Periodontol. 2000* 62, 218–31. doi:10.1111/prd.12008

Hernández, M., Dutzan, N., García-Sesnich, J., Abusleme, L., Dezerega, a, Silva, N., González, F.E., Vernal, R., Sorsa, T., Gamonal, J., 2011. Host-pathogen interactions in progressive chronic periodontitis. *J. Dent. Res.* 90, 1164–70. doi:10.1177/0022034511401405

Hinz, B., Dugina, V., Ballestrem, C., Wehrle-Haller, B., Chaponnier, C., 2003. Alpha-smooth muscle actin is crucial for focal adhesion maturation in myofibroblasts. *Mol. Biol. Cell* 14, 2508–19. doi:10.1091/mbc.E02-11-0729

Hinz, B., Phan, S.H., Thannickal, V.J., Galli, A., Bochaton-Piallat, M.-L., Gabbiani, G., 2007. The myofibroblast: one function, multiple origins. *Am. J. Pathol.* 170, 1807–16. doi:10.2353/ajpath.2007.070112

Hirasawa, M., Takada, K., Makimura, M., Otake, S., 2002. Improvement of periodontal status by green tea catechin using a local delivery system: a clinical pilot study., Journal of periodontal research. doi:1o640 [pii]

Ho, W.-F., Chen, W.-K., Wu, S.-C., Hsu, H.-C., 2008. Structure, mechanical properties, and grindability of dental Ti-Zr alloys. *J. Mater. Sci. Mater. Med.* 19, 3179–86. doi:10.1007/s10856-008-3454-x

Holmberg, K. V, Abdolhosseini, M., Li, Y., Chen, X., Gorr, S.-U., Aparicio, C., 2013. Bio-inspired stable antimicrobial peptide coatings for dental applications. *Acta Biomater.* 9, 8224–31. doi:10.1016/j.actbio.2013.06.017

Hormia, M., Könönen, M., 1994. Immunolocalization of fibronectin and vitronectin receptors in human gingival fibroblasts spreading on titanium surfaces. *J. Periodontal Res.* 29, 146–152. doi:10.1111/j.1600-0765.1994.tb01103.x

Hosokawa, Y., Hosokawa, I., Shindo, S., Ozaki, K., Nakanishi, T., Nakae, H., Matsuo, T., 2011. Black tea polyphenol inhibits CXCL10 production in oncostatin M-stimulated human gingival fibroblasts. *Int. Immunopharmacol.* 11, 670–4. doi:10.1016/j.intimp.2011.01.009

Hrishi, T., Kundapur, P., Naha, A., Thomas, B., Kamath, S., Bhat, G., 2015. Effect of adjunctive use of green tea dentifrice in periodontitis patients - A Randomized Controlled Pilot Study. *Int. J. Dent. Hyg.* doi:10.1111/idh.12131

Hughes, F.J., 2015. Periodontium and Periodontal Disease, in: Stem Cell Biology and Tissue Engineering in Dental Sciences. Elsevier Inc., pp. 433–444. doi:10.1016/B978-0-12-397157-9.00038-2

Iwasaki, M., Manz, M.C., Taylor, G.W., Yoshihara, A., Miyazaki, H., 2012. Relations of serum ascorbic acid and α-tocopherol to periodontal disease. *J. Dent. Res.* 91, 167–72. doi:10.1177/0022034511431702

Iwasaki, M., Moynihan, P., Manz, M.C., Taylor, G.W., Yoshihara, A., Muramatsu, K., Watanabe, R., Miyazaki, H., 2013. Dietary antioxidants and periodontal disease in community-based older Japanese: a 2-year follow-up study. *Public Health Nutr.* 16, 330–8. doi:10.1017/S1368980012002637

Izzi, V., Masuelli, L., Tresoldi, I., Sacchetti, P., Modesti, A., Galvano, F., Bei, R., 2012. The effects of dietary flavonoids on the regulation of redox inflammatory networks. *Front. Biosci. (Landmark Ed.* 17, 2396–418. doi:10.2741/4061

Jaiswal, N., Haynesworth, S.E., Caplan, A.I., Bruder, S.P., 1997. Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells in vitro. *J. Cell. Biochem.* 64, 295–312. doi:10.1002/(SICI)1097-4644(199702)64:2<295::AID-JCB12>3.0.CO;2-I

Jeong, H.M., Han, E.H., Jin, Y.H., Choi, Y.H., Lee, K.Y., Jeong, H.G., 2011. Xanthohumol from the hop plant stimulates osteoblast differentiation by RUNX2 activation. *Biochem. Biophys. Res. Commun.* 409, 82–89. doi:10.1016/j.bbrc.2011.04.113

Jia, T.L., Wang, H.Z., Xie, L.P., Wang, X.Y., Zhang, R.Q., 2003. Daidzein enhances osteoblast growth that may be mediated by increased bone morphogenetic protein (BMP) production. *Biochem. Pharmacol.* 65, 709–715. doi:10.1016/S0006-2952(02)01585-X

Jin, C., Ren, L., Ding, H., Shi, G., Lin, H., Zhang, F., 2012. Enhanced attachment, proliferation, and differentiation of human gingival fibroblasts on titanium surface modified with biomolecules. *J. Biomed. Mater. Res. B. Appl. Biomater.* 100, 2167–77. doi:10.1002/jbm.b.32784

Jung, R.E., Pjetursson, B.E., Glauser, R., Zembic, A., Zwahlen, M., Lang, N.P., 2008. A systematic review of the 5-year survival and complication rates of implant-supported single crowns. *Clin. Oral Implants Res.* 19, 119–30. doi:10.1111/j.1600-0501.2007.01453.x

Kähkönen, M.P., Hopia, a I., Vuorela, H.J., Rauha, J.P., Pihlaja, K., Kujala, T.S., Heinonen, M., 1999. Antioxidant activity of plant extracts containing phenolic compounds. *J. Agric. Food Chem.* 47, 3954–3962. doi:10.1021/jf990146l

Kaigler, D., Avila, G., Wisner-Lynch, L., Nevins, M.L., Nevins, M., Rasperini, G., Lynch, S.E., Giannobile, W. V, 2011. Platelet-derived growth factor applications in periodontal and peri-implant bone regeneration. *Expert Opin. Biol. Ther.* 11, 375–385. doi:10.1517/14712598.2011.554814

Kajiya, M., Giro, G., Taubman, M. a, Han, X., Mayer, M.P. a, Kawai, T., 2010. Role of periodontal pathogenic bacteria in RANKL-mediated bone destruction in periodontal disease. *J. Oral Microbiol.* 2, 1–16. doi:10.3402/jom.v2i0.5532

Kantarci, A., Hasturk, H., Van Dyke, T.E., 2015. Animal models for periodontal regeneration and peri-implant responses. *Periodontol. 2000* 68, 66–82. doi:10.1111/prd.12052

Kassebaum, N.J., Bernabé, E., Dahiya, M., Bhandari, B., Murray, C.J.L., Marcenes, W., 2014. Global Burden of Severe Tooth Loss: A Systematic Review and Meta-analysis. *J. Dent. Res.* 93, 20S–28S. doi:10.1177/0022034514537828

Kats, A., Båge, T., Georgsson, P., Jönsson, J., Quezada, H.C., Gustafsson, A., Jansson, L., Lindberg, C., Näsström, K., Yucel-Lindberg, T., 2013. Inhibition of microsomal prostaglandin E synthase-1 by aminothiazoles decreases prostaglandin E2 synthesis in vitro and ameliorates experimental periodontitis in vivo. *FASEB J.* 27, 2328–41. doi:10.1096/fj.12-214445

Kawaguchi, H., Hirachi, A., Hasegawa, N., Iwata, T., Hamaguchi, H., Shiba, H., Takata, T., Kato, Y., Kurihara, H., 2004. Enhancement of periodontal tissue regeneration by transplantation of bone marrow mesenchymal stem cells. *J. Periodontol.* 75, 1281–1287. doi:10.1902/jop.2004.75.9.1281

Kearns, V.R., Williams, R.L., Mirvakily, F., Doherty, P.J., Martin, N., Vr, K., Rl, W., Pj, D., Guided, M.N., Rachel, L., 2013. Guided gingival fibroblast attachment to titanium surfaces: an in vitro study. *J. Clin. Periodontol.* 40, 99–108. doi:10.1111/jcpe.12025

Khan, M.A., Williams, R.L., Williams, D.F., 1999. Conjoint corrosion and wear in titanium alloys. *Biomaterials* 20, 765–772. doi:10.1016/S0142-9612(98)00229-4

Kida, Y., Kobayashi, M., Suzuki, T., Takeshita, A., Okamatsu, Y., Hanazawa, S., Yasui, T., Hasegawa, K., 2005. Interleukin-1 stimulates cytokines, prostaglandin E2 and matrix metalloproteinase-1 production via activation of MAPK/AP-1 and NF-kappaB in human gingival fibroblasts. *Cytokine* 29, 159–68. doi:10.1016/j.cyto.2004.10.009

Kim, H.-Y., Yeo, I.-S., Lee, J.-B., Kim, S.-H., Kim, D.-J., Han, J.-S., 2012. Initial in vitro bacterial adhesion on dental restorative materials. *Int. J. Artif. Organs* 35, 773–79. doi:10.5301/ijao.5000153

Kim, H.P., Son, K.H., Chang, H.W., Kang, S.S., 2004. Anti-inflammatory Plant Flavonoids and Cellular Action Mechanisms. *J. Pharmacol. Sci.* 96, 229–245. doi:10.1254/jphs.CRJ04003X

Kim, J.W., Yang, H., Cho, N., Kim, B., Kim, Y.C., Sung, S.H., 2015. Hepatoprotective constituents of Firmiana simplex stem bark against ethanol insult to primary rat hepatocytes. *Pharmacogn. Mag.* 11, 55–60. doi:10.4103/0973-1296.149704

Kim, S.-Y., Oh, N., Lee, M.-H., Kim, S.-E., Leesungbok, R., Lee, S.-W., 2009. Surface microgrooves and acid etching on titanium substrata alter various cell behaviors of cultured human gingival fibroblasts. *Clin. Oral Implants Res.* 20, 262–72. doi:10.1111/j.1600-0501.2008.01652.x

Kinane, D.F., Attström, R., 2005. Advances in the pathogenesis of periodontitis. Group B consensus report of the fifth European Workshop in Periodontology. *J. Clin. Periodontol.* 32 Suppl 6, 130–131. doi:10.1111/j.1600-051X.2005.00823.x

Kinane, D.F., Preshaw, P.M., Loos, B.G., 2011. Host-response: understanding the cellular and molecular mechanisms of host-microbial interactions--consensus of the Seventh European Workshop on Periodontology. *J. Clin. Periodontol.* 38 Suppl 1, 44–8. doi:10.1111/j.1600-051X.2010.01682.x

Kirkwood, K.L., Cirelli, J. a, Rogers, J.E., Giannobile, W. V, 2007. Novel host response therapeutic approaches to treat periodontal diseases. *Periodontol. 2000* 43, 294–315. doi:10.1111/j.1600-0757.2006.00166.x

Kobayashi, E., Matsumoto, S., Doi, H., Yoneyama, T., Hamanaka, H., 1995. Mechanical properties of the binary titanium-zirconium alloys and their potential for biomedical materials. *J. Biomed. Mater. Res.* 29, 943–50. doi:10.1002/jbm.820290805

Koeberle, A., Werz, O., 2014. Multi-target approach for natural products in inflammation. *Drug Discov. Today* 19, 1871–1882. doi:10.1016/j.drudis.2014.08.006

Kokubu, E., Hamilton, D.W., Inoue, T., Brunette, D.M., 2009. Modulation of human gingival fibroblast adhesion, morphology, tyrosine phosphorylation, and ERK 1/2 localization on polished, grooved and SLA substratum topographies. *J. Biomed. Mater. Res. A* 91, 663–70. doi:10.1002/jbm.a.32273

Kramer, P.R., Nares, S., Kramer, S.F., Grogan, D., Kaiser, M., 2004. Mesenchymal stem cells acquire characteristics of cells in the periodontal ligament in vitro. *J. Dent. Res.* 83, 27–34. doi:10.1177/154405910408300106

Lamolle, S.F., Monjo, M., Rubert, M., Haugen, H.J., Lyngstadaas, S.P., Ellingsen, J.E., 2009. The effect of hydrofluoric acid treatment of titanium surface on nanostructural and chemical changes and the growth of MC3T3-E1 cells. *Biomaterials* 30, 736–742. doi:10.1016/j.biomaterials.2008.10.052

Lampugnani, M.G., 1999. Cell migration into a wounded area in vitro. *Methods Mol. Biol.* 96, 177–82. doi:10.1385/1-59259-258-9:177

Langenbach, F., Handschel, J., 2013. Effects of dexamethasone, ascorbic acid and β-glycerophosphate on the osteogenic differentiation of stem cells in vitro. *Stem Cell Res. Ther.* 4, 117. doi:10.1186/scrt328

Lee, C.H., Huang, Y.L., Liao, J.F., Chiou, W.F., 2011. Ugonin K promotes osteoblastic differentiation and mineralization by activation of p38 MAPK- and ERK-mediated

expression of Runx2 and osterix. *Eur. J. Pharmacol.* 668, 383–389. doi:10.1016/j.ejphar.2011.06.059

Lee, S.-W., Kim, S.-Y., Lee, M.-H., Lee, K.-W., Leesungbok, R., Oh, N., 2009. Influence of etched microgrooves of uniform dimension on in vitro responses of human gingival fibroblasts. *Clin. Oral Implants Res.* 20, 458–66. doi:10.1111/j.1600-0501.2008.01671.x

Lequin, R.M., 2005. Enzyme immunoassay (EIA)/enzyme-linked immunosorbent assay (ELISA). *Clin. Chem.* 51, 2415–8. doi:10.1373/clinchem.2005.051532

Lévesque, C.M., Voronejskaia, E., Huang, Y.C.C., Mair, R.W., Ellen, R.P., Cvitkovitch, D.G., 2005. Involvement of sortase anchoring of cell wall proteins in biofilm formation by Streptococcus mutans. *Infect. Immun.* 73, 3773–3777. doi:10.1128/IAI.73.6.3773-3777.2005

Li, B., Jin, Y., 2015. Periodontal Tissue Engineering : Current Approaches and Future Therapies, in: Stem Cell Biology and Tissue Engineering in Dental Sciences. Elsevier Inc., pp. 471–482. doi:10.1016/B978-0-12-397157-9.00041-2

Liang, C.-C., Park, A.Y., Guan, J.-L., 2007. In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. *Nat. Protoc.* 2, 329–33. doi:10.1038/nprot.2007.30

Lieder, R., Petersen, P.H., Sigurjónsson, Ó.E., 2013. Endotoxins-the invisible companion in biomaterials research. *Tissue Eng. Part B. Rev.* 19, 391–402. doi:10.1089/ten.TEB.2012.0636

Lindhe, J., Meyle, J., 2008. Peri-implant diseases: Consensus Report of the Sixth European Workshop on Periodontology. *J. Clin. Periodontol.* 35, 282–5. doi:10.1111/j.1600-051X.2008.01283.x

Linkevicius, T., Apse, P., 2008. Influence of abutment material on stability of peri-implant tissues: a systematic review. *Int. J. Oral Maxillofac. Implants* 23, 449–456. doi:10.1016/S0022-3913(09)60012-3

Lissoni, P., Fumagalli, L., Paolorossi, F., Rovelli, F., Roselli, M.G., Maestroni, G.J.M., 1997. Anticancer neuroimmunomodulation by pineal hormones other than melatonin: Preliminary phase II study of the pineal indole 5-methoxytryptophol in association with low-dose IL-2 and melatonin. *J. Biol. Regul. Homeost. Agents* 11, 119–122.

Lissoni, P., Pittalis, S., Rovelli, F., Zecchini, S., Casati, M., Tremolada, M., Pelizzoni, F., 1996. Immunomodulatory properties of a pineal indole hormone other than melatonin, the 5-methoxytryptophol. *J. Biol. Regul. Homeost. Agents* 10, 27–30.

Lissoni, P., Rovelli, F., Frassineti, A., Fumagalli, L., Malysheva, O., Conti, A., Maestroni, G., 2000. Oncostatic activity of pineal neuroendocrine treatment with the pineal indoles melatonin and 5-methoxytryptamine in untreatable metastatic

cancer patients progressing on melatonin alone. *Neuroendocrinol. Lett.* 21, 319–323.

Liu, J., Huang, F., He, H.-W., 2013. Melatonin effects on hard tissues: bone and tooth. *Int. J. Mol. Sci.* 14, 10063–74. doi:10.3390/ijms140510063

Liu, X., Chu, P., Ding, C., 2004. Surface modification of titanium, titanium alloys, and related materials for biomedical applications. *Mater. Sci. Eng. R Reports* 47, 49–121. doi:10.1016/j.mser.2004.11.001

Luo, C., Wang, A., Wang, X., Li, J., Liu, H., Wang, M., Wang, L., Lai, D., Zhou, L., 2015. A new proline-containing flavonol glycoside from Caragana leucophloea Pojark. *Nat. Prod. Res.* 1–9. doi:10.1080/14786419.2015.1007974

Lyngstadaas, S.P., Lundberg, E., Ekdahl, H., Andersson, C., Gestrelius, S., 2001. Autocrine growth factors in human periodontal ligament cells cultured on enamel matrix derivative. *J. Clin. Periodontol.* 28, 181–8. doi:10.1034/j.1600-051x.2001.028002181.x

Mack, D., Rohde, H., Harris, L.G., Davies, A.P., Horstkotte, M.A., Knobloch, J.K.M., 2006. Biofilm formation in medical device-related infection. *Int. J. Artif. Organs* 29, 343–359.

Mahanonda, R., Pichyangkul, S., 2007. Toll-like receptors and their role in periodontal health and disease. *Periodontol. 2000* 43, 41–55. doi:10.1111/j.1600-0757.2006.00179.x

Manach, C., Scalbert, A., Morand, C., Rémésy, C., Jiménez, L., 2004. Polyphenols: Food sources and bioavailability. *Am. J. Clin. Nutr.*

Manwaring, M.E., Walsh, J.F., Tresco, P. a., 2004. Contact guidance induced organization of extracellular matrix. *Biomaterials* 25, 3631–3638. doi:10.1016/j.biomaterials.2003.10.043

Marín-Pareja, N., Salvagni, E., Guillem-Marti, J., Aparicio, C., Ginebra, M.-P., 2014. Collagen-functionalised titanium surfaces for biological sealing of dental implants: Effect of immobilisation process on fibroblasts response. *Colloids Surfaces B Biointerfaces* 122, 601–610. doi:10.1016/j.colsurfb.2014.07.038

Martin, H.J., Schulz, K.H., Bumgardner, J.D., Walters, K.B., 2007. XPS study on the use of 3-aminopropyltriethoxysilane to bond chitosan to a titanium surface. *Langmuir* 23, 6645–51. doi:10.1021/la063284v

Maruyama, T., Tomofuji, T., Endo, Y., Irie, K., Azuma, T., Ekuni, D., Tamaki, N., Yamamoto, T., Morita, M., 2011. Supplementation of green tea catechins in dentifrices suppresses gingival oxidative stress and periodontal inflammation. *Arch. Oral Biol.* 56, 48–53. doi:10.1016/j.archoralbio.2010.08.015

Mathur, A., Mathur, L., Manohar, B., Mathur, H., Shankarapillai, R., Shetty, N., Bhatia, A., 2013. Antioxidant therapy as monotherapy or as an adjunct to treatment of

periodontal diseases. *J. Indian Soc. Periodontol.* 17, 21–4. doi:10.4103/0972-124X.107469

McKnight, H., Kelsey, W.P., Hooper, D.A., Hart, T.C., Mariotti, A., 2014. Proteomic analyses of human gingival and periodontal ligament fibroblasts. *J. Periodontol.* 85, 810–8. doi:10.1902/jop.2013.130161

Mei, S., Wang, H., Wang, W., Tong, L., Pan, H., Ruan, C., Ma, Q., Liu, M., Yang, H., Zhang, L., Cheng, Y., Zhang, Y., Zhao, L., Chu, P.K., 2014. Antibacterial effects and biocompatibility of titanium surfaces with graded silver incorporation in titania nanotubes. *Biomaterials* 35, 4255–65. doi:10.1016/j.biomaterials.2014.02.005

Middleton, E., Kandaswami, C., Theoharides, T.C., 2000. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. *Pharmacol. Rev.* 52, 673–751.

Mogi, M., Otogoto, J., Ota, N., Togari, A., 2004. Differential expression of RANKL and osteoprotegerin in gingival crevicular fluid of patients with periodontitis. *J. Dent. Res.* 83, 166–169. doi:10.1177/154405910408300216

Moharamzadeh, K., Brook, I.M., Van Noort, R., Scutt, a. M., Thornhill, M.H., 2007. Tissue-engineered Oral Mucosa: a Review of the Scientific Literature. *J. Dent. Res.* 86, 115–124. doi:10.1177/154405910708600203

Monsarrat, P., Vergnes, J.-N., Nabet, C., Sixou, M., Snead, M.L., Planat-Bénard, V., Casteilla, L., Kémoun, P., 2014. Concise Review: Mesenchymal stromal cells used for periodontal regeneration: a systematic review. *Stem Cells Transl. Med.* 3, 768–774. doi:10.5966/sctm.2013-0183

Nablo, B.J., Rothrock, A.R., Schoenfisch, M.H., 2005. Nitric oxide-releasing sol-gels as antibacterial coatings for orthopedic implants. *Biomaterials* 26, 917–924. doi:10.1016/j.biomaterials.2004.03.031

Nagai, M., Hayakawa, T., Fukatsu, A., Yamamoto, M., Fukumoto, M., Nagahama, F., Mishima, H., Yoshinari, M., Nemoto, K., Kato, T., 2002. In vitro study of collagen coating of titanium implants for initial cell attachment. *Dent. Mater. J.* 21, 250–260. doi:10.4012/dmj.21.250

Naik, E., Dixit, V.M., 2011. Mitochondrial reactive oxygen species drive proinflammatory cytokine production. *J. Exp. Med.* 208, 417–20. doi:10.1084/jem.20110367

Noguchi, K., Ishikawa, I., 2007. The roles of cyclooxygenase-2 and prostaglandin E2 in periodontal disease. *Periodontol. 2000* 43, 85–101. doi:10.1111/j.1600-0757.2006.00170.x

Nokhbehsaim, M., Winter, J., Rath, B., Jäger, A., Jepsen, S., Deschner, J., 2011. Effects of enamel matrix derivative on periodontal wound healing in an inflammatory environment in vitro. *J. Clin. Periodontol.* 38, 479–90. doi:10.1111/j.1600-051X.2010.01696.x

Okawachi, H., Ayukawa, Y., Atsuta, I., Furuhashi, A., Sakaguchi, M., Yamane, K., Koyano, K., 2012. Effect of titanium surface calcium and magnesium on adhesive activity of epithelial-like cells and fibroblasts. *Biointerphases* 7, 27. doi:10.1007/s13758-012-0027-9

Ono, M., Kantoh, K., Ueki, J., Shimada, A., Wakabayashi, H., Matsuta, T., Sakagami, H., Kumada, H., Hamada, N., Kitajima, M., Oizumi, H., Oizumi, T., 2011. Quest for anti-inflammatory substances using IL-1β-stimulated gingival fibroblasts. *In Vivo* 25, 763–8.

Ouzir, M., Bouhaddou, N., Khalki, H., Lakhdar-Ghazal, N., 2013. Physiological and pharmacological properties of 5-methoxytryptophol. *Expert Rev. Endocrinol. Metab.* 8, 355–364. doi:10.1586/17446651.2013.811866

Pae, A., Lee, H., Kim, H.-S., Kwon, Y.-D., Woo, Y.-H., 2009. Attachment and growth behaviour of human gingival fibroblasts on titanium and zirconia ceramic surfaces. *Biomed. Mater.* 4, 025005. doi:10.1088/1748-6041/4/2/025005

Page, R.C., Schroeder, H.E., 1976. Pathogenesis of inflammatory periodontal disease. A summary of current work. *Lab. Invest.* 34, 235–249.

Palaiologou, A.A., Yukna, R.A., Moses, R., Lallier, T.E., 2001. Gingival, dermal, and periodontal ligament fibroblasts express different extracellular matrix receptors. *J. Periodontol.* 72, 798–807. doi:10.1902/jop.2001.72.6.798

Palaska, I., Papathanasiou, E., Theoharides, T.C., 2013. Use of polyphenols in periodontal inflammation. *Eur. J. Pharmacol.* 720, 77–83. doi:10.1016/j.ejphar.2013.10.047

Paster, B.J., Olsen, I., Aas, J.A., Dewhirst, F.E., 2006. The breadth of bacterial diversity in the human periodontal pocket and other oral sites. *Periodontol. 2000* 42, 80–7. doi:10.1111/j.1600-0757.2006.00174.x

Petti, S., Scully, C., 2009. Polyphenols, oral health and disease: A review. *J. Dent.* 37, 413–23. doi:10.1016/j.jdent.2009.02.003

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 29, e45. doi:10.1093/nar/29.9.e45

Pfaffl, M.W., Tichopad, A., Prgomet, C., Neuvians, T.P., 2004. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper - Excel-based tool using pair-wise correlations. *Biotechnol. Lett.* 26, 509–515. doi:10.1023/B:BILE.0000019559.84305.47

Pietta, P.G., 2000. Flavonoids as antioxidants. *J. Nat. Prod.* doi:10.1021/np9904509

Pittenger, M.F., Mackay, a M., Beck, S.C., Jaiswal, R.K., Douglas, R., Mosca, J.D., Moorman, M. a, Simonetti, D.W., Craig, S., Marshak, D.R., 1999. Multilineage potential of adult human mesenchymal stem cells. *Science (80-.).* 284, 143–147. doi:10.1126/science.284.5411.143

Ponsonnet, L., Comte, V., Othmane, A., Lagneau, C., Charbonnier, M., Lissac, M., Jaffrezic, N., 2002. Effect of surface topography and chemistry on adhesion, orientation and growth of fibroblasts on nickel–titanium substrates. *Mater. Sci. Eng. C* 21, 157–165. doi:10.1016/S0928-4931(02)00097-8

Preshaw, P.M., 2008. Host response modulation in periodontics. *Periodontol. 2000* 48, 92–110. doi:10.1111/j.1600-0757.2008.00252.x

Preshaw, P.M., Hefti, A.F., Jepsen, S., Etienne, D., Walker, C., Bradshaw, M.H., 2004. Subantimicrobial dose doxycycline as adjunctive treatment for periodontitis. A review. *J. Clin. Periodontol.* 31, 697–707. doi:10.1111/j.1600-051X.2004.00558.x

Qiao, L., Sun, Y., Chen, R., Fu, Y., Zhang, W., Li, X., Chen, J., Shen, Y., Ye, X., 2014. Sonochemical effects on 14 flavonoids common in citrus: relation to stability. *PLoS One* 9, e87766. doi:10.1371/journal.pone.0087766

Ramseier, C. a, Rasperini, G., Batia, S., Giannobile, W. V, 2012. Advanced reconstructive technologies for periodontal tissue repair. *Periodontol. 2000* 59, 185–202. doi:10.1111/j.1600-0757.2011.00432.x

Rao, J., Otto, W.R., 1992. Fluorimetric DNA assay for cell growth estimation. *Anal. Biochem.* 207, 186–192. doi:10.1016/0003-2697(92)90521-8

Reiter, R.J., Calvo, J.R., Karbownik, M., Qi, W., Tan, D.X., 2000. Melatonin and its relation to the immune system and inflammation. *Ann. N. Y. Acad. Sci.* 917, 376–386. doi:10.1111/j.1749-6632.2000.tb05402.x

Rice-Evans, C. a., Miller, N.J., Paganga, G., 1996. Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radic. Biol. Med.* 20, 933–956. doi:10.1016/0891-5849(95)02227-9

Rodriguez, C., Mayo, J.C., Sainz, R.M., Antolin, I., Herrera, F., Martin, V., Reiter, R.J., 2004. Regulation of antioxidant enzymes: a significant role for melatonin. *J. Pineal Res.* 36, 1–9. doi:10.1046/j.1600-079X.2003.00092.x

Rodriguez-Naranjo, M.I., Moyá, M.L., Cantos-Villar, E., Garcia-Parrilla, M.C., 2012. Comparative evaluation of the antioxidant activity of melatonin and related indoles. *J. Food Compos. Anal.* 28, 16–22. doi:10.1016/j.jfca.2012.07.001

Rohdewald, P., 2002. A review of the French maritime pine bark extract (Pycnogenol), a herbal medication with a diverse clinical pharmacology. *Int. J. Clin. Pharmacol. Ther.* 40, 158–168. doi:10.5414/CPP40158

Rompen, E., 2012. The impact of the type and configuration of abutments and their (repeated) removal on the attachment level and marginal bone. *Eur. J. Oral Implantol.* 5 Suppl, S83–90.

Rompen, E., Domken, O., Degidi, M., Pontes, A.E.F., Piattelli, A., 2006. The effect of material characteristics, of surface topography and of implant components and

connections on soft tissue integration: a literature review. *Clin. Oral Implants Res.* 17 Suppl 2, 55–67. doi:10.1111/j.1600-0501.2006.01367.x

Rupp, F., Scheideier, L., Olshanska, N., De Wild, M., Wieland, M., Geis-Gerstorfer, J., 2006. Enhancing surface free energy and hydrophilicity through chemical modification of microstructured titanium implant surfaces. *J. Biomed. Mater. Res. - Part A* 76, 323–334. doi:10.1002/jbm.a.30518

Sakagami, G., Sato, E., Sugita, Y., Kosaka, T., Kubo, K., Maeda, H., Kameyama, Y., 2006. Effects of nifedipine and interleukin-1alpha on the expression of collagen, matrix metalloproteinase-1, and tissue inhibitor of metalloproteinase-1 in human gingival fibroblasts. *J Periodontal Res* 41, 266–272. doi:10.1111/j.1600-0765.2006.00867.x

Salvi, G.E., Lang, N.P., 2005. Host response modulation in the management of periodontal diseases. *J. Clin. Periodontol.* 32 Suppl 6, 108–29. doi:10.1111/j.1600-051X.2005.00785.x

Sanz, M., D'Aiuto, F., Deanfield, J., Fernandez-Aviles, F., 2010. European workshop in periodontal health and cardiovascular disease-scientific evidence on the association between periodontal and cardiovascular diseases: a review of the literature. *Eur. Hear. J. Suppl.* 12, B3–B12. doi:10.1093/eurheartj/suq003

Satish, L., Kathju, S., 2010. Cellular and Molecular Characteristics of Scarless versus Fibrotic Wound Healing. *Dermatol. Res. Pract.* 2010, 790234. doi:10.1155/2010/790234

Satué, M., Arriero, M.D.M., Monjo, M., Ramis, J.M., 2013. Quercitrin and taxifolin stimulate osteoblast differentiation in MC3T3-E1 cells and inhibit osteoclastogenesis in RAW 264.7 cells. *Biochem. Pharmacol.* 86, 1476–86. doi:10.1016/j.bcp.2013.09.009

Saulacic, N., Bosshardt, D.D., Bornstein, M.M., Berner, S., Buser, D., 2012. Bone apposition to a titanium-zirconium alloy implant, as compared to two other titanium-containing implants. *Eur. Cells Mater.* 23, 273–288.

Sbordone, L., Bortolaia, C., 2003. Oral microbial biofilms and plaque-related diseases: microbial communities and their role in the shift from oral health to disease. *Clin. Oral Investig.* 7, 181–8. doi:10.1007/s00784-003-0236-1

Schulz, S., Tomakidi, P., Mauth, C., Kohal, R., Steinberg, T., 2012. Interactive fibroblast-keratinocyte co-cultures: an in vivo-like test platform for dental implant-based soft tissue integration. *Tissue Eng. Part C. Methods* 18, 785–96. doi:10.1089/ten.TEC.2012.0027

Schwarz, F., Ferrari, D., Herten, M., Mihatovic, I., Wieland, M., Sager, M., Becker, J., 2007a. Effects of surface hydrophilicity and microtopography on early stages of soft and hard tissue integration at non-submerged titanium implants: an immunohistochemical study in dogs. *J. Periodontol.* 78, 2171–84. doi:10.1902/jop.2007.070157

Schwarz, F., Herten, M., Sager, M., Wieland, M., Dard, M., Becker, J., 2007b. Histological and immunohistochemical analysis of initial and early subepithelial connective tissue attachment at chemically modified and conventional SLA titanium implants. A pilot study in dogs. *Clin. Oral Investig.* 11, 245–55. doi:10.1007/s00784-007-0110-7

Sculean, A., Gruber, R., Bosshardt, D.D., 2014. Soft tissue wound healing around teeth and dental implants. *J. Clin. Periodontol.* 41 Suppl 1, S6–22. doi:10.1111/jcpe.12206

Sculean, A., Schwarz, F., Becker, J., Brecx, M., 2007. The application of an enamel matrix protein derivative (Emdogain) in regenerative periodontal therapy: a review. *Med. Princ. Pract.* 16, 167–80. doi:10.1159/000100386

Sen, C.K., Packer, L., 1996. Antioxidant and redox regulation of gene transcription. *FASEB J.* 10, 709–720.

Shin, S.Y., Rios, H.F., Giannobile, W. V, Oh, T., 2015. Periodontal Regeneration : Current Therapies, in: Stem Cell Biology and Tissue Engineering in Dental Sciences. Elsevier Inc., pp. 459–469. doi:10.1016/B978-0-12-397157-9.00040-0

Sies, H., 1997. Oxidative stress: oxidants and antioxidants. *Exp. Physiol.* 82, 291–295. doi:10.1113/expphysiol.1997.sp004024

Slimestad, R., Fossen, T., Vågen, I.M., 2007. Onions: a source of unique dietary flavonoids. *J. Agric. Food Chem.* 55, 10067–80. doi:10.1021/jf0712503

Sodek, J., McKee, M.D., 2000. Molecular and cellular biology of alveolar bone. *Periodontol. 2000* 24, 99–126. doi:10.1034/j.1600-0757.2000.2240106.x

Soell, M., Elkaim, R., Tenenbaum, H., 2002. Cathepsin C, Matrix Metalloproteinases, and their Tissue Inhibitors in Gingiva and Gingival Crevicular Fluid from Periodontitis-affected Patients. *J. Dent. Res.* 81, 174–178. doi:10.1177/154405910208100306

Spatafora, C., Tringali, C., 2012. Natural-derived Polyphenols as Potential Anticancer Agents. *Anticancer. Agents Med. Chem.* 12, 902–918. doi:10.2174/187152012802649996

Srinivasan, V., Mohamed, M., Kato, H., 2012. Melatonin in Bacterial and Viral Infections with Focus on Sepsis: A Review. *Recent Pat. Endocr. Metab. Immune Drug Discov.* doi:10.2174/187221412799015317

Stacey, G., Bar, P., Granville, R., 2009. Primary Cell Cultures and Immortal Cell Lines, in: Encyclopedia of Life Sciences. John Wiley & Sons, Ltd, pp. 1–6. doi:10.1038/npg.els.0003960

Staudte, H., Sigusch, B.W., Glockmann, E., 2005. Grapefruit consumption improves vitamin C status in periodontitis patients. *Br. Dent. J.* 199, 213–217. doi:10.1038/sj.bdj.4812613

Steinemann, S.G., 1998. Titanium--the material of choice? *Periodontol. 2000* 17, 7–21. doi:10.1111/j.1600-0757.1998.tb00119.x

Stoddart, M.J., Geoff Richards, R., Alini, M., 2012. In vitro experiments with primary mammalian cells: To pool or not to pool? *Eur. Cells Mater.* 24. doi:vol024a00 [pii]

Subramani, K., Jung, R.E., Molenberg, A., Hammerle, C.H.F., 2009. Biofilm on dental implants: a review of the literature. *Int. J. Oral Maxillofac. Implants* 24, 616–626.

Taba, J., Jin, Q., Sugai, J. V., Giannobile, W. V., 2005. Current concepts in periodontal bioengineering. *Orthod. Craniofacial Res.* 8, 292–302. doi:10.1111/j.1601-6343.2005.00352.x

Tan, D.-X., Manchester, L.C., Hardeland, R., Lopez-Burillo, S., Mayo, J.C., Sainz, R.M., Reiter, R.J., 2003. Melatonin: a hormone, a tissue factor, an autocoid, a paracoid, and an antioxidant vitamin. *J. Pineal Res.* 34, 75–78. doi:10.1034/j.1600-079X.2003.02111.x

Tanner, A., Maiden, M.F.J., Lee, K., Shulman, L.B., Weber, H.P., 1997. Dental Implant Infections. *Clin. Infect. Dis.* 25, S213–S217. doi:10.1086/516243

Tek-en, G., 2015. (Own work, derivative of Basic anatomy tooth, tooth) [CC BY-SA 3.0 (http://creativecommons.org/licenses/by-sa/3.0)] [WWW Document]. *Wikimedia Commons*. URL http://commons.wikimedia.org/wiki/File%3ACross_sections_of_teeth.svg (accessed 3.20.15).

