Dental Materials xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Dental Materials

journal homepage: www.elsevier.com/locate/dental

# Dexamethasone and doxycycline functionalized nanoparticles enhance osteogenic properties of titanium surfaces

Manuel Toledano-Osorio[a,b], Elvira de Luna-Bertos[c,d,*], Manuel Toledano[a,d], Francisco Javier Manzano-Moreno[d,e], Victor Costela-Ruiz[d,f], Concepción Ruiz[c,d,g], Javier Gil[h], Raquel Osorio[a,d]

[a] University of Granada, Faculty of Dentistry, Colegio Máximo de Cartuja s/n, Granada 18071, Spain
[b] Medicina Clínica y Salud Pública PhD Programme, Spain
[c] Biomedical Group (BIO277). Department of Nursing, Faculty of Health Sciences. University of Granada, Spain
[d] Instituto Investigación Biosanitaria, IBS. Granada, Granada, Spain
[e] Biomedical Group (BIO277). Department of Stomatology, School of Dentistry, University of Granada, Spain
[f] Biomedical Group (BIO277). Department of Nursing, Faculty of Health Sciences, Campus de Ceuta. University of Granada, Spain
[g] Institute of Neuroscience, University of Granada, Centro de Investigación Biomédica (CIBM). Parque de Tecnológico de la Salud (PTS), Granada, Spain
[h] International University of Cataluña (UIC), Barcelona, Spain

ARTICLE INFO

*Keywords:*
Titanium
Nanoparticles
Osteoblasts
Osteogenic
Dexamethasone
Doxycycline
Polymers

ABSTRACT

*Objectives:* To evaluate the effect of doxycycline and dexamethasone doped nanoparticles covering titanium surfaces, on osteoblasts proliferation and differentiation.

*Methods:* Doxycycline and dexamethasone doped polymeric nanoparticles were applied on titanium discs (Ti-DoxNPs and Ti-DexNPs). Undoped NPs and uncovered Ti discs were used as control. Human MG-63 osteoblast-like cells were cultured. Osteoblasts proliferation was tested by MTT assay. Alkaline phosphatase activity was analyzed. Differentiation gene expression was assessed by real-time quantitative polymerase chain reaction. Scanning Electron Microscopy was performed to assess osteoblasts morphology. Mean comparisons were conducted by ANOVA and Wilcoxon or Tukey tests ($p < 0.05$).

*Results:* No differences in osteoblasts proliferation were found. Osteoblasts grown on Ti-DoxNPs significantly increased alkaline phosphatase activity. Doxycycline and dexamethasone nanoparticles produced an over-expression of the main osteogenic proliferative genes (TGF-β1, TGF-βR1 and TGF-βR2). The expression of Runx-2 was up-regulated. The osteogenic proteins (AP, OSX and OPG) were also overexpressed on osteoblasts cultured on Ti-DoxNPs and Ti-DexNPs. The OPG/RANKL ratio was the highest when DoxNPs were present (75-fold increase with respect to the control group). DexNPs also produced a significantly higher OPG/RANKL ratio with respect to the control (20 times higher). Osteoblasts grown on titanium discs were mainly flat and polygonal in shape, with inter-cellular connections. In contrast, osteoblasts cultured on Ti-DoxNPs or Ti-DexNPs were found to be spindle-shaped and had abundant secretions on their surfaces.

*Significance:* DoxNPs and DexNPs were able to stimulate osteoblasts differentiation when applied on titanium surfaces, being considered potential inducers of osteogenic environment when performing regenerative procedures around titanium dental implants.

## 1. Introduction

Peri-implantitis is an inflammatory process associated, among others, with a microbial challenge around dental implants, leading to a progressive destruction of the supporting bone [1]. Two factors are considered crucial in the development of this disease: i) a pathogenic biofilm development at the implant surface [1] and ii) the activation of the immune system which is leaded by bacteria and titanium elutes, ions and particles provoking an exacerbated inflammatory reaction against implants [2]. At this stage, bone destruction together with a proliferation of connective and granulation tissue are produced, resulting in tissue fibrosis, thus leading to the implant loss if incomplete

* Correspondence to: Department of Nursing, Faculty of Health Sciences. University of Granada, Avenida de la Ilustración, S/N., 18016 Granada, Spain.
*E-mail address:* elviradlb@ugr.es (E. de Luna-Bertos).

https://doi.org/10.1016/j.dental.2023.05.004
Received 1 May 2023; Accepted 2 May 2023
0109-5641/© 2023 The Authors. Published by Elsevier Inc. on behalf of The Academy of Dental Materials. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Please cite this article as: M. Toledano-Osorio, E. de Luna-Bertos, M. Toledano et al., Dexamethasone and doxycycline functionalized nanoparticles enhance osteogenic properties of titanium surfaces, Dental Materials, https://doi.org/10.1016/j.dental.2023.05.004

or no treatment is applied [3]. Reported prevalence of peri-implantitis is variable, but it is considered to be around 20% of the placed dental implants [4].

Considering the importance of inflammation for fibrosis formation, immunomodulatory and anti-inflammatory drugs, as dexamethasone have been proposed for the fibrosis treatment [3]. Peri-implantitis is also an infectious disease. Therefore, a wide spectrum antibiotic as doxycycline may be efficacious as an adjuvant to the treatment and prevention of this disease [5,6]. Dexamethasone is not only an immunomodulator and anti-inflammatory, but also an osteogenic drug used for cell culture experiments to induce proliferation and maturation of Human Bone Marrow Stem Cells [7] as well as to increase extracellular matrix mineralization of osteoblasts [8,9]. Doxycycline has also been reported to exert osteogenic effect when immobilized on membranes for bone regeneration [10]. These antibiotic and anti-inflammatory drugs may ideally be administered through a system which is able to permit their local release. It will not only reduce the non-specific effects of the drugs on other tissues, but also enable to perform an effective therapy by mild and sustained dosages [5,6].

Surface functionalization of dental implants has already been intended. However, it was not successfully performed, mainly due to the fact that these surfaces are chemically inert. The lack of reactive functional groups at implants surfaces hampers the grafting of drugs or any other molecule. There are some chemical techniques which may be able to modify implants surfaces. Nevertheless, these techniques involve multiple laboratory steps, high costs [3], and the may limit their clinical applications.

Our proposal is the immobilization of polymeric nanoparticles (NPs) doped with dexamethasone or doxycycline onto implant surfaces in order to facilitate the local delivery of the drugs. This may be performed applying an easy implant surface modification method, which may be performed at the clinical environment, thus enhancing the effective loading and selective release of the antibiotics and/or anti-inflammatory drugs at the implant site.

The present work aimed to evaluate if doxycycline and dexamethasone-doped polymeric nanoparticles, applied onto titanium surfaces, may exert effects on the osteoblasts' proliferation and differentiation.

## 2. Materials and methods

### 2.1. Specimens preparation

Nanoparticles production: four different NPs have been manufactured as described by Osorio et al. [11]: (a) Undoped NPs (Un-NPs); (b) NPs doped with doxycycline (Dox-NPs) and (c) NPs loaded with dexamethasone (Dex-NPs). NPs were produced through a polymerization/precipitation procedure with a composition of 2-hydroxyethyl methacrylate (backbone monomer), ethylene glycol dimethacrylate (cross-linker) and methacrylic acid (functional monomer), with a final diameter of approximately 220 nm [12]. For the functionalizing process, 30 mg of NPs were immersed during 30 min at room temperature, and under continuous agitation, in an aqueous solution of dexamethasone ($40mgL^{-1}$) (Sigma-Aldrich, Chemie Gmbh, Riedstr, Germany) and doxycycline hyclate ($40mgL^{-1}$) (Sigma-Aldrich) until reaching the adsorption equilibrium. The suspensions were centrifuged, and the NPs were separated from the supernatant and re-suspended in phosphate buffered saline (PBS) [13]. Doped NPs were previously shown to effectively liberate drugs [13].