Tekbas, O.F., Ogur, R., Korkmaz, A., Kilic, A., Reiter, R.J., 2008. Melatonin as an antibiotic: new insights into the actions of this ubiquitous molecule. *J. Pineal Res.* 44, 222–6. doi:10.1111/j.1600-079X.2007.00516.x

Teughels, W., Van Assche, N., Sliepen, I., Quirynen, M., 2006. Effect of material characteristics and/or surface topography on biofilm development. *Clin. Oral Implants Res.* 17 Suppl 2, 68–81. doi:10.1111/j.1600-0501.2006.01353.x

Thoma, D.S., Jones, A.A., Dard, M., Grize, L., Obrecht, M., Cochran, D.L., 2011. Tissue integration of a new titanium-zirconium dental implant: a comparative histologic and radiographic study in the canine. *J. Periodontol.* 82, 1453–61. doi:10.1902/jop.2010.100737

Tipton, D.A., Flynn, J.C., Stein, S.H., Dabbous, M.K., 2003. Cyclooxygenase-2 inhibitors decrease interleukin-1beta-stimulated prostaglandin E2 and IL-6 production by human gingival fibroblasts. *J. Periodontol.* 74, 1754–63. doi:10.1902/jop.2003.74.12.1754

Tomofuji, T., Ekuni, D., Irie, K., Azuma, T., Endo, Y., Tamaki, N., Sanbe, T., Murakami, J., Yamamoto, T., Morita, M., 2009. Preventive effects of a cocoa-enriched diet on gingival oxidative stress in experimental periodontitis. *J. Periodontol.* 80, 1799–808. doi:10.1902/jop.2009.090270

Tonetti, M.S., Chapple, I.L.C., Jepsen, S., Sanz, M., 2015a. Primary and Secondary Prevention of Periodontal and Peri-Implant Diseases Introduction to, and Objectives of the Consensus from the 11 th European Workshop on Periodontology. *J. Clin. Periodontol.* 42, S1–S4. doi:10.1111/jcpe.12382

Tonetti, M.S., Eickholz, P., Loos, B.G., Papapanou, P., van der Velden, U., Armitage, G., Bouchard, P., Deinzer, R., Dietrich, T., Hughes, F., Kocher, T., Lang, N.P., Lopez, R., Needleman, I., Newton, T., Nibali, L., Pretzl, B., Ramseier, C., Sanz-Sanchez, I., Schlagenhauf, U., Suvan, J.E., Fabrikant, E., Fundak, A., 2015b. Principles in Prevention of Periodontal Diseases. *J. Clin. Periodontol.* 42, S5–S11. doi:10.1111/jcpe.12368

Totilo, S., 2007. Burning the Virtual Shoe Leather: Does journalism in a computer world matter? *Columbia J. Rev.* 46, 38–44. doi:10.1007/s12272-015-0585-8

Toworfe, G.K., Bhattacharyya, S., Composto, R.J., Adams, C.S., Shapiro, I.M., Ducheyne, P., 2009. Effect of functional end groups of silane self-assembled monolayer surfaces on apatite formation, fibronectin adsorption and osteoblast cell function. *J. Tissue Eng. Regen. Med.* 3, 26–36. doi:10.1002/term.131

Treccani, L., Yvonne Klein, T., Meder, F., Pardun, K., Rezwan, K., 2013. Functionalized ceramics for biomedical, biotechnological and environmental applications. *Acta Biomater.* 9, 7115–7150. doi:10.1016/j.actbio.2013.03.036

Trofin, E. a, Monsarrat, P., Kémoun, P., 2013. Cell therapy of periodontium: from animal to human? *Front. Physiol.* 4, 325. doi:10.3389/fphys.2013.00325

Trzeciakiewicz, A., Habauzit, V., Horcajada, M.-N., 2009. When nutrition interacts with osteoblast function: molecular mechanisms of polyphenols. *Nutr. Res. Rev.* 22, 68–81. doi:10.1017/S095442240926402X

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., Speleman, F., 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* 3, research0034.1–research0034.11. doi:10.1186/gb-2002-3-7-research0034

Villar, C.C., Huynh-Ba, G., Mills, M.P., Cochran, D.L., 2011. Wound healing around dental implants. *Endod. Top.* 25, 44–62. doi:10.1111/etp.12018

Waddington, R.J., Moseley, R., Embery, G., 2000. Reactive oxygen species: a potential role in the pathogenesis of periodontal diseases. *Oral Dis.* 6, 138–151.

Wallace, S.N., Carrier, D.J., Clausen, E.C., 2005. Batch solvent extraction of flavanolignans from milk thistle (Silybum marianum L. Gaertner). *Phytochem. Anal.* 16, 7–16. doi:10.1002/pca.803

Walsh, B.J., Thornton, S.C., Penny, R., Breit, S.N., 1992. Microplate reader-based quantitation of collagens. *Anal. Biochem.* 203, 187–190. doi:10.1016/0003-2697(92)90301-M

Wang, H., Joseph, J.A., 1999. Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader. *Free Radic. Biol. Med.* 27, 612–616. doi:10.1016/S0891-5849(99)00107-0

Wang, H.-L., Greenwell, H., Fiorellini, J., Giannobile, W., Offenbacher, S., Salkin, L., Townsend, C., Sheridan, P., Genco, R.J., 2005. Periodontal regeneration. *J. Periodontol.* 76, 1601–22. doi:10.1902/jop.2005.76.9.1601

Wei, H., Wu, S., Feng, Z., Zhou, W., Dong, Y., Wu, G., Bai, S., Zhao, Y., 2012. Increased fibroblast functionality on CNN2-loaded titania nanotubes. *Int. J. Nanomedicine* 7, 1091–100. doi:10.2147/IJN.S28694

Weidmann, A.E., 2012. Dihydroquercetin: More than just an impurity? *Eur. J. Pharmacol.* 684, 19–26. doi:10.1016/j.ejphar.2012.03.035

Weinreb, M., Nemcovsky, C.E., 2015. In vitro models for evaluation of periodontal wound healing/regeneration. *Periodontol. 2000* 68, 41–54. doi:10.1111/prd.12079

Wieland, M., Chehroudi, B., Textor, M., Brunette, D.M., 2002. Use of Ti-coated replicas to investigate the effects on fibroblast shape of surfaces with varying roughness and constant chemical composition. *J. Biomed. Mater. Res.* 60, 434–44. doi:10.1002/jbm.10059

Williams, R.J., Spencer, J.P.E., Rice-Evans, C., 2004. Flavonoids: Antioxidants or signalling molecules? *Free Radic. Biol. Med.* 36, 838–849. doi:10.1016/j.freeradbiomed.2004.01.001

Yarrow, J.C., Perlman, Z.E., Westwood, N.J., Mitchison, T.J., 2004. A high-throughput cell migration assay using scratch wound healing, a comparison of image-based readout methods. *BMC Biotechnol.* 4, 21. doi:10.1186/1472-6750-4-21

Yin, Y., Li, W., Son, Y.-O., Sun, L., Lu, J., Kim, D., Wang, X., Yao, H., Wang, L., Pratheeshkumar, P., Hitron, A.J., Luo, J., Gao, N., Shi, X., Zhang, Z., 2013. Quercitrin protects skin from UVB-induced oxidative damage. *Toxicol. Appl. Pharmacol.* 269, 89–99. doi:10.1016/j.taap.2013.03.015

Yucel-Lindberg, T., Båge, T., 2013. Inflammatory mediators in the pathogenesis of periodontitis. *Expert Rev. Mol. Med.* 15, e7. doi:10.1017/erm.2013.8

Zhao, B., Yi, Z., Lu, Z., Tian, W., Cui, F., Feng, H., Hu, H., Kawakami, T., Takagi, T., Nagai, N., 2006. Improvement of Adhesion and Spreading of Human Gingival Fibroblasts to RGD-grafted Titanium Surface. *J. Hard Tissue Biol.* 15, 65–68. doi:10.2485/jhtb.15.65

Zhao, L., Chu, P.K., Zhang, Y., Wu, Z., 2009. Antibacterial coatings on titanium implants. *J. Biomed. Mater. Res. - Part B Appl. Biomater.* 91, 470–480. doi:10.1002/jbm.b.31463

Zhao, L., La, V.D., Grenier, D., 2013. Antibacterial, Antiadherence, Antiprotease, and Anti-Inflammatory Activities of Various Tea Extracts: Potential Benefits for Periodontal Diseases. *J. Med. Food* 16, 428–436. doi:10.1089/jmf.2012.0207

Zhou, L., Lai, Y., Huang, W., Huang, S., Xu, Z., Chen, J., Wu, D., 2015. Biofunctionalization of microgroove titanium surfaces with an antimicrobial peptide to enhance their bactericidal activity and cytocompatibility. *Colloids Surfaces B Biointerfaces* 128, 552–560. doi:10.1016/j.colsurfb.2015.03.008

Zimmermann, D.R., Dours-Zimmermann, M.T., Schubert, M., Bruckner-Tuderman, L., 1994. Versican is expressed in the proliferating zone in the epidermis and in association with the elastic network of the dermis. *J. Cell Biol.* 124, 817–825. doi:10.1083/jcb.124.5.817

APPENDIX 1

Paper I

Anti-fibrotic and anti-inflammatory properties of melatonin on human gingival fibroblasts in vitro

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Metabolic Disorders and Endocrinology

Anti-fibrotic and anti-inflammatory properties of melatonin on human gingival fibroblasts *in vitro*

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ABSTRACT

Melatonin (MEL) has been proposed as a therapeutic agent for the oral cavity, due to its antioxidant and anti-inflammatory effects since periodontal diseases are aggravated by free radicals, and by disproportionate immunological response to plaque microorganism. In addition, MEL promotes bone formation. This study aimed to investigate the effects of MEL and 5-methoxytryptophol (5-MTX), an indole derivative related to MEL, on the growth rate of *Staphylococcus epidermidis* and on human gingival fibroblasts (HGF) for potential use in periodontal applications. HGF were treated with MEL and 5-MTX and the effects were evaluated on cell viability, gene expression, collagen production, wound healing and matrix metalloproteinase-1/tissue inhibitor of metalloproteinases-1 (MMP1/TIMP1) production. MEL showed the most promising biological effects in relation to periodontal applications. Thus, MEL was not toxic for HGF, increased collagen IIIα1, decorin and IL10, it down-regulated the expression of pro-fibrotic markers during wound healing, decreased the MMP1/TIMP1 protein ratio. These results suggest that MEL could contribute to protect and recover the integrity of gingival tissues, thus, displaying a potential use for periodontal disease treatment or to functionalize dental implant abutments to improve soft tissue integration.

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1. Introduction

The periodontium is composed of hard (cementum and bone) and soft tissue (periodontal ligament and gingiva), which maintain teeth function [1]. Periodontal disease is directly related to an increased production of free radicals by periodontopathic bacteria [2] and is indirectly caused by inflammation and by an immune response against these bacteria that results in destruction of periodontal tissues and, in severe cases, is accompanied by the loss of alveolar bone with eventual loss of the tooth [3]. Gingival fibroblasts are the major constituents of gingival and maintain tissue integrity by regulating collagen and proteoglycan metabolism [1]. Together with matrix metalloproteinases, free radicals induce collagen degradation and cell damage [4], in addition to be pro-inflammatory [5].

Melatonin (MEL) is synthesized from serotonin in the pineal gland and in a variety of other tissues. Many of its effects differ to its primary neurohormonal functions owing to its properties as anti-inflammatory, effective free radical scavenger and stimulator of antioxidant enzymes [6–9]. The anabolic action of MEL on bone metabolism has been proved both *in vitro* and *in vivo* [10], as it promotes osteoblast proliferation and differentiation [11,12], decreases osteoclast-related bone resorption [13,14] and accelerates osseointegration of dental implants [15]. Once in blood, MEL reaches saliva and protects the oral cavity from oxidative stress-related tissue damage [16–18]. Moreover, MEL levels correlate with the periodontal health status indicating that it may have a protective role against periodontal disease [19–21]. *In vitro*, MEL decreases dental materials' toxic effects on gingival fibroblasts [22]. Thus, many authors have suggested potential beneficial effects of MEL in dentistry, for instance, on periodontal disease or in dental implantology [17,18,23,24].

Although MEL effects on bone metabolism seem to be clear, the effects on gingival fibroblasts remain unstudied. The objective of the present research was study the effects of MEL on primary human gingival fibroblasts (HGF) and on the growth rate of *Staphyloccocus epidermidis* and compare them with the effect of 5-methoxytryptophol (5-MTX), an indole derivative related to MEL. 5-MTX is also present at the pineal gland [25], prevents the oxidative damage *in vitro* [26], inhibits osteoclastogenesis and promotes osteoblast differentiation in a higher degree than MEL [27]. Thus, it may also affect HGF in a different manner than MEL.

In the present study, we aimed at evaluating the effect of MEL and 5-MTX for potential use in periodontal applications. Therefore, we systematically compared the effect of increasing doses of the 5-methoxyindoles on the growth rate of *S. epidermidis* and screened their effect on cell viability and on gene expression in human

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gingival fibroblasts. Then, selected doses were further evaluated by measuring their effect on collagen production, wound healing and matrix metalloproteinase-1 and tissue inhibitor of metalloproteinases 1 production.

2. Material and methods

2.1. Treatments

All chemical reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise specified. MEL and 5-MTX were dissolved in absolute ethanol (Scharlab, Barcelona, Spain), aliquoted and stored at −20 °C. MEL and 5-MTX were further dissolved in culture media, prior to each media change. Final concentration of ethanol (0.88%) was included as vehicle control group in the experiments.

2.2. Culture and proliferation assay of Staphylococcus epidermidis

S. epidermidis 4814 (CECT, Valencia, Spain) were grown from frozen stocks in LB broth (Scharlab). Following an overnight incubation, 0.2 mL of bacterial suspensions (1×10^6 bacteria/mL) were incubated with the different treatments in 96-well flat-bottomed plates for 24 h. Vehicle control groups served as negative control and 6 μg/mL vancomycin served as positive control for bacterial growth inhibition. Plates were incubated in a microplate reader (PowerWave HT, Biotek, Winooski, VT, USA) with temperature control and shaking function at 37 °C for 24 h. At regular time intervals, the optical density was measured at 600 nm to determine bacterial proliferation. The assays were carried out in triplicate, with three replicates at each condition. Bacterial growth rate (μ) was calculated during the exponential growth phase following the equation $\ln OD_t - \ln OD_0 = \mu(t - t_0)$.

2.3. Cell culture

Three different donors of primary human gingival fibroblasts (Provitro GmbH, Berlin, Germany) were used: HGF-A (27 years, Caucasian, female, lot number 313X100401), HGF-B (19 years, Caucasian, male, lot number 322X070501) and HGF-C (47 years, Caucasian, male, lot number 323X070501). HGF cells were routinely cultured at 37 °C in a humidified atmosphere of 5% CO2, and maintained in fibroblast growth medium (Provitro GmbH) supplemented with 10% fetal calf serum (FCS), 50 ng amphotericin/mL and 50 μg gentamicin/mL (Provitro GmbH). Cells were subcultured 1:4 before reaching confluency using PBS and trypsin/EDTA (PAA Laboratories GmbH, Pasching, Austria), as recommended by suppliers. Experiments were performed with HGF cells at between passages 7 and 8 after isolation. Trypan blue stain was used to determine total and viable cell number.

Three replicate wells for each donor were seeded in 48-well plates at a density of 1.0×10^4 cells/well for all control and test samples ($n = 9$). For collagen quantification, cells from one donor were seeded in 96-well plates at a density of 7.0×10^3 ($n = 6$). Cells were treated 48 h after seeding with 0.001 to 2.5 mM MEL or 5-MTX. Cells were grown for 2 days (cytotoxicity and wound healing assay) and for 14 days (gene expression and protein quantification).Treatments were added at every media change (every other day).

2.4. Determination of cytotoxicity

The presence of LDH activity in the culture media after 48 h of cell culture was used as an index of cell death. LDH activity was determined spectrophotometrically after 30 min of incubation at 25 °C of 50 μL of culture media and 50 μL of the reaction mixture, by measuring the oxidation of NADH at 490 nm in the presence of pyruvate, according to the manufacturer's kit instructions (Cytotoxicity Detection kit, Roche Diagnostics, Mannheim, Germany). Results from all the samples were presented relative to the LDH activity in the medium of cells treated with the vehicle control (low control, 0% of cell death) and of cells treated with 1% Triton X-100 (high control, 100% cell death). The percentage of LDH activity was calculated using the following equation: Cytotoxicity (%) = (exp. value − low control)/(high control − low control) × 100.

2.5. RNA isolation and real-time RT-PCR analysis

Total RNA was isolated after 14 days of cell culture using Tripure (Roche Diagnostics), according to the manufacturer's protocol. Total RNA was quantified at 260 nm using a Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The same amount of RNA (0.2 μg) was reverse transcribed to cDNA at 42 °C for 60 min using High Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA, USA), according to the protocol of the supplier. Aliquots of each cDNA were frozen (−20 °C) until the PCR reactions were carried out.

Real-time PCR was performed for two reference genes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and beta-actin (ACTBL2), and target genes, collagen III α1 (COL3A1), decorin (DCN), integrin α2 (ITGA2), interleukin-6 (IL6), matrix metalloproteinase-1 (MMP1), tissue inhibitor of metalloproteinases 1 (TIMP1), α-smooth muscle actin (ACTA2), endothelin-1 (EDN1) and transforming growth factor-β1 (TGFB1). Real-time PCR was performed in the Lightcycler 480® (Roche Diagnostics) using SYBR green detection. Primer sequences and real-time conditions are described elsewhere [28]. Target genes are covering different aspects of gingival fibroblast function like extracellular matrix (ECM) production and organization, regulation of cell adhesion and inflammation.

All samples were normalized by the geometric mean of the expression levels of ACTBL2 and GAPDH and fold changes were related to the control groups using the mathematical model described by Pfaffl [29]:

$$\text{ratio} = E_{\text{target}}{}^{\Delta \text{Cp target (mean control}-\text{sample)}} / E_{\text{reference}}{}^{\Delta \text{Cp target (mean control}-\text{sample)}}$$

where *Cp* is the is the crossing point of the reaction amplification curve as determined by the LightCycler 480 software. Stability of reference genes was calculated using the BestKeeper tool [30]. The crossing point variation of the reference genes among samples was lower than 0.28. Moreover, a good consistence of the bestkeeper index was proved as its contributing reference genes were tightly correlated with it ($0.815 < r < 0.822$), with a significance level of $P = 0.001$ for all reference genes.

2.6. Collagen quantification

After 14 days of cell culture, cells were washed with PBS and dryed overnight at 37 °C in a humidified atmosphere plus 24 h at 37 °C in a dry atmosphere. Collagen was stained with Sirius Red F3BA 0.1% in saturated picric acid for 1 h. Unbounded dye was removed with 10 mM HCl washes and dye was solibilized with 100 mM NaOH. Absorbance was measured with a microplate reader at 540 nm. Readings were compared with a calf-skin collagen standard included in the assay.

2.7. Wound healing assay

48 h after seeding, the monolayer was scraped in a straight line to create a scratch, with a 200 μL sterile pipette tip, and cells were

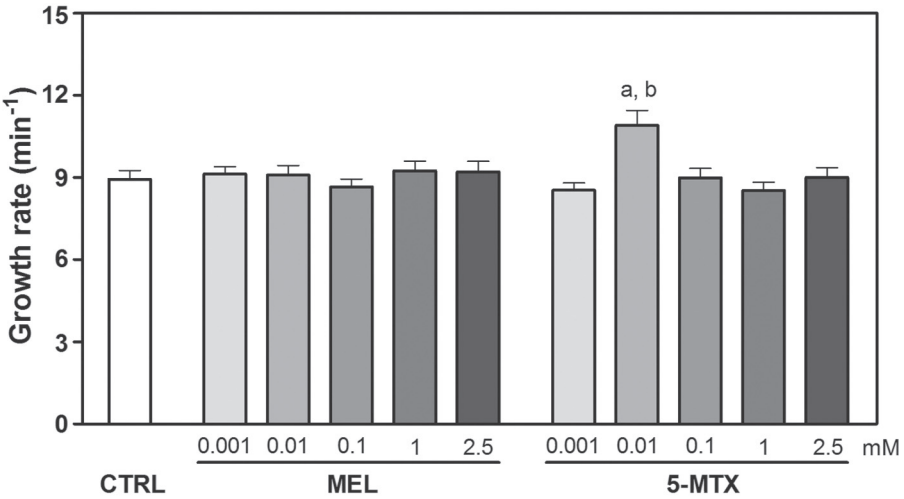

Fig. 1. *S. epidermidis* growth rate cultured with 0.001 to 2.5 mM MEL and 5-MTX. Data represent the mean ± SEM of three independent experiments ($n = 9$). Differences between groups were assessed by Student *t*-test: $^{a}P < 0.05$ treatment *versus* control (ethanol); $^{b}P \leq 0.05$ MEL *versus* 5-MTX for the same dose.

washed once with growth medium to remove debris and detached cells. Cells were then treated with 0.1 and 1 mM MEL or 5-MTX. Immediately after treatment and 48 h later, images of the same areas were taken using a brightfield inverted microscope (Leica DM IRB, Wetzlar, Germany). Then, culture media was removed and RNA was isolated as described before to study gene expression of MMP1, ACTA2, EDN1 and TGFB1. The images were quantitatively analyzed with TScratch software using the default parameter settings [31]. The open wound area (%) was defined as 100 × (uncovered image area at 48 h/uncovered image area at 0 h).

2.8. Enzyme-linked immunosorbent assays (ELISA)

The detection of TIMP1 and MMP1 was performed from cell culture media after 14 d of cell culture ($n = 9$) by commercially available ELISA kits according to the manufacturer instructions (Sigma-Aldrich).

2.9. Statistics

All data are presented as mean values ± SEM (standard error of the mean). The Kolmogorov–Smirnov test was done to assume parametric or non-parametric distributions for the normality tests. Differences between treatment and control groups were assessed by Mann–Whitney-test, Wilcoxon test, Student *t*-test or by paired *t*-test. SPSS® program for Windows, version 17.0 (Chicago, IL, USA) was used. Results were considered statistically significant at *p*-values (P) ≤ 0.05.

3. Results

First, we evaluated the effect of MEL and 5-MTX on *S. epidermidis* growth. Increasing concentrations of both MEL and 5-MTX did not affect *S. epidermidis* growth when compared with control (Fig. 1). However, bacterial growth rate increased with 0.01 mM 5-MTX compared with vehicle control and with 0.01 mM MEL. Vancomycin, used as a positive control for bacterial growth inhibition, totally inhibited the growth of bacteria (data not shown).

LDH activity, an index of cytotoxicity, showed that the highest doses (2.5 mM) of both biomolecules and the lowest dose (0.001 mM) of 5-MTX produced a higher LDH release compared with control on HGF (Fig. 2). LDH activity was higher for 5-MTX compared to MEL for the same doses used. LDH activity values were in all cases under 12%.

Then, we analyzed the effect of the molecules on gene expression in HGF. We found that 0.1 and 1 mM MEL and 5-MTX produced the most relevant changes at mRNA level of the different studied genes (Fig. 3). The higher dose of 5-MTX (2.5 mM) was not analyzed for gene expression due to their higher cytotoxic effects and visual inspection of HGF after 14 days of cell culture. COL3A1 mRNA levels significantly increased in cells cultured with 1 mM MEL and 0.1 mM 5-MTX compared with control group. However, 0.1 and 1 mM 5-MTX stimulated COL3A1 in a lower degree than the corresponding MEL doses. Similarly, 0.1 and 1 mM MEL and 0.1 mM 5-MTX increased DCN gene expression. While MMP1 expression decreased in cells cultured from 0.01 to 2.5 mM MEL and from 0.1 mM to 1 mM 5-MTX compared with control group, TIMP1 expression only increased with MEL treatments. Thus, for both MMP1 and TIMP1 mRNA levels greater effects of MEL over 5-MTX were found in the corresponding doses. IL6 and ITGA2 expression remained statistically steady until 1 mM MEL compared with control, and decreased or increased, respectively, with 2.5 mM MEL. For the same genes, 0.01 and 0.1 mM 5-MTX decreased IL6 and 0.1 and 1 mM increased ITGA2 compared with control, and also when compared with MEL

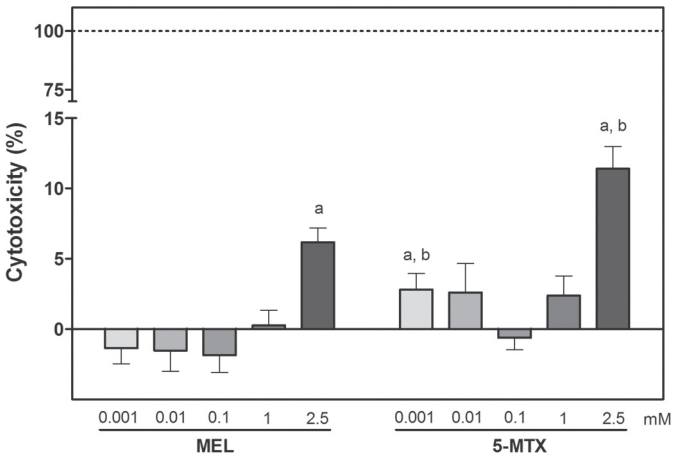

Fig. 2. LDH activity measured in human gingival fibroblasts culture media collected after 48 h of treatment with 0.001 to 2.5 mM MEL or 5-MTX. High control (100% cytotoxicity) was cell culture media from cells treated with 1% Triton X-100. Low control (0% cytotoxicity) was cell culture media from control vehicle cells. Values represent the mean ± SEM ($n = 9$). Paired *t*-test was performed to assess differences between treatments and control groups: $^{a}P < 0.05$ treatment *versus* control (ethanol); $^{b}P \leq 0.05$ MEL *versus* 5-MTX for the same dose.

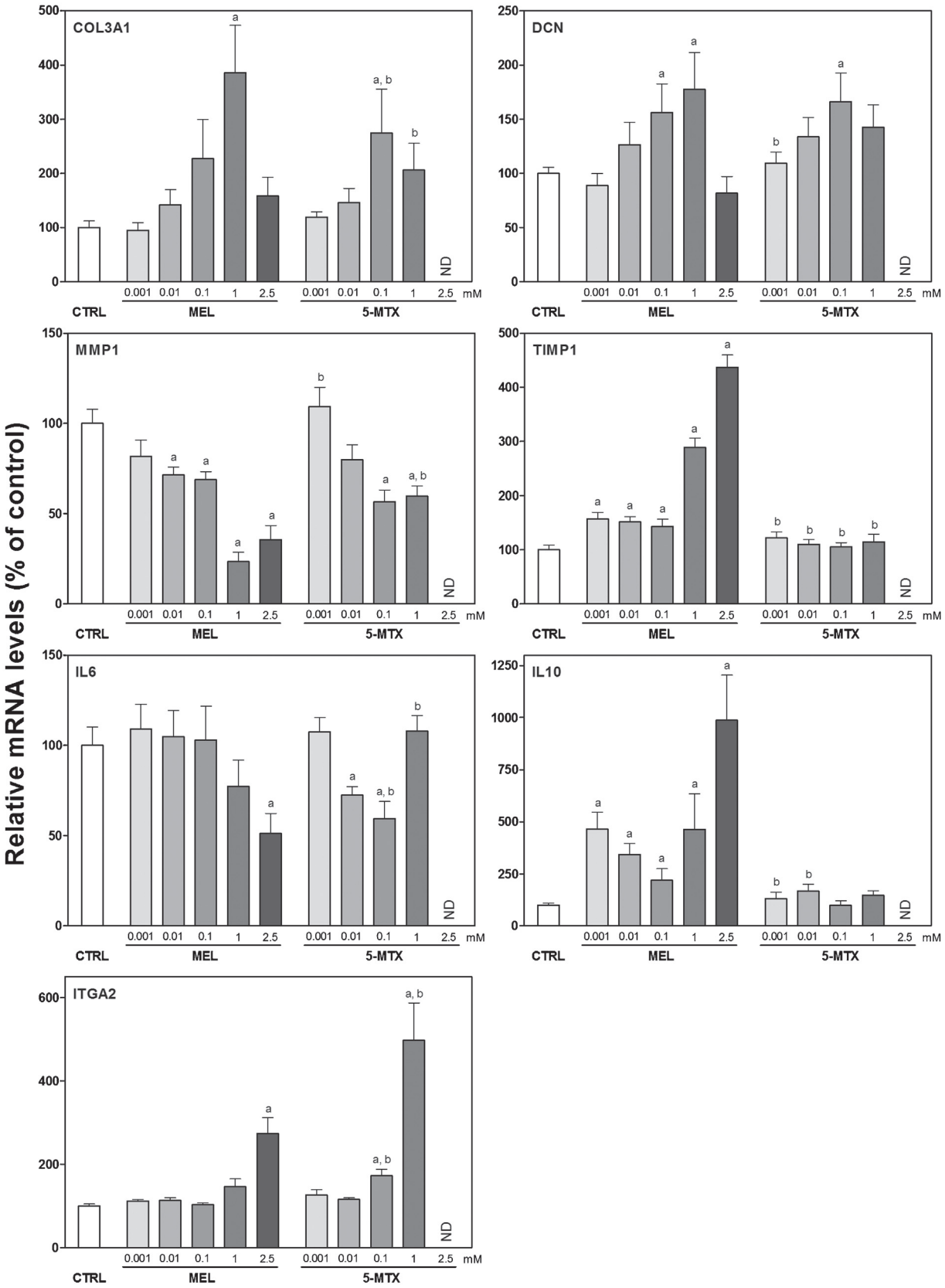

Fig. 3. Effect of 0.001 to 2.5 mM MEL or 5-MTX treatment for 14 days on the mRNA levels of COL3A1, DCN, MMP1, TIMP1, IL6, IL10 and ITGA2 in human gingival fibroblasts. ND = not determined. Data were normalized to reference genes (beta-actin and GAPDH), expressed as percentage of control which was set to 100%. Values represent the mean ± SEM ($n = 9$). Paired *t*-test was performed to assess differences between treatments and control groups: [a]$P \leq 0.05$ treatment *versus* control (ethanol); [b]$P \leq 0.05$ MEL *versus* 5-MTX for the same dose.

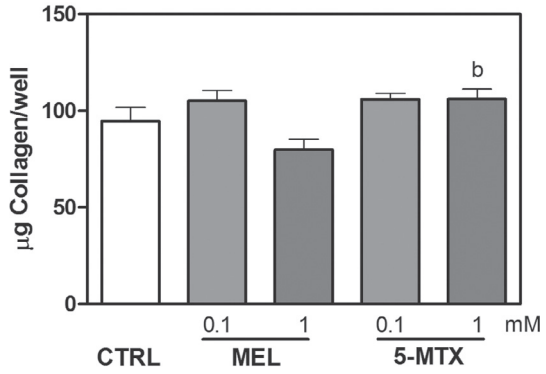

Fig. 4. Effect of 0.1 and 1 mM MEL or 5-MTX treatment for 14 days on collagen production in human gingival fibroblasts. Values represent the mean ± SEM ($n = 6$). Student's t test was performed to assess differences between treatments and control groups: $^{a}P \leq 0.05$ treatment *versus* control (ethanol); $^{b}P \leq 0.05$ MEL *versus* 5-MTX for the same dose.

for ITGA2. IL10 expression increased with all MEL doses when compared with control, while 5-MTX showed no important effects on IL10 mRNA levels.

Fig. 4 shows that total collagen remained statistically steady compared with control group. However, 1 mM 5-MTX induced more collagen deposition than 1 mM MEL. Regarding wound healing assay, MEL and 5-MTX did not affect HGF wound healing closure in the present study. However, 1 mM MEL significantly decreased ACTA2, EDN1 and TGFB1 compared with control and 1 mM 5-MTX (Fig. 5).

Finally, we found that 1 mM MEL decreased MMP1 production while it increased TIMP1 production resulting in a decreased

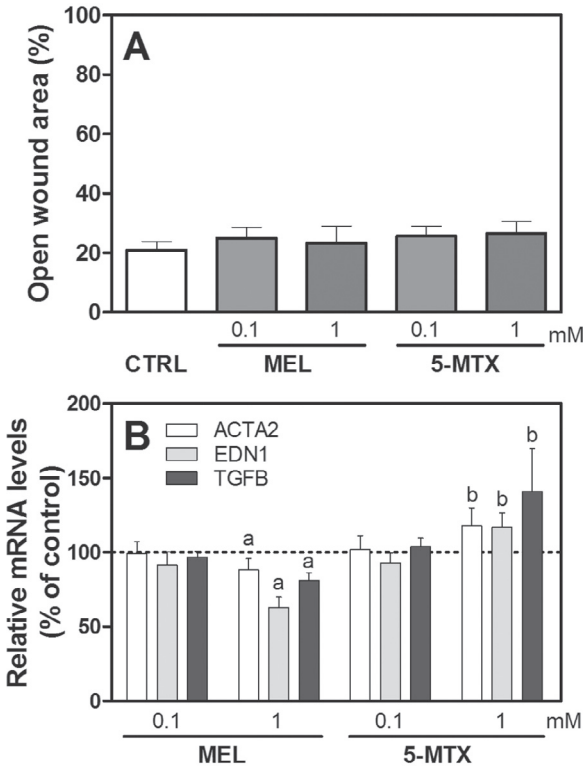

Fig. 5. Wound healing assay. Wound open area (A) and gene expression levels of ACTA2, EDN1 and TGFB1 (B) after 48 h of healing in presence of 0.1 and 1 mM MEL or 5-MTX. Data represent fold changes of target genes normalized to beta-actin and GAPDH (reference genes) expressed relative to control group that was set to 100% (dotted line). Values represent the mean ± SEM ($n = 9$). Paired t-test was performed to assess differences between treatments and control groups: $^{a}P \leq 0.05$ treatment *versus* control (ethanol); $^{b}P \leq 0.05$ MEL *versus* 5-MTX for the same dose.

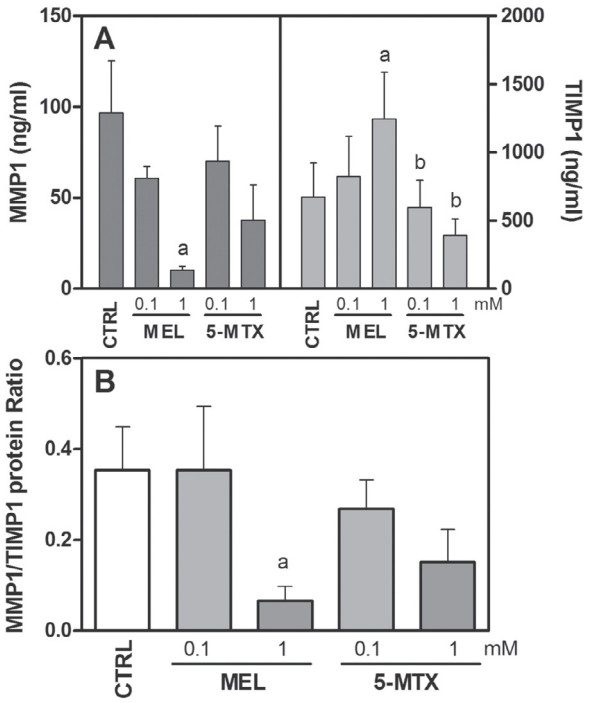

Fig. 6. Effect of 0.1 and 1 mM MEL or 5-MTX treatment for 14 days on MMP1 and TIMP1 production (A) and on the MMP1/TIMP1 ratio (B) in human gingival fibroblasts. Values represent the mean ± SEM ($n = 9$). Significant differences were assessed by paired t-test for MMP1 and TIMP1 production and by Wilcoxon test for the MMP1/TIMP1 ratio: $^{a}P \leq 0.05$ treatment *versus* control (ethanol); $^{b}P \leq 0.05$ MEL *versus* 5-MTX for the same dose.

MMP1/TIMP1 ratio. Furthermore, cells treated with MEL showed increased TIMP1 release than those treated with 5-MTX (Fig. 6).

4. Discussion

This study reports for the first time the effects of melatonin in human gingival fibroblasts. We show that the treatment with MEL is safe for HGF, it increases COL3A1, DCN, TIMP1, IL10 mRNA levels while downregulates MMP1 expression, decreases the expression of pro-fibrotic markers during wound healing and decreases MMP1/TIMP1 protein ratio. Additionally, our results confirm recent *in vivo* studies in which topical administration of MEL showed favorable effects on the progression of periodontal disease [32,33]. Moreover, we have compared the effects of MEL with a biologically- and chemically-related 5-methoxyindole, 5-MTX, which has shown lower potential than MEL for stimulated periodontal regeneration.

In periodontal disease, the response to oral pathogens results in a strong release of inflammatory mediators, such as ROS, IL6 and MMPs, inducing collagen degradation which ends in tissue destruction. Together with this process, failed tissue repair occurs, resulting in fibrosis and scarring [1]. In searching for new agents for potential use in periodontal disease treatment, those biomolecules capable of (a) increasing collagen synthesis, (b) promoting scarless tissue repair (c) decreasing ROS, (d) stimulating immune response, (e) reducing the progressive loss of alveolar bone, in addition to (f) antibacterial activity, might constitute potential therapeutic agents. Since MEL is an endogen, non-toxic, powerful antioxidant, it has anti-inflammatory and immunoenhancing actions and it promotes proliferation and bone remodeling besides its antimicrobial effects, it may benefit the progression of periodontal disease.

We found that MEL and 5-MTX were safe for human gingival fibroblasts up to the concentration of 1 mM. The only side effect

found for MEL long-term treatment is sleepiness following oral ingestion [34]. Only 2.5 mM MEL and 5-MTX treatments increased LDH activity by 6 and up to 11%, respectively, which may be related to a higher metabolic activity after 48 h of culture. However, HGF treatment with 2.5 mM 5-MTX for 14 d decreased cell viability, as observed visually under the phase-contrast microscope.