Doxycycline and dexamethasone loading efficacy: it was evaluated by testing the concentration in the aqueous solution of dexamethasone and doxycycline hyclate before and after NPs immersion, during the NPs doping process execution. Concentrations were analysed by a Waters mass spectrometer with a C18 UPLC column (UPLC Synapt G2 Mass Spectrometer Waters, Waters Corp. Milford, MA, USA). In the case of dexamethasone, values were obtained with an UV-Vis detector at 242-nm wavelength (PDA 200–500-nm). Calibration curves were created by plotting the attained values against previously known concentrations. The quantities of released drugs in each well were determined using these calibration curves. The loading efficacy for the drug was the result of discounting the final drug amount in the supernatants, to the initial quantity present in the loading solutions.

Specimens' production: Sterile titanium discs (TiD) (grade 2) of 6 mm of diameter were manufactured and donated by Klockner (Klockner S.A., Barcelona, Spain), surfaces were comparable to the commercially available SLActive® surface. Titanium discs were placed inside the 24-well plate, and covered with 10 μL of the different nanoparticles suspensions diluted in PBS ($10mgmL^{-1}$)[6]. The control group did only receive 10 μL of phosphate buffer solution (PBS).

### 2.2. Cell Culture

The surface over TiD covered with Un-NPs, Dox-NPs, Dex-NPs and PBS were used for the establishment of the cell cultures. The human MG63 osteosarcoma cell line was employed. Osteoblasts were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained in Dulbecco's modified Eagle medium (DMEM; Invitrogen Gibco Cell Culture Products, Carlsbad, CA, USA). It was added to the culture medium: Penicillin $100IUmL^{-1}$ (Lab Roger SA, Barcelona, Spain), amphotericin B $2.5mgmL^{-1}$ (Sigma, St. Louis, MO, USA), gentamicin $50mgmL^{-1}$ (Braum Medical SA, Jaen, Spain), 1% glutamine (Sigma), and 2% HEPES (Sigma) supplemented with 10% fetal bovine serum (FBS; Gibco, Paisley, UK) [14]. Then, cultures were kept in a humidified atmosphere of 95% air and 5% $CO_2$, at 37 °C. Cells were detached from the flask using 0.05% trypsin (Sigma) and 0.02% ethylenediaminetetraacetic acid solution (EDTA; Sigma). Cells were then rinsed and re-suspended in complete culture medium with 10% FBS [14].

### 2.3. Cell Proliferation Assay

Osteoblasts were seeded at $1 \times 10^4$ cells/mL per well. The cells were cultured in a humid atmosphere 95% air and 5% $CO_2$ at 37 C. After 48 h, cell proliferation was assayed using the 3-(4,5-dimethylthiazol-2-yl) − 2,5-diphenyltetrazolium (MTT) test (Sigma). The media was replaced by phenol red-free DMEM with MTT $0.5mgmL^{-1}$ and after incubated for 4 h. MTT cellular reduction resulted in the formation of insoluble crystal deposits of formazan, that were dissolved by adding dimethyl sulfoxide (Merck Biosciences, Darmstadt, Germany). Absorbance was measured with a spectrophotometer (Sunrise, Tecan, Männedorf, Switzerland) at 570-nm [14]. The results were expressed as mean absorbance and standard deviation (SD). At least three experiments performed for each type of nanoparticles.

### 2.4. Alkaline Phosphatase Activity measurement

The alkaline phosphatase (AP) activity was used to evaluate the early osteoblasts differentiation by a colorimetric assay (Diagnostic kit 104-LL, Sigma). The conversion of the colourless substrate p-nitrophenyl phosphate to the yellow p-nitrophenol was measured by this test; it was accomplished by the AP enzyme. The rate of colour shift corresponded with the amount of AP enzyme present in the culture. Some standards of p nitrophenol (0–250 μM) were also prepared from dilutions of a 1000 μM stock solution, they were assayed in parallel [14]. After 72 h, cell cultures on the discs placed inside the 24-well plate were lysed in 100 μL of triton x-100 and then with 1 M Tris pH 8.00 by ultrasonication for 4 min. The suspension was then mixed with a 7.6 mM p-nitrophenylphosphate solution, at a proportion of 1:10 and finally incubated for 15 min at 37°C. A substrate solution was prepared by merging an aqueous solution of $4mgmL^{-1}$ of 4-nitrophenyl phosphate disodium salt (Sigma) with an