The progression of periodontal disease is characterized by increased collagen solubility and degradation taxes, producing a net connective tissue loss [1]. We found that MEL and to a lesser extend 5-MTX increase COL3A1 and DCN mRNA levels. Decorin, a small leucine-rich proteoglycan highly expressed in human gingiva [35], regulates fibril organization of collagens [36], including type I and type III, the major protein components of the gingival connective tissue ECM [35]. Furthermore, COL3A1 mRNA increases during human gingival fibroblasts differentiation [28], which is associated with scarless wound healing [37]. In periodontal disease, the breakdown of collagen parallels a decrease in decorin [38]. Therefore, our mRNA expression results suggest a potential use of MEL and 5-MTX in the maintenance of connective tissue structure during periodontal disease. The mechanism of action of MEL and its metabolites include melatonin membrane receptors (MT1, MT2), cytosolic binding sites (MT3, calmodulin) and nuclear receptors (retinoid-related orphan receptors), which are expressed in gingival fibroblasts [39]. Previous studies suggest that MEL activates melatonin membrane receptors which in turn, stimulate collagen synthesis [40]. Despite of the increased COL3A1 mRNA levels, we found similar collagen production among groups. This discrepancy can be explained by the lack of ascorbic acid in the fibroblasts growth media, leading to the accumulation of unprocessed procollagen in the cell culture medium and to a minimal deposition of secreted collagen [41,42]. Under the described culture media conditions collagen deposition was slowed down [42], we consider that the gene expression results represent the effect of MEL and 5-MTX on collagen production.

ECM undergoes through a constant turnover of its components, in both normal and diseased conditions, mainly through MMP-mediated collagen degradation [1]. Excess MMP production and activity leads to an accelerated ECM degradation associated with periodontitis [43]. The activity of MMPs is controlled by proteolytic activation, production regulation and neutralization by TIMPs [44]. An imbalance of MMPs over their inhibitors results in a fibrolytic situation [45]. In the present study, MEL treatment decreased MMP1/TIMP1 mRNA and protein ratio, suggesting that MEL may decrease MMP-related ECM destruction. Regulation the MMPs through melatonin has been proposed as a therapeutic option [46], since oxidative stress plays an important role in regulating the activities of MMPs. Previous studies have shown that MEL inhibited MMP-9 activity via binding to its active site [47] and via MT1 receptor signaling pathway [48]. In addition, we also found that ITGA2 expression, a collagen receptor needed for matrix remodeling [49] and which increased levels are associated with a fibrotic phenotype [50], was up-regulated for 5-MTX treatments, while MEL did not increase it.

Wound healing response depends on the ability of fibroblasts to produce and arrange new collagen and other ECM components in similar quantities and ratios to unwounded tissue [37]. TGFB, the major activator of ECM synthesis, plays a crucial role in healing by activating the expression of collagen and other ECM components to reconstitute the tissue matrix [1]. However, accumulation of TGF-β1 and endothelin-1 initiates the differentiation of fibroblasts into myofibroblasts, which express highly contractile proteins including ACTA2 [51], a hallmark of fibrogenic conditions. This process can produce either a scarred (repaired) tissue or a structural and functional regenerated tissue [52,53]. Here, ACTA2, END1 and TGFB mRNA levels after wound healing decreased for MEL treated cells, suggesting a more-regenerative/less-scarring response that would reduce tissue damage. In addition, we found that MEL up-regulated IL10 expression, an anti-inflammatory and anti-fibrotic cytokine [1], thus, contributing to decrease the inflammatory reaction, another way to prevent and control periodontal disease [54]. The anti-inflammatory properties of MEL [55] could explain this response since scarless healing is characterized by markedly diminished or minimal inflammatory response [37].

A marked characteristic of periodontal disease is a great production of free radicals, derived from bacteria themselves or from the immune system [2] that coexists with a decrease in the antioxidant systems [18]. When balance between free radicals and antioxidant systems is disrupted, oxidative stress occurs. In the present research we did not study MEL and 5-MTX ROS-scavenging properties. However, MEL also has receptor-independent activity [39] and can directly neutralize a variety of free radicals, indirectly inhibit expression and activity of nitric oxide synthase and cyclooxigenase and increase gene expression and activities of antioxidant enzymes [56–60]. MEL structurally-related compounds showed different antioxidant properties [26,61,62]. Moreover, MEL blocked oxidative stress, inflammation and tissue damage in periodontitis induced-rats [63] and topical melatonin treatment decreased the extend of periodontal disease, pocket depth and bone-related periodontitis markers in humans [32,33]. Therefore, the antioxidant and anti-inflammatory properties of MEL might justify its therapeutic use in diseases in which free radical damage is a major aspect of the condition, as periodontal disease [18] and might also explain the differential effects of 5-MTX compared with MEL.

MEL showed antibacterial effects against gram-positive and gram-negative bacteria by reducing intracellular substrates availability, such as free iron and fatty acids [64,65]. However, in the present study, MEL neither 5-MTX showed antibacterial activity against *S. epidermidis*, one of the initial colonizers during dental plaque formation and involved in implant surface biofilm formation [66,67]. This finding may be attributed to the species specificity of the antibacterial activity of the molecules tested or the method used in the present study.

In summary, MEL increases collagen, decorin and IL10 expression, decreases the MMP1/TIMP1 ratio and promotes scarless wound healing, in combination with its antioxidant properties and beneficial effect on bone metabolism, all in all, suggesting that it could contribute to protect and recover the integrity of gingival tissues. However, further studies with oral administration of MEL should confirm these findings at oral mucosa level to evaluate its possible applications in the prevention of periodontal disease and as coating for dental implant abutments to improve soft tissue integration.

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References

[1] Bartold PM, Narayanan AS. Molecular and cell biology of healthy and diseased periodontal tissues. Periodontology 2000 2006;40:29–49.
[2] Gustafsson A, Åsman B. Increased release of free oxygen radicals from peripheral neutrophils in adult periodontitis after Feγ-receptor stimulation. J Clin Periodontol 1996;23:38–44.
[3] Smalley JW. Pathogenic mechanisms in periodontal disease. Adv Dent Res 1994;8:320–8.
[4] Waddington RJ, Moseley R, Embery G. Reactive oxygen species: a potential role in the pathogenesis of periodontal diseases. Oral Dis 2000;6:138–51.
[5] Naik E, Dixit VM. Mitochondrial reactive oxygen species drive proinflammatory cytokine production. J Exp Med 2011;208:417–20.

[6] Allegra M, Reiter RJ, Tan DX, Gentile C, Tesoriere L, Livrea MA. The chemistry of melatonin's interaction with reactive species. J Pineal Res 2003;34:1–10.
[7] Tan DX, Manchester LC, Hardeland R, Lopez-Burillo S, Mayo JC, Sainz RM, et al. Melatonin: a hormone, a tissue factor, an autocoid, a paracoid, and an antioxidant vitamin. J Pineal Res 2003;34:75–8.
[8] Rodriguez C, Mayo JC, Sainz RM, Antolin I, Herrera F, Martin V, et al. Regulation of antioxidant enzymes: a significant role for melatonin. J Pineal Res 2004;36:1–9.
[9] Reiter RJ, Calvo JR, Karbownik M, Qi W, Tan DX. Melatonin and its relation to the immune system and inflammation. Ann NY Acad Sci 2000;917:376–86.
[10] Liu J, Huang F, He HW. Melatonin effects on hard tissues: bone and tooth. Int J Mol Sci 2013;14:10063–74.
[11] Nakade O, Koyama H, Ariji H, Yajima A, Kaku T. Melatonin stimulates proliferation and type I collagen synthesis in human bone cells in vitro. J Pineal Res 1999;27:106–10.
[12] Roth JA, Kim BG, Lin WL, Cho MI. Melatonin promotes osteoblast differentiation and bone formation. J Biol Chem 1999;274:22041–47.
[13] Koyama H, Nakade O, Takada Y, Kaku T, Lau KHW. Melatonin at pharmacologic doses increases bone mass by suppressing resorption through down-regulation of the RANKL-mediated osteoclast formation and activation. J Bone Miner Res 2002;17:1219–29.
[14] Suzuki N, Hattori A. Melatonin suppresses osteoclastic and osteoblastic activities in the scales of goldfish. J Pineal Res 2002;33:253–8.
[15] Cutando A, Gómez-Moreno G, Arana C, Muñoz F, Lopez-Peña M, Stephenson J, et al. Melatonin stimulates osteointegration of dental implants. J Pineal Res 2008;45:174–9.
[16] Blasiak J, Kasznicki J, Drzewoski J, Pawlowska E, Szczepanska J, Reiter RJ. Perspectives on the use of melatonin to reduce cytotoxic and genotoxic effects of methacrylate-based dental materials. J Pineal Res 2011;51:157–62.
[17] Gomez-Moreno G, Guardia J, Ferrera MJ, Cutando A, Reiter RJ. Melatonin in diseases of the oral cavity. Oral Dis 2010;16:242–7.
[18] Cutando A, Gomez-Moreno G, Arana C, Acuna-Castroviejo D, Reiter RJ. Melatonin: potential functions in the oral cavity. J Periodontol 2007;78:1094–102.
[19] Almughrabi OM, Marzouk KM, Hasanato RM, Shafik SS. Melatonin levels in periodontal health and disease. J Periodontal Res 2013;48:315–21.
[20] Srinath R, Acharya AB, Thakur SL. Salivary and gingival crevicular fluid melatonin in periodontal health and disease. J Periodontol 2010;81:277–83.
[21] Gómez-Moreno G, Cutando-Soriano A, Arana C, Galindo P, Bolaños J, Acuña-Castroviejo D, et al. Melatonin expression in periodontal disease. J Periodontal Res 2007;42:536–40.
[22] Blasiak J, Synowiec E, Tarnawska J, Czarny P, Poplawski T, Reiter RJ. Dental methacrylates may exert genotoxic effects via the oxidative induction of DNA double strand breaks and the inhibition of their repair. Mol Biol Rep 2012;39:7487–96.
[23] Cutando A, Arana C, Gómez-Moreno G, Escames G, López A, Ferrera MJ, et al. Local application of melatonin into alveolar sockets of beagle dogs reduces tooth removal—induced oxidative stress. J Periodontol 2007;78:576–83.
[24] Carrillo-Vico A, Guerrero JM, Lardone PJ, Reiter RJ. A review of the multiple actions of melatonin on the immune system. Endocrine 2005;27:189–200.
[25] Delvings P, McIsaac WM, Taborsky RG. The metabolism of 5-methoxytryptophol. J Biol Chem 1965;240:348–50.
[26] Millán-Plano S, Piedrafita E, Miana-Mena FJ, Fuentes-Broto L, Martínez-BallarÃín E, López-Pingarrón L, et al. Melatonin and structurally-related compounds protect synaptosomal membranes from free radical damage. Int J Mol Sci 2010;11:312–28.
[27] Satué M, Arriero MM, Ramis JM, Monjo M. Comparative effects of melatonin and 5-methoxytryptophol on osteoblast and osteoclast function in vitro. J Endocrinol 2013. Submitted.
[28] Gomez-Florit M, Ramis JM, Xing R, Taxt-Lamolle S, Haugen HJ, Lyngstadaas SP, et al. Differential response of human gingival fibroblasts to titanium- and titanium–zirconium-modified surfaces. J Periodontal Res 2013. http://dx.doi.org/10.1111/jre.12121.
[29] Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 2001;29:e45.
[30] Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper—Excel-based tool using pair-wise correlations. Biotechnol Lett 2004;26:509–15.
[31] Geback T, Schulz MM, Koumoutsakos P, Detmar M. TScratch: a novel and simple software tool for automated analysis of monolayer wound healing assays. Biotechniques 2009;46:265–74.
[32] Cutando A, Lopez-Valverde A, de Diego RG, de Vicente J, Reiter R, Herrero Fernandez M, et al. Effect of topical application of melatonin to the gingiva on salivary osteoprotegerin RANKL and melatonin levels in patients with diabetes and periodontal disease. Odontology 2013.
[33] Cutando A, Lopez-Valverde A, Gomez-de-Diego R, Arias-Santiago S, de Vicente-Jimenez J. Effect of gingival application of melatonin on alkaline and acid phosphatase, osteopontin and osteocalcin in patients with diabetes and periodontal disease. Med Oral Patol Oral Cir Bucal 2013;18:e657–63.
[34] Arendt J. Safety of melatonin in long-term use(?). J Biol Rhythms 1997;12:673–81.
[35] Bartold PM, Walsh LJ, Narayanan AS. Molecular and cell biology of the gingiva. Periodontology 2000 2000;24:28–55.
[36] Hakkinen L, Strassburger S, Kahari VM, Scott PG, Eichstetter I, Lozzo RV, et al. A role for decorin in the structural organization of periodontal ligament. Lab Invest 2000;80:1869–80.
[37] Satish L, Kathju S. Cellular and molecular characteristics of scarless versus fibrotic wound healing. Dermatol Res Pract 2010;2010:790234.
[38] Oksala O, Haapasalmi K, Hakkinen L, Uitto VJ, Larjava H. Expression of heparan sulphate and small dermatan/chondroitin sulphate proteoglycans in chronically inflamed human periodontium. J Dent Res 1997;76:1250–9.
[39] Cutando A, Aneiros-Fernández J, López-Valverde A, Arias-Santiago S, Aneiros-Cachaza J, Reiter RJ. A new perspective in oral health: potential importance and actions of melatonin receptors MT1, MT2, MT3, and RZR/ROR in the oral cavity. Arch Oral Biol 2011;56:944–50.
[40] Drobnik J, Owczarek K, Piera L, Tosik D, Olczak S, Ciosek J, et al. Melatonin-induced augmentation of collagen deposition in cultures of fibroblasts and myofibroblasts is blocked by luzindole—a melatonin membrane receptors inhibitor. Pharmacol Rep 2013;65:642–9.
[41] Lareu RR, Arsianti I, Subramhanya HK, Yanxian P, Raghunath M. In vitro enhancement of collagen matrix formation and crosslinking for applications in tissue engineering: a preliminary study. Tissue Eng 2007;13:385–91.
[42] Chen C, Raghunath M. Focus on collagen: in vitro systems to study fibrogenesis and antifibrosis—state of the art. Fibrog Tissue Repair 2009;2:7.
[43] Birkedal-Hansen H. Role of matrix metalloproteinases in human periodontal diseases. J Periodontol 1993;64:474–84.
[44] Sakagami G, Sato E, Sugita Y, Kosaka T, Kubo K, Maeda H, et al. Effects of nifedipine and interleukin-1alpha on the expression of collagen, matrix metalloproteinase-1, and tissue inhibitor of metalloproteinase-1 in human gingival fibroblasts. J Periodontal Res 2006;41:266–72.
[45] Soell M, Elkaim R, Tenenbaum H, Cathepsin C. Matrix metalloproteinases, and their tissue inhibitors in gingiva and gingival crevicular fluid from periodontitis-affected patients. J Dent Res 2002;81:174–8.
[46] Swarnakar S, Paul S, Singh LP, Reiter RJ. Matrix metalloproteinases in health and disease: regulation by melatonin. J Pineal Res 2011;50:8–20.
[47] Rudra DS, Pal U, Maiti NC, Reiter RJ, Swarnakar S. Melatonin inhibits matrix metalloproteinase-9 activity by binding to its active site. J Pineal Res 2013;54:398–405.
[48] Mao L, Yuan L, Slakey L, Jones F, Burow M, Hill S. Inhibition of breast cancer cell invasion by melatonin is mediated through regulation of the p38 mitogen-activated protein kinase signaling pathway. Breast Cancer Res 2010;12:R107.
[49] Heino J. The collagen receptor integrins have distinct ligand recognition and signaling functions. Matrix Biol 2000;19:319–23.
[50] Guo F, Carter DE, Mukhopadhyay A, Leask A. Gingival fibroblasts display reduced adhesion and spreading on extracellular matrix: a possible basis for scarless tissue repair? PLoS One 2011;6:e27097.
[51] Hinz B, Phan SH, Thannickal VJ, Galli A, Bochaton-Piallat ML, Gabbiani G. The myofibroblast: one function, multiple origins. Am J Pathol 2007;170:1807–16.
[52] Gurtner GC, Werner S, Barrandon Y, Longaker MT. Wound repair and regeneration. Nature 2008;453:314–21.
[53] Lin NH, Gronthos S, Bartold PM. Stem cells and periodontal regeneration. Aust Dent J 2008;53:108–21.
[54] Morton RS, Dongari-Bagtzoglou AI. Regulation of gingival fibroblast interleukin-6 secretion by cyclosporine A. J Periodontol 1999;70:1464–71.
[55] Mauriz JL, Collado PS, Veneroso C, Reiter RJ, Gonzalez-Gallego J. A review of the molecular aspects of melatonin's anti-inflammatory actions: recent insights and new perspectives. J Pineal Res 2012.
[56] Reiter RJ, Melchiorri D, Sewerynek E, Poeggeler B, Barlow-Walden L, Chuang J, et al. A review of the evidence supporting melatonin's role as an antioxidant. J Pineal Res 1995;18:1–11.
[57] Tan DX, Manchester LC, Reiter RJ, Plummer BF, Limson J, Weintraub ST, et al. Melatonin directly scavenges hydrogen peroxide: a potentially new metabolic pathway of melatonin biotransformation. Free Radical Biol Med 2000;29:1177–85.
[58] Aydogan S, Yerer MB, Goktas A. Melatonin and nitric oxide. J Endocrinol Invest 2006;29:281–7.
[59] Tomás-Zapico C, Coto-Montes A. A proposed mechanism to explain the stimulatory effect of melatonin on antioxidative enzymes. J Pineal Res 2005;39:99–104.
[60] Galano A, Tan DX, Reiter RJ. Melatonin as a natural ally against oxidative stress: a physicochemical examination. J Pineal Res 2011;51:1–16.
[61] Rodriguez-Naranjo MI, Moyá ML, Cantos-Villar E, Garcia-Parrilla MC. Comparative evaluation of the antioxidant activity of melatonin and related indoles. J Food Compos Anal 2012;28:16–22.
[62] Galano A, Tan DX, Reiter RJ. On the free radical scavenging activities of melatonin's metabolites, AFMK and AMK. J Pineal Res 2013;54:245–57.
[63] Kara A, Akman S, Ozkanlar S, Tozoglu U, Kalkan Y, Canakci CF, et al. Immune modulatory and antioxidant effects of melatonin in experimental periodontitis in rats. Free Radical Biol Med 2013;55:21–6.
[64] Tekbas OF, Ogur R, Korkmaz A, Kilic A, Reiter RJ. Melatonin as an antibiotic: new insights into the actions of this ubiquitous molecule. J Pineal Res 2008;44:222–6.
[65] Srinivasan V, Mohamed M, Kato H. Melatonin in bacterial and viral infections with focus on sepsis: a review. Recent Pat Endocr Metab Immune Drug Discov 2012;6:30–9.
[66] Mack D, Rohde H, Harris LG, Davies AP, Horstkotte MA, Knobloch JK. Biofilm formation in medical device-related infection. Int J Artif Organs 2006;29:343–59.
[67] Sbordone L, Bortolaia C. Oral microbial biofilms and plaque-related diseases: microbial communities and their role in the shift from oral health to disease. Clin Oral Investig 2003;7:181–8.

Paper II

Identification of quercitrin as potential therapeutic agent for periodontal applications

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Identification of Quercitrin as a Potential Therapeutic Agent for Periodontal Applications

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Background: Flavonoids are natural phenolic compounds with antioxidant, anti-inflammatory, and antimicrobial capacity. This study aims to investigate the effects of different flavonoids for potential use in periodontal applications.

Methods: Cultures of *Staphylococcus epidermidis* or primary human gingival fibroblasts (HGFs) were treated with different doses of chrysin, diosmetin, galangin, quercitrin, and taxifolin. The effect of these molecules was evaluated on *S. epidermidis* growth rate and HGF viability, gene expression, collagen production, reactive oxygen species (ROS) levels, wound healing, and production of matrix metalloproteinase (MMP)-1 and tissue inhibitor of MMP-1 (TIMP1).

Results: Among all the screened flavonoids, quercitrin showed the most promising biologic effects, in both HGFs and *S. epidermidis*. Thus, quercitrin was not toxic for HGFs; increased collagen IIIα1 and decorin levels; downregulated interleukin-6 messenger RNA levels; decreased the expression of profibrotic markers during wound healing; decreased ROS levels in basal and stimulated conditions; and decreased the MMP1/TIMP1 ratio. Quercitrin also decreased the bacterial growth rate.

Conclusions: Results suggest that quercitrin could contribute to protect and recover the integrity of gingival tissues, thus displaying a potential use for periodontal disease treatment or to functionalize dental implant abutments to improve soft tissue integration. Further studies are required to confirm the role of quercitrin in gingival tissues. *J Periodontol 2014;85:966-974.*

KEY WORDS

Antioxidants; fibroblasts; flavonoids; periodontal diseases; TIMP1 protein, human; wound healing.

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Periodontal disease is an inflammation and infection of the gingiva that leads to the breakdown of periodontal tissues and, in severe cases, is accompanied by the loss of alveolar bone with eventual loss of the tooth.[1] Gingival fibroblasts are the major constituents of periodontal connective tissue. They maintain gingival tissue integrity by regulating collagen and proteoglycan metabolism.[2] The tissue damage induced by periodontal disease is caused directly by bacteria and indirectly by inflammation and an immune response against these bacteria. During the inflammatory process, large quantities of reactive oxygen species (ROS) are produced[3] that, together with matrix metalloproteinases (MMPs), cause the breakdown of periodontal tissues. ROS have been shown to induce collagen degradation and cell damage[4] in addition to being pro-inflammatory.[5]

Those biomolecules capable of suppressing ROS and promoting collagen synthesis might constitute potential therapeutic agents for periodontal disease. Flavonoids are natural phenolic compounds present in fruit and vegetables with antioxidant,[6] anti-inflammatory,[7] and antimicrobial capacity,[8-12] among other biologic functions.[13,14] Flavonoids have structures consisting of three rings (two of them aromatic), which are labeled A, B, and C (see supplementary Fig. 1 in online *Journal of Periodontology*). The various classes of

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flavonoids differ in the level of oxidation and pattern of substitution of the C ring, whereas individual compounds within a class differ in the pattern of substitution of the A and B rings. Some flavonoids, mixtures of them, or polyphenol plant extracts have been shown to have beneficial effects on human gingival fibroblasts (HGFs).[15-21] In fact, cranberry pro-anthocyanidins (a group of flavonoids) have been suggested as natural weapons against periodontal disease.[22] However, to the best of the authors' knowledge, no previous data exist on the effect of any of the selected flavonoids on HGFs. Interestingly, chrysin and diosmetin were selected as candidate biomolecules due to their substantial antimicrobial activity.[12] Furthermore, it has been shown that galangin has an antibacterial effect in resistant strains of diverse bacteria,[9-11,23] that taxifolin enhances the efficacy of conventional antibiotics in methicillin-resistant *Staphylococcus aureus*,[8] and that quercitrin has been used as an antibacterial agent.[24]

In the present study, with the aim to identify potential agents for periodontal applications, the authors screened different flavonoids. Two flavones (chrysin and diosmetin), two flavonols (galangin and quercitrin), and a flavanone (taxifolin, also known as dihydroquercetin) were tested for their effect on HGFs (the major constituents of periodontal connective tissue) and for their antimicrobial properties.

MATERIALS AND METHODS

Biomolecules Used for Testing

All reagents were obtained from one commercial source,† unless otherwise specified. Galangin, quercitrin, and taxifolin were dissolved in absolute ethanol, and chrysin and diosmetin were dissolved in dimethyl sulfoxide (DMSO). Treatment concentrations were prepared by diluting stock solutions in culture media before use. Final concentrations of ethanol (1%) or DMSO (0.6%) were included as vehicle control groups in the experiments.

Culture and Proliferation Assay of Staphylococcus epidermidis

S. epidermidis 4184‡ were grown from frozen stocks in lysogeny broth. After an overnight incubation, 0.2-mL bacterial suspensions (1×10^6 bacteria/mL) were incubated with the different treatments in 96-well flat-bottomed plates for 24 hours. Vehicle control groups served as negative control, and 6-μg/mL vancomycin served as positive control for bacterial growth inhibition. Plates were incubated in a microplate reader§ with temperature control and shaking function at 37°C for 24 hours. At regular intervals, the optical density (OD) was measured at 600 nm to determine bacterial proliferation. The assays were carried out in triplicate, with three replicates at each condition. Bacterial growth rate (μ) was calculated during the exponential growth phase following the equation $\ln OD_t - \ln OD_0 = \mu(t - t_0)$.

Cell Culture

Three different donors of primary HGFs‖ were used: HGF-A (aged 27 years, white, female, lot number 313X100401), HGF-B (aged 19 years, white, male, lot number 322X070501), and HGF-C (aged 47 years, white, male, lot number 323X070501). All donors provided written informed consent, and the investigators were not able to ascertain the identity of the donors. The study was conducted in accordance with the Helsinki Declaration of 1975, as revised in 2000. HGF cells were routinely cultured at 37°C in a humidified atmosphere of 5% CO_2 and maintained in fibroblast growth medium supplemented with 10% fetal calf serum, 50 ng amphotericin/mL, and 50 μg gentamicin/mL.¶ Cells were subcultured 1:4 before reaching confluence using phosphate-buffered saline (PBS) and trypsin/EDTA,# as recommended by the suppliers. Experiments were performed with HGF cells between passages 7 and 8 after isolation. Trypan blue stain was used to determine total and viable cell number.

Three replicate wells for each donor were seeded in 48-well plates at a density of 1.0×10^4 cells/well for all control and test samples (n = 9). For collagen and ROS quantification, cells from one donor were seeded in 96-well plates at a density of 7.0×10^3 (n = 6). Forty-eight hours after seeding, cells were treated with different doses (ranging from 0.1 to 500 μM) of the selected flavonoids diluted in complete fibroblast growth medium. Medium was changed every other day. Treatments were added at every medium change to keep the concentration of biomolecules in solution throughout the whole experiment.

Determination of Cytotoxicity

The presence of lactate dehydrogenase (LDH) activity in the culture media after 48 hours of incubation was used as an index of cell death. LDH activity was determined spectrophotometrically after 30 min of incubation at 25°C of 50 μL culture medium and 50 μL reaction mixture, by measuring the oxidation of nicotinamide adenine dinucleotide at 490 nm in the presence of pyruvate, according to the kit manufacturer's instructions.** Results from all the samples were presented relative to the LDH activity in the medium of cells treated with the vehicle control

† Sigma-Aldrich, St. Louis, MO.
‡ Colección Española de Cultivos Tipo, Valencia, Spain.
§ PowerWave HT, Biotek Instruments, Winooski, VT.
‖ Provitro, Berlin, Germany.
¶ Provitro.
# Provitro.
** Cytotoxicity Detection kit, Roche Diagnostics, Mannheim, Germany.

for each case, 1% ethanol or 0.6% DMSO (low control, 0% of cell death), and of cells treated with 1% non-ionic surfactant[††] (high control, 100% cell death). The percentage of LDH activity was calculated using the following equation: cytotoxicity (%) = [(expected value – low control)/(high control – low control)] × 100.

RNA Isolation and Real-Time Polymerase Chain Reaction

Total RNA was isolated after 14 days of incubation using an isolation reagent,[‡‡] according to the manufacturer's protocol. Total RNA was quantified at 260 nm using a nanodrop spectrophotometer.[§§] The same amount of RNA (0.2 μg) was reverse transcribed to complementary DNA (cDNA) at 42°C for 60 min, according to the protocol of the supplier.[‖‖] Aliquots of each cDNA were frozen (–20°C) until the polymerase chain reaction (PCR) was carried out.

Real-time PCR was performed for two reference genes, *GAPDH* and β-actin (*ACTBL2*), and target genes, collagen III α1 (*COL3A1*), decorin (*DCN*), integrin α2 (*ITGA2*), interleukin-6 (*IL6*), *MMP1*, tissue inhibitor of MMP-1 (*TIMP1*), α-smooth muscle actin (*ACTA2*), endothelin-1 (*EDN1*), and transforming growth factor-β1 (*TGFB1*). Real-time PCR was performed in a thermocycler[¶¶] using SYBR green detection. Each reaction contained 7 μL master mix,[##] 0.5 μM of each gene (except 0.25 μM for *COL3A1* and *ITGA2*), the sense and antisense specific primers (see supplementary Table 1 in online *Journal of Periodontology*), and 3 μL cDNA dilution in a final volume of 10 μL. The amplification program consisted of a preincubation step for denaturation of the template cDNA (5 minutes at 95°C), followed by 45 cycles consisting of a denaturation step (10 seconds at 95°C), an annealing step (10 seconds at 60°C), and an extension step (10 seconds at 72°C). After each cycle, fluorescence was measured at 72°C. A negative control without cDNA template was run in each assay.

All samples were normalized by the geometric mean of the expression levels of *ACTBL2* and *GAPDH*, and fold changes were related to the control groups using the mathematic model described by Pfaffl:[25] ratio = $E_{\text{target}}^{\Delta Cp \text{ target (mean control – sample)}} / E_{\text{reference}}^{\Delta Cp \text{ reference (mean control – sample)}}$, where Cp is the crossing point of the reaction amplification curve as determined by the software.[***] Stability of reference genes was calculated using a statistical tool.[†††] The crossing point variation of the reference genes among samples was <0.56. Moreover, a good consistency of the pair-wise correlation index[‡‡‡] was proved by the fact that its contributing reference genes were tightly correlated with it ($0.831 < r < 0.917$), with a significance level of $P = 0.001$ for all reference genes.

Collagen Quantification

After 14 days of incubation, cells were washed with PBS and dried overnight at 37°C in a humidified atmosphere plus 24 hours at 37°C in a dry atmosphere. Collagen was stained with Sirius red F3BA 0.1% in saturated picric acid for 1 hour. Unbound dye was removed by washing with 10 mM HCl, and dye was solubilized with 100 mM NaOH. Absorbance was measured with a microplate reader at 540 nm. Readings were compared with a calfskin collagen standard included in the assay.

ROS Assay

When they reached confluence, cells were incubated for 0.5 hours with 100 μM H_2O_2 (H_2O_2-stimulated group) or in normal conditions (basal group). Before treatment, cells were washed with PBS and treated with quercitrin, taxifolin, or vehicle control overnight under standard cell culture conditions. Growth medium was removed, and cells were stained with 10 μM dichlorodihydrofluorescein diacetate[§§§] (H2DCF-DA) in growth medium without fetal calf serum for 0.5 hours at 37°C. Then, a fluorometer[‖‖‖] was set to record fluorescence of H2DCF-DA according to the manufacturer's protocol.

Wound Healing Assay

Forty-eight hours after seeding, the monolayer was scraped with a 200-μL sterile pipette tip in a straight line to create a scratch, and cells were washed once with growth medium to remove debris and detached cells. Cells were then treated with 200 and 500 μM quercitrin or 10 and 100 μM taxifolin. Immediately after treatment and 48 hours later, images of the same areas were taken using a bright-field inverted microscope.[¶¶¶] Then, culture medium was removed, and RNA was isolated as described previously to study gene expression of *MMP1*, *ACTA2*, *EDN1*, and *TGFB1*. The images were quantitatively analyzed[###] using the default parameter settings.[26] The open wound area (%) was defined as 100 × (uncovered image area at 48 hours/uncovered image area at 0 hours).

†† Triton X-100, Sigma Aldrich.
‡‡ TriPure, Roche Diagnostics.
§§ NanoDrop Technologies, Wilmington, DE.
‖‖ High Capacity RNA-to-cDNA kit, Applied Biosystems, Thermo Fisher Scientific, Waltham, MA.
¶¶ LightCycler 480, Roche Diagnostics.
LightCycler 480 SYBR Green I Master, Roche Diagnostics.
*** LightCycler 480 software, Roche Diagnostics.
††† BestKeeper software, Technical University of Munich, Weihenstephan, Germany.
‡‡‡ BestKeeper software, Technical University of Munich.
§§§ Molecular Probes, Thermo Fisher Scientific.
‖‖‖ Cary Eclipse, Agilent Technologies, Santa Clara, CA.
¶¶¶ Leica DM IRB, Wetzlar, Germany.
TScratch, Swiss Federal Institute of Technology, Zürich, Switzerland.

Enzyme-Linked Immunosorbent Assays

The detection of proteins TIMP1 and MMP1 was performed from cell culture media after 14 days of cell culture (n = 9) by commercially available enzyme-linked immunosorbent assay kits according to the instructions.

Statistical Analyses

All data are presented as mean values ± SEM. The Kolmogorov–Smirnov test was done to test parametric or non-parametric distributions for the normality tests. Effect of dose was assessed by analysis of variance (ANOVA) test. Differences between treatment and control groups were assessed by Mann–Whitney *U* test, Wilcoxon test for ratio, Student *t* test, or paired *t* test. A specific computer program**** was used. Results were considered statistically significant at $P \leq 0.05$.

RESULTS

Effect of the Different Flavonoids on S. epidermidis *Growth*

To calculate the growth rate, the exponential growth phase was set from 8 to 16 hours of culture (data not shown). Although some chrysin, diosmetin, and quercitrin doses inhibited bacterial growth rate (9%, 12% to 16%, and 33% inhibition, respectively), galangin and 200 μM taxifolin increased it (Fig. 1). Vancomycin, used as a positive control for bacterial growth inhibition, totally inhibited the growth of bacteria (data not shown).

Effect of the Different Flavonoids on Cytotoxicity and Cell Morphology in HGFs

Figure 2 shows that treatment with quercitrin induced a significantly lower release of LDH compared with low control (cells treated with the vehicle control). On the other hand, 100 and 200 μM chrysin, 500 μM galangin, and doses ≥10 μM taxifolin produced a significantly higher release of LDH to the culture media compared with their respective vehicle low controls. However, visual observation of cell morphology (data not shown) indicated that doses of 100, 200, and 500 μM galangin and of 100 and 200 μM chrysin were toxic, since cells had a round and not a fibroblastic shape. For that reason, those treatments were not further processed. Although no toxic effect was found after 48 hours of treatment, cell morphology and cell number indicated that treatment for 14 days with doses >10 μM diosmetin and 200 μM taxifolin was also toxic for the cells.

Effect of the Different Flavonoids on Gene Expression in HGFs

COL3A1 messenger RNA (mRNA) levels significantly increased in cells cultured with several conditions (reaching a three-fold increase for 500 μM quercitrin), although cells treated with 10 μM

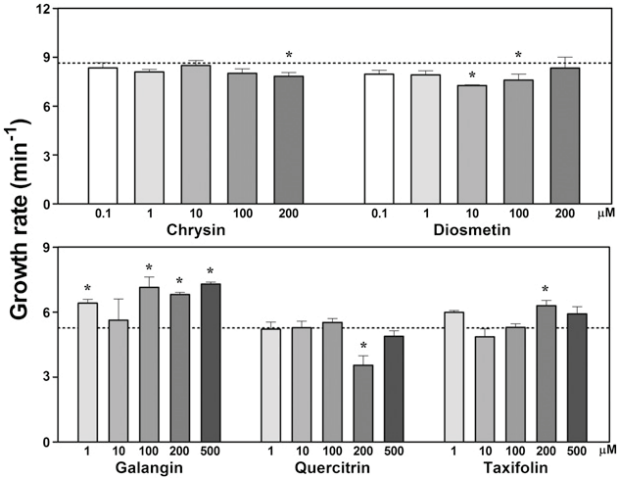

Figure 1.
*S. epidermidis growth rate cultured with different doses of the selected flavonoids. Data represent the mean ± SEM of three independent experiments (n = 9). Dotted line indicates control group growth rate. Differences between groups were assessed by Student t test: *P <0.05 treatment versus control (DMSO for chrysin and diosmetin; ethanol for galangin, quercitrin, and taxifolin).*

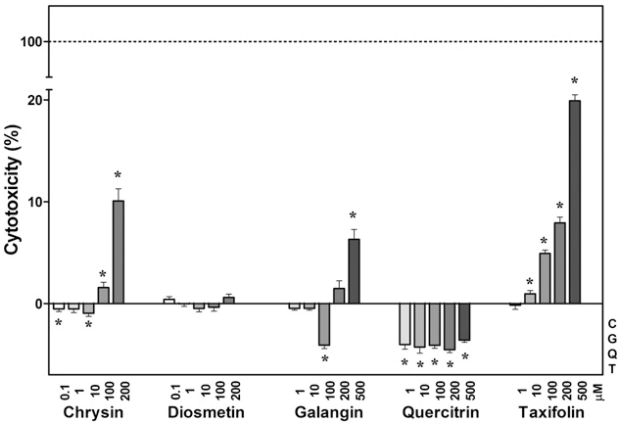

Figure 2.
*LDH activity measured in culture media collected after 48 hours of treatment in HGFs. High control (100% cytotoxicity) was cell culture media from cells treated with 1% non-ionic surfactant. Low control (0% cytotoxicity) was cell culture media from control vehicle cells. Values represent the mean ± SEM (n = 9). Effect of dose was assessed by ANOVA. Significant differences (P <0.05): C = effect of chrysin, G = galangin, Q= quercitrin, T = taxifolin. Moreover, paired t test was performed to assess differences between treatments and control groups: *P <0.05 treatment versus control (DMSO for chrysin and diosmetin; ethanol for galangin, quercitrin, and taxifolin).*

chrysin showed decreased expression compared with the control (Fig. 3). *DCN* expression increased in cells treated with chrysin, galangin, quercitrin, and taxifolin. Again, 500 μM quercitrin achieved the highest expression increase, and 10 μM chrysin decreased *DCN* expression compared with the

**** SPSS for Windows, v.17.0, IBM, Chicago, IL.

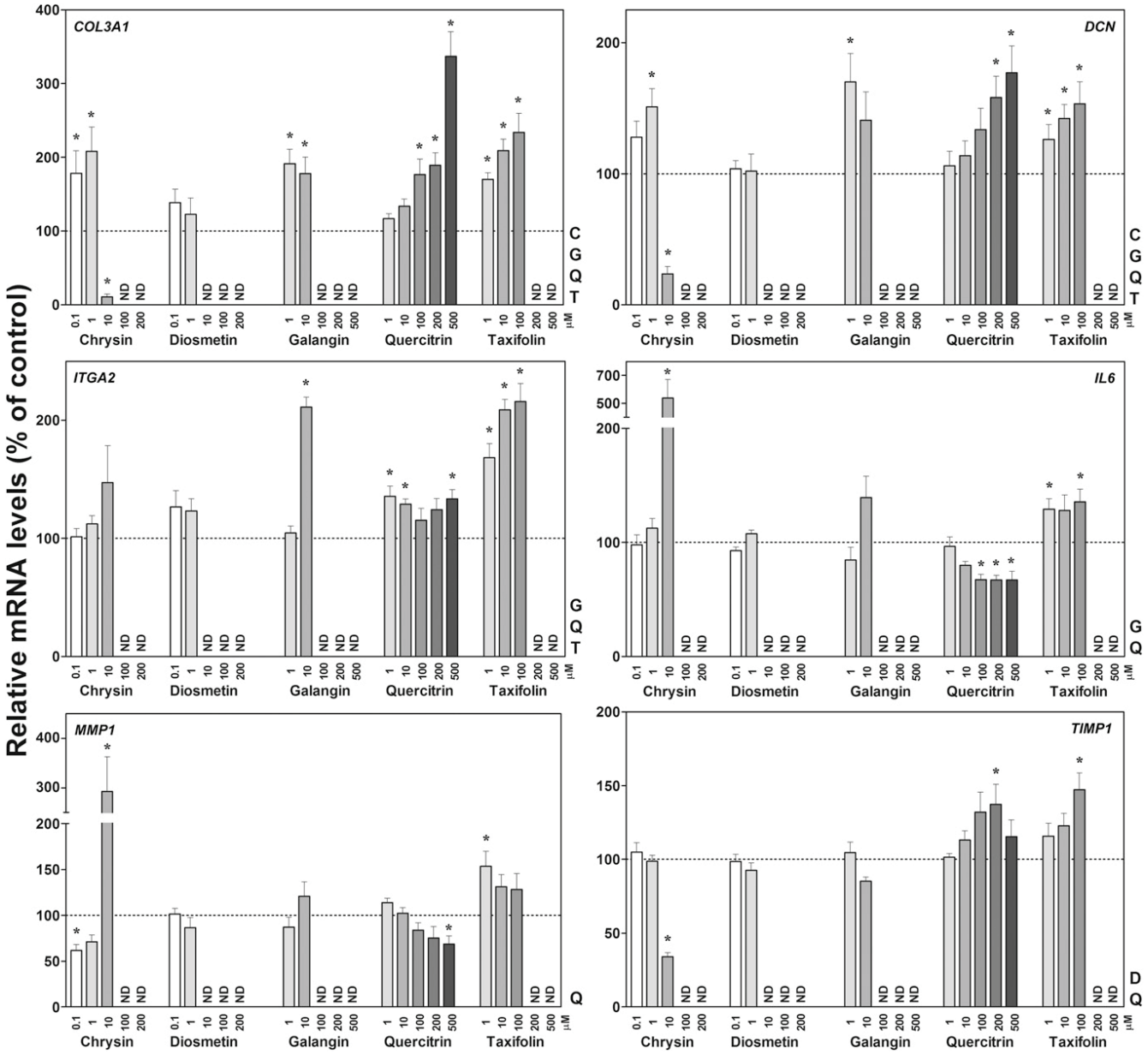

Figure 3.

Effect of treatment with flavonoids for 14 days on the mRNA levels of COL3A1, DCN, MMP1, TIMP1, ITGA2, *and* IL6 *in HGFs. ND = not determined. Data were normalized to reference genes (β-actin and* GAPDH*), expressed as percentage of control, which was set to 100%. Values represent the mean ± SEM (n = 9). Effect of dose was assessed by ANOVA. Significant differences (P <0.05): C = effect of chrysin, G = galangin, Q= quercitrin, T = taxifolin. Paired t test was performed to assess differences between treatments and control groups:* *****P ≤*0.05 treatment versus control (DMSO for chrysin and diosmetin; ethanol for galangin, quercitrin, and taxifolin).*

control. It was found that *ITGA2* expression increased in cells treated with galangin and taxifolin, and to a lesser extent with quercitrin. *IL6* and *MMP1* achieved the highest expression levels in cells cultured with 10 µM chrysin, being also upregulated with taxifolin treatments. Cells treated with quercitrin showed decreased *IL6* and *MMP1* expression levels. On the other hand, cells treated with quercitrin and taxifolin showed increased *TIMP1* expression levels.

Effects of Flavonoids on Collagen Deposition and ROS Levels in HGFs

After 14 days, total collagen decreased in cells treated with 10 µM taxifolin compared with control (Fig. 4A). Quercitrin and taxifolin reduced the oxidative stress in basal (0 µM H_2O_2) and stimulated (100 µM H_2O_2) situations, compared with the control group. Furthermore, 10 µM taxifolin decreased oxidative stress compared with 1 µM taxifolin (Fig. 4B).

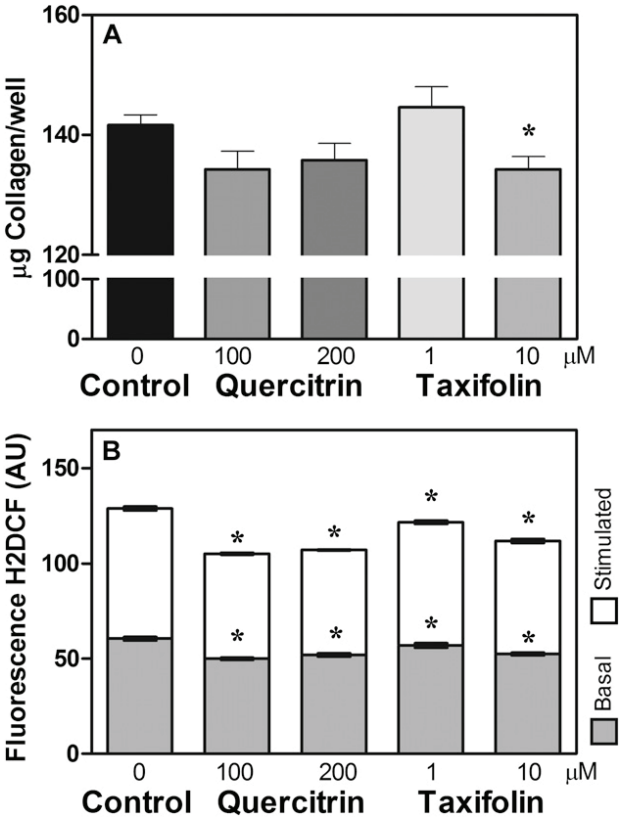

Figure 4.
Effects of quercitrin and taxifolin on collagen production ***(A)*** *and ROS levels* ***(B)****. AU = arbitrary units. Values represent the mean ± SEM (n = 6). ROS were evaluated in basal (0 µM H_2O_2) and stimulated (100 µM H_2O_2) situations. Student t test was performed to assess differences between treatments and control groups: *P ≤0.05 treatment versus control (ethanol).*

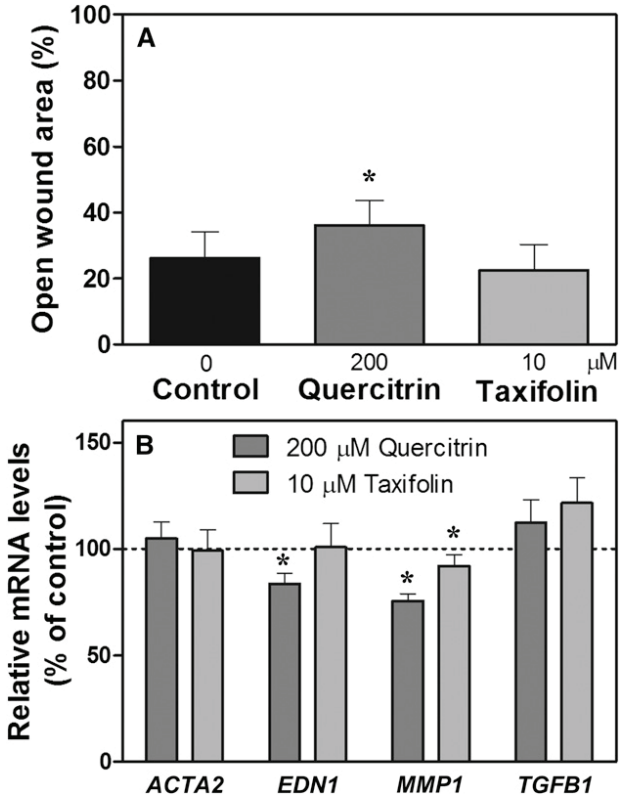

Figure 5.
Wound healing assay. Wound open area ***(A)*** *and gene expression levels of* ACTA2, EDN1, MMP1, *and* TGFB1 ***(B)*** *after 48 hours of healing in the presence of quercitrin and taxifolin. Data represent fold changes of target genes normalized to* β-actin *and* GAPDH *(reference genes) expressed relative to a control group that was set to 100%. Values represent the mean ± SEM (n = 9). Paired t test was performed to assess differences between treatments and control groups: *P ≤0.05 treatment versus control (ethanol).*

Effects of Flavonoids on Wound Healing in HGFs

The effects of 200 µM quercitrin and 10 µM taxifolin on wound healing were analyzed. Although 200 µM quercitrin increased open wound areas compared with control groups (Fig. 5), it significantly decreased the expression of *EDN1* and *MMP1* after 48 hours of healing, whereas taxifolin only decreased *MMP1* expression compared with untreated cells.

Effects of Flavonoids on TIMP1 and MMP1 Production in HGFs

Production of TIMP1 increased by treatment with 200 µM quercitrin and 10 µM taxifolin, whereas the MMP1/TIMP1 ratio only decreased in cells treated with 200 µM quercitrin compared with control (Fig. 6).

DISCUSSION

This study demonstrates for the first time that quercitrin displays a potential use for periodontal disease treatment; the authors show that quercitrin is not toxic for HGFs, increases collagen IIIα1 and decorin levels, downregulates *IL-6* mRNA levels, decreases the expression of profibrotic markers during wound healing, decreases ROS levels in basal and stimulated conditions, and decreases the MMP1/TIMP1 ratio. Quercitrin also decreases the bacterial growth rate.

In periodontal disease, the response to oral pathogens results in a strong release of inflammatory mediators, such as ROS, IL6, and MMPs, inducing collagen degradation, which ends in tissue breakdown. Together with this process, failed tissue repair occurs, resulting in fibrosis and scarring.[2] In searching for new agents for potential use in periodontal disease treatment, those biomolecules capable of: 1) increasing collagen synthesis; 2) promoting scarless tissue repair; and 3) decreasing ROS, in addition to 4) antibacterial activity, might constitute potential therapeutic agents. Flavonoids can act as antioxidant, anti-inflammatory, and ROS-scavenging molecules. However, their effects on gingival fibroblasts are unknown.

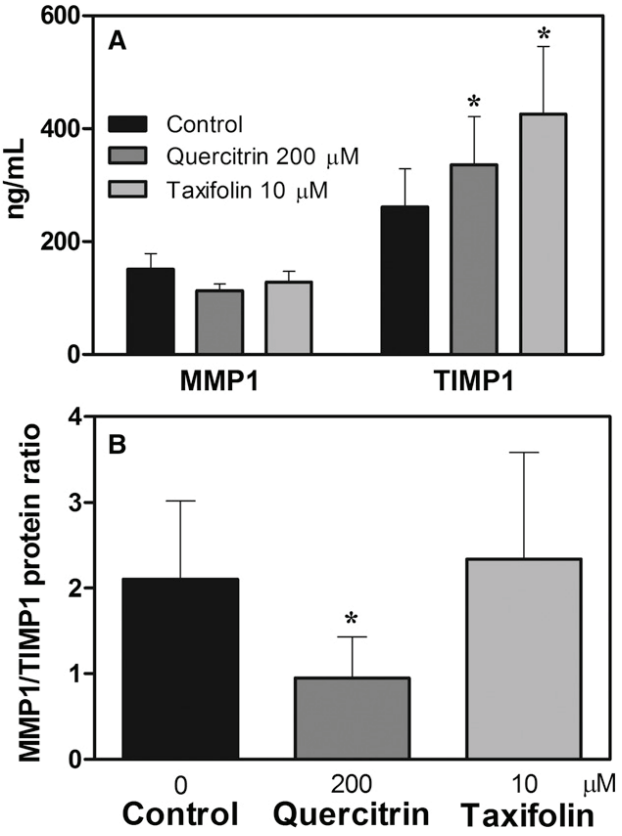

Figure 6.
Effect of quercitrin and taxifolin on MMP1 and TIMP1 production ***(A)*** *and on the MMP1/TIMP1 ratio* ***(B)*** *after 14 days of cell culture. Values represent the mean ± SEM (n = 9). Significant differences were assessed by paired t test for MMP1 and TIMP1 production and by Wilcoxon test for the MMP1/TIMP1 ratio:* *******P ≤0.05 treatment versus control (ethanol).*

The progression of periodontal disease is characterized by increased collagen solubility and degradation rate, producing a net connective tissue loss.[2] Quercitrin and taxifolin increased *COL3A1* and *DCN* mRNA levels. Decorin, a small leucine-rich proteoglycan highly expressed in human gingiva,[27] regulates fibril organization of collagens,[28] including type I and type III, the major protein components of the gingival connective tissue extracellular matrix (ECM).[27] Furthermore, *COL3A1* mRNA increases during HGF differentiation,[29] which is associated with scarless wound healing.[30] In periodontal disease, the breakdown of collagen parallels a decrease in decorin.[31] Therefore, the present mRNA expression results suggest a potential use of quercitrin and taxifolin in periodontal disease treatment. Despite the *COL3A1* mRNA levels, collagen production was similar among groups. This discrepancy can be explained by the lack of ascorbic acid in the fibroblast growth media, leading to the accumulation of unprocessed procollagen in the cell culture medium and a minimal deposition of secreted collagen.[32,33] Under the described culture media conditions, collagen deposition was slowed down;[33] therefore, gene expression results represent the effect of the studied flavonoids on collagen production.

ECM undergoes a constant turnover of its components, in both normal and diseased conditions, mainly through MMP-mediated collagen degradation.[2] Excess MMP production leads to accelerated ECM degradation associated with periodontitis.[34] The activity of MMPs is controlled by proteolytic activation, production regulation, and neutralization by TIMPs.[35] An imbalance of MMPs over their inhibitors results in a fibrolytic situation.[36] In the present study, quercitrin treatment decreased MMP1/TIMP1 mRNA and protein ratios, suggesting that quercitrin may decrease MMP-related ECM breakdown, as previously suggested for other flavonoids.[37] Targeting the MMPs has been used as a therapeutic option[38] because in vivo studies suggest that subantimicrobial doses of doxycycline, an MMP activity inhibitor, in combination with surgery reduce periodontal disease and inhibit early-stage bone resorption.[39] In addition, quercitrin downregulated *IL6* expression, thus contributing to a decrease in the inflammatory reaction, another way to prevent and control periodontal disease,[40] since increased *IL6* levels are associated with periodontal disease–related bone resorption.[20]

Wound healing response depends on the ability of fibroblasts to produce and arrange new collagen and other ECM components in similar quantities and ratios to unwounded tissue.[30] This process can produce either a scarred (repaired) tissue or a structural and functional regenerated tissue.[41,42] Furthermore, contrary to scarring, efficient regeneration requires inhibition of inflammation and wound contraction.[43] Here, the mRNA levels of the profibrotic markers *END1* and *MMP1* after wound healing decreased for cells treated with 200 μM quercitrin, and *IL6* mRNA levels decreased, together with a slowed-down wound healing, suggesting a more-regenerative/less-scarring response. In addition, expression of *ITGA2*—a collagen receptor needed for matrix remodeling,[44] increased levels of which are associated with a fibrotic phenotype[45]—was upregulated for taxifolin treatments, whereas 200 μM quercitrin did not increase it.

Quercitrin and taxifolin decrease oxidative stress levels on HGFs in the present research, as previously demonstrated with other antioxidants.[46] Prolonged ROS release produces tissue damage and contributes to the development of many chronic inflammatory diseases. Previous studies have shown that selected phenolic compounds protect tissues from ROS-mediated damage to proteoglycans, lipids, collagens, and TIMPs.[47] Future work should explore the

effects of the flavonoids selected in the present study in oxidation products.

In addition, the different flavonoids exhibited different antibacterial properties toward *S. epidermidis*, which is one of the initial colonizers during dental plaque formation and is involved in implant surface biofilm formation.[48,49] Among the tested biomolecules, 200 μM quercitrin showed the strongest bacterial growth inhibition, although it diminished at higher concentrations, probably because of solubility problems of flavonoids at high concentrations.[9] Previous studies have hypothesized that the hydroxylation degree might affect the antibacterial activity of phenolic compounds,[50] which is in agreement with the present finding with quercitrin. However, it was not the scope of the present research to study structure–activity relationships.

CONCLUSIONS

In summary, quercitrin increases collagen and decorin expression, decreases the MMP1/TIMP1 ratio and *IL-6* and ROS levels, and promotes scarless wound healing, all in all suggesting that it could contribute to protect and recover the integrity of gingival tissues. Furthermore, quercitrin also decreases the *S. epidermidis* growth rate. Further studies are required to evaluate the possible applications in the prevention of periodontal disease and as coating for dental implant abutments to improve soft tissue integration.

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REFERENCES

1. Smalley JW. Pathogenic mechanisms in periodontal disease. *Adv Dent Res* 1994;8:320-328.
2. Bartold PM, Narayanan AS. Molecular and cell biology of healthy and diseased periodontal tissues. *Periodontol 2000* 2006;40:29-49.
3. Chapple IL, Matthews JB. The role of reactive oxygen and antioxidant species in periodontal tissue destruction. *Periodontol 2000* 2007;43:160-232.
4. Waddington RJ, Moseley R, Embery G. Reactive oxygen species: A potential role in the pathogenesis of periodontal diseases. *Oral Dis* 2000;6:138-151.
5. Naik E, Dixit VM. Mitochondrial reactive oxygen species drive proinflammatory cytokine production. *J Exp Med* 2011;208:417-420.
6. Pietta P-G. Flavonoids as antioxidants. *J Nat Prod* 2000;63:1035-1042.
7. Izzi V, Masuelli L, Tresoldi I, et al. The effects of dietary flavonoids on the regulation of redox inflammatory networks. *Front Biosci (Landmark Ed)* 2012;17:2396-2418.
8. An J, Zuo GY, Hao XY, Wang GC, Li ZS. Antibacterial and synergy of a flavanonol rhamnoside with antibiotics against clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA). *Phytomedicine* 2011;18:990-993.
9. Cushnie TP, Hamilton VE, Lamb AJ. Assessment of the antibacterial activity of selected flavonoids and consideration of discrepancies between previous reports. *Microbiol Res* 2003;158:281-289.
10. Cushnie TP, Lamb AJ. Assessment of the antibacterial activity of galangin against 4-quinolone resistant strains of *Staphylococcus aureus*. *Phytomedicine* 2006;13:187-191.
11. Lee YS, Kang OH, Choi JG, et al. Synergistic effects of the combination of galangin with gentamicin against methicillin-resistant *Staphylococcus aureus*. *J Microbiol* 2008;46:283-288.
12. Uzel A, Sorkun K, Onçağ O, Cogŭlu D, Gençay O, Salih B. Chemical compositions and antimicrobial activities of four different Anatolian propolis samples. *Microbiol Res* 2005;160:189-195.
13. Domitrovic R. The molecular basis for the pharmacological activity of anthocyans. *Curr Med Chem* 2011;18:4454-4469.
14. Pilšáková L, Riečanský I, Jagla F. The physiological actions of isoflavone phytoestrogens. *Physiol Res* 2010;59:651-664.
15. Hosokawa Y, Hosokawa I, Shindo S, et al. Black tea polyphenol inhibits CXCL10 production in oncostatin M-stimulated human gingival fibroblasts. *Int Immunopharmacol* 2011;11:670-674.
16. Ara T, Honjo K, Fujinami Y, Hattori T, Imamura Y, Wang PL. Preventive effects of a kampo medicine, orento on inflammatory responses in lipopolysaccharide treated human gingival fibroblasts. *Biol Pharm Bull* 2010;33:611-616.
17. Tomofuji T, Ekuni D, Irie K, et al. Preventive effects of a cocoa-enriched diet on gingival oxidative stress in experimental periodontitis. *J Periodontol* 2009;80:1799-1808.
18. Hosokawa Y, Hosokawa I, Ozaki K, Nakanishi T, Nakae H, Matsuo T. Catechins inhibit CCL20 production in IL-17A-stimulated human gingival fibroblasts. *Cell Physiol Biochem* 2009;24:391-396.
19. Gutiérrez-Venegas G, Jiménez-Estrada M, Maldonado S. The effect of flavonoids on transduction mechanisms in lipopolysaccharide-treated human gingival fibroblasts. *Int Immunopharmacol* 2007;7:1199-1210.
20. Bodet C, Chandad F, Grenier D. Cranberry components inhibit interleukin-6, interleukin-8, and prostaglandin E production by lipopolysaccharide-activated gingival fibroblasts. *Eur J Oral Sci* 2007;115:64-70.
21. Al-Shaher A, Wallace J, Agarwal S, Bretz W, Baugh D. Effect of propolis on human fibroblasts from the pulp and periodontal ligament. *J Endod* 2004;30:359-361.
22. Feghali K, Feldman M, La VD, Santos J, Grenier D. Cranberry proanthocyanidins: Natural weapons against periodontal diseases. *J Agric Food Chem* 2012;60:5728-5735.
23. Eumkeb G, Sakdarat S, Siriwong S. Reversing β-lactam antibiotic resistance of *Staphylococcus aureus* with galangin from *Alpinia officinarum* Hance and synergism with ceftazidime. *Phytomedicine* 2010;18:40-45.
24. Soberón JR, Sgariglia MA, Sampietro DA, Quiroga EN, Vattuone MA. Antibacterial activity of plant extracts

from northwestern Argentina. *J Appl Microbiol* 2007; 102:1450-1461.
25. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 2001;29:e45.
26. Gebäck T, Schulz MM, Koumoutsakos P, Detmar M. TScratch: A novel and simple software tool for automated analysis of monolayer wound healing assays. *Biotechniques* 2009;46:265-274.
27. Bartold PM, Walsh LJ, Narayanan AS. Molecular and cell biology of the gingiva. *Periodontol 2000* 2000;24: 28-55.
28. Häkkinen L, Strassburger S, Kähäri VM, et al. A role for decorin in the structural organization of periodontal ligament. *Lab Invest* 2000;80:1869-1880.
29. Gómez-Florit M, Ramis JM, Xing R, et al. Differential response of human gingival fibroblasts to titanium- and titanium-zirconium-modified surfaces [published online ahead of print August 6, 2013]. *J Periodontal Res*. 10.1111/jre.12121.
30. Satish L, Kathju S. Cellular and molecular characteristics of scarless versus fibrotic wound healing. *Dermatol Res Pract* 2010;2010:790234.
31. Oksala O, Haapasalmi K, Häkkinen L, Uitto VJ, Larjava H. Expression of heparan sulphate and small dermatan/chondroitin sulphate proteoglycans in chronically inflamed human periodontium. *J Dent Res* 1997;76: 1250-1259.
32. Lareu RR, Arsianti I, Subramhanya HK, Yanxian P, Raghunath M. In vitro enhancement of collagen matrix formation and crosslinking for applications in tissue engineering: A preliminary study. *Tissue Eng* 2007;13: 385-391.
33. Chen CZ, Raghunath M. Focus on collagen: In vitro systems to study fibrogenesis and antifibrosis state of the art. *Fibrogenesis Tissue Repair* 2009;2:7.
34. Birkedal-Hansen H. Role of matrix metalloproteinases in human periodontal diseases. *J Periodontol* 1993;64 (Suppl. 5):474-484.
35. Sakagami G, Sato E, Sugita Y, et al. Effects of nifedipine and interleukin-1alpha on the expression of collagen, matrix metalloproteinase-1, and tissue inhibitor of metalloproteinase-1 in human gingival fibroblasts. *J Periodontal Res* 2006;41:266-272.
36. Soell M, Elkaim R, Tenenbaum H. Cathepsin C, matrix metalloproteinases, and their tissue inhibitors in gingiva and gingival crevicular fluid from periodontitis-affected patients. *J Dent Res* 2002;81:174-178.
37. Lim H, Kim HP. Inhibition of mammalian collagenase, matrix metalloproteinase-1, by naturally-occurring flavonoids. *Planta Med* 2007;73:1267-1274.
38. Fingleton B. MMPs as therapeutic targets — Still a viable option? *Semin Cell Dev Biol* 2008;19:61-68.
39. Gapski R, Hasturk H, Van Dyke TE, et al. Systemic MMP inhibition for periodontal wound repair: Results of a multi-centre randomized-controlled clinical trial. *J Clin Periodontol* 2009;36:149-156.
40. Morton RS, Dongari-Bagtzoglou AI. Regulation of gingival fibroblast interleukin-6 secretion by cyclosporine A. *J Periodontol* 1999;70:1464-1471.
41. Gurtner GC, Werner S, Barrandon Y, Longaker MT. Wound repair and regeneration. *Nature* 2008;453: 314-321.
42. Lin NH, Gronthos S, Bartold PM. Stem cells and periodontal regeneration. *Aust Dent J* 2008;53:108-121.
43. Metcalfe AD, Ferguson MW. Tissue engineering of replacement skin: The crossroads of biomaterials, wound healing, embryonic development, stem cells and regeneration. *J R Soc Interface* 2007;4:413-437.
44. Heino J. The collagen receptor integrins have distinct ligand recognition and signaling functions. *Matrix Biol* 2000;19:319-323.
45. Guo F, Carter DE, Mukhopadhyay A, Leask A. Gingival fibroblasts display reduced adhesion and spreading on extracellular matrix: A possible basis for scarless tissue repair? *PLoS ONE* 2011;6:e27097.
46. Matsui S, Tsujimoto Y, Ozawa T, Matsushima K. Antioxidant effects of antioxidant biofactor on reactive oxygen species in human gingival fibroblasts. *J Clin Biochem Nutr* 2011;48:209-213.
47. Pradeep AR, Rao NS, Bajaj P, Agarwal E. 8-Isoprostane: A lipid peroxidation product in gingival crevicular fluid in healthy, gingivitis and chronic periodontitis subjects. *Arch Oral Biol* 2013;58:500-504.
48. Mack D, Rohde H, Harris LG, Davies AP, Horstkotte MA, Knobloch JK. Biofilm formation in medical device-related infection. *Int J Artif Organs* 2006;29:343-359.
49. Sbordone L, Bortolaia C. Oral microbial biofilms and plaque-related diseases: Microbial communities and their role in the shift from oral health to disease. *Clin Oral Investig* 2003;7:181-188.
50. Puupponen-Pimiä R, Nohynek L, Meier C, et al. Antimicrobial properties of phenolic compounds from berries. *J Appl Microbiol* 2001;90:494-507.

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Paper III

Quercitrin for periodontal regeneration: effects on human gingival fibroblasts and human mesenchymal stem cells

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Quercitrin for periodontal regeneration: effects on human gingival fibroblasts and mesenchymal stem cells

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Keywords

flavonoids; periodontal diseases; periodontal regeneration; therapeutic uses; antioxidants; inflammation; connective tissue; osteogenesis

Abstract

Periodontal disease (PD) is the result of an infection and chronic inflammation of the gingiva that may lead to its destruction and, in severe cases, alveolar bone and tooth loss. The ultimate goal of periodontal treatment is to achieve periodontal soft and hard tissues regeneration. We previously selected quercitrin, a catechol-containing flavonoid, as a potential agent for periodontal applications. In this study, we tested the effects of quercitrin as a bioactive molecule to address periodontal regeneration on primary human gingival fibroblasts (hGF) and primary human mesenchymal stem cells (hMSC) cultured under basal and inflammatory conditions. To mimic PD inflammatory status, interleukin-1 beta (IL-1β) was used. The expression of different genes related to inflammation and extracellular matrix were evaluated and prostaglandin E2 (PGE2) production was quantified in hGFs; alkaline phosphatase activity and calcium content were analysed in hMSCs. Quercitrin decreased the release of the inflammatory mediator PGE2 and partially re-established the impaired collagen metabolism induced by IL-1β treatment in hGFs. Quercitrin also increased ALP activity and mineralization in hMSCs, thus, it increased hMSCs differentiation towards the osteoblastic lineage. These findings suggest quercitrin as a novel bioactive molecule to enhance both soft and hard tissue regeneration of the periodontium.

1. Introduction

Periodontal disease (PD) is the result from an infection and chronic inflammation of tooth supporting tissues. Gingival inflammation or gingivitis is the first manifestation, which may lead to soft tissue destruction. In some subjects, this progresses to periodontitis, which is defined by alveolar bone and in severe cases tooth loss [1]. Current strategies for PD treatment are generally successful in eliminating active disease and some of them have achieved a certain degree of regeneration. To eliminate the active disease, mechanical and antimicrobial approaches [2] are used although the effects of local antimicrobial therapy are modest and mostly temporary, while the use of systemic antibiotics can induce the development of bacterial resistance [3]. Furthermore, these strategies alone are insufficient since PD is the result of destructive inflammation; thus, if successful, treatment frequently results in a process of gingival fibrosis and limited bone remodelling, rather than in true regeneration of the periodontal tissues [4], [5]. In fact, there is considerable data of successful results in periodontal regeneration using guided tissue regeneration, enamel matrix derivative and platelet-derived growth factor, although the outcomes of such modalities are not always predictable [6]–[8].

The ideal periodontal treatment should damp down the inflammatory response to either decrease the excessive production of proinflammatory mediators, destructive enzymes and free radicals and/or to stimulate tissue regeneration, allowing for the restoration of soft tissue attachment and bone formation [9]. Tissue engineering, which involves the use of appropriate cells, signals and engineering materials, provides an alternative approach to further facilitate and optimize periodontal regeneration [5]. Recent strategies for periodontal regeneration include the use of graft materials, barrier membranes, gene therapy or growth factors through topical delivery. However, gene therapy may have undesired host immune reactions or potential tumorigenesis and growth factors are unstable and have short half-life [10], [11]. Hence, there is an urgent need for finding bioactive molecules with both soft and hard tissue healing activities for periodontal tissue regeneration applications.

Nature is often a source of inspiration for finding new bioactive molecules. Natural phenolic compounds, such as flavonoids, are ubiquitous, abundant and offer a range of properties and an array of functions [12] such as antioxidant [13], anti-inflammatory [14] and antimicrobial capacity [15], [16], among others [17]. Actually, in two independent previous screenings we searched for candidate molecules for soft [18] and hard [19] tissue applications. From both studies, quercitrin was selected as the one showing better performance. Quercitrin decreased extracellular matrix (ECM) degradation, reduced oxidative stress levels, promoted scarless wound healing in human gingival fibroblasts and showed antibacterial properties [18]. Furthermore, it also stimulated the differentiation of mouse pre-osteoblastic cells and inhibited osteoclastogenesis from mouse monocytes, which could prevent bone resorption [19].

Moreover, as a catechol-containing flavonoid, quercitrin could help to control the inflammatory response in PD progression, since molecules with catechol moieties have shown potent anti-inflammatory effects [20]–[22].

In the present study, we hypothesized that quercitrin could address different aspects of periodontal regeneration due to its positive effects on soft and hard tissue. Thus, we set

an inflammatory environment, mimicking PD, and we investigated the effects of quercitrin on primary human gingival fibroblasts (hGF) and primary human mesenchymal stem (hMSC) cells.

2. Materials and Methods

2.1. Cell culture

Three different donors of primary human gingival fibroblasts (hGF; Provitro GmbH, Berlin, Germany) were used (range 19-47 years; male:female ratio 2:1). Provitro assured that cells were obtained ethically and legally and that all donors provided written informed consent. Cells were routinely cultured at 37˚C/5% CO_2, and maintained in fibroblast growth medium (FGM; Provitro GmbH) supplemented with 10% foetal calf serum, 50 ng/ml amphotericin and 50 µg/ml gentamicin (Provitro GmbH). Experiments were performed with hGF between passages 7 and 8 after isolation. Three replicate wells for each donor were seeded in 96-well plates at a density of $7.0x10^3$ cells per well. Media was supplemented with ascorbic acid (100 µM; Sigma-Aldrich, St. Louis, MO, USA). Experiments were run in duplicate (n=18).

Two different donors of human bone marrow-mesenchymal stem cells (hMSCs; Stemcell Technologies, Grenoble, France) were used (20 years; male:female ratio 0:2). Stemcell Technologies assured that cells were obtained ethically and legally and that all donors provided written informed consent. Cells were routinely cultured at 37˚C/5% CO_2, and maintained in low glucose DMEM GlutaMAX (Life Technologies, Carlsbad, CA, USA) supplemented with 10% stem cell-tested foetal bovine serum (Biosera, Boussens, France), 100 U/ml penicillin and 100 µg/ml streptomycin (Biowest, Nuaille, France). Experiments were performed with hMSCs between passages 5 and 7 after isolation. Six replicate wells for each donor were seeded in 48-well plates at a density of $8.5x10^3$ cells per well and grown for 2 days prior to media supplementation without (control) or with additives. Experiments were run in duplicate (n=12).

2.2. Establishment of an inflammatory stimulus

At confluence (three days after seeding), hGF were treated for 1 d with 0.1, 1 and 10 ng/ml of interleukin-1 beta (IL-1β; R&D systems, Abingdon, UK) diluted in complete FGM. After 1 d of treatment, cells were harvested for gene expression analysis. After the analysis, 1 ng/ml IL-1β concentration was selected for further studies.

2.3. Effect of quercitrin on hGF cultured under an inflammatory stimulus

Quercitrin (PubChem CID: 5280459; Sigma-Aldrich) was dissolved in absolute ethanol and kept at -20 ºC. Stock solutions were further dissolved in culture medium, prior to use. Final concentration of ethanol (1%) was included as vehicle control group in all the experiments.

At confluence (three days after seeding), hGF were treated for short (1 and 3 d) and long term (14 d) with 200 µM quercitrin diluted in complete FGM. Quercitrin was added at every media change (3 times per week). The inflammatory stimulus was

created by the addition of 1 ng/ml IL-1β. Four different scenarios were set either using a therapeutic (T) or a preventive approach (P) (**Figure 1 A**): Treatment for 1d with IL-1β and quercitrin; treatment for 3 d with IL-1β and quercitrin; treatment with quercitrin for 14 d plus IL-1β during the first 3 d (T); and treatment with quercitrin for 14 d plus IL-1β during the last 3 d (P).

2.4. Effect of quercitrin on osteogenic differentiation of hMSCs

hMSCs were grown under six different conditions: basal media (control); basal media with 200 μM quercitrin (QUER); osteogenic media (OS) containing 50 μg/ml ascorbic acid, 10 nM dexamethasone and 10 mM β-glycerol phosphate (Sigma-Aldrich); osteogenic media with 200 μM quercitrin (OS+QUER); osteogenic media supplemented with IL-1β (OS+IL-1β); and osteogenic media supplemented with IL-1β and quercitrin (OS+IL-1β+QUER). Supplements were added at every media change for 19 days and cells were harvested after 19 days for analysis.

2.5. RNA isolation and real-time RT-PCR analysis

Total RNA was isolated using Tripure (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's protocol. Total RNA was quantified at 260 nm using a nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The same amount of RNA (0.2 μg) was reverse transcribed to cDNA at 42 ºC for 60 min, according to the protocol of the supplier (High Capacity RNA-to-cDNA kit, Applied Biosystems, Foster City, CA, USA). Aliquots of each cDNA were frozen (-20 °C) until the PCR reactions were carried out.

Real-time PCR was performed for two reference genes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and beta-actin (ACTBL2), and target genes (Table 1). Real-time PCR was performed in a thermocycler (Lightcycler 480, Roche Diagnostics) using SYBR green detection. Each reaction contained 7 μl of master mix (Lightcycler 480 SYBR Green I Master, Roche Diagnostics), 0.5 μM of each, the sense and the antisense specific primers and 3 μl of the cDNA dilution in a final volume of 10 μl. The amplification program consisted of a pre-incubation step for denaturation of the template cDNA (5 min 95°C), followed by 45 cycles consisting of a denaturation step (10 s 95 °C), an annealing step (10 s 60 °C) and an extension step (10 s 72 °C). After each cycle, fluorescence was measured at 72 °C. A negative control without cDNA template was run in each assay.

All samples were normalized by the geometric mean of the expression levels of ACTBL2 and GAPDH and fold changes were related to the control groups using the following equation: ratio = $E_{target}^{\Delta Cp \text{ target (mean control – sample)}} / E_{reference}^{\Delta Cp \text{ reference (mean control – sample)}}$, where Cp is the is the crossing point of the reaction amplification curve and E the effciency from the given slopes using serial dilutions, as determined by the software (Lightcycler 480 software, Roche Diagnostics). Stability of reference genes was calculated using a statistical tool (BestKeeper software, Technical University of Munich, Weihenstephan, Germany).

Table 1. Sense (S) and antisense (A) primers used in the real-time PCR of reference and target genes.