**Table 1**
Sequences of Primers for the amplification of osteoblasts' cDNA by real-time polymerase chain reaction.

| Gene | Sense Primer (5′ − 3′) | Antisense Primer |
|---|---|---|
| TGFβ1 | TGAACCGGCCTTTCCTGCTTCTCATG | GCGGAAGTCAATGTACAGCTGCCGC |
| TGFβ-R1 | ACTGGCAGCTGTCATTGCTGGACCAG | CTGAGCCAGAACCTGACGTTGTCATATCA |
| TGFβ-R2 | GGCTCAACCACCAGGGCATCCAGAT | CTCCCCGAGAGCCTGTCCAGATGCT |
| TGFβ-R3 | ACCGTGATGGGCATTGCGTTTGCA | GTGCTCTGCGTGCTGCCGA TGCTGT |
| Runx-2 | TGGTTAATCTCCGCAGGTCAC | ACTGTGCTGAAGAGGCTGTTTG |
| VEGF | CCTTGCTGCTCTACCTCCAC | CACACAGGATGGCTTGAAGA |
| OSX | TGCCTAGAAGCCCTGAGAAA | TTTAACTTGGGGCCTTGAGA |
| BMP-2 | TCGAAATTCCCCGTGACCAG | CCACTTCCACCACGAATCCA |
| BMP-7 | CTGGTCTTTGTCTGCAGTGG | GTACCCCTCAACAAGGCTTC |
| AP | CCAACGTGGCTAAGAATGTCATC | TGGGCATTGGTGTTGTACGTC |
| Col-1 | AGAACTGGTACATCAGCAAG | GAGTTTACAGGAAGCAGACA |
| OSC | CCATGAGAGCCCTCACACTCC | GGTCAGCCAACTCGTCACAGTC |
| OPG | ATGCAACACAGCACAACATA | GTTGCCGTTTTATCCTCTCT |
| RANKL | ATACCCTGATGAAAGGAGGA | GGGGCTCAATCTATATCTCG |
| UBC | TGGGATGCAAATCTTCGTGAAGACCCTGAC | ACCAAGTGCAGAGTGGACTCTTTCTGGATG |
| PPIA | CCATGGCAAATGCTGGACCCAACACAAATG | TCCTGAGCTACAGAAGGAATGATCTGGTGG |
| RPS13 | GGTGTTGCACAAGTACGTTTTGTGACAGGC | TCATATTTCCAATTGGGAGGGAGGACTCGC |

equal volume of 1.5 M alkaline buffer (Sigma). The reaction was stopped, by adding 1 mL 0.05 N NaOH, then, the final absorbance was measured with a spectrophotometer at 405-nm (Sunrise, Tecan, Männedorf, Switzerland). The total protein content was estimated by the Bradford method using a protein assay kit from Bio-Rad Laboratories (Bio-Rad Laboratories, Nazareth-Eke, Belgium). Experiments were conducted in triplicate.

### 2.5. RNA extraction and real-time polymerase chain reaction (RT-PCR) assessments

After 48 h of culturing, the Qiagen RNeasy extraction kit (Qiagen Inc., Hilden, Germany) was used in order to extract the osteoblastic mRNA. The amount of extracted mRNA from the cells was measured by means of UV spectrophotometry at 260-nm (Eppendorf AG, Hamburg, Germany). Afterwards, 1 μg of mRNA from each group was brought to 40 μL of total volume, reverse-transcribed to cDNA and amplified with iScriptTM cDNA Synthesis Kit (Bio-Rad laboratories, Hercules, CA, USA) by polymerase chain reaction [10].