Gene	Primer sequence (5´- 3´)	Product size (bp)
Beta-actin (ACTBL2)	S: CTGGAACGGTGAAGGTGACA A: AAGGGACTTCCTGTAACAATGCA	136
Collagen III α1 (COL3A1)	S: GGCCTACTGGGCCTGGTGGT A: CCACGTTCACCAGGGGCACC	190
Cyclooxygenase-2 (COX2)	S: ATGGGGTGATGAGCAGTTGT A: GAAAGGTGTCAGGCAGAAGG	221
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	S: TGCACCACCAACTGCTTAGC A: GGCATGGACTGTGGTCATGAG	87
Interleukin-6 (IL6)	S: AGGAGACTTGCCTGGTGAAA A: GCATTTGTGGTTGGGTCAG	196
Interleukin-8 (IL8)	S: GGTGCAGTTTTGCCAAGGAG A: TTCCTTGGGGTCCAGACAGA	183
Matrix metalloproteinase-1 (MMP1)	S: TGTCAGGGGAGATCATCGGGACA A: TGGCCGAGTTCATGAGCTGCA	177
Metalloproteinase inhibitor-1 (TIMP1)	S: TTCCGACCTCGTCATCAGGG A: TAGACGAACCGGATGTCAGC	144

2.6. Enzyme-linked immunosorbent assays (ELISA)

Commercially available ELISA kits were run to quantify interleukin-8 (eBioscience, San Diego, CA, USA), and prostaglandin E2 (PGE2; Thermo Scientific, Rockford, IL, USA) from cell culture media according to supplier instructions.

2.7. Alkaline phosphatase (ALP) activity and calcium quantification

Cells were washed with PBS and lysated with 0.1% Triton X-100. ALP activity was quantified by measuring the cleavage of p-Nitrophenyl Phosphate (pNPP; Sigma-Aldrich) in a soluble yellow end product that absorbs at 405 nm. Twenty-five microliters of sample were incubated with 100 µl of pNPP. In parallel to the samples, a standard curve with calf intestinal alkaline phosphatase (Promega, Madison, WI, USA) was constructed. On the other hand, two-hundred microliters of sample were incubated with 1 N HCl overnight, followed by centrifugation at 500 xg for 2 min for the subsequent determination of Ca^{2+} content in the supernatant by inductively coupled plasma atomic emission spectrometer (ICP-AES) Optima 5300 DV (PerkinElmer, Waltham, MA, USA). Data were compared with $CaCl_2$ standards included in the assay.

2.8. Statistical analysis

All data are presented as mean values ± standard error of the mean (SEM). The Kolmogorov-Smirnov test was done to assume parametric or non-parametric distributions. Differences between groups were assessed by paired t-test or Wilcoxon test, depending on data distribution. A specific computer program (SPSS version 17.0, Chicago, IL, USA) was used. Results were considered statistically significant at $P \leq 0.05$.

3. Results

3.1. IL-1β induces the expression of inflammatory markers on hGFs

The addition of IL-1β for 1 day significantly stimulated the expression of IL6, IL8 and MMP1, reaching a plateau at 1 ng/ml IL-1β (**Table 2**). Moreover, hGF viability, measured by the lactate dehydrogenase activity released to culture media, as described in [18], after 1 and 3 days of stimulation with IL-1β remained similar to the controls (data not shown). Based on this data, we selected 1 ng/ml IL-1β as inflammatory stimulator for the subsequent experiments.

Table 2. Effect of IL-1β stimulation on human gingival fibroblasts. Data were normalized to reference genes, expressed as percentage of control (no stimulation) which was set to 100%. Values represent the mean ± SEM of two independent experiments. Differences between treatment and control groups: *$P \leq 0.05$ treatment versus control.

IL-1β (ng/ml)	mRNA levels (% of control)		
	IL6	IL8	MMP1
0	100±8	100±9	100±4
0.1	3952±509*	81456±12260*	333±27*
1	3832±477*	80414±11765*	369±37*
10	3709±410*	76026±11161*	362±36*

3.2. Quercitrin decreases damage on hGFs in an inflammatory environment

First, we studied the expression of genes related to ECM (**Figure 1 B**). We found that quercitrin treatment downregulated MMP1 IL-1β-induced expression at day 1 and 14, and upregulated TIMP1 IL-1β-decreased expression at day 3 and 14, resulting in a decreased MMP1/TIMP1 mRNA ratio at short- and long-term (data not shown). Also, we found that COL3A1 expression increased in all quercitrin-treated groups. Furthermore, quercitrin prevented the decrease in TIMP1 and COL3A1 after IL-1β stimulation (preventive approach).

COX2 mRNA was highly upregulated after IL-1β stimulation. Treatment with quercitrin significantly decreased the COX2 IL-1β-induced expression at day 1 and 14for both, the therapeutic and the preventive scenarios (**Figure 1 B**). Furthermore, to assess whether the effect of quercitrin on COX2 expression was functionally significant, the release of PGE2, a proinflammatory COX2 end product, was determined. Our results show that quercitrin decreased the release of PGE2 on all IL-1β treated groups (**Figure 2**).

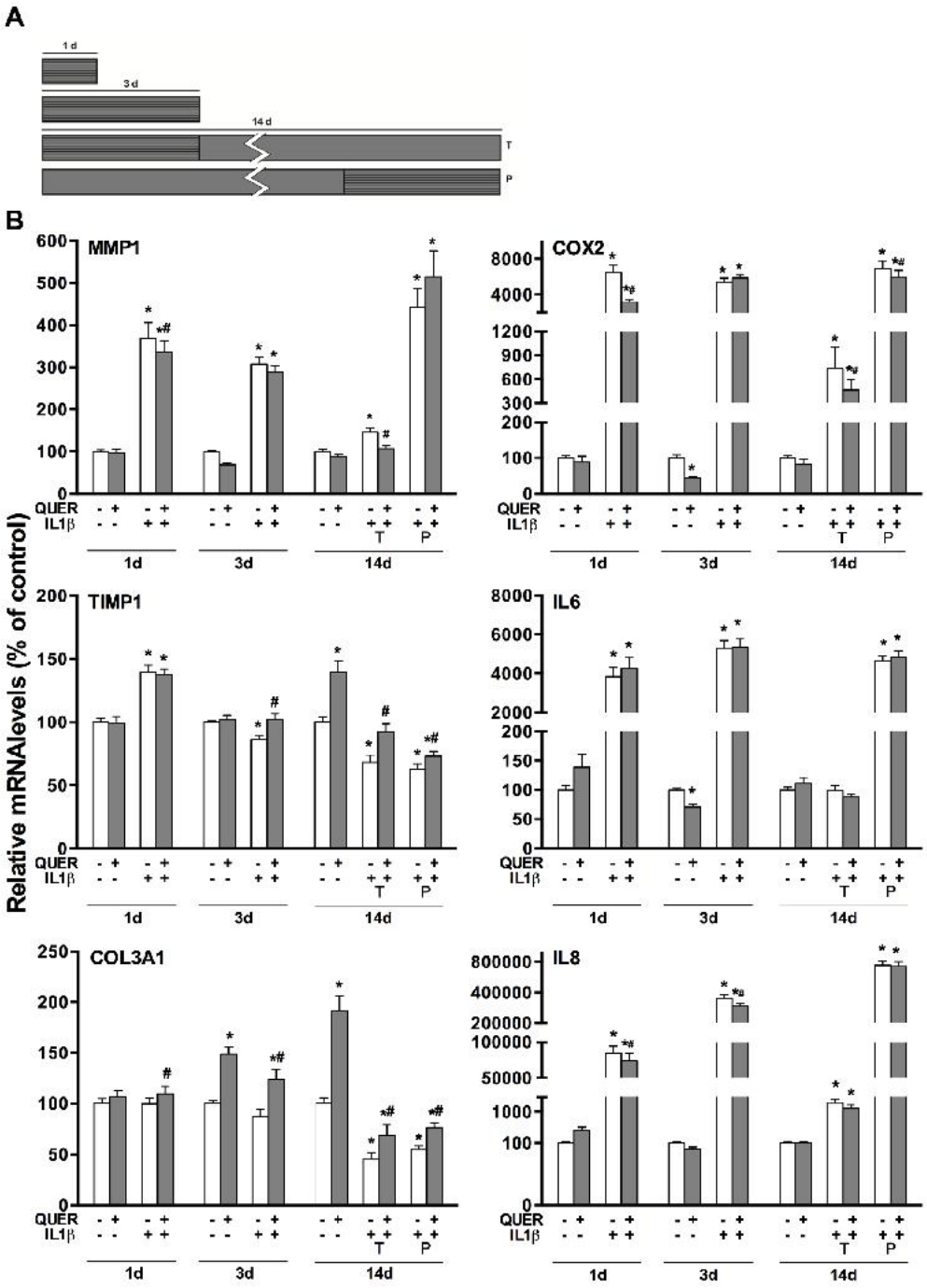

Figure 1. Effect of quercitrin on hGF stimulated with IL-1β. (A) Experimental design: four different inflammatory scenarios were set either using a therapeutic (T) or a preventive approach (P) by treating with IL-1β (stripped pattern); treatment for 1d with IL-1β; treatment for 3 d with IL-1β; treatment with IL-1β during the first 3 d and culturing the cells for 14 d (T); and culturing the cells for 14 d plus IL-1β treatment during the last 3 d (P). The effect of quercitrin treatment during the entire culture period under these four scenarios was evaluated and compared to vehicle treatment (control). **(B)** Gene expression results: cells were treated with quercitrin (grey bars) or without (white bars), following the experimental design. Data were normalized to reference genes, expressed as percentage of control (vehicle), which was set to 100%. Values represent the mean ± SEM of two independent experiments. Differences between treatment and control groups: $*P \leq 0.05$ treatments versus control (vehicle without IL-1β); $^{\#}P \leq 0.05$ quercitrin versus vehicle in the presence of IL-1β for each time point. Abbreviations: quercitrin (QUER), interleukin-1 beta (IL-1β).

We also studied the gene expression of different inflammatory markers (**Figure 1 B**). IL-1β highly upregulated the mRNA levels of IL6 and IL8 at short- and long-term. This increased gene expression was not reversed by quercitrin treatment but for IL8 mRNA levels at short-term (days 1 and 3). However, IL-8 protein levels remained unchanged among groups (data not shown).

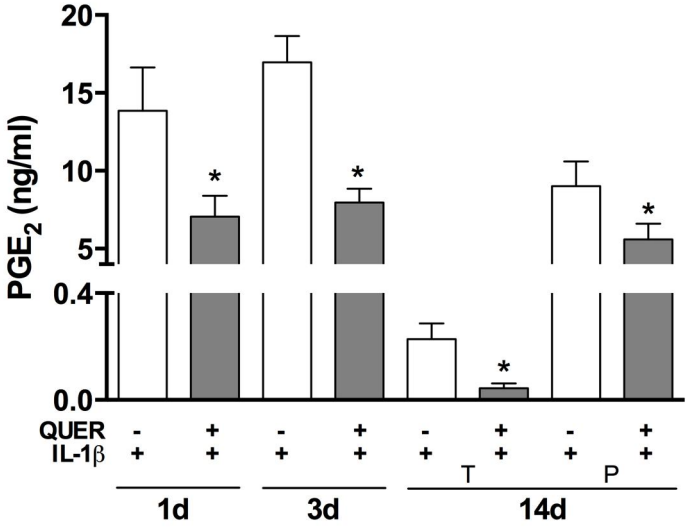

Figure 2. Quercitrin decreased PGE2 release on hGF stimulated with IL-1β. Cells were treated with IL-1β with (grey bars) or without quercitrin (white bars). Four different scenarios were set either using a therapeutic (T) or a preventive approach (P): treatment for 1d with IL-1β and quercitrin; treatment for 3 d with IL-1β and quercitrin; treatment with quercitrin for 14 d plus IL-1β during the first 3 d (T); and treatment with quercitrin for 14 d plus IL-1β during the last 3 d (P). Values represent the mean ± SEM of two independent experiments. Differences between treatment and control groups: $*P \leq 0.05$ quercitrin versus vehicle in the presence of IL-1β for each time point. Abbreviations: quercitrin (QUER), interleukin-1 beta (IL-1β).

3.3. Quercitrin increases osteogenic differentiation of hMSCs

Analysis of ALP showed significantly increased ALP activity in quercitrin treated cells on basal and osteogenic situations. Additionally, quercitrin increased cell calcium content in osteogenic and inflammatory situations. No significant differences were observed in calcium content of cells treated with quercitrin in basal media compared to control. As expected, culture of hMSCs with osteogenic media induced a significant increase of both, ALP activity and calcium content compared to control. Furthermore, IL-1β stimulation significantly induced an increase in cell calcium content although no significant changes were seen in ALP activity (**Figure 3**).

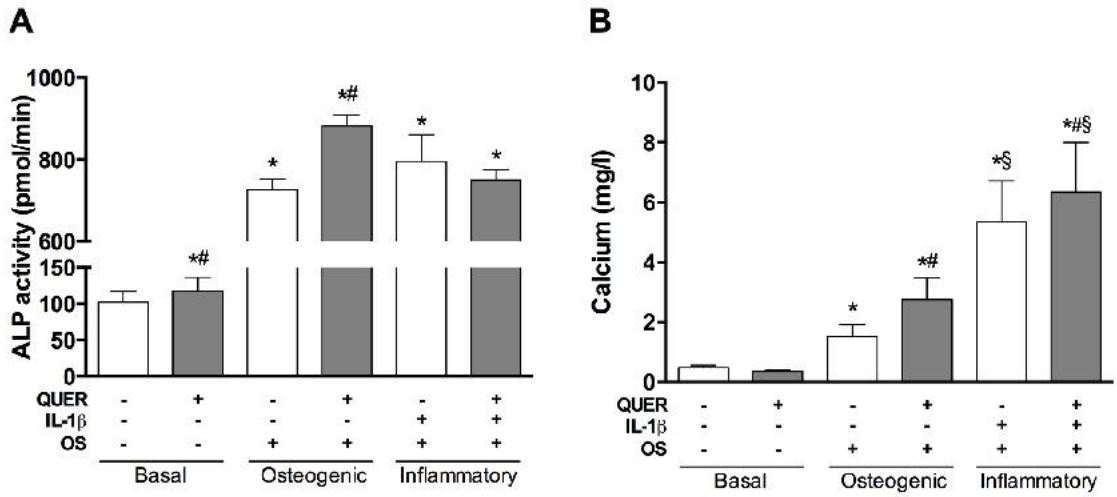

Figure 3. Quercitrin increased the osteoblastic differentiation of hMSCs. (A) ALP activity and **(B)** calcium content determined after 19 days of hMSCs growth under six different conditions: basal media (control); basal media with 200 μM quercitrin (QUER); osteogenic media (OS); osteogenic media with 200 μM quercitrin (OS+QUER); osteogenic media supplemented with IL-1β (OS+IL-1β); and osteogenic media supplemented with IL-1β and quercitrin (OS+IL-1β+QUER). Values represent the mean ± SEM of two independent experiments. Differences between treatment and control groups: $^{*}P \leq 0.05$ treatment versus control (vehicle); $^{\#}P \leq 0.05$ quercitrin versus vehicle; $^{\S}P \leq 0.05$ inflammation groups versus OS group.

4. Discussion

In this study we demonstrate the positive effects of quercitrin on soft and hard tissue under inflammatory conditions using an in vitro model with human primary cultures of gingival fibroblasts and mesenchymal stem cells. We show that quercitrin decreases the release of the inflammatory mediator PGE2 and partially re-establishes the impaired collagen metabolism induced by IL-1β stimulation on primary human gingival fibroblasts. Furthermore, quercitrin enhances human mesenchymal stem cells differentiation towards the osteoblastic lineage. These findings suggest quercitrin as a novel bioactive molecule to address soft and hard tissue regeneration of the periodontium.

The presence of pathogens in the oral cavity causes the activation of a inflammatory response that stimulates the recruitment of immune cells and the production of cytokines, chemokines, cyclooxygenase products and MMPs, which enhance the inflammatory process [2]. As a consequence, the gingival connective tissue is substituted by a chronic inflammatory infiltrate that can ultimately spread to deeper periodontal tissues leading to soft and hard tissues destruction [4]. Here we used the inflammatory mediator IL-1β to mimic *in vitro* the inflammatory process in PD progression. IL-1β is broadly used to induce experimental inflammation and to enhance the proinflammatory response [23]–[25], imitating the inflammatory pathways activated in response to oral pathogens [26]. The concentrations used here are in in the range with the IL-1β levels usually found in patients with periodontitis [27]. However, although the stimulation of IL-1β with periodontal characteristics was used here to simulate a

periodontitis condition, the only addition of IL-1β does not reflect the entire chronic inflammatory process in periodontal disease *in vivo*, and can only help to investigate *in vitro* the effects of quercitrin. Here, the addition of IL-1β produced a potent up-regulation of IL6, IL8 and MMP1 mRNA levels in hGF, key inflammatory mediators in the progression of periodontal disease [26] and consistent with previous reports in the literature [25], [28]. Modifying the local environment to reduce inflammation is one requirement to achieve complete periodontal regeneration [29]. Inducible COX2 and PGE2 production are highly related to PD status [30]. Here we show that quercitrin treatment effectively inhibited COX2 expression and its functional product PGE2 under an inflammatory stimulus, in agreement with a previous study with LPS-stimulated HGF with different flavonoids [31]. Moreover, previous studies showed that quercitrin can also downregulate inducible nitric oxide synthase, an enzyme highly expressed by inflammatory stimuli that produces the inflammatory mediator NO [32], [33]. Unexpectedly, the upregulation of IL6 and IL8 induced by IL-1β was not reversed by quercitrin treatment contrary to what was previously shown in LPS-stimulated macrophages [34] and *in vivo* [32]. This finding might be explained by the stimulation with IL-1β that activates the complex NF-κB signalling pathway and positive and negative feedback mechanisms that regulate cytokines expression [35], [36], thus, masking quercitrin anti-inflammatory effect. Furthermore, different investigations have provided strong evidence that phenolic compounds act by direct modulation of mitogen-activated protein kinase/activator protein-1 and NF-κB pathways, which have central roles regulating cell differentiation and inflammation, among other downstream targets [37], [38].

During the progress of gingivitis, the inflammatory process results in connective tissue breakdown as the result of an imbalance of MMPs over their inhibitors, TIMPs [39]. Here, our IL-1β inflammatory *in vitro* model decreased COL3A1 mRNA expression and increased MMP1/TIMP1 mRNA ratio, mimicking the molecular events observed in PD. Our results show that quercitrin treatment partially re-established the control expression levels of COL3A1 and the MMP1/TIMP1 mRNA ratio under an inflammatory stimulus in line with our previous results without IL-1β stimulation [18]. Therefore, quercitrin could contribute to the regeneration of functional connective tissue destroyed by inflammation.

Further progression of gingivitis leads to periodontitis, which is characterised by alveolar bone resorption. This study proves that quercitrin increases ALP activity in basal and osteogenic media, and mineralization in osteogenic and inflammatory situations in hMSCs; thus, quercitrin enhanced the osteoblastic differentiation of MSCs, another requisite for complete periodontal regeneration [29]. This finding is supported by our previous study with mouse pre-osteoblasts [19]. Under IL-1β stimulation, we observed that final calcium content increased while ALP activity decreased. During osteoblastic differentiation, ALP peaks at the maturation phase and decreases at the mineralization phase. Therefore, the decreased ALP activity together with the increased mineralization point to a shifted hMSCs differentiation to earlier time points in the presence of IL-1β and osteogenic media, in agreement with previous reports [40], [41]. It has been demonstrated that IL-1β without osteogenic media increases osteoblast mineralization through an atypical biochemical mechanism without differentiating into osteoblasts [42] and that when osteogenic media is added, the combination of stimuli leads to additive effects, where dexametasone leads to an increase in ALP activity, whereas IL-1β leads to increased calcification [40], [41]. Here, quercitrin addition to the

IL-1β-stimulated osteogenic group increased the final calcium content almost 20%, while quercitrin addition to the osteogenic group increased it 80%. This result indicates that quercitrin increases mineralization of hMSCs even in the presence of IL-1β, although the mineralization mechanism of IL-1β might have obscured any direct effect that quercitrin might have had. Therefore, our results suggest that quercitrin may enhance bone formation even in inflammatory conditions.

Though periodontal ligament derived MSCs would have been the preferred cells for this study, it has been demonstrated that bone-marrow MSCs have the capacity to promote the regeneration of alveolar bone, cementum and periodontal ligament [11], [43] and that bone marrow MSCs are comparable to periodontal ligament MSCs in their differentiation capacity and ability to regenerate periodontal bone [44], [45]. Actually, bone marrow MSCs showed periodontal ligament-like cell features when cocultured with the periodontal ligament *in vivo* [46].

Current research in periodontal regeneration is focused in discovering new bioactive molecules, improving stem cell implantation and developing biomaterials that act as scaffolds or as drug delivery systems [47]. Quercitrin might have different applications in periodontal regeneration since it is a bioactive molecule with antibacterial, anti-oxidant and anti-inflammatory properties and it promotes soft and hard tissue regeneration [18], [19], [34], [48]. Besides, quercitrin could decrease the antibiotic administration and increase the safety of anti-inflammatory drugs currently used for periodontal disease treatment [2]. Regarding to bone regeneration, stem cell-based periodontal regeneration is a promising therapeutic option [11]. Furthermore, the inhibition of osteoblastic differentiation of bone progenitor cells from the periodontal tissue has been suggested to be dependent on the local environment [47], [49]. Here, we have shown that quercitrin promotes the osteoblastic differentiation of hMSCs even in an inflammatory situation; therefore quercitrin could help hard tissue to regenerate itself. Furthermore, previous results of our group show that quercitrin decreased osteoclast formation in RAW264.7 cells [19], together with the present results, quercitrin might show an anabolic effect on bone formation. Regarding the development of new biomaterials, we have also used quercitrin to functionalize Ti-surfaces, showing promising results [50]. Therefore, we hypothesize that quercitrin could be used in combination with other biomaterials to further improve periodontal regeneration. Future studies should confirm the effects of quercitrin *in vivo*.

5. Conclusion

Quercitrin decreased the release of the proinflammatory mediator PGE2 under inflammatory stimulus and also reversed the collagenolitic situation created by IL-1β by increasing COL3A1 expression and decreasing MMP1/TIMP1 mRNA ratio in human gingival fibroblasts. Furthermore, quercitrin increased the osteoblastic differentiation of mesenchymal stem cells in basal, osteogenic and inflammatory conditions. Our data suggest quercitrin as a bioactive molecule that could create a microenvironment suitable for soft and hard tissue regeneration and therefore enhance periodontal regeneration.

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References

[1] M. S. Tonetti, P. Eickholz, B. G. Loos, P. Papapanou, U. van der Velden, G. Armitage, P. Bouchard, R. Deinzer, T. Dietrich, F. Hughes, T. Kocher, N. P. Lang, R. Lopez, I. Needleman, T. Newton, L. Nibali, B. Pretzl, C. Ramseier, I. Sanz-Sanchez, U. Schlagenhauf, J. E. Suvan, E. Fabrikant, and A. Fundak, "Principles in Prevention of Periodontal Diseases," *J. Clin. Periodontol.*, vol. 42, no. S16, pp. S5–S11, 2015.

[2] A. Dentino, S. Lee, J. Mailhot, and A. F. Hefti, "Principles of periodontology.," *Periodontol. 2000*, vol. 61, no. 1, pp. 16–53, Feb. 2013.

[3] L. J. a Heitz-Mayfield and N. P. Lang, "Surgical and nonsurgical periodontal therapy. Learned and unlearned concepts.," *Periodontol. 2000*, vol. 62, no. 1, pp. 218–31, Jun. 2013.

[4] F. J. Hughes, "Periodontium and Periodontal Disease," in *Stem Cell Biology and Tissue Engineering in Dental Sciences*, Elsevier Inc., 2015, pp. 433–444.

[5] J. Han, D. Menicanin, S. Gronthos, and P. Bartold, "Stem cells, tissue engineering and periodontal regeneration.," *Aust. Dent. J.*, pp. 1–14, 2013.

[6] M. Esposito, M. G. Grusovin, N. Papanikolaou, P. Coulthard, and H. V Worthington, "Enamel matrix derivative (Emdogain(R)) for periodontal tissue regeneration in intrabony defects.," *Cochrane database Syst. Rev.*, no. 4, p. CD003875, Jan. 2009.

[7] C. a Ramseier, G. Rasperini, S. Batia, and W. V Giannobile, "Advanced reconstructive technologies for periodontal tissue repair.," *Periodontol. 2000*, vol. 59, no. 1, pp. 185–202, Jun. 2012.

[8] D. Kaigler, G. Avila, L. Wisner-Lynch, M. L. Nevins, M. Nevins, G. Rasperini, S. E. Lynch, and W. V Giannobile, "Platelet-derived growth factor applications in periodontal and peri-implant bone regeneration.," *Expert Opin. Biol. Ther.*, vol. 11, no. 3, pp. 375–385, 2011.

[9] P. M. Preshaw, "Host response modulation in periodontics.," *Periodontol. 2000*, vol. 48, pp. 92–110, Jan. 2008.

[10] S. Y. Shin, H. F. Rios, W. V Giannobile, and T. Oh, "Periodontal Regeneration : Current Therapies," in *Stem Cell Biology and Tissue Engineering in Dental Sciences*, Elsevier Inc., 2015, pp. 459–469.

[11] B. Li and Y. Jin, "Periodontal Tissue Engineering : Current Approaches and Future Therapies," in *Stem Cell Biology and Tissue Engineering in Dental Sciences*, Elsevier Inc., 2015, pp. 471–482.

[12] W. E. Bentley and G. F. Payne, "Nature's Other Self-Assemblers," *Science (80-.).*, vol. 341, no. 6142, pp. 136–137, 2013.

[13] P. G. Pietta, “Flavonoids as antioxidants,” *Journal of Natural Products*, vol. 63, no. 7. American Chemical Society, pp. 1035–1042, 2000.

[14] V. Izzi, L. Masuelli, I. Tresoldi, P. Sacchetti, A. Modesti, F. Galvano, and R. Bei, “The effects of dietary flavonoids on the regulation of redox inflammatory networks.,” *Front. Biosci. (Landmark Ed.*, vol. 17, pp. 2396–418, Jan. 2012.

[15] J. An, G. Y. Zuo, X. Y. Hao, G. C. Wang, and Z. S. Li, “Antibacterial and synergy of a flavanonol rhamnoside with antibiotics against clinical isolates of methicillin-resistant Staphylococcus aureus (MRSA),” *Phytomedicine*, vol. 18, no. 11, pp. 990–993, 2011.

[16] T. P. T. Cushnie and A. J. Lamb, “Recent advances in understanding the antibacterial properties of flavonoids.,” *Int. J. Antimicrob. Agents*, vol. 38, no. 2, pp. 99–107, Aug. 2011.

[17] M. Friedman, “Overview of antibacterial, antitoxin, antiviral, and antifungal activities of tea flavonoids and teas.,” *Mol. Nutr. Food Res.*, vol. 51, no. 1, pp. 116–34, Jan. 2007.

[18] M. Gómez-Florit, M. Monjo, and J. M. Ramis, “Identification of Quercitrin as Potential Therapeutic Agent for Periodontal Applications.,” *J. Periodontol.*, vol. 85, no. 7, pp. 966–974, Feb. 2014.

[19] M. Satué, M. D. M. Arriero, M. Monjo, and J. M. Ramis, “Quercitrin and taxifolin stimulate osteoblast differentiation in MC3T3-E1 cells and inhibit osteoclastogenesis in RAW 264.7 cells.,” *Biochem. Pharmacol.*, vol. 86, no. 10, pp. 1476–86, Nov. 2013.

[20] D. Luo, T. C. T. Or, C. L. H. Yang, and A. S. Y. Lau, “Anti-Inflammatory Activity of Iridoid and Catechol Derivatives from Eucommia ulmoides Oliver,” *ACS Chem. Neurosci.*, vol. 5, no. 9, pp. 855–866, 2015.

[21] M. T. Heneka, F. Nadrigny, T. Regen, A. Martinez-Hernandez, L. Dumitrescu-Ozimek, D. Terwel, D. Jardanhazi-Kurutz, J. Walter, F. Kirchhoff, U.-K. Hanisch, and M. P. Kummer, “Locus ceruleus controls Alzheimer’s disease pathology by modulating microglial functions through norepinephrine.,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 107, no. 13, pp. 6058–63, Mar. 2010.

[22] L. T. Zheng, G.-M. Ryu, B.-M. Kwon, W.-H. Lee, and K. Suk, “Anti-inflammatory effects of catechols in lipopolysaccharide-stimulated microglia cells: inhibition of microglial neurotoxicity.,” *Eur. J. Pharmacol.*, vol. 588, no. 1, pp. 106–13, Jun. 2008.

[23] I. Furuhashi, S. Iwata, S. Shibata, T. Sato, and H. Inoue, “Inhibition by licochalcone A, a novel flavonoid isolated from liquorice root, of IL-1beta-induced PGE2 production in human skin fibroblasts.,” *J. Pharm. Pharmacol.*, vol. 57, no. 12, pp. 1661–6, Dec. 2005.

[24] Y. Kida, M. Kobayashi, T. Suzuki, A. Takeshita, Y. Okamatsu, S. Hanazawa, T. Yasui, and K. Hasegawa, “Interleukin-1 stimulates cytokines, prostaglandin E2 and matrix metalloproteinase-1 production via activation of MAPK/AP-1 and NF-kappaB in human gingival fibroblasts.,” *Cytokine*, vol. 29, no. 4, pp. 159–68, Feb. 2005.

[25] M. Ono, K. Kantoh, J. Ueki, A. Shimada, H. Wakabayashi, T. Matsuta, H. Sakagami, H. Kumada, N. Hamada, M. Kitajima, H. Oizumi, and T. Oizumi,

"Quest for anti-inflammatory substances using IL-1β-stimulated gingival fibroblasts," *In Vivo (Brooklyn).*, vol. 25, no. 5, pp. 763–768, 2011.

[26] T. Yucel-Lindberg and T. Båge, "Inflammatory mediators in the pathogenesis of periodontitis.," *Expert Rev. Mol. Med.*, vol. 15, no. August, p. e7, Jan. 2013.

[27] M. Nokhbehsaim, J. Winter, B. Rath, A. Jäger, S. Jepsen, and J. Deschner, "Effects of enamel matrix derivative on periodontal wound healing in an inflammatory environment in vitro.," *J. Clin. Periodontol.*, vol. 38, no. 5, pp. 479–90, May 2011.

[28] P. Palmqvist, P. Lundberg, I. Lundgren, L. Hanstrom, and U. H. Lerner, "IL-1 and TNF- Regulate IL-6-type Cytokines in Gingival Fibroblasts," *J. Dent. Res.*, vol. 87, no. 6, pp. 558–563, Jun. 2008.

[29] D. A. Reed and T. G. H. Diekwisch, "Biological Aspects of Periodontal Repair," in *Stem Cell Biology and Tissue Engineering in Dental Sciences*, Elsevier Inc., 2015, pp. 445–458.

[30] K. Noguchi and I. Ishikawa, "The roles of cyclooxygenase-2 and prostaglandin E2 in periodontal disease.," *Periodontol. 2000*, vol. 43, pp. 85–101, Jan. 2007.

[31] G. Gutiérrez-Venegas and A. Contreras-Sánchez, "Luteolin and fisetin inhibit the effects of lipopolysaccharide obtained from Porphyromonas gingivalis in human gingival fibroblasts.," *Mol. Biol. Rep.*, vol. 40, no. 1, pp. 477–85, Jan. 2013.

[32] M. Comalada, D. Camuesco, S. Sierra, I. Ballester, J. Xaus, J. Gálvez, and A. Zarzuelo, "In vivo quercitrin anti-inflammatory effect involves release of quercetin, which inhibits inflammation through down-regulation of the NF-κB pathway," *Eur. J. Immunol.*, vol. 35, no. 2, pp. 584–592, 2005.

[33] X. Dai, Y. Ding, Z. Zhang, X. Cai, and Y. Li, "Quercetin and quercitrin protect against cytokine-induced injuries in RINm5F β-cells via the mitochondrial pathway and NF-κB signaling," *Int. J. Mol. Med.*, vol. 31, no. 1, pp. 265–271, Jan. 2013.

[34] S.-H. Fang, Y. K. Rao, and Y.-M. Tzeng, "Anti-oxidant and inflammatory mediator's growth inhibitory effects of compounds isolated from Phyllanthus urinaria.," *J. Ethnopharmacol.*, vol. 116, no. 2, pp. 333–40, Mar. 2008.

[35] S. Ghosh and M. S. Hayden, "New regulators of NF-kappaB in inflammation.," *Nat. Rev. Immunol.*, vol. 8, no. 11, pp. 837–48, Nov. 2008.

[36] B. Hoesel and J. A. Schmid, "The complexity of NF-κB signaling in inflammation and cancer.," *Mol. Cancer*, vol. 12, no. 1, p. 86, Jan. 2013.

[37] H. P. Kim, K. H. Son, H. W. Chang, and S. S. Kang, "Anti-inflammatory Plant Flavonoids and Cellular Action Mechanisms," *J. Pharmacol. Sci.*, vol. 96, no. 3, pp. 229–245, 2004.

[38] A. Trzeciakiewicz, V. Habauzit, and M.-N. Horcajada, "When nutrition interacts with osteoblast function: molecular mechanisms of polyphenols.," *Nutr. Res. Rev.*, vol. 22, no. 1, pp. 68–81, Jun. 2009.

[39] M. Soell, R. Elkaim, and H. Tenenbaum, "Cathepsin C, Matrix Metalloproteinases, and their Tissue Inhibitors in Gingiva and Gingival Crevicular Fluid from Periodontitis-affected Patients," *J. Dent. Res.*, vol. 81, no. 3, pp. 174–178, Mar. 2002.

[40] K. Sonomoto, K. Yamaoka, K. Oshita, S. Fukuyo, X. Zhang, K. Nakano, Y. Okada, and Y. Tanaka, "Interleukin-1β induces differentiation of human mesenchymal stem cells into osteoblasts via the Wnt-5a/receptor tyrosine kinase-like orphan receptor 2 pathway.," *Arthritis Rheum.*, vol. 64, no. 10, pp. 3355–63, Oct. 2012.

[41] C. Loebel, E. M. Czekanska, J. Staudacher, G. Salzmann, R. G. Richards, M. Alini, and M. J. Stoddart, "The calcification potential of human MSCs can be enhanced by interleukin-1 β in osteogenic medium," *J. Tissue Eng. Regen. Med.*, p. n/a–n/a, Sep. 2014.

[42] E. Ferreira, R. M. Porter, N. Wehling, R. P. O'Sullivan, F. Liu, a Boskey, D. M. Estok, M. B. Harris, M. S. Vrahas, C. H. Evans, and J. W. Wells, "Inflammatory cytokines induce a unique mineralizing phenotype in mesenchymal stem cells derived from human bone marrow," *J. Biol. Chem.*, vol. 288, no. 41, pp. 29494–29505, 2013.

[43] H. Kawaguchi, A. Hirachi, N. Hasegawa, T. Iwata, H. Hamaguchi, H. Shiba, T. Takata, Y. Kato, and H. Kurihara, "Enhancement of periodontal tissue regeneration by transplantation of bone marrow mesenchymal stem cells.," *J. Periodontol.*, vol. 75, no. 9, pp. 1281–1287, 2004.

[44] F. M. Chen, L. A. Wu, M. Zhang, R. Zhang, and H. H. Sun, "Homing of endogenous stem/progenitor cells for in situ tissue regeneration: Promises, strategies, and translational perspectives," *Biomaterials*, vol. 32, no. 12, pp. 3189–3209, 2011.

[45] P. Monsarrat, J.-N. Vergnes, C. Nabet, M. Sixou, M. L. Snead, V. Planat-Bénard, L. Casteilla, and P. Kémoun, "Concise Review: Mesenchymal stromal cells used for periodontal regeneration: a systematic review," *Stem Cells Transl. Med.*, vol. 3, pp. 768–774, 2014.

[46] P. R. Kramer, S. Nares, S. F. Kramer, D. Grogan, and M. Kaiser, "Mesenchymal stem cells acquire characteristics of cells in the periodontal ligament in vitro.," *J. Dent. Res.*, vol. 83, no. 1, pp. 27–34, 2004.

[47] F. J. Hughes, M. Ghuman, and A. Talal, "Periodontal regeneration: a challenge for the tissue engineer?," *Proc. Inst. Mech. Eng. H.*, vol. 224, no. 12, pp. 1345–58, Dec. 2010.

[48] X. Dai, Y. Ding, Z. Zhang, X. Cai, and Y. Li, "Quercetin and quercitrin protect against cytokine-induced injuries in RINm5F β-cells via the mitochondrial pathway and NF-κB signaling," *Int. J. Mol. Med.*, vol. 31, no. 1, pp. 265–271, Jan. 2013.

[49] D. L. Carnes, C. L. Maeder, and D. T. Graves, "Cells with osteoblastic phenotypes can be explanted from human gingiva and periodontal ligament.," *J. Periodontol.*, vol. 68, pp. 701–707, 1997.

[50] A. Córdoba, M. Satué, M. Gómez-Florit, M. Hierro-Oliva, C. Petzold, S. P. Lyngstadaas, M. L. González-Martín, M. Monjo, and J. M. Ramis, "Flavonoid-Modified Surfaces: Multifunctional Bioactive Biomaterials with Osteopromotive, Anti-Inflammatory, and Anti-Fibrotic Potential," *Adv. Healthc. Mater.*, vol. 4, no. 4, pp. 540–549, Oct. 2015.

Paper IV

Differential response of human gingival fibroblasts to titanium- and titanium-zirconium-modified surfaces

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Differential response of human gingival fibroblasts to titanium- and titanium-zirconium-modified surfaces

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Gómez-Florit M, Ramis JM, Xing R, Taxt-Lamolle S, Haugen HJ, Lyngstadaas S P, Monjo M. Differential response of human gingival fibroblasts to titanium- and titanium-zirconium-modified surfaces. J Periodont Res 2013; doi: 10.1111/jre.12121. © 2013 John Wiley & Sons A/S. Published by John Wiley & Sons Ltd

Background and Objective: Gingival fibroblasts are responsible for the constant adaptation, wound healing and regeneration of gingival connective tissue. New titanium-zirconium (TiZr) abutment surfaces have been designed to improve soft tissue integration and reduce implant failure compared with titanium (Ti). The aim of the present study was first to characterize a primary human gingival fibroblast (HGF) model and secondly to evaluate their differential response to Ti and TiZr polished (P), machined (M) and machined + acid-etched (modMA) surfaces, respectively.

Material and Methods: HGF were cultured on tissue culture plastic or on the different Ti and TiZr surfaces. Cell morphology was evaluated through confocal and scanning electron microscopy. A wound healing assay was performed to evaluate the capacity of HGF to close a scratch. The expression of genes was evaluated by real-time RT-PCR, addressing: (i) extracellular matrix organization and turnover; (ii) inflammation; (iii) cell adhesion and structure; and (iv) wound healing. Finally, cells on Ti/TiZr surfaces were immunostained with anti-ITGB3 antibodies to analyze integrin β3 production. Matrix metalloproteinase-1 (MMP1) and inhibitor of metallopeptidases-1 (TIMP1) production were analyzed by enzyme-linked immunosorbent assays.

Results: On tissue culture plastic, HGF showed no differences between donors on cell proliferation and on the ability for wound closure; α-smooth muscle actin was overexpressed on scratched monolayers. The differentiation profile showed increased production of extracellular matrix components. Ti and TiZr showed similar biocompatibility with HGF. TiZr increased integrin-β3 mRNA and protein levels, compared with Ti. Cells on TiZr surfaces showed higher MMP1 protein than Ti surfaces, although similar TIMP1 protein production. In this *in vitro* experiment, P and M surfaces from both Ti and TiZr showed better HGF growth than modMA.

Conclusion: Taking into account the better mechanical properties and bioactivity of TiZr compared with Ti, the results of the present study show that TiZr is a potential clinical candidate for soft tissue integration and implant success.

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Soft tissue integration (STI) is one of the critical points for dental implant success as it establishes an effective biological seal between the oral cavity and the implant. This integration at the dental implant's abutments protects bone and implant from bacterial penetration, avoiding gingival recession and inflammation-driven bone resorption (1), and inhibits epithelial downgrowth (2). A soft tissue barrier, with gingival tissue attached to the implant's abutment, may improve this protective function.

Several cell types have been identified within gingival connective tissue, among these, gingival fibroblasts account for most connective tissue cells. They synthesize many extracellular matrix (ECM) components and are responsible for the constant adaptation of gingival connective tissue as well as for wound healing repair and regeneration (3,4). Fibroblasts are mesenchymal cells with many vital functions during development and in adult organisms. Although fibroblasts are among the most accessible mammalian cells to culture *in vitro*, they remain poorly defined in molecular terms. Cultured fibroblasts from diverse sites displayed different and characteristic transcriptional patterns although they showed similar morphology, appearing as spindle-shaped cells, positive for vimentin immunostaining and without markers for other cell lineages, thus, suggesting that fibroblasts at different locations in the body should be considered differentiated cell types (5).

Titanium (Ti) is the most widely used material in implantology because of its mechanical strength, resistance to corrosion and excellent biocompatibility (6). However, its mechanical properties are limited in the case of small diameter implants when placed in a narrow bone space (e.g. maxilla front) (7). To enhance its strength, Ti can be alloyed with other elements. Ti alloys containing zirconium (Zr) have shown improved tensile and fatigue strength than Ti (8–10) and with similar biocompatibility (11,12). The surface properties of implants influence adhesion and differentiation of cells surrounding implants, including gingival fibroblasts (13,14). Different modifications, such as generation of nano-topographies and surface roughness, have been used to improve osteoblast cell attachment and osseointegration of oral dental implants (15). Unlike osteoblasts, nano-topography reduces the adhesion and spreading of fibroblasts and epithelial cells, which have a higher affinity for smooth or finely grooved surfaces (1,16).

Although the same surface modifications can be applied to Ti and TiZr, it is possible that these two materials may end up with different surface characteristics. The physicochemical surface characteristics of TiZr implant abutments might thus elicit different fibroblastic responses compared with Ti. The purposes of the present research were to characterize three different donors of primary human gingival fibroblasts (HGF) through analysis over time of cell attachment, growth, morphology, wound healing and gene expression of several genes to establish a valuable model for screening new abutment surfaces, addressing: (i) ECM organization and turnover; (ii) cell adhesion and structure; (iii) inflammation; and (iv) wound healing. Then, the response of HGF to surface-modified TiZr implant abutments was compared to Ti surfaces.

Material and methods

Ti and TiZr discs

Coin-shaped samples (diameter 4.39 mm, thickness 2 mm) made of grade IV Ti and TiZr, containing 13–17% zirconium, were kindly provided by Institut Straumann (Basel, Switzerland) as machined (M) and machined + acid-etched (modMA) surfaces. Mechanical polishing was used to fabricate the polished (P) surfaces.

The P samples were prepared by grinding and polishing M surfaces using a polishing machine (Phoenix 4000; Buehler GmbH, Dusseldorf, Germany), and then washed in ultrasonic baths with NaOH (40 wt.%) and HNO_3 (50 wt.%) as reported earlier (17). The modMA surfaces were produced from M surfaces acid-etched in HCl/H_2SO_4 at 125–130°C for 5 min. They were then rinsed in NaCl under N_2 protection and stored in 0.9% NaCl solution, producing a hydrophilic surface (18). All samples were dipped in phosphate-buffered saline (PBS) before placing them in wells before cell seeding.

Surface characterization

Scanning electron microscope (Quanta 200F, Eindhoven, The Netherlands) was used to visualize surface topography of the modified samples.

Topographical parameters were recorded by using a blue light laser profilometer (Plμ 2300; Sensofar, Terrassa, Spain) to scan areas of $255 \times 191\ \mu m^2$. Two samples from each group were scanned on four areas randomly selected. Surface area roughness (S_a, absolute average height deviation from a mean plane) was calculated from the scanned areas with SensoMap software (SensoMap Plus 4.1; Sensofar).

Cell culture

Three different donors of primary HGF (Provitro GmbH, Berlin, Germany) were used: HGF-A (27 years, Caucasian, female, lot number 313X100401), HGF-B (19 years, Caucasian, male, lot number 322X070501) and HGF-C (47 years, Caucasian, male, lot number 323X070501). HGF cells were cultured at 37°C in a humidified atmosphere of 5% CO_2, and maintained in fibroblast growth medium (Provitro GmbH) supplemented with 10% fetal calf serum and 50 ng amphotericin/mL and 50 μg gentamicin/mL (Provitro GmbH). Culture media was changed every other day. Cells were subcultured before reaching confluence using PBS and trypsin/EDTA, as recommended by the supplier. Trypan blue stain was used to determine total and viable cell number. Experiments were performed with HGF cells between passages 7 and 8 after isolation.

To characterize different HGF donors, 2.0×10^4 cells were seeded on to tissue culture plastic surfaces

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(TCPS, ø 1.9 cm^2). HGF cells were maintained in complete fibroblast growth medium and harvested after 1, 7, 14, 21 or 28 d to perform the experiments.

To test the performance of the different surfaces, coin-shaped implant abutments were placed in 96-well half area plates (Corning, Lowell, MA, USA). HGF-A was used to perform the experiments and 3.5×10^3 cells were seeded on each coin-shaped implant. The same number of cells was cultured in parallel on TCPS. HGF cells were maintained up to 14 d in complete fibroblast growth medium.

Cell number determination by DNA quantification

Culture media was removed from wells, and 100 or 200 μL of distilled water were added to each well for cells on implant abutments or on TCPS, respectively. Plates were incubated for 1 h at room temperature. Plates were frozen at −80°C to lysate cells. Plates were thawed until reaching room temperature and 100 or 200 μL of Hoechst 33258 (Sigma-Aldrich, St. Louis, MO, USA) at 20 μg/mL in TNE buffer were added, for cells on implant abutments or on TCPS, respectively. Two hundred μL aliquots were transferred to 96-well fluorescence plates and a spectrophotometer was set to record fluorescence. Relative fluorescence units are correlated with the cell number using a linear standard curve.

Cell cytotoxicity: lactate dehydrogenase activity in the culture media

The presence of lactate dehydrogenase (LDH) activity in the culture media after 24 h of incubation of HGF on the implant abutment surfaces was used as an index of cell death. LDH activity was determined spectrophotometrically after 30 min of incubation at 25°C of 50 μL of culture media and 50 μL of the reaction mixture, by measuring the oxidation of NADH at 490 nm in the presence of pyruvate, according to the manufacturer's kit instructions (Roche Diagnostics, Mannheim, Germany). Toxicity was presented relative to the LDH activity in the media of cells seeded on TCPS without treatment (low control, 0% of cell death) and on cells grown on TCPS treated with 1% Triton X-100 (high control, 100% of death), using the following equation:Cytotoxicity (%) = (exp. value − low control)/(high control − low control) × 100.

Immunofluorescence

Cells grown for 2 and/or 14 d on the surfaces were fixed for 15 min with 4% formaldehyde in PBS at room temperature. For actin cytoskeleton visualization, cells were stained with phalloidin–fluorescein isothiocyanate 5 μg/mL (Sigma, St. Louis, MO, USA) in PBS with Triton X-100 0.2% for 30 min. For integrin beta-3 visualization, cells were incubated with 0.1% Triton X-100 for 5 min, 1% bovine serum albumin for 30 min and then with anti-integrin beta-3 antibody (AB47584, Abcam, Cambridge, UK) for 1 h at 1 : 50 dilution in PBS. Then, a Cy3-conjugated goat antirabbit IgG (Molecular Probes, Eugene, OR, USA) was used as secondary antibody for 1.5 h at 1 : 200 dilution in PBS. Cells were washed with PBS and coin-shaped samples were placed on slides. Finally, a drop of Fluoroshield™ with DAPI (Sigma) was added and cover glasses were mounted on the samples.

Two samples of each group were used to perform the experiment and two images of each sample were taken with the confocal microscope (Leica DMI 4000B equipped with a Leica TCS SPE laser system).

RNA isolation and real-time RT-PCR analysis

Total RNA was isolated using Tripure® (Roche Diagnostics) and was quantified at 260 nm using a Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The same amount of RNA was reverse transcribed to cDNA at 42°C for 60 min using High Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA, USA), according to the protocol of the supplier. Aliquots of each cDNA were frozen (−20°C) until the PCR reactions were carried out.

Real-time PCR was performed for two reference genes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and beta-actin (ACTBL2), and several target genes (Table 1). Real-time PCR was performed in the Lightcycler 480® (Roche Diagnostics) using SYBR green detection. Each reaction contained 7 μL Lightcycler-FastStart DNA MasterPLUS SYBR Green I (containing Fast Start Taq polymerase, reaction buffer, dNTPs mix, SYBR-Green I dye and MgCl2), 0.5 μM (except for COL3A1, COL5A1 and ITGA2 that was 0.25 μM) of each, the sense and the antisense specific primers (Table 1) and 3 μL of the cDNA dilution in a final volume of 10 μL. The amplification program consisted of a pre-incubation step for denaturation of the template cDNA (5 min 95°C), followed by 45 cycles consisting of a denaturation step (10 s 95°C), an annealing step (10 s 60°C, except for ITGB3, which was 62°C) and an extension step (10 s 72°C). After each cycle, fluorescence was measured at 72°C. A negative control without a cDNA template was run in each assay.

Real-time efficiencies (E) were calculated from the given slopes in the LightCycler 480 software using serial dilutions, showing all the investigated transcripts high real-time PCR efficiency rates, and high linearity when different concentrations were used. PCR products were subjected to a melting curve analysis on the LightCycler and subsequently 2% agarose/TAE gel electrophoresis to confirm amplification specificity, Tm and amplicon size, respectively.

All samples were normalized by the geometric mean of the expression levels of ACTBL2 and GAPDH and fold changes were related to the control groups using the mathematical model described by Pfaffl (19), where *C*p is the crossing point of the reaction amplification curve as determined by the LightCycler 480 software. The stability of reference genes was calculated using the BestKeeper tool (20). The *C*p variation of the reference genes among samples was lower than 0.6. Moreover, a good consistence of

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Table 1. Sense (S) and antisense (A) primers used in real-time PCR of reference and target genes

Related function	Gene	Primer sequence (5′–3′)	Product size (bp)	GenBank accession number
ECM component	Collagen I α1 (COL1A1)	S: AGAGCATGACCGATGGATTC A: TTCTTGAGGTTGCCAGTC	122	NM_000088.3
ECM component	Collagen III α1 (COL3A1)	S: GGCCTACTGGGCCTGGTGGT A: CCACGTTCACCAGGGGCACC	190	NM_000090.3
ECM component	Collagen IV α2 (COL4A2)	S: CCAGAGCGTCTTGGCGGGTG A: TACGTCCCTGCAGTCCCGGG	176	NM_001846.2
ECM component	Collagen V α1 (COL5A1)	S: CTCCAGCAGGCCAGGTTGGC A: ATCACTCCCAGCCCGACCCC	198	NM_000093.3
ECM component	Collagen XII α1 (COL12A1)	S: GCGGCCCTGCTCCTGTCTTC A: AGGCCCATCCGTTGTAGGGTCC	159	NM_004370.5
ECM component	Decorin (DCN)	S: ATCTCAGCTTTGAGGGCTCC A: GCCTCTCTGTTGAAACGGTC	146	NM_001920.3
ECM component	Versican (VCAN)	S: GCCGCCTTCCAAGGCCAAGA A: GAGAGGGAGCCCCTCACCGG	129	NM_004385.4
ECM component	Osteonectin (SPARC)	S: GCGGTCCTTCAGACTGCCCG A: CTTGCTGAGGGGCTGCCAAGG	138	NM_003118
ECM turnover	Matrix metalloproteinase-1 (MMP1)	S: TGTCAGGGGAGATCATCGGGACA A: TGGCCGAGTTCATGAGCTGCA	177	NM_002421.3
ECM turnover	Metallopeptidase inhibitor 1 (TIMP1)	S: TTCCGACCTCGTCATCAGGG A: TAGACGAACCGGATGTCAGC	144	NM_003254.2
ECM component/cell adhesion	Fibronectin (FN1)	S: CGGAGAGACAGGAGGAAATAGCCCT A: TTGCTGCTTGCGGGGCTGTC	150	NM_212482.1
Cell adhesion	Integrin α2 (ITGA2)	S: AGCCTCGGCTTGATCCTCACCA A: GGGCAGGGCTGAGTTGCAGG	170	NM_002203.3
Cell adhesion	Integrin α8 (ITGA8)	S: GCCCGCACAGCGAGTGTCTT A: TCTGCCTGCACTGCGCAGAC	124	NM_003638.1
Cell adhesion	Integrin β3 (ITGB3)	S: GAGGCGGACGAGATGCGAGC A: GCCCAGAGGCAGGGCCTCAT	192	NM_000212.2
Cell structure	Vimentin (VIM)	S: GGCCGCCTGCAGGATGAGATTC A: CAGAGAAATCCTGCTCTCCTCGC	153	NM_003380.3
Pro-inflamatory cytokine	Interleukin-6 (IL6)	S: AGGAGACTTGCCTGGTGAAA A: GCATTTGTGGTTGGGTCAG	196	NM_000600.3
Pro-inflamatory cytokine	Tumor necrosis factor-alpha (TNF)	S: CTATCTGGGAGGGGTCTTCC A: GGGGGTAATAAAGGGATTGG	181	NM_000594.3
Anti-inflammatory cytokine	Interleukin-10 (IL10)	S: TTATCTTGTCTCTGGGCTTGG A: ATGAAGTGGTTGGGGAATGA	139	NM_000572.2
Wound healing/fibrogenic	α-smooth muscle actin (ACTA2)	S: TAAGACGGGAATCCTGTGAAGC A: TGTCCCATTCCCACCATCAC	184	NM_001141945.1
Wound healing/fibrogenic	Transforming growth factor-β1 (TGFB1)	S: TGTCACCGGAGTTGTGCGGC A: GGCCGGTAGTGAACCCGTTG	131	NM_000660.4
Wound healing/fibrogenic	Endothelin-1 (EDN1)	S: ACGGCGGGGAGAAACCCACT A: ACGGAACAACGTGCTCGGGA	147	NM_001955.4
Reference gene	Beta-actin (ACTBL2)	S: CTGGAACGGTGAAGGTGACA A: AAGGGACTTCCTGTAACAATGCA	136	NM_001101.3
Reference gene	Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	S: TGCACCACCAACTGCTTAGC A: GGCATGGACTGTGGTCATGAG	87	NM_002046.3

ECM, extracellular matrix.

the BestKeeper index was proved, as its contributing reference genes were tightly correlated with it ($0.841 < r < 0.894$), with a p value of 0.001 for both reference genes.

For each gene, the coefficient of variation (CV) of the expression was calculated. The CV equals the standard deviation divided by the mean (expressed as a percentage). The CV is used as a statistic for comparing the degree of variation between genes, even if the mean expressions are drastically different from each other (21).

Wound healing assay and scanning electron microscopy

When HGF cells were confluent, the monolayer was scraped in a straight line to create a scratch. Two hundred μL sterile pipette tips were used to scratch the monolayers grown on 24-well plates, and 10 μL tips to scratch monolayers grown on coin-shaped implant abutments. Then, monolayers were washed once with growth medium to remove debris and detached cells.

A bright field inverted microscope (Leica DM IRB, Wetzlar, Germany)

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was used to take images 0, 24 and 48 h after the scratch of the same areas from cells grown on TCPS. Then, culture media was removed and RNA was isolated as described before to study gene expression. The images were quantitatively analyzed with TScratch software using the default parameter settings (22). The open wound area (%) at a time point was defined as 100 × (uncovered image area at 24 or 48 h/uncovered image area at 0 h).

A scanning electron microscope (Hitachi S-3400N, Krefeld, Germany) using back-scattered electrons, 40 Pa of pressure and 10 kV of voltage was used to acquire images of cells grown on samples. Cells were washed twice with PBS and fixed with glutaraldehyde 4% in PBS for 2 h. The fixative solution was removed, and the cells were washed with distilled water three times. At 30 min intervals, the cells were dehydrated by the addition of 50%, 70%, 90% and 100% ethanol solutions. Finally, the ethanol was removed, and the cells were left at room temperature to evaporate the remaining ethanol before analysis.

Enzyme-linked immunosorbent assays

The detection of TIMP1 and MMP1 was performed from cell lysates after 14 d of cell culture ($n = 6$) by commercially available ELISA kits (Sigma) according to kit instructions. Briefly, cells were washed with PBS and lysed with 1% Nonidet P-40, 20 mM Tris–HCl pH 7.4, 100 mM NaCl and protease inhibitors (Complete; Roche Diagnostics). The absorbance was measured with a microplate reader at 450 nm.

Statistical analysis

All data are presented as means ± SEM. The Kolmogorov–Smirnov test was done to assume parametric or non-parametric distributions for the normality tests. Differences between groups were assessed by two-way analysis of variance (ANOVA) test followed by post-hoc pairwise comparisons using the DMS test. When data were not parametric or ANOVA test was not suitable, the Mann–Whitney test or Student *t*-test were run depending on their normal distribution. CV over time was calculated to analyze stability of gene expression. To measure correlation among variables, Pearson or Spearman correlation analysis were used. SPSS® program for Windows, version 17.0 (SPSS Inc., Chicago, IL, USA) was used. Results were considered statistically significant at $p \leq 0.05$.

Results

Human gingival fibroblasts characterization

We first characterized HGF from three different donors through the analysis over time of cell number, cell adhesion, gene expression and a wound healing assay. Cells reached confluence after 7 d of culture and continued to proliferate as multilayers until day 21. No differences between the three donors were found in cell proliferation on TCPS (data not shown).

The expression of different target genes was studied over time (Fig. 1). Only VIM and ITGA8 expression was maintained over time. Although the ANOVA test revealed significant differences in gene expression between donors, it can be observed that the three donors followed similar expression patterns for all the genes analyzed. Expression of genes coding for ECM proteins followed different patterns during differentiation. Thus, while relative mRNA levels of COL3A1, COL5A1, COL12A1, DCN, VCAN, SPARC and TIMP1 increased over time, the mRNA levels of COL4A2 and MMP1 decreased. It is noteworthy that COL3A1 and DCN were highly upregulated over time (up to eightfold at the last time point) and that the MMP1/TIMP1 mRNA ratio decreased over time (Fig. S1A). Regarding expression of genes involved in cell adhesion and cell integrity, the expression levels of VIM together with FN1 and ITGA8 tended to be stable over time (CV of 21%, 25% and 19%, respectively). Nevertheless, gene expression of ITGA2 and ITGB3 was upregulated after 7 d of cell culture. Among the pro-fibrotic-related genes, while EDN1 expression decreased, ACTA2 expression increased after 7 d of cell culture.

To study the recovery of the monolayer after a scratch, a wound healing assay was performed (Fig. S2). We found that the percentage of open wound area after 24 and 48 h was statistically lower than the initial scratched area at 0 h (Fig. S2A). In the healed monolayer, 48 h after the scratch, ACTA2 expression increased and MMP1 expression decreased, while EDN1 and TGFB1 did not change, compared with the unscratched monolayer (Fig. S2B).

Surface characterization

Six candidate surfaces were created by three methods and two materials with different surface area roughness (S_a) (Fig. 2). Roughness of M surfaces was not statistically different when comparing Ti and TiZr. However, despite the same treatment, P and modMA surfaces displayed significantly different S_a depending on the material, but, while roughness was higher in TiZr compared with Ti for P treatment, modMA Ti was higher (above 1 μm) compared with modMA TiZr (190 nm).

In accordance to S_a values, scanning electron microscope images (Fig. 2) revealed similar grooved textures on M Ti and M TiZr surfaces and different smooth surfaces for P Ti and P TiZr samples. For modMA surfaces, scanning electron microscope images showed that TiZr preserved the original grooved morphology of M and was less roughened than modMA Ti.

Cell behavior on Ti- and TiZr-modified surfaces

Performance of TiZr surfaces was compared with that of Ti surfaces through the analysis of cell cytotoxicity, DNA content, morphology, recovery after a scratch and gene expression.

We found no differences in cell toxicity when comparing Ti and TiZr

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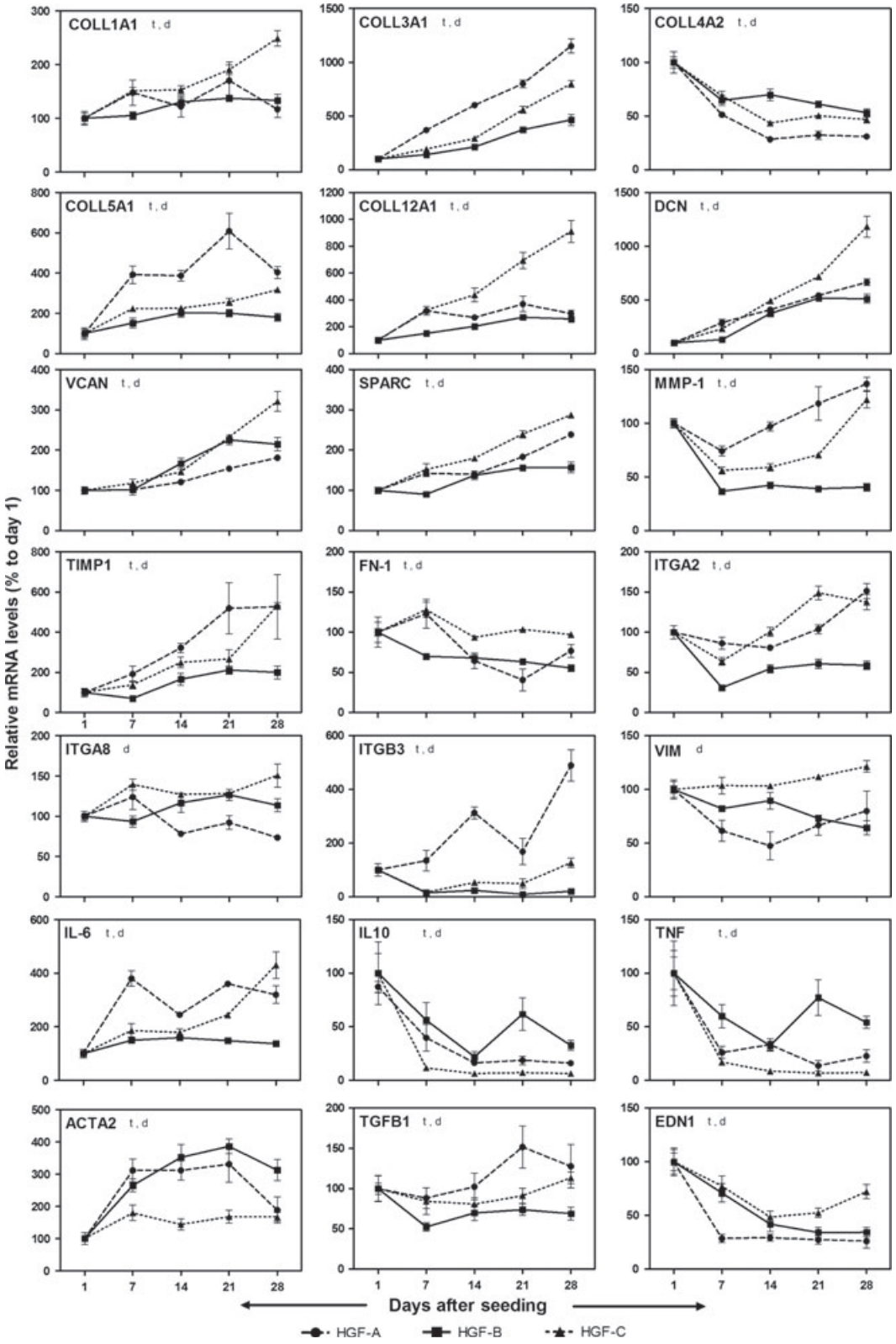

Fig. 1. Gene expression levels at different times of COL1A1, COL3A1, COL4A2, COL5A1, COL12A1, DCN, VCAN, SPARC, TIMP1, VIM, ITGA2, ITGA8, ITGB3, FN1, MMP1, IL6, IL10, TNF, ACTA2, EDN1 and TGFB1 of HGF cells seeded on to tissue culture plastic surfaces. Data represent fold changes of target genes normalized to beta-actin and GAPDH (reference genes) expressed relative to cells at day 1 that were set at 100%. Values represent the mean ± SEM ($n = 6$ for each donor). (*t*) effect of time ($p \leq 0.05$); (d) effect of donor ($p \leq 0.05$), as assessed by ANOVA. HGF, human gingival fibroblasts.

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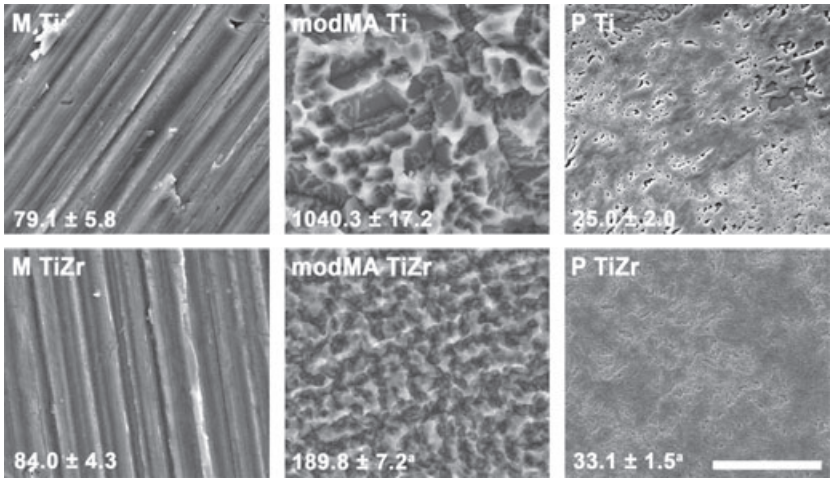

Fig. 2. Scanning electron microscope images and surface roughness (nm) of the Ti and TiZr surfaces. Data represent mean ± SEM. Significant differences were assessed by Student *t*-test: (a) $p \leq 0.05$ Ti vs. TiZr for the same surface modification. Bar scale = 10 μm. M, machined; modMA, machined + acid-etched; P, polished.

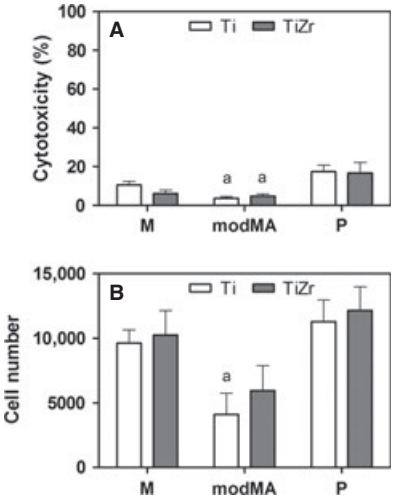

Fig. 3. Analysis of cytotoxicity (A) and proliferation (B) of human gingival fibroblast cells seeded on Ti and TiZr surfaces. (A) Lactic dehydrogenase activity measured from culture media of human gingival fibroblast cells seeded on Ti and TiZr implants after 24 h of cell culture. Positive control (100%) was cell culture media from cells seeded on to plastic culture dishes and incubated with Triton X-100 at 1%. Negative control (0%) was cell culture media from cells seeded on plastic culture dishes without any treatment. Values represent the mean ± SEM ($n = 12$). M Ti surfaces were compared against other surfaces; significant differences were assessed by the Mann–Whitney test: (a) $p \leq 0.05$. (B) DNA content after 48 h was determined by fluorimetric DNA quantification. Values represent the mean ± SEM ($n = 4$). M Ti surfaces were compared against the other surfaces, significant differences were assessed by Student *t*-test: (a) $p \leq 0.05$. M, machined; modMA, machined + acid-etched; P, polished.

surfaces (Fig. 3A). Cytotoxicity was lower than 20% in all cases although modMA Ti and modMA TiZr showed less cytotoxicity than M Ti. Moreover, M and P Ti and TiZr surfaces supported better cell attachment after 48 h than did modMA surfaces (Fig. 3B). Images show the same cell behavior within each group for both Ti and TiZr surfaces (Fig. 4). HGF showed spindle-shaped morphology in M and P surfaces. However, while cells grew aligned to M surfaces cells grew disorderly on P surfaces. On modMA surfaces, few HGF were found and showed round-shaped morphology, indicating poor cell attachment, in agreement with cell number results.

When cell monolayers on the implant abutment surfaces were confluent (time 0), they were scratched. After 48 h, wounds in cell monolayers were healed with no differences among Ti/TiZr in M and P surfaces. Very few cells grew on modMA surfaces, either on Ti or TiZr; therefore, wound healing closure could not be assessed (Fig. S3).

Expression of different genes was analyzed after 14 d of cell culture on the different surfaces (Fig. 5). This time point was chosen based on previous characterization of HGF cultured on TCPS, where most changes in gene expression were observed and considering that HGF were still proliferating in multilayers until 21 d (data not shown). First, we compared gene expression of HGF cultured on Ti to the expression on TiZr for the same surface modification. ITGB3 expression was significantly increased in all TiZr surfaces (fourfold on average) compared with Ti surfaces. For M surfaces, gene expression response to Ti and TiZr followed a similar pattern, except for ITGB3. For P surfaces, several genes were upregulated on P TiZr compared with P Ti surfaces, including some collagens and TIMP1. For modMA surfaces, the expression of COL5A1, VIM, FN1, TGFB1, EDN1 and ACTA2 was significantly downregulated in TiZr compared with Ti.

When comparing different surfaces with M Ti, modMA were the surfaces where more changes on gene expression were seen. While gene expression of several ECM components decreased in both Ti and TiZr modMA surfaces (including TIMP1), the expression of COL4A2, MMP1, ITGA2, ITGA8 and ACTA2 increased in modMA Ti/TiZr surfaces. Both Ti and TiZr P surfaces behaved similarly to M Ti although some differences were found. Relative mRNA levels of COL1A1, COL12A1 and TGB1 significantly decreased in P Ti, while the mRNA levels of ITGB3 significantly increased in P TiZr, compared with M Ti. It is worth highlighting that expression of interleukin 6 (IL6), MMP1 and TIMP1 increased in both Ti and TiZr P surfaces compared with M Ti, though it was only significant for TIMP1 in both surfaces and IL6 in P TiZr. The MMP1/TIMP1 mRNA ratio statistically increased on modMA Ti and TiZr surfaces compared with M Ti (Fig. S1B).

Consistent with the observation that ITGB3 expression was increased on TiZr surfaces after 14 d of cell culture, cells on TiZr showed increased integrin β3 protein levels, except on

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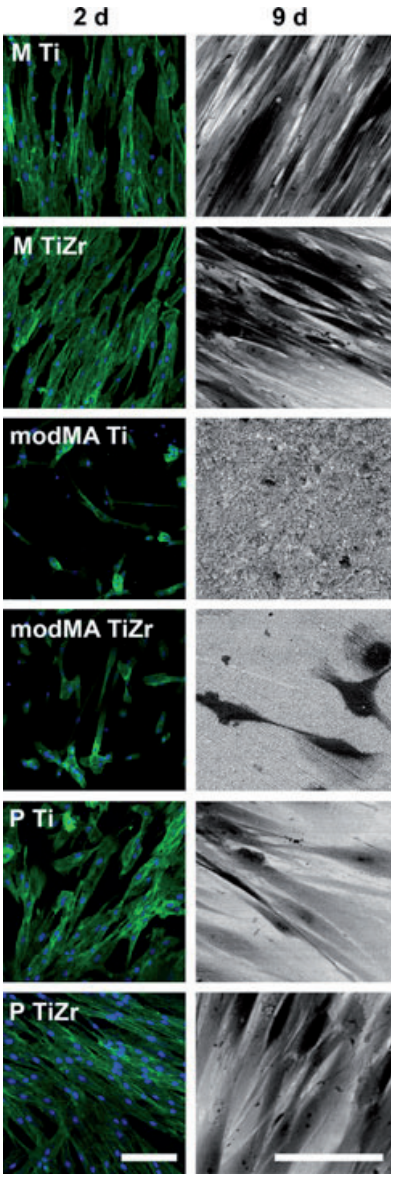

Fig. 4. Cell morphology on the different Ti and TiZr surfaces. Confocal microscopy was used to visualize actin immunostaining (green) and nuclei (blue) after 48 h (left column). Scanning electron microscopy was used to visualize human gingival fibroblasts on the surfaces after 9 d (right column). Bar scale = 100 μm. M, machined; modMA, machined + acid-etched; P, polished.

modMA TiZr surfaces (Fig. 6). M Ti/TiZr surfaces showed similar integrin β3 after 2 d of cell culture, although after 14 d its presence decreased on M Ti while on M TiZr was maintained. P TiZr increased ITGB3 levels after 2 and 14 d of cell culture compared with P Ti. In general, ITGB3 immunostaining was low on modMA surfaces, as there were few cells.

MMP1 production significantly increased on TiZr surfaces compared with Ti (1.5 vs. 0.9 ng/mL) although TiZr did not affect TIMP1 production compared with Ti. Compared with M Ti, MMP1 increased on M TiZr, modMA TiZr and P Ti/TiZr surfaces (Fig. 7A); TIMP1 decreased on modMA Ti (Fig. 7B); and the MMP1/TIMP1 ratio increased on modMA Ti/TiZr and on M TiZr (Fig. 7C). Interestingly, the production of TIMP1 was higher than the production of MMP1 (an average of 55 ng/mL for TIMP1 vs. 1.2 ng/mL for MMP1), as also found at mRNA levels (a difference of sixfold, taking into account similar PCR efficiencies for both pairs of primers).

Discussion

STI and hard tissue integration is mandatory for dental implant success. While over the past years, research and development of dental implant biomaterials has been focused on osseointegration, nowadays STI is gaining ground, as dental implants require a soft tissue barrier to prevent bacterial penetration and inhibit epithelial downgrowth. After installation of a dental implant, fibroblasts from the oral connective tissue are the preferred cells to form a collagen-rich connective tissue to repopulate the wound (23). In the present research, we first established an *in vitro* model with primary HGF for later evaluation of their biological response to surface-modified TiZr implant abutments and compare it with the gold standard in implantology, Ti.

We show that HGF produce an organized ECM, do not tend to fibrolytic or proinflammatory situations and have the capacity for *in vitro* differentiation into myofibroblasts. This was characterized by increased gene expression over time of ECM constituents such as several collagens, proteoglycans and glycoproteins (production of an organized ECM); decreased MMP1/TIMP1 mRNA ratio over time [lack of tendency to a fibrolytic situation (24)]; steady expression of IL6 together with decreased expression of tumor necrosis factor-alpha [lack of a proinflammatory situation (25)]; re-establishment of the full monolayer architecture in 48 h after a wound scratch and increased expression of ACTA2 [hallmark of myofibroblast differentiation and fibrogenic conditions (26)]. However, the elevated levels of ITGA2 could be a feature of a fibrotic phenotype (27).

Regarding the *in vitro* test system, monolayers of either gingival epithelial keratinocytes or fibroblasts have been used to test STI (1). Complex interactive co-cultures of gingival keratinocytes and fibroblasts have also been used as a test platform for STI evaluation (28), although keratinocytes induce fibroblasts to differentiate into myofibroblasts (29). Cell co-cultures are more time and resource consuming than single cell type cultures; therefore, we considered that co-cultures were not the optimal test system to perform high-throughput analysis.