The RNA was then reverse transcribed and after the NCBI- nucleotide library and Primer3-design were used in order to design the primers to detect mRNA of: runt-related transcription factor 2 (Runx-2), osterix (OSX), alkaline phosphatase (AP), osteocalcin (OSC), osteoprotegerin (OPG), ligand for RANK (RANKL), type I collagen (Col-I), bone morphogenetic proteins 2 and 7 (BMP-2 and BMP-7), TGF-β1 and TGF-β receptors (TGF-βR1, TGF-βR2, and TGF-βR3) and vascular endothelial growth factor (VEGF). Results were always normalized using ubiquitin C (UBC), peptidylprolyl isomerase A (PPIA), and ribosomal protein S13 (RPS13) as housekeeping genes [10]. The primer sequences are included in Table 1.

The RT-qPCR was conducted using the SsoFast™ EvaGreen® Supermix Kit (Bio-Rad laboratories). The obtained cDNA (5 μL per sample) was placed in 96-well microplates and amplified by means of an IQ5-Cycler (Bio-Rad laboratories). The annealing and elongating temperatures were set at 60–65ºC and 72ºC, respectively. Over 40 cycles were performed. The Ct values were plotted against the log cDNa dilution in order to obtain a standard curve for each of the targeted gene. Then, nonspecific PCR products and primer dimers were ruled out creating a melting profile and carrying out an agarose gel electrophoresis. The results were expressed as the proportion of ng of mRNA per average ng of housekeeping mRNA [10]. The whole process was performed in triplicate.

### 2.6. Scanning Electron Microscopy (SEM)

Osteoblasts were seeded at $1 \times 10^4$ cells/mL per well, onto the discs previously placed inside the 24-well plate, and covered with the distinct nanoparticles. The cells were cultured for 48 h, at 37°C and in a humid atmosphere of 95% air and 5% $CO_2$. Two discs of each experimental group were submitted to critical drying point and were covered with carbon. SEM (GEMINI, Carl Zeiss SMT, Oberkochen, Germany) analysis was performed [14].

### 2.7. Statistical Analysis

Normal distribution was probed by Kolmogorv–Smirnov tests ($p > 0.05$). Mean comparisons were conducted by one-way ANOVA for variables following a normal distribution and by Kruskal-Wallis for non-parametric data distribution. Bonferroni multiple comparisons were carried out. Significance was set at $p < 0.05$. Data were always expressed as means ± standard deviation (SD).

## 3. Results

### 3.1. NPs loading efficacy

Doxycycline and dexamethasone loading efficacy was 92% and 91.63%, respectively.

### 3.2. Cell Proliferation Assay

The results of the MTT assay are presented in Fig. 1, as mean and standard deviations of the osteoblastic cells proliferation. No differences between groups were found ($p = 0.122$).

### 3.3. Alkaline Phosphatase (AP) Activity

Mean and standard deviations of AP activity are expressed as international units (IU) of AP per mg of total proteins and they are presented in Fig. 2. Statistically significant differences were found between Ti-DoxNPs (0.08 IU) and the other groups (with values ranging from 0.02 to 0.001).

### 3.4. Real-time Quantitative Polymerase Chain Reaction

Results from the RT-qPCR analysis are displayed in Fig. 3. In general, it can be seen that the incorporation of doxycycline and dexamethasone to the experimental NPs caused an up-regulation of the

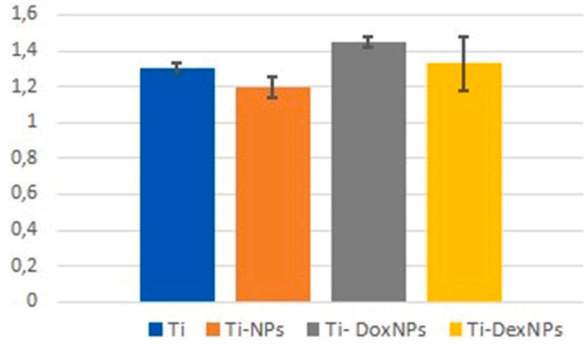


**Fig. 1.** Mean and standard deviation of absorbance values obtained after the MTT assay for the different experimental groups. No statistical differences were found between groups. Ti: titanium discs, Ti-NPs: Titanium discs covered with un-doped NPs, Ti-DoxNPs: Titanium discs with doxycycline doped NPs, Ti-DexNPs: Titanium discs with dexamethasone doped NPs.

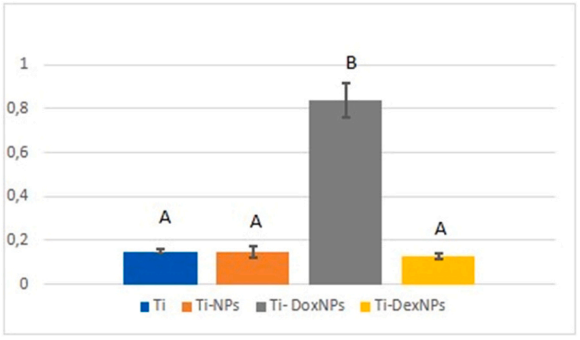


**Fig. 2.** Mean and standard deviation of international units of AP per mg of proteins values obtained at the different tested discs. Distinct letter indicates significant differences between groups after ANOVA and post-hoc comparisons ($p < 0.05$). Ti: titanium discs, Ti-NPs: Titanium discs covered with un-doped NPs, Ti-DoxNPs: Titanium discs with doxycycline doped NPs, Ti-DexNPs: Titanium discs with dexamethasone doped NPs.

expression of the main osteogenic genes. Regarding the most relevant proliferation-related genes, it can be observed how Dox-NPs and Dex-NPs up-regulated TGF-β1, being overexpressed about 2 and 4 times, respectively, if compared to the control titanium discs ($p < 0.05$). TGF-βR1 genes were also overexpressed about 8 and 4 times (Dox-NPs and Dex-NPs, respectively) and TGF-βR2 about 5 (Dox-NPs) and 2 (Dex-NPs) times respect to the control discs ($p < 0.05$). TGF-βR3 was not upregulated in any case ($p > 0.05$).

The expression of AP and Runx-2 was largely increased when osteoblasts were cultured in the presence of Dox-NPs and Dex-NPs (4-fold and 8-fold increases, respectively, for AP; and 3-fold and 4-fold times for Runx-2 respect to the control group; $p < 0.05$). In both cases, differences between osteoblasts grown with Dex-NPs and Dox-NPs were also significant ($p < 0.05$). In the case of OSX gene expression, Dox-NPs and Dex-NPs produced an up-regulation (5-fold and 3-fold increases respect to the control titanium discs, respectively) with significant differences between both groups ($p < 0.05$). In relation to bone morphogenetic protein BMP-2, Dox-NPs and Dex-NPs equally produced a double-fold up-regulation respect to the control group. However, for BMP-7, Dex-NPs produced positive up-regulation about 7-fold change and Dox-NPs (with significant differences with Dex-NPs) up-regulated BMP-7 about 3.5 times. OSC expression was only significantly up-regulated in the group of Dex-NPs, being overexpressed about four times ($p < 0.05$).

Dox-NPs and Dex-NPs groups attained a marked down-regulation of the expression of RANKL, and in the case of Dox-NPs, OPG was also overexpressed. Therefore, The OPG/RANKL ratio was significantly higher when Dox-NPs were present (300-fold increase respect to the control group). Dex-NPs did not produce a significantly higher OPG/RANKL ratio respect to the control group.

VEGF and COL-I genes expression were not modified by the presence of doxycycline nor dexamethasone doped NPs onto the titanium discs ($p = 0.12$ and $p = 0.38$, respectively).