In this study, we compared different Ti and TiZr surfaces to evaluate their applicability for STI. TiZr was as biocompatible as Ti, as previously demonstrated (11,12). In addition, TiZr supported cell attachment and had little influence on either cell morphology or alignment. We found that integrin β3 mRNA and protein levels increased on TiZr compared with Ti. Integrin αvβ3 is involved in cell attachment (30), thus, this finding could be related to the slight but not significant, enhanced cell attachment on to TiZr compared with Ti. Besides the strong ITGB3 increase, Ti and TiZr caused different responses in HGF, as previously found with osteoblasts (31). Regarding P and modMA surfaces, although Ti and TiZr showed different roughness, we only found few differences among materials, including the decreased expression of the pro-fibrotic markers in modMA TiZr (ACTA2, EDN1 and TGFB1) compared with modMA Ti and increased expression of some ECM markers in P TiZr (COL1A1, COL3A1, DCN, VCAN and TIMP1) compared with P Ti. For M surfaces, with a similar roughness, MMP1

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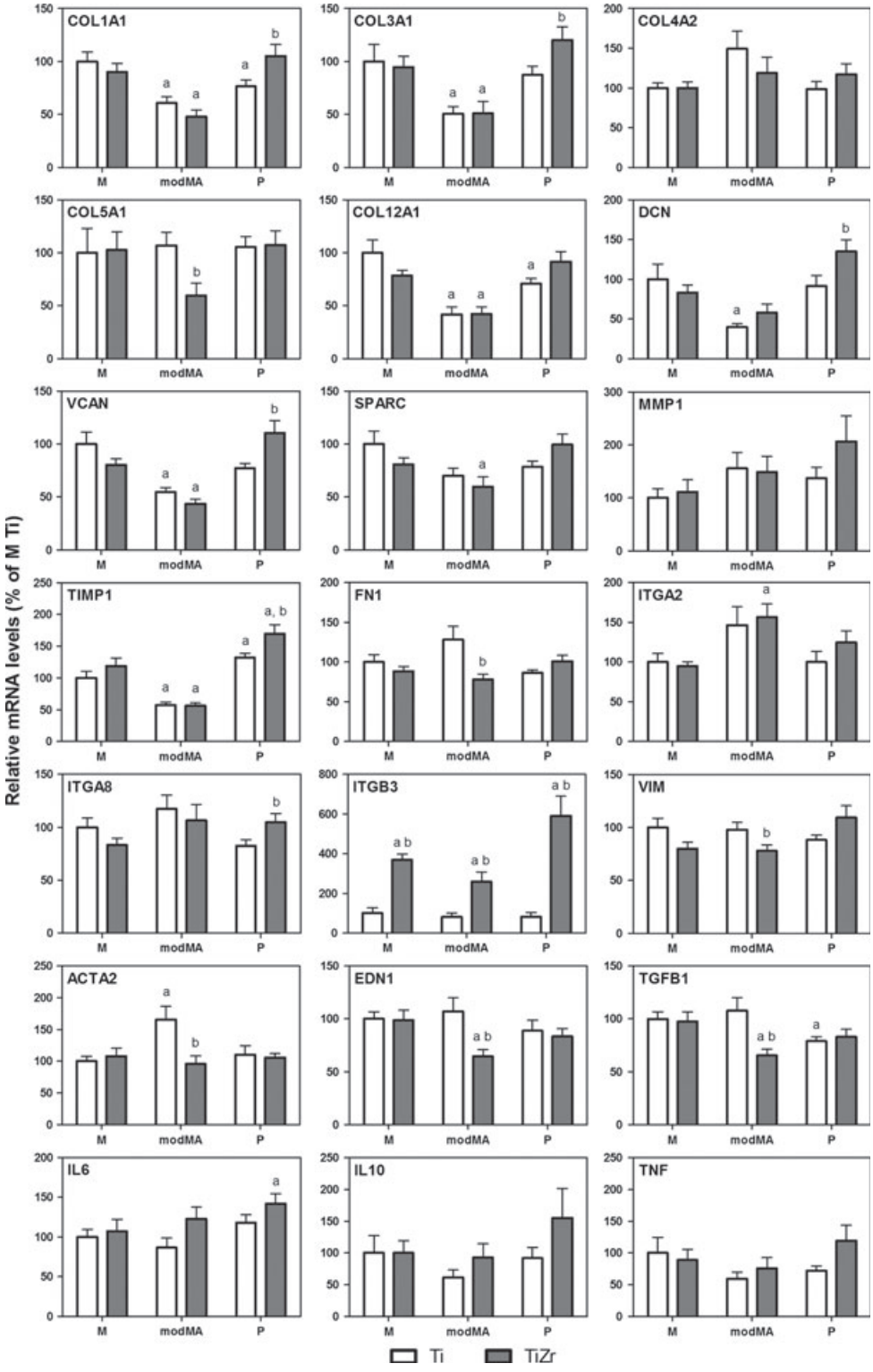

Fig. 5. Gene expression levels of human gingival fibroblasts grown for 14 d on Ti and TiZr. Data represent fold changes of target genes normalized to beta-actin and GAPDH (reference genes) expressed relative to cells grown on M Ti that were set at 100%. Values represent the mean ± SEM ($n = 8$). Significant differences were assessed by Student *t*-test: (a) $p \leq 0.05$ vs. M Ti surfaces; (b) $p \leq 0.05$ Ti vs. TiZr for the same surface. M, machined; modMA, machined + acid-etched; P, polished.

production increased on M TiZr resulting in an increased MMP1/TIMP1 ratio. MMPs degrade native fibrillar collagens during ECM turnover, in both normal and diseased conditions, and their activity is controlled mainly by metallopeptidase inhibitors (TIMPs) (32), which balance controls of tissue remodeling

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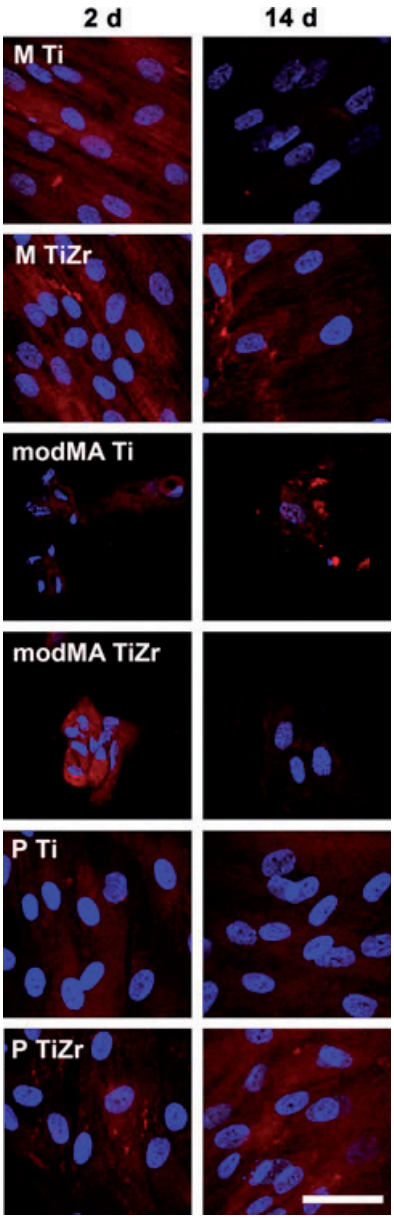

Fig. 6. Confocal microscopy was used to visualize integrin β3 (red) and nuclei (blue) after 2 and 14 d of cell culture on the Ti and TiZr surfaces. Bar scale = 50 μm. M, machined; modMA, machined + acid-etched; P, polished.

(33). An increased MMP1/TIMP1 ratio in M TiZr surfaces could reflect either the remodeling of newly synthesized collagen (34) or an accelerated ECM degradation, which has been associated with periodontitis (35,36). Interestingly, it has been shown that MMP production is regulated by the ECM through integrin receptor signaling (37) and that MMP1 increases

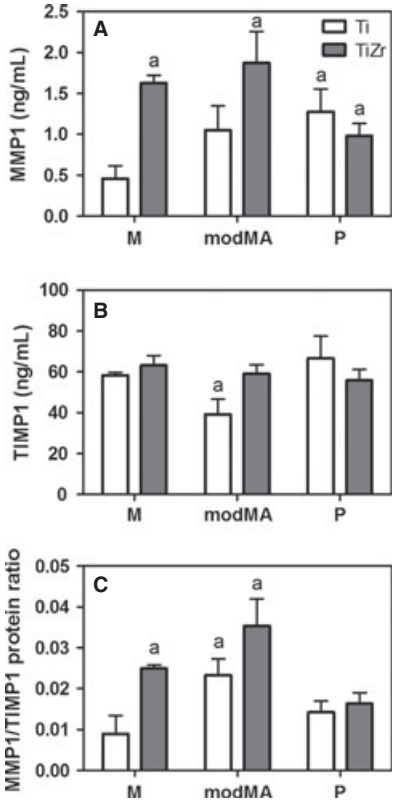

Fig. 7. Effect of Ti and TiZr surfaces on MMP1 (A) and TIMP1 (B) production and the MMP1/TIMP1 ratio (C), after 14 d of cell culture. Values represent the mean ± SEM ($n = 6$). Significant differences were assessed by Student *t*-test: (a) $p \leq 0.05$ vs. M Ti surfaces. M, machined; MMP1, matrix metalloproteinase 1; modMA, machined + acid-etched; P, polished; TIMP1, metallopeptidase inhibitor 1.

in parallel with ITGB3 expression in smooth muscle cells from wounding sites (38). Thus, the greater integrin β3 production on TiZr surfaces could be responsible for the increased MMP1 production on TiZr surfaces. Furthermore, MMP1 and ITGB3 expression were positively correlated (Pearson's correlation coefficient = 0.365; $p = 0.012$). Future studies should explore the pathways between MMP1 and ITGB3 in gingival fibroblasts to understand further the mechanisms implicated under this hypothesis.

We also evaluated the performance of the different surface modifications comparing with the reference surface, M Ti. We considered M Ti the reference surface as machined is the standard implant neck and abutment surface of dental products in the market and Ti is the gold standard in implantology (6). For modMA surfaces, the decreased cell number, abnormal morphology and easy-stained condensed nuclei suggest impaired growth and even apoptosis. Moreover, modMA surfaces decreased gene expression of several ECM components and vimentin and increased the mRNA levels of MMP1, ITGA2, ITGA8, IL6, the pro-fibrotic markers and the MMP1/TIMP1 mRNA and protein ratio suggesting impaired HFG growth and a pro-fibrotic situation on to these surfaces. As the initial cell attachment to a surface is a critical step for cell survival, for this *in vitro* study we hypothesize that although modMA surfaces were not cytotoxic, initial cell attachment was impaired, and was, in consequence, cell survival and growth. This can be explained by the increased roughness of modMA surface as cell number and the expression of several genes encoding for ECM proteins (COL1A1, COL3A1, COL12A1, DCN, VCAN and TIMP1) were negatively correlated with surface roughness (Spearman's correlation coefficient $-0.425 \leq r \leq -0.771$; $p < 0.05$). This impairment in cell attachment and growth might be also explained by surface hydrophilicity (14,39), even though we did not measure the contact angle in the present study. Thus, although modMA do not seem to be applicable for STI, hydrophilic rough surfaces downregulated the gene expression of key proinflammatory cytokines of macrophages (40), increased the osteogenic differentiation of osteoblasts (15) and, *in vivo*, favored blood wetting and the formation of a fibrin network, resulting in superior osseointegrative properties (41) and connective tissue attachment (42,43).

For M and P surfaces, data suggest a normal fibroblast growth, M surfaces created a pattern of mechanical stress that caused cell alignment (44) to the grooves, which is related to a higher density of focal contacts (45), while no clear orientation was seen on P surfaces. Previous studies have shown similar results with fibroblasts

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and rough surfaces (S_a from 500 nm to 1 μm) compared with M (S_a = 119 nm) and smooth surfaces (S_a from 14 to 293 nm) (14,28,46). However, some studies have demonstrated that it is the combination of micro-roughness with further acid etching that promotes HGF adhesion and proliferation (47,48).

In conclusion, we show that monocultures of HGF are a valuable *in vitro* model for high-throughput screening of new abutment surfaces designed to improve STI. We also prove in this *in vitro* study that: (i) TiZr has the same biocompatibility than Ti surfaces on primary HGF; (ii) TiZr surfaces show higher integrin β3 mRNA and protein levels together with increased MMP1 protein compared with Ti; and (iii) M surfaces caused better alignment than the other studied surfaces. Taking into account the better mechanical properties and bioactivity of TiZr compared with Ti, the results of the present study show that TiZr is a potential clinical candidate for STI and implant success.

Acknowledgements

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Ratio MMP1/TIMP1. (A) MMP1/TIMP1 mRNA ratio over time of HGF cells seeded on to TCPS. Values represent the mean ± SEM (n = 6 for each donor); (t) effect of time ($p < 0.05$). (B) MMP1/TIMP1 mRNA ratio of HGF cells seeded on to Ti/TiZr-modified surfaces. Values represent the mean ± SEM (n = 8). Significant differences were assessed by Student t-test: (a) $p \leq 0.05$ *versus* machined Ti surfaces (M Ti).

Figure S2 Wound healing assay. Cell monolayers were scratched at 0 h (C) and allowed to heal up to 48 h (D, E; dotted line indicates scratch mark). (A) The open wound area was calculated for each donor and then the mean ± SEM (n = 3) of the three donors was calculated. (B) Gene expression levels 48 h after scratching HGF monolayers of MMP1, ACTA2, EDN1 and TGFB1 of HGF cells seeded on to TCPS. Data represent fold changes of target genes normalized to beta-actin and GAPDH (reference genes) expressed relative to cells before scratching the monolayers that were set at 100%. Values represent the median and the 5–95 percentile (n = 3 for each donor). Significant differences were assessed by Student t-test: (a) $p \leq 0.05$.

Figure S3 Wound healing assay. Scanning electron microscopy images of HGF grown on Ti and TiZr implants 48 h after scratching the surface. Dotted line indicates the scratch mark. Bar scale = 2 mm.

References

1. Rompen E, Domken O, Degidi M, Pontes AE, Piattelli A. The effect of material characteristics, of surface topography and of implant components and connections on soft tissue integration: a literature review. *Clin Oral Implants Res* 2006;**17**(Suppl 2):55–67.
2. Chehroudi B, Gould TR, Brunette DM. The role of connective tissue in inhibiting epithelial downgrowth on titanium-coated percutaneous implants. *J Biomed Mater Res* 1992;**26**:493–515.
3. Bartold PM, Walsh LJ, Narayanan AS. Molecular and cell biology of the gingiva. *Periodontol 2000* 2000;**24**:28–55.
4. Abiko Y, Hiratsuka K, Kiyama-Kishikawa M, Tsushima K, Ohta M, Sasahara H. Profiling of differentially expressed genes in human gingival epithelial cells and fibroblasts by DNA microarray. *J Oral Sci* 2004;**46**:19–24.
5. Chang HY, Chi JT, Dudoit S *et al.* Diversity, topographic differentiation, and positional memory in human fibroblasts. *Proc Natl Acad Sci USA* 2002;**99**:12877–12882.
6. Steinemann SG. Titanium—the material of choice? *Periodontol 2000* 1998;**17**:7–21.
7. Saulacic N, Bosshardt DD, Bornstein MM, Berner S, Buser D. Bone apposition to a titanium-zirconium alloy implant, as compared to two other titanium-containing implants. *Eur Cells Mater* 2012;**23**:273–286; discussion 286-278.
8. Ho WF, Chen WK, Wu SC, Hsu HC. Structure, mechanical properties, and grindability of dental Ti-Zr alloys. *J Mater Sci Mater Med* 2008;**19**:3179–3186.
9. Kobayashi E, Matsumoto S, Doi H, Yoneyama T, Hamanaka H. Mechanical properties of the binary titanium-zirconium alloys and their potential for biomedical materials. *J Biomed Mater Res* 1995;**29**:943–950.
10. Thoma DS, Jones AA, Dard M, Grize L, Obrecht M, Cochran DL. Tissue integration of a new titanium-zirconium dental implant: a comparative histologic and radiographic study in the canine. *J Periodontol* 2011;**82**:1453–1461.
11. Linkevicius T, Apse P. Influence of abutment material on stability of peri-implant tissues: a systematic review. *Int J Oral Maxillofac Implants* 2008;**23**:449–456.
12. Pae A, Lee H, Kim HS, Kwon YD, Woo YH. Attachment and growth behaviour of human gingival fibroblasts on titanium and zirconia ceramic surfaces. *Biomed Mater* 2009;**4**:025005.
13. Kokubu E, Hamilton DW, Inoue T, Brunette DM. Modulation of human gingival fibroblast adhesion, morphology, tyrosine phosphorylation, and ERK 1/2 localization on polished, grooved and SLA substratum topographies. *J Biomed Mater Res A* 2009;**91**:663–670.
14. Oates CJ, Wen W, Hamilton DW. Role of titanium surface topography and surface wettability on focal adhesion kinase mediated signaling in fibroblasts. *Materials* 2011;**4**:893–907.
15. Le Guehennec L, Soueidan A, Layrolle P, Amouriq Y. Surface treatments of titanium dental implants for rapid osseointegration. *Dent Mater* 2007;**23**:844–854.
16. Kononen M, Hormia M, Kivilahti J, Hautaniemi J, Thesleff I. Effect of surface processing on the attachment, orientation, and proliferation of human gingival fibroblasts on titanium. *J Biomed Mater Res* 1992;**26**:1325–1341.
17. Lamolle SF, Monjo M, Lyngstadaas SP, Ellingsen JE, Haugen HJ. Titanium implant surface modification by cathodic reduction in hydrofluoric acid: surface characterization and in vivo performance. *J Biomed Mater Res A* 2009;**88**:581–588.
18. Rupp F, Scheideler L, Olshanska N, de Wild M, Wieland M, Geis-Gerstorfer J. Enhancing surface free energy and hydrophilicity through chemical modification of

microstructured titanium implant surfaces. *J Biomed Mater Res A* 2006;**76A**: 323–334.
19. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 2001;**29**:e45.
20. Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper–Excel-based tool using pairwise correlations. *Biotechnol Lett* 2004;**26**:509–515.
21. Novak JP, Sladek R, Hudson TJ. Characterization of variability in large-scale gene expression data: implications for study design. *Genomics* 2002;**79**:104–113.
22. Geback T, Schulz MM, Koumoutsakos P, Detmar M. TScratch: a novel and simple software tool for automated analysis of monolayer wound healing assays. *Biotechniques* 2009;**46**:265–274.
23. Palaiologou AA, Yukna RA, Moses R, Lallier TE. Gingival, dermal, and periodontal ligament fibroblasts express different extracellular matrix receptors. *J Periodontol* 2001;**72**:798–807.
24. Soell M, Elkaim R, Cathepsin TH, C, . Matrix metalloproteinases, and their tissue inhibitors in gingiva and gingival crevicular fluid from periodontitis-affected patients. *J Dent Res* 2002;**81**:174–178.
25. Bartold PM, Narayanan AS. Molecular and cell biology of healthy and diseased periodontal tissues. *Periodontol 2000* 2006;**40**:29–49.
26. Hinz B, Phan SH, Thannickal VJ, Galli A, Bochaton-Piallat ML, Gabbiani G. The myofibroblast: one function, multiple origins. *Am J Pathol* 2007;**170**:1807–1816.
27. Guo F, Carter DE, Mukhopadhyay A, Leask A. Gingival fibroblasts display reduced adhesion and spreading on extracellular matrix: a possible basis for scarless tissue repair? *PLoS ONE* 2011;**6**:e27097.
28. Schulz S, Tomakidi P, Mauth C, Kohal R, Steinberg T. Interactive fibroblast-keratinocyte co-cultures: an in vivo-like test platform for dental implant-based soft tissue integration. *Tissue Eng Part C Methods* 2012;**18**:785–796.
29. Shephard P, Martin G, Smola-Hess S, Brunner G, Krieg T, Smola H. Myofibroblast differentiation is induced in keratinocyte-fibroblast co-cultures and is antagonistically regulated by endogenous transforming growth factor-beta and interleukin-1. *Am J Pathol* 2004;**164**:2055–2066.
30. Hormia M, Kononen M. Immunolocalization of fibronectin and vitronectin receptors in human gingival fibroblasts spreading on titanium surfaces. *J Periodontal Res* 1994;**29**:146–152.
31. Chakravorty N, Ivanovski S, Prasadam I, Crawford R, Oloyede A, Xiao Y. The microRNA expression signature on modified titanium implant surfaces influences genetic mechanisms leading to osteogenic differentiation. *Acta Biomater* 2012;**8**: 3516–3523.
32. Sakagami G, Sato E, Sugita Y *et al*. Effects of nifedipine and interleukin-1alpha on the expression of collagen, matrix metalloproteinase-1, and tissue inhibitor of metalloproteinase-1 in human gingival fibroblasts. *J Periodontal Res* 2006;**41**:266–272.
33. Domeij H, Modeer T, Yucel-Lindberg T. Matrix metalloproteinase-1 and tissue inhibitor of metalloproteinase-1 production in human gingival fibroblasts: the role of protein kinase C. *J Periodontal Res* 2004;**39**:308–314.
34. Dasu MR, Hawkins HK, Barrow RE, Xue H, Herndon DN. Gene expression profiles from hypertrophic scar fibroblasts before and after IL-6 stimulation. *J Pathol* 2004;**202**:476–485.
35. Birkedal-Hansen H. Role of matrix metalloproteinases in human periodontal diseases. *J Periodontol* 1993;**64**:474–484.
36. Kubota T, Nomura T, Takahashi T, Hara K. Expression of mRNA for matrix metalloproteinases and tissue inhibitors of metalloproteinases in periodontitis-affected human gingival tissue. *Arch Oral Biol* 1996;**41**:253–262.
37. Werb Z. ECM and cell surface proteolysis: regulating cellular ecology. *Cell* 1997;**91**:439–442.
38. Bendeck MP, Irvin C, Reidy M *et al*. Smooth muscle cell matrix metalloproteinase production is stimulated via αvβ3 integrin. *Arterioscler Thromb Vasc Biol* 2000;**20**:1467–1472.
39. Ponsonnet L, Reybier K, Jaffrezic N *et al*. Relationship between surface properties (roughness, wettability) of titanium and titanium alloys and cell behaviour. *Mater Sci Eng C-Mater Biol Appl* 2003;**23**:551–560.
40. Hamlet S, Alfarsi M, George R, Ivanovski S. The effect of hydrophilic titanium surface modification on macrophage inflammatory cytokine gene expression. *Clin Oral Implants Res* 2012;**23**:584–590.
41. Donos N, Hamlet S, Lang NP *et al*. Gene expression profile of osseointegration of a hydrophilic compared with a hydrophobic microrough implant surface. *Clin Oral Implants Res* 2011;**22**: 365–372.
42. Schwarz F, Ferrari D, Herten M *et al*. Effects of surface hydrophilicity and microtopography on early stages of soft and hard tissue integration at non-submerged titanium implants: an immunohistochemical study in dogs. *J Periodontol* 2007;**78**: 2171–2184.
43. Schwarz F, Herten M, Sager M, Wieland M, Dard M, Becker J. Histological and immunohistochemical analysis of initial and early subepithelial connective tissue attachment at chemically modified and conventional SLA®titanium implants. A pilot study in dogs. *Clin Oral Investig* 2007;**11**:245–255.
44. Walboomers XF, Jansen JA. Cell and tissue behavior on micro-grooved surfaces. *Odontology* 2001;**89**:2–11.
45. Eisenbarth E, Meyle J, Nachtigall W, Breme J. Influence of the surface structure of titanium materials on the adhesion of fibroblasts. *Biomaterials* 1996;**17**:1399–1403.
46. Yamano S, Ma AK, Shanti RM, Kim SW, Wada K, Sukotjo C. The influence of different implant materials on human gingival fibroblast morphology, proliferation, and gene expression. *Int J Oral Maxillofac Implants* 2011;**26**:1247–1255.
47. Lee SW, Kim SY, Lee MH, Lee KW, Leesungbok R, Oh N. Influence of etched microgrooves of uniform dimension on in vitro responses of human gingival fibroblasts. *Clin Oral Implants Res* 2009;**20**:458–466.
48. Kim SY, Oh N, Lee MH, Kim SE, Leesungbok R, Lee SW. Surface microgrooves and acid etching on titanium substrata alter various cell behaviors of cultured human gingival fibroblasts. *Clin Oral Implants Res* 2009;**20**:262–272.

Paper V

Flavonoid-functionalised titanium surfaces favour gingival cells in the race for the surface against oral bacteria

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Flavonoid-functionalised titanium surfaces favour gingival cells in the race for the surface against oral bacteria

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Keywords

quercitrin; inflammation; gingiva; surface modification; flavonoids; peri-implantitis; Streptococcus mutans.

Abstract

Success of a dental implant depends on osseo- and soft tissue-integration. Many dental implants fail due to the infection and inflammation that walk hand in hand with poor healing and soft tissue integration. We have previously biofunctionalised titanium surfaces with quercitrin, a natural flavonoid with multi-target actions with the aim to improve soft tissue integration and increase dental implants long-term success. Here, bacterial attachment and biofilm formation using *Streptococcus mutans* was analysed. Then, the anti-inflammatory properties and the potential of quercitrin-functionalised surfaces to boost soft tissue regeneration were tested using primary human gingival fibroblasts. An inflammatory situation was mimicked adding interleulin-1 beta to cell culture. We found that quercitrin-functionalised surfaces decreased initial bacterial adhesion while increasing human gingival fibroblasts attachment. Furthermore, quercitrin-functionalised Ti increased collagen mRNA levels and decreased matrix metalloproteinase-1/tissue inhibitor of metalloproteinanse-1 mRNA ratio while also decreasing cyclooxygenase-2 mRNA and prostaglandin E_2 release under basal and inflammatory conditions. These results suggest that surfaces biofunctionalised with quercitrin could enhance the soft tissue integration and increase dental implants success.

1. Introduction

Success of a dental implant depends on osseo- and soft tissue-integration. Many dental implants fail due to the infection and inflammation that walk hand in hand with poor healing and soft tissue integration [1], [2]. Soft tissue integration to dental implants establishes a biological seal between the oral environment and the implant which prevents microbial invasion, gingival recession and inflammatory peri-implantitis [2]–[4]. Therefore, the development of antibacterial, anti-inflammatory and tissue-regenerative surface modifications constitutes a major challenge to guarantee implant success.

Research efforts to develop a new generation of dental implants are focused in bioactive surface coatings intended to provoke a defined cellular reaction [5]. A number of modified surfaces with integrated antibiotics, growth factors, antiresorptive drugs, synthetic peptides or complex formulations of artificial extracellular matrix (ECM) components have been developed. However, biocompatibility of most anti-bacterial surfaces is still uncertain while the lack of stability of growth factors after implantation is the main reason for the absence of such a product from the current implant market [6], [7].

An interesting option for surface modification is the use of natural multi-target molecules. Flavonoids are natural phenolic compounds derived from plants with antioxidant [8], anti-inflammatory [9] and antimicrobial capacity [10], [11], among other functions [12]. In previous studies, we screened among different flavonoids and selected quercitrin due to its potential in soft and hard periodontal tissues regeneration and its anti-inflammatory activity [13], [14]. Furthermore, we successfully grafted quercitrin to titanium (Ti) surfaces and observed that the resulting surfaces had promising biological activities *in vitro* [15] . In a posterior work, to increase the flavonoid coating stability and reproducibility, quercitrin grafting conditions were optimized. The optimized quercitrin-functionalised Ti surfaces enhanced human mesenchymal stem cells initial adhesion and promoted osteoblastic differentiation [16]. One step closer for the exploitation of quercitrin-functionalised Ti-surfaces for their use in dental implant abutments would be the evaluation of their antibacterial, anti-inflammatory and soft tissue-regenerative properties.

In the present study, we aimed at evaluating the effects of quercitrin-functionalised Ti-surfaces in bacterial adhesion and biofilm formation using *Staphylococcus mutans*. Furthermore, human gingival fibroblasts were used to evaluate the effects of quercitrin-functionalised Ti-surfaces in cell adhesion, anti-inflammatory properties in normal and in inflammatory microenvironments and soft tissue regeneration.

2. Materials and Methods

2.1. Materials

Machined Ti disks, c.p. grade IV, 6.2 mm diameter and 2 mm height were purchased from Implantmedia (Lloseta, Spain). (3-Aminopropyl)triethoxysilane (APTES), quercitrin and sodium cyanoborohydride ($NaCNBH_3$) were purchased from Sigma–

Aldrich (St. Louis, MO, USA). Deionized water was obtained from a Millipore system (Billerica, MA, USA). Technical acetone was purchased to Acros Organics (Geel, Belgium) and nitric acid, absolute ethanol, and anhydrous toluene to Scharlab (Barcelona, Spain).

2.2. Surface functionalization

Surfaces were prepared as previously described [15], [16]. Briefly, Ti disks were passivated by immersion in 30% (v/v) HNO_3 for 30 min followed by immersion in H_2O for 24 h (Ti). Immediately after Ti passivation, aminosilanization was performed with 2% (v/v) APTES solution in dry toluene for 24 h under a dry nitrogen atmosphere. Then, disks were gently rinsed with dry toluene, acetone, and ethanol, and finally dried under a nitrogen flow. Aminosilanised Ti disks (A samples) were then immersed either in 1 mM quercitrin at pH 5.0 (QUER), in 100 µM $NaCNBH_3$ at pH 7.5 (A Red) or in 1 mM quercitrin and 100 µM $NaCNBH_3$ at pH 7.5 (QUER Red) aqueous solutions. Samples were stirred for 1 h (150 rpm), gently rinsed with water, and dried under a nitrogen flow. $NaCNBH_3$ was used to reduce the imine bond, resulting from the reaction between the terminal amino group of APTES and the ketone group of quercitrin, to a single -C–N- bond to decrease bond reactivity and increase coating reproductibility [16]. This reduction is known to be selective for iminium ions at mildly basic pH. For bacterial experiments, surfaces were polished and cleaned [17] prior to surface modification. Disinfection of surfaces was conducted with dry heat (100˚C, 30 min) previous to bacteria/cell seeding.

2.3. Bacterial experiments

The bacterial strain used in this study was *Streptococcus mutans* ATCC 25175 (*S. mutans*). The strain was maintained in brain heart infusion (BHI) agar plates and cultured in BHI broth for 24 h at 37˚C/5% CO_2.

2.3.1. Bacterial adhesion

Bacterial suspension was homogenized through a 3 min ultrasonic bath (Ultrasons; JP Selecta, Barcelona, Spain) and harvested by centrifugation, washed two times with artificial saliva [18] preconditioned at 37˚C, and resuspended at a concentration of 3 x 10^8 bacteria/ml in artificial saliva. The flow system was a modified Robbins device where disks were fixed to removable ports that allow contact between the surfaces under study and a flow of suspended bacteria in laminar conditions. Before each experiment, the whole system was filled with artificial saliva and preconditioned at 37˚C. Afterward, the bacterial suspension was allowed to flow through the system at a flow rate of 2 ml/min, corresponding to a shear rate at the wall of the flow chamber of 0.97 s^{-1} (unpublished results). This shear rate is such that the specific interactions that could occur between the cells and the different substrata dominate over the drag force of the fluid flow. The bacterial suspension was perfused through the system with recirculation for 30, 60 and 90 min. Samples were carefully removed from the flow chamber and bacteria were stained with the kit Live/Dead Baclight L-7012 (Invitrogen, Camarillo, CA, USA) according to the manufacturer. Then, 5-6 pictures at randomly chosen locations from each sample were taken under fluorescence and the number of bacteria per unit area was counted. The experiments were run in triplicate from separately grown bacterial suspensions.

2.3.2. Biofilm formation

Bacteria were resuspended at a concentration of 3 x 10^8 bacteria/ml in BHI. Ti-modified surfaces were disinfected and placed in 96-well plates in sterile conditions. Then, 200 µl of bacterial suspension were cultured on the surfaces at 37˚C and gentle shaking (20 rpm). After 90 min, bacterial suspension was carefully removed and fresh BHI was added. Samples were cultured at 37˚C and gentle shaking (20 rpm) for 24 h. Biofilm formation was quantified using BacTiter-Glo (Promega, Fitchburg, WI, USA) according to the manufacturer's instructions. Culture media was removed and 200 µl of BacTiter-Glo were added to the wells and incubated for 10 min. Then, the content of each well was transferred to wells 96-well white polystyrene flat-bottomed microtiter plates. The light emission (luciferin-luciferase reaction) was measured in a microplate reader (FLx800; BioTek Instruments, Winooski, VT, USA). Each assay was performed in duplicate and repeated two times from separately grown bacterial suspension in order to confirm reproducibility.

2.3.3. Scanning electron microscopy

To visualise biofilm formation on the surfaces, bacteria were fixed with 4% glutaraldehyde in PBS for 2 h and washed with distilled water. At 30 min intervals, the samples were dehydrated by the addition of 50%, 70%, 90% and 100% ethanol solutions. Samples were left at room temperature to evaporate the remaining ethanol before sputter gold coating. A scanning electron microscope (SEM; Hitachi S-3400N, Krefeld, Germany) using secondary electrons, low vacuum conditions and 15 kV of voltage was used to acquire images.

2.4. Cell culture

Three different donors of primary human gingival fibroblasts (hGF; Provitro GmbH, Berlin, Germany) were used (range 19-47 years; male:female ratio 2:1). Provitro assures that cells were obtained ethically and legally and that all donors provided written informed consent. Cells were routinely cultured at 37˚C/5% CO_2, and maintained in fibroblast growth medium (Provitro GmbH) supplemented with 10% foetal calf serum, 50 ng/ml amphotericin and 50 µg/ml gentamicin (Provitro GmbH). Experiments were performed with hGF between passages 7 and 8 after isolation and media was supplemented with ascorbic acid (100 µM; Sigma-Aldrich).

The different coins were placed in 96-well plates in sterile conditions. Three replicates for each donor were seeded at a density of 7.0x10^3 cells on each coin (n = 9). For cell adhesion experiments, four replicates from one randomly selected donor were used (n = 4).

In order to create inflammatory conditions, 1 ng/ml interleukin-1 beta (IL-1β; R&D systems, Abingdon, UK) was added 1 d after cell seeding and kept until day 3, according to previous studies [14].

2.5. Cell adhesion and vinculin immunostaining

Once seeded, hGFs were allowed to adhere for 15, 30, 60 and 120 minutes to the different surfaces. Then, unbounded cells were removed by washing twice with PBS.

Cells were fixed with 4% paraformaldehyde for 15 min and permeabilised with 0.25% Triton X-100 for 10 min. For nucleus counting, cells were stained for 30 min with 5 µg/ml Phalloidin-FITC. For vinculin immunostaining, cells were blocked with 5% bovine serum albumin for 1 h followed by incubation with anti-vinculin recombinant rabbit monoclonal antibody at 4 µg/ml (Invitrogen) for 3 h and then labelled with Alexa Flour® 488 goat anti-rabbit IgG secondary antibody at 5 µg/ml (Thermo Scientific, Rockford, IL, USA) for 30 min. Samples were then mounted with DAPI-Fluoroshield and visualized under a fluorescence microscope and a confocal microscope. For nucleus counting, two pictures from the same positions of each coin were taken. Cells were counted using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

2.6. RNA isolation and real-time RT-PCR analysis

Total RNA was isolated using Tripure (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's protocol. Total RNA was quantified at 260 nm using a nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The same amount of RNA (0.2 µg) was reverse transcribed to cDNA at 42 ºC for 60 min, according to the protocol of the supplier (High Capacity RNA-to-cDNA kit, Applied Biosystems, Foster City, CA, USA). Aliquots of each cDNA were frozen (-20 °C) until the PCR reactions were carried out.

Real-time PCR was performed for two reference genes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and beta-actin (ACTBL2), and target genes (**Table 1**). Real-time PCR was performed in a thermocycler (Lightcycler 480, Roche Diagnostics) using SYBR green detection. Each reaction contained 7 µl of master mix (Lightcycler 480 SYBR Green I Master, Roche Diagnostics), 0.5 µM of each, the sense and the antisense specific primers and 3 µl of the cDNA dilution in a final volume of 10 µl. The amplification program consisted of a pre-incubation step for denaturation of the template cDNA (5 min 95°C), followed by 45 cycles consisting of a denaturation step (10 s 95 °C), an annealing step (10 s 60 °C) and an extension step (10 s 72 °C). After each cycle, fluorescence was measured at 72 °C. A negative control without cDNA template was run in each assay.

All samples were normalized by the geometric mean of the expression levels of ACTBL2 and GAPDH and fold changes were related to the control groups using the following equation: ratio = $E_{target}^{\Delta Cp\ target\ (mean\ control\ -\ sample)}$ / $E_{reference}^{\Delta Cp\ reference\ (mean\ control\ -\ sample)}$ adapted from [19], where Cp is the is the crossing point of the reaction amplification curve and E is the eficiency from the given slopes using serial dilutions, as determined by the software (Lightcycler 480 software, Roche Diagnostics). Stability of reference genes was calculated using a statistical tool (BestKeeper software, Technical University of Munich, Weihenstephan, Germany) [20].

2.7. Prostaglandin E2 quantification

A commercially available enzyme-linked immunosorbent assay kit was run to quantify PGE2 (Thermo Scientific) from cell culture media according to supplier instructions

2.8. Statistical analysis

All data are presented as mean values ± standard error of the mean (SEM). The Kolmogorov-Smirnov test was done to assume parametric or non-parametric distributions. Differences between groups were assessed by paired t-test or Wilcoxon test, depending on data distribution. Two-way ANOVA test using Fisher's LSD comparisons was used for adhesion experiments. SPSS software (version 17.0, Chicago, IL, USA) and GraphPad Prism (version 6, La Jolla, CA, USA) were used. Results were considered statistically significant at p values ≤ 0.05.

Table 1. Sense (S) and antisense (A) primers used in the real-time PCR of reference and target genes.

Gene	Primer sequence (5´- 3´)	Product size (bp)
Beta-actin (ACTBL2)	S: CTGGAACGGTGAAGGTGACA A: AAGGGACTTCCTGTAACAATGCA	136
Collagen I α1 (COL1A1)	S: AGAGCATGACCGATGGATTC A: TTCTTGAGGTTGCCAGTC	122
Collagen III α1 (COL3A1)	S: GGCCTACTGGGCCTGGTGGT A: CCACGTTCACCAGGGGCACC	190
Cyclooxygenase-2 (COX2)	S: ATGGGGTGATGAGCAGTTGT A: GAAAGGTGTCAGGCAGAAGG	221
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	S: TGCACCACCAACTGCTTAGC A: GGCATGGACTGTGGTCATGAG	87
Matrix metalloproteinase-1 (MMP1)	S: TGTCAGGGGAGATCATCGGGACA A: TGGCCGAGTTCATGAGCTGCA	177
Metalloproteinase inhibitor-1 (TIMP1)	S: TTCCGACCTCGTCATCAGGG A: TAGACGAACCGGATGTCAGC	144

3. Results

3.1. Bacterial adhesion and biofilm formation

QUER surfaces significantly decreased *S. mutans* adhesion after 30 min compared to Ti and A controls. After 60 and 90 min A and QUER surfaces decreased bacterial adhesion compared to Ti control. Unexpectedly, A Red and QUER Red surfaces increased bacterial adhesion at all time points compared to Ti control and to non-reduced surfaces (**Figure 1 A**). Moreover, in all surfaces studied and times, the bacterial viability was not compromised.

We did not find statistical differences in biofilm formation between the different groups. However, A and QUER groups decreased biofilm formation by 12 and 6% respectively, compared to Ti control (**Figure 1 B**). Higher biofilm formation was observed for A Red and QUER Red surfaces compared to the non-reduced surfaces. Furthermore, SEM images confirmed these trends (**Figure 1 C**).

Due to the increased bacterial adhesion of A Red and QUER Red surfaces, we decided to select A and QUER surfaces for further analysis.

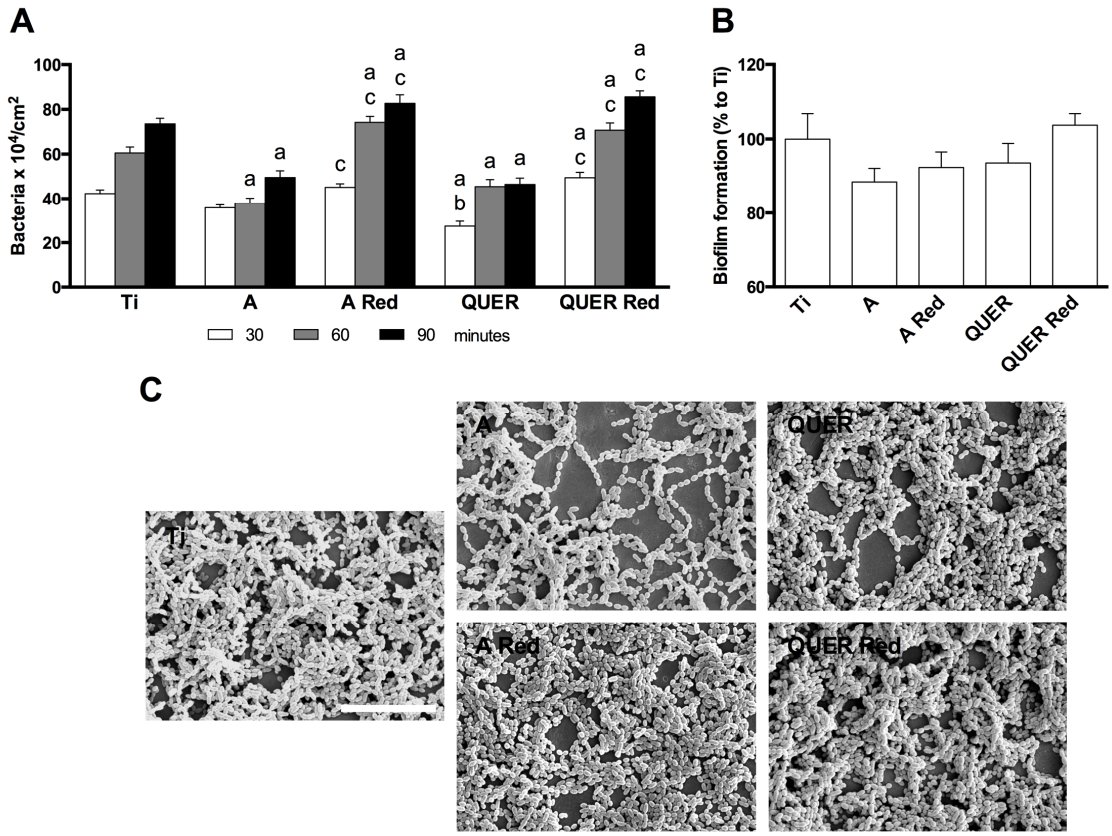

Figure 1. Bacterial adhesion and biofilm formation: (A) Dinamic bacterial adhesion: quantitative analysis of the live bacteria adhered to the control and modified surfaces after 30, 60 and 90 min. **(B)** Biofilm formation: quantitative analysis of the percentage of biofilm formation on the different surfaces after 24 hours. **(C)** Scanning electron microscope images of the biofilm formed on the different surfaces (scale bar = 100 µm). Statistical comparisons $p \leq 0.05$: (a) versus Ti control at each time point; (b) versus A control at each time point; (c) effect of reduction within each group at each time point.

3.2. Cell adhesion

We found that QUER surfaces increased hGF adhesion compared to Ti and A controls after 30 min although we did not observe this clear effect at longer adhesion times, i.e. 60 and 120 min (**Figure 2 A**). When comparing the total number of cells adhered to the surfaces, A surfaces decreased cell number by almost the half while QUER surfaces increased it almost 20% compared to Ti. Furthermore, QUER surfaces increased cell adhesion compared to A control surfaces.

Confocal images reveal that after 30 min, hGFs on QUER surfaces showed the typical spindle-shaped fibroblastic morphology while on Ti and A surfaces were more rounded. Also, hGFs on QUER surfaces showed higher vinculin staining and filopodia than cells on Ti and A surfaces (**Figure 2 B**).

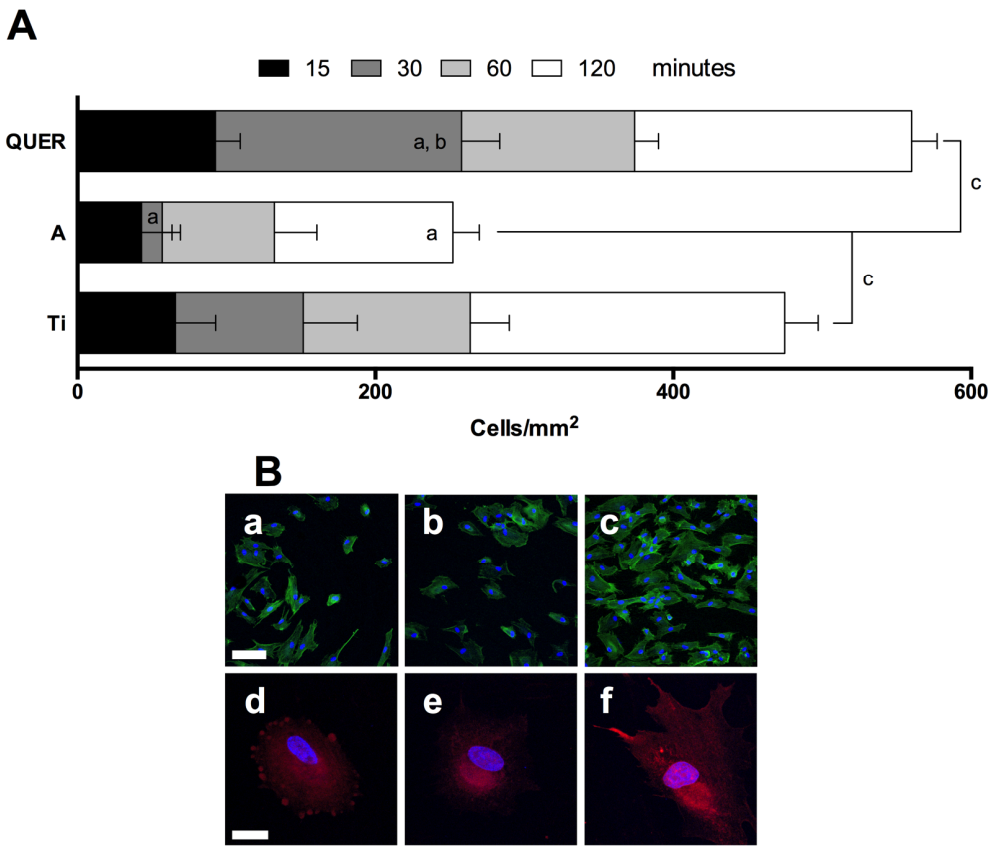

Figure 2. hGF adhesion: (A) Quantitative analysis of the cells adhered to the different surfaces after 15, 30, 60 and 120 minutes. **(B)** Cells were allowed to adhere to the surfaces for 30 min. Then, cells were stained with phalloidin-FITC (a-c; green) or with anti-vinculin antibodies (d-f; red) and nuclei were stained with DAPI (blue). Ti (a, d); A (b, e); QUER (c, f) surfaces (scale bars: upper = 100 µm; bottom = 25 µm). Statistical comparisons $p \leq 0.05$: (a) versus Ti for each time point; (b) versus A for each time point; (c) the total number of cells on the different surfaces was compared.

3.3. Response of hGF to the different surfaces

In basal conditions, QUER surfaces increased collagens mRNA levels compared to Ti and A controls and decreased MMP1/TIMP1 mRNA ratio and COX2 mRNA levels compared to Ti. When hGF growing on Ti surfaces were challenged with IL-1β, we observed decreased collagens and TIMP1 mRNA levels while increasing COX2 mRNA. Under this challenging condition, QUER surfaces increased the mRNA levels of collagens while decreasing MMP1/TIMP1 mRNA ratio and COX2 mRNA compared to Ti and A controls (**Figure 3**).

After 14 days of cell culture, PGE2 released to culture media was increased on the groups cultured under inflammatory conditions (**Figure 4**). Furthermore, A and QUER surfaces decreased PGE2 production, compared to Ti surfaces.

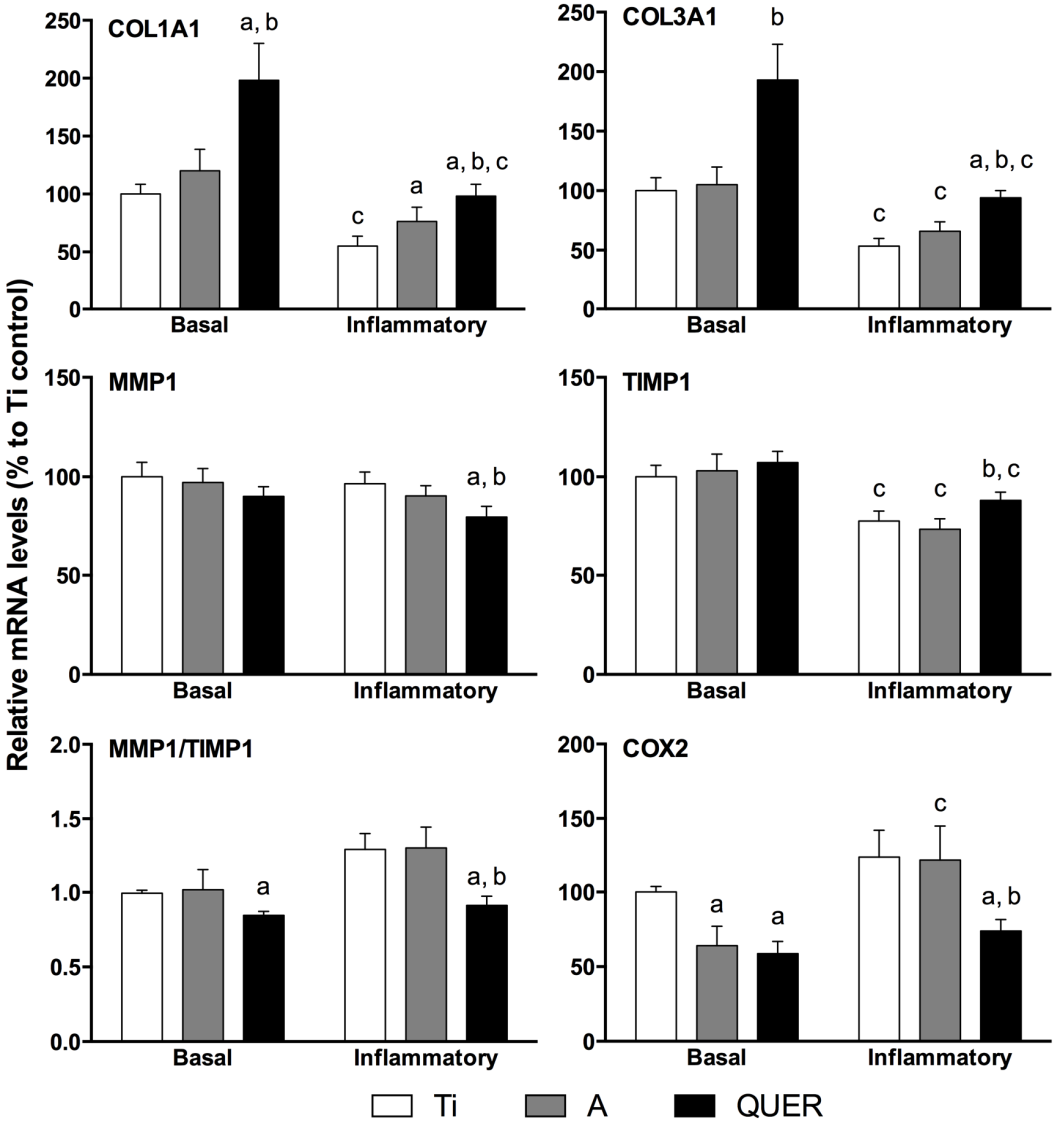

Figure 3. Gene expression: Analysis of gene expression after 14 days of hGF culture on the different surfaces. Cells were cultured with IL-1β to mimic inflammation (inflammatory group) or without it (basal group). Statistical comparisons $p \leq 0.05$: (a) versus Ti within each condition; (b) versus A within each condition; (c) effect of IL-1β addition for each surface.

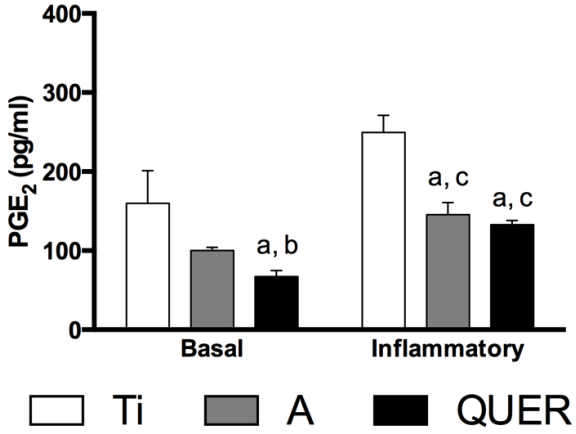

Figure 4. PGE2 release: The release of PGE2 to the culture media was evaluated after 14 days of culture of hGF on the different surfaces. Cells were cultured with IL-1β to mimic inflammation (inflammatory group) or without it (basal group). Statistical comparisons $p \leq 0.05$: (a) versus Ti within each condition; (b) versus A within each condition; (c) effect of IL-1β addition for each surface.

3.4. Effect of disinfection on the bioactivity of the different surfaces

We hypothesized that the disinfection process with dry heat at 100 ºC would not affect the coatings since the melting temperature of quercitrin is 176-179 ºC and the boiling temperatures for APTES and quercitrin are 223 ºC and 316 ºC respectively. To confirm that the disinfection did not alter the bioactivity of the coatings, we analysed the response of hGF on them and we compared it to the control group (**Supp. Fig. 1**). We only found that COX2 mRNA on Ti disinfected surfaces was decreased compared to Ti control.

4. Discussion

In this study we present a surface biofunctionalised with quercitrin, a natural flavonoid with multi-target actions, designed to improve soft tissue integration and to increase dental implants long-term success. Quercitrin-functionalised Ti surfaces decreased initial bacterial adhesion while increasing human gingival fibroblasts attachment. Furthermore, quercitrin-functionalised Ti increased collagen mRNA levels and decreased MMP1/TIMP1 mRNA ratio while also decreasing COX2 mRNA and PGE2 release under basal and inflammatory conditions. These results suggest that surfaces biofunctionalised with quercitrin could enhance the soft tissue integration and increase dental implants success.

Peri-implant soft tissue healing follows the four overlapping phases of wound healing [3]. After an implant placement a blood coagulum is formed which provides a provisional ECM. Inflammatory cells migrate, phagocytise foreign particles and release inflammatory mediators to either finalise the inflammation or to amplify it. Fibroblasts

then invade the fibrin network and produce collagen fibres to form a connective tissue in contact with the implant surface. The final phase involves collagen remodelling which can produce either a scarred (repaired) tissue or a structural and functional regenerated tissue, depending on the microenvironmental signals. During this process, bacterial accumulation (dental plaque formation), may trigger the inflammatory process. However, it depends on the soft tissue integration to resist the deletereous effects of plaque accumulation, thus, avoiding peri-implantitis to guarantee long-term efficacy of dental implants.

Peri-implant tissue is colonized by the same flora as the periodontium [21]. Among oral micro flora, *S. mutans* is one of the initial colonizers of dental plaque and is one of the best known biofilm forming bacterium [22]. We found that quercitrin-functionalised surfaces inhibited initial *S. mutans* attachment, in line with the results obtained on glass and hydroxyapatite surfaces using *S. mutants* suspensions in tea extract solutions (containing flavonoids, tannins and indolic compounds) [23]. Biofilm formation was slightly inhibited on quercitrin-coated surfaces. In previous studies, quercitrin had a bacteriostatic effect on *Staphylococcus epidermidis* [13] while Hasan et al. [24] found that quercitrin decreased *S. mutans* biofilm formation. However, in both studies quercitrin concentrations were much higher than the amount of biomolecule grafted to the surfaces, which was 0.2 to 0.5 nmol of quercitrin per Ti coin on QUER surfaces [16]. It is interesting to highlight that aminosilanized surfaces (A group) also decreased bacterial adhesion, in agreement with previous reports [25]. It has been shown that bacterial adhesion depends on the surface's terminal functionality and that, in fact, decreases on amino (NH_2)-terminated surfaces [26].

Our surfaces reduced with sodium cyanoborohydride showed an increased bacterial adhesion and biofilm formation. In a previous work, the amount of quercitrin grafted on QUER Red surfaces was around 0.1 nmol per Ti coin, less than that grafted to the non-reduced QUER surfaces [16]. The hydrolytic stability of siloxanes at pH 7.5 has been reported to be poor [27]. Therefore, the grafting conditions used to produce QUER Red and A Red samples, at pH 7.5, may favour the hydrolisis of APTES grafted to Ti; on one hand, decreasing the availability of amino-terminal groups on A Red surfaces, and, on the other hand, decreasing the amount of quercitrin that can be grafted on QUER Red surfaces, all in all, increasing bacterial adhesion and biofilm formation.

After installation of a dental implant, gingival fibroblasts are the preferred cells to repopulate the wound and to form a collagen-rich connective tissue [28]. We found that quercitrin-functionalised surfaces increased initial hGF attachment. This fact together with the decreased bacterial attachment on quercitrin-functionalised surfaces, increase hGF possibilites to win the "race for the surface" against oral bacteria [29] since it depends on both an increased biomaterial surface area cell coverage and a decreased number of bacteria present on the surface [30], [31]. Compared with often-reported monofunctional surface coatings on which bacterial adhesion and biofilm formation is discouraged or they promote host tissue integration [7], [32], quercitrin-functionalised surfaces decreased bacterial adhesion while increased cell attachment. Furthermore, quercitrin-functionalised surfaces avoid toxicity concerns of some anti-bacterial coatings. Therefore, the surfaces presented herein may form the required connective tissue around the implant to establish the biological seal that could prevent further bacterial colonization and ultimately preventing the implant loss by peri-implantitis.

Inflammation is necessary for the effective defence against pathogens and to set in motion tissue repair following injury [33]. However, excessive inflammation has been shown to delay healing and to result in increased scarring, compromising tissue regeneration [34]. Thus, a controlled inflammatory process after an implant placement is critical for their success. Here, we found that quercitrin-functionalised surfaces effectively inhibited COX2 expression and decreased its functional product PGE2 under basal and inflammatory conditions, in agreement with our previous results [14]. Furthermore, the antioxidant properties of quercitrin [13], could also contribute to the inflammation resolution since oxidative stress is considered one of the major causes of inflammation and inhibition of tissue regeneration [35].

Collagen synthesis and remodelling is a requisite for complete wound healing. Here we found that quercitrin-functionalised surfaces increased COL1A1 and COL3A1 mRNA levels in basal and inflammatory conditions. Moreover, in inflammatory conditions MMPs are upregulated while TIMPs, their inhibitors, are downregulated, all together boosting collagen degradation [36]. Quercitrin-functionalised surfaces decreased MMP1 and increased TIMP1 mRNA levels in inflammatory conditions, thus decreasing inflammation-driven ECM-destruction. Noteworthy, quercitrin decreased the expression of pro-fibrotic markers in a wound healing *in vitro* model [13] and higher proportions of type III collagen are related to more-regenerative than more-scarring responses [37]. Thus, data suggest that quercitrin-functionalised surfaces may induce connective tissue formation around a dental implant even in the inflammatory conditions found after a dental implant installation. Furthermore, in previous studies of our group, quercitrin increased the osteoblastic differentiation of mesenchymal stem cells when added to the culture media and when grafted to Ti surfaces [16]. Therefore, quercitrin-functionalised surfaces could also increase the osseointegration process of dental implants.

Natural-derived products with multi-target actions, which promise higher therapeutic efficacy and safety [38], represent an alternative to pharmaceuticals and animal-derived compounds due to low immunogenicity and toxicity [12]. Furthermore, the presence of the bioactive molecule in the place itself may offer the advantages of local theraphy compared to systemic administration. Further clinical studies should confirm the potential of quercitrin-functionalised surfaces in soft tissue integration.

5. Conclusions

A stable peri-implant soft tissue attachment, in the presence of bone support, has a critical impact on the long-term success of implant therapy. Biofunctionalization of titanium surfaces with quercitrin decreased initial bacterial adhesion while increasing human gingival fibroblasts attachment. Furthermore, quercitrin-functionalised Ti increased collagen mRNA levels and decreased MMP1/TIMP1 mRNA ratio while also decreasing COX2 mRNA and PGE2 release under basal and inflammatory conditions. These results suggest that surfaces biofunctionalised with quercitrin could enhance the soft tissue integration and increase dental implants success.

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References

[1] R. E. Jung, B. E. Pjetursson, R. Glauser, A. Zembic, M. Zwahlen, and N. P. Lang, "A systematic review of the 5-year survival and complication rates of implant-supported single crowns.," *Clin. Oral Implants Res.*, vol. 19, no. 2, pp. 119–30, Feb. 2008.

[2] A. Sculean, R. Gruber, and D. D. Bosshardt, "Soft tissue wound healing around teeth and dental implants.," *J. Clin. Periodontol.*, vol. 41 Suppl 1, pp. S6–22, Apr. 2014.

[3] C. C. Villar, G. Huynh-Ba, M. P. Mills, and D. L. Cochran, "Wound healing around dental implants," *Endod. Top.*, vol. 25, no. 1, pp. 44–62, Sep. 2011.

[4] P. Martin and S. J. Leibovich, "Inflammatory cells during wound repair: The good, the bad and the ugly," *Trends in Cell Biology*, vol. 15. pp. 599–607, 2005.

[5] R. Gruber and D. D. Bosshardt, "Dental Implantology and Implants - Tissue Interface," in *Stem Cell Biology and Tissue Engineering in Dental Sciences*, Elsevier Inc., 2015, pp. 735–747.

[6] N. Bhatavadekar, "Peri-implant soft tissue management: Where are we?," *J. Indian Soc. Periodontol.*, vol. 16, no. 4, pp. 623–7, 2012.

[7] H. J. Busscher, H. C. van der Mei, G. Subbiahdoss, P. C. Jutte, J. J. a. M. van den Dungen, S. a. J. Zaat, M. J. Schultz, and D. W. Grainger, "Biomaterial-Associated Infection: Locating the Finish Line in the Race for the Surface," *Sci. Transl. Med.*, vol. 4, no. 153, pp. 153rv10–153rv10, 2012.

[8] P. G. Pietta, "Flavonoids as antioxidants," *Journal of Natural Products*, vol. 63, no. 7. American Chemical Society, pp. 1035–1042, 2000.

[9] V. Izzi, L. Masuelli, I. Tresoldi, P. Sacchetti, A. Modesti, F. Galvano, and R. Bei, "The effects of dietary flavonoids on the regulation of redox inflammatory networks.," *Front. Biosci. (Landmark Ed.*, vol. 17, pp. 2396–418, Jan. 2012.

[10] J. An, G. Y. Zuo, X. Y. Hao, G. C. Wang, and Z. S. Li, "Antibacterial and synergy of a flavanonol rhamnoside with antibiotics against clinical isolates of methicillin-resistant Staphylococcus aureus (MRSA)," *Phytomedicine*, vol. 18, no. 11, pp. 990–993, 2011.

[11] T. P. T. Cushnie and A. J. Lamb, "Recent advances in understanding the antibacterial properties of flavonoids.," *Int. J. Antimicrob. Agents*, vol. 38, no. 2, pp. 99–107, Aug. 2011.

[12] M. Friedman, “Overview of antibacterial, antitoxin, antiviral, and antifungal activities of tea flavonoids and teas.,” *Mol. Nutr. Food Res.*, vol. 51, no. 1, pp. 116–34, Jan. 2007.

[13] M. Gómez-Florit, M. Monjo, and J. M. Ramis, “Identification of Quercitrin as Potential Therapeutic Agent for Periodontal Applications.,” *J. Periodontol.*, vol. 85, no. 7, pp. 966–974, Feb. 2014.

[14] M. Gómez-Florit, M. Monjo, and J. M. Ramis, “Quercitrin for periodontal regeneration: effects on human gingival fibroblasts and mesenchymal stem cells,” *Submitt. to "Journal Tissue Eng. Regen. Med.*, 2015.

[15] A. Córdoba, M. Satué, M. Gómez-Florit, M. Hierro-Oliva, C. Petzold, S. P. Lyngstadaas, M. L. González-Martín, M. Monjo, and J. M. Ramis, “Flavonoid-Modified Surfaces: Multifunctional Bioactive Biomaterials with Osteopromotive, Anti-Inflammatory, and Anti-Fibrotic Potential,” *Adv. Healthc. Mater.*, vol. 4, no. 4, pp. 540–549, Oct. 2015.

[16] A. Córdoba, M. Monjo, M. Hierro-Oliva, M. L. González-Martín, and J. M. Ramis, “Bioinspired quercitrin nanocoatings enhance stem cell adhesion and osteoblast mineralization,” *Manuscript*, 2015.

[17] M. Gómez-Florit, J. M. Ramis, R. Xing, S. Taxt-Lamolle, H. J. Haugen, S. P. Lyngstadaas, and M. Monjo, “Differential response of human gingival fibroblasts to titanium- and titanium-zirconium-modified surfaces.,” *J. Periodontal Res.*, vol. 49, no. 4, pp. 425–36, Aug. 2014.

[18] J. Y. Gal, Y. Fovet, and M. Adib-Yadzi, “About a synthetic saliva for in vitro studies,” *Talanta*, vol. 53, pp. 1103–1115, 2001.

[19] M. W. Pfaffl, “A new mathematical model for relative quantification in real-time RT-PCR.,” *Nucleic Acids Res.*, vol. 29, no. 9, p. e45, May 2001.

[20] M. W. Pfaffl, A. Tichopad, C. Prgomet, and T. P. Neuvians, “Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper--Excel-based tool using pair-wise correlations.,” *Biotechnol. Lett.*, vol. 26, no. 6, pp. 509–15, Mar. 2004.

[21] M. Hultin, A. Gustafsson, H. Hallström, L. Johansson, A. Ekfeldt, and B. Klinge, “Microbiological findings and host response in patients with peri-implantitis.,” *Clin. Oral Implants Res.*, vol. 13, no. 4, pp. 349–58, Aug. 2002.

[22] C. M. Lévesque, E. Voronejskaia, Y. C. C. Huang, R. W. Mair, R. P. Ellen, and D. G. Cvitkovitch, “Involvement of sortase anchoring of cell wall proteins in biofilm formation by Streptococcus mutans,” *Infect. Immun.*, vol. 73, pp. 3773–3777, 2005.

[23] Y. Wang, S. M. Lee, and G. A. Dykes, “Potential mechanisms for the effects of tea extracts on the attachment, biofilm formation and cell size of Streptococcus mutans.,” *Biofouling*, vol. 29, no. 3, pp. 307–18, Jan. 2013.

[24] S. Hasan, K. Singh, M. Danisuddin, P. K. Verma, and A. U. Khan, “Inhibition of major virulence pathways of Streptococcus mutans by quercitrin and deoxynojirimycin: a synergistic approach of infection control.,” *PLoS One*, vol. 9, no. 3, p. e91736, 2014.

[25] A. Rodríguez-Cano, P. Cintas, M. C. Fernández-Calderón, M. Á. Pacha-Olivenza, L. Crespo, L. Saldaña, N. Vilaboa, M. L. González-Martín, and R.

Babiano, “Controlled silanization-amination reactions on the Ti6Al4V surface for biomedical applications,” *Colloids Surfaces B Biointerfaces*, vol. 106, pp. 248–257, 2013.

[26] M. G. Katsikogianni and Y. F. Missirlis, “Bacterial adhesion onto materials with specific surface chemistries under flow conditions,” in *Journal of Materials Science: Materials in Medicine*, 2010, vol. 21, no. 3, pp. 963–968.

[27] B. M. Silverman, K. a. Wieghaus, and J. Schwartz, “Comparative properties of siloxane vs phosphonate monolayers on a key titanium alloy,” *Langmuir*, vol. 21, no. 1, pp. 225–228, 2005.

[28] A. A. Palaiologou, R. A. Yukna, R. Moses, and T. E. Lallier, “Gingival, dermal, and periodontal ligament fibroblasts express different extracellular matrix receptors.,” *J. Periodontol.*, vol. 72, no. 6, pp. 798–807, Jun. 2001.

[29] A. G. Gristina, “Biomaterial-centered infection: microbial adhesion versus tissue integration.,” *Science*, vol. 237, no. 4822, pp. 1588–1595, 1987.

[30] G. Subbiahdoss, R. Kuijer, D. W. Grijpma, H. C. van der Mei, and H. J. Busscher, “Microbial biofilm growth vs. tissue integration: ‘the race for the surface’ experimentally studied.,” *Acta Biomater.*, vol. 5, no. 5, pp. 1399–404, Jun. 2009.

[31] G. Subbiahdoss, R. Kuijer, H. J. Busscher, and H. C. Van Der Mei, “Mammalian cell growth versus biofilm formation on biomaterial surfaces in an in vitro post-operative contamination model,” *Microbiology*, vol. 156, no. 10, pp. 3073–3078, 2010.

[32] T. He, Y. Zhang, A. C. K. Lai, and V. Chan, “Engineering bio-adhesive functions in an antimicrobial polymer multilayer,” *Biomed. Mater.*, vol. 10, no. 1, p. 015015, 2015.

[33] G. C. Gurtner, S. Werner, Y. Barrandon, and M. T. Longaker, “Wound repair and regeneration,” *Nature*, vol. 453, no. 7193, pp. 314–321, May 2008.

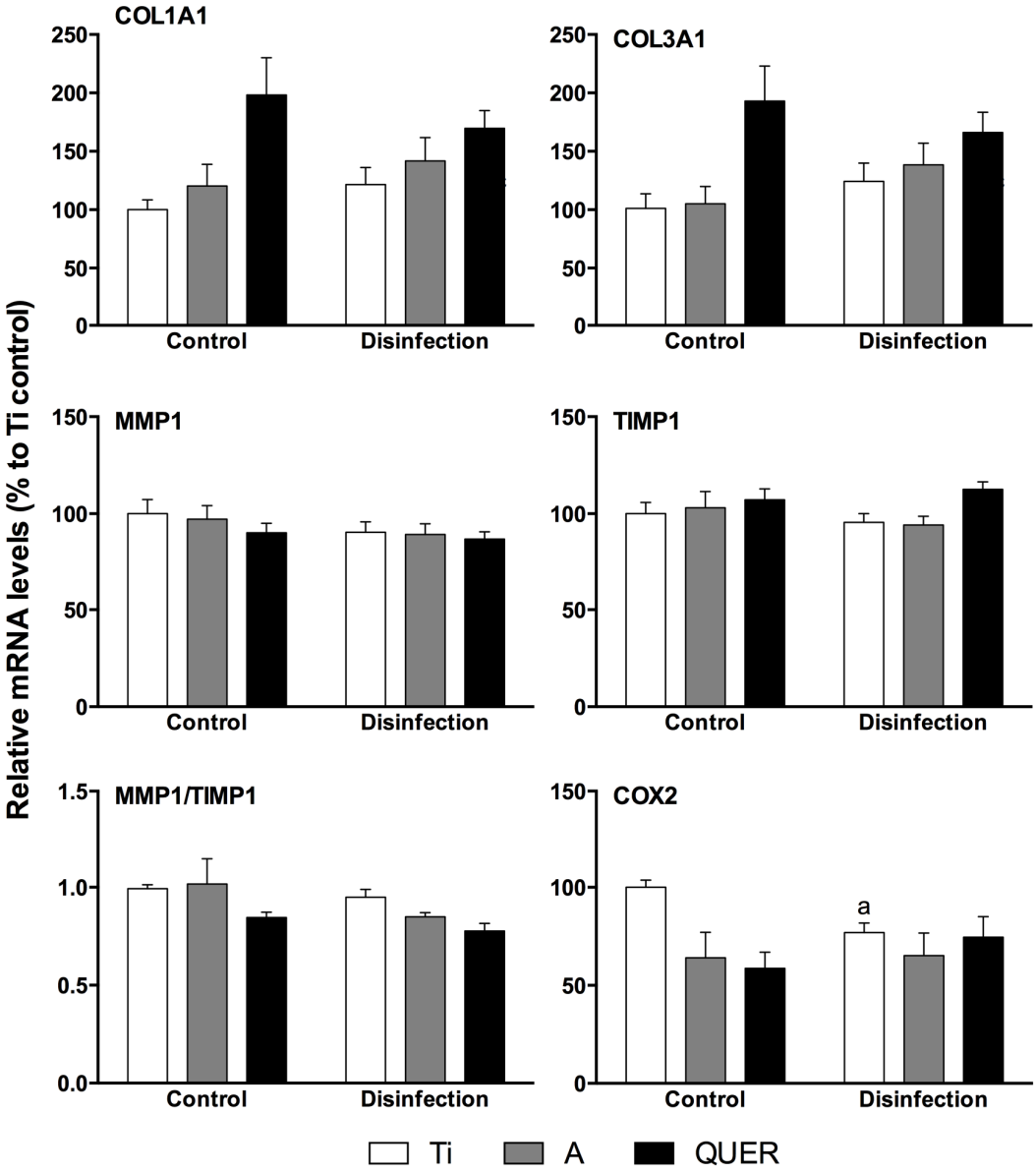
[34] S. a Eming, T. Krieg, and J. M. Davidson, “Inflammation in wound repair: molecular and cellular mechanisms.,” *J. Invest. Dermatol.*, vol. 127, no. September 2006, pp. 514–525, 2007.

[35] I. L. C. Chapple and J. B. Matthews, “The role of reactive oxygen and antioxidant species in periodontal tissue destruction,” *Periodontol. 2000*, vol. 43, no. 1, pp. 160–232, Jan. 2007.

[36] M. Soell, R. Elkaim, and H. Tenenbaum, “Cathepsin C, Matrix Metalloproteinases, and their Tissue Inhibitors in Gingiva and Gingival Crevicular Fluid from Periodontitis-affected Patients,” *J. Dent. Res.*, vol. 81, no. 3, pp. 174–178, Mar. 2002.

[37] L. Satish and S. Kathju, “Cellular and Molecular Characteristics of Scarless versus Fibrotic Wound Healing.,” *Dermatol. Res. Pract.*, vol. 2010, p. 790234, Jan. 2010.

[38] A. Koeberle and O. Werz, “Multi-target approach for natural products in inflammation,” *Drug Discov. Today*, vol. 19, no. 12, pp. 1871–1882, 2014.



Supplementary Figure 1. Analysis of gene expression after 14 days of hGF culture on the different surfaces. Disinfection of surfaces was conducted with dry heat (100˚C, 30 min) (disinfection group) or without it (control group). Statistical comparisons $p \leq 0.05$: (a) versus Ti within each condition; (b) versus A within each condition; (c) effect of IL-1β addition for each surface.