### 3.5. Scanning electron microscopy

Selected images from the SEM analysis are displayed in Fig. 4. A general (Fig. 4 A to 4D) and a higher magnification view (Figs. 4E to 4H) of the four groups are provided. Cells were grown in every experimental group, and difference in number of cells is not appreciable (Fig. 4 A to 4D). Osteoblasts grown on titanium discs were mainly flat and polygonal in shape, with inter-cellular connections (Fig. 4 A and 4E). Mineral depositions may be observed on the surfaces of osteoblasts cultured in the presence of Dex-NPs and Dox-NPs (Fig. 4 C and 4D). At a higher magnification, it may be observed that osteoblasts grown in the presence of Dox-NPs and Dex-NPs are spindle-shaped and have more abundant secretions on their surfaces (Fig. 4 G and 4 H). Cytoplasmatic extensions between cells and towards each other are clearly evidenced in all groups (Figs. 4E to 4H).

## 4. Discussion

This study aimed to evaluate if doxycycline and dexamethasone-doped polymeric nanoparticles, applied onto titanium surfaces, may exert effects on the osteoblasts' proliferation and differentiation. For this purpose, a MG-63 osteoblast-like cell model was designed. Proliferation was studied by means of MTT assay and also by the expression of proliferation-related genes using RT-qPCR (e.g., TGF-β1, TGFβ-R1, TGFβ-R2 or TGFβ-R3). Osteoblasts' differentiation was quantitatively tested by AP activity analysis. Moreover, the expression of differentiation-related genes (e.g., AP, OSC, BMP-2, BMP-7, Runx-2 or OSX) was also measured. The ratio of the genes expression OPG/RANKL was analyzed in order to ascertain bone remodelling activity and the potential effect on bone mass preservation [15]. Finally, SEM images of the cultured osteoblasts onto the titanium surfaces were evaluated as indirect evidence of NPs-to-cells and cell-to-cell interactions. Cell morphology was also analyzed, provided the previously reported association between changes in osteoblast morphology and its differentiation stages [16].

In the present research, doped polymeric nanoparticles have been applied onto titanium surfaces. Previously, various novel nanostructured biomaterials and drug carriers, including titanium-based nanotubes, polymer micelles, liposomes vesicle, peptides, multifunctional polymers and other bioactive materials have also been researched for implant surface modification, trying to enhance the bone healing process and to avoid pathological bone loss [17].

The MG-63 osteoblast-like cell line was selected since these cells share their main characteristics with primary human osteoblasts and there are no interspecies differences with primary human osteoblasts. Tumoral line cells need a shorter isolation time and there is unlimited accessibly [18,19]. Proliferation of the MG-63 osteoblasts was studied by MTT assay, and no differences were found among the experimental groups and the control (titanium discs) (Fig. 1). This result is crucial, as at this point, it is demonstrated that osteoblasts are able to grow onto titanium surfaces treated with the experimental NPs as much as they grown in the titanium surface per se. Therefore, it may be inferred that the NPs are not harmful to the osteoblastic cells, and may be proposed as carriers for therapeutic agents. To analyze these results, it should be taken into account that SLA titanium surfaces are considered as the 'gold standard' for osteoblasts proliferation [20].

The present NPs are polymeric in nature. The NPs have a final diameter of approximately 220 nm [12], but even when the mean diameter of these NPs exceed 100 nm, following recommendations on biopolymers terminology they can be considered as nanoparticles

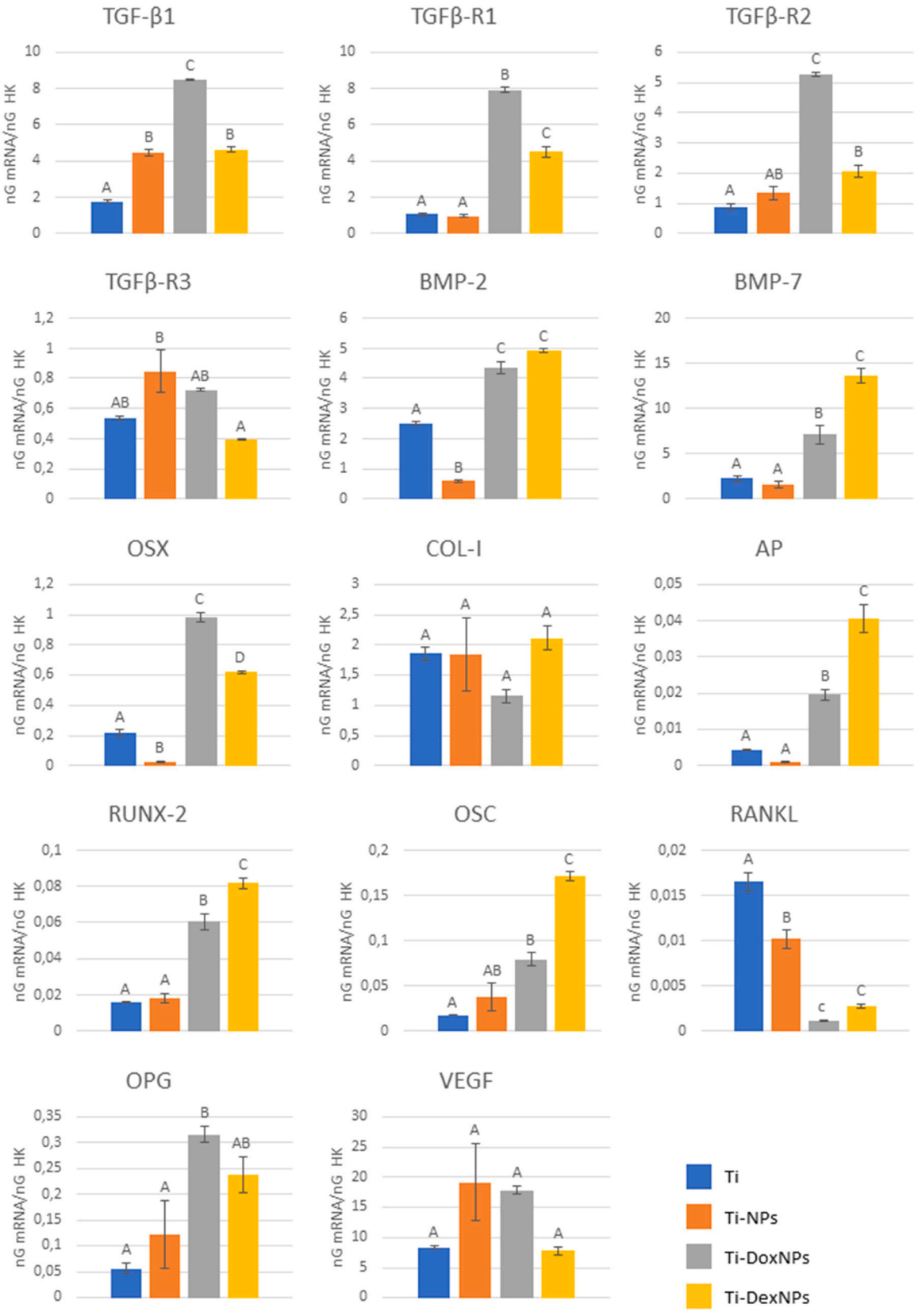


**Fig. 3.** Quantitative real-time PCR gene expression analysis of TGFβ-1, TGFβ-R1, TGFβ-R2, TGFβ-R3, VEGF, BMP2, BMP7, OSC, RANKL, OPG, OSX, Col-I, AP, Runx-2 established for cultured osteoblasts seeded on the several experimental NPs covered titanium discs, after 48 h. Results were expressed as mean and standard deviation and presented in ng mRNA/ng HK. Different letters indicate significant differences after One-way ANOVA and post-hoc multiple comparisons ($p \leq 0.05$). Ti: titanium discs, Ti-NPs: Titanium discs covered with undoped NPs, Ti-DoxNPs: Titanium discs with doxycycline doped NPs, Ti-DexNPs: Titanium discs with dexamethasone doped NPs.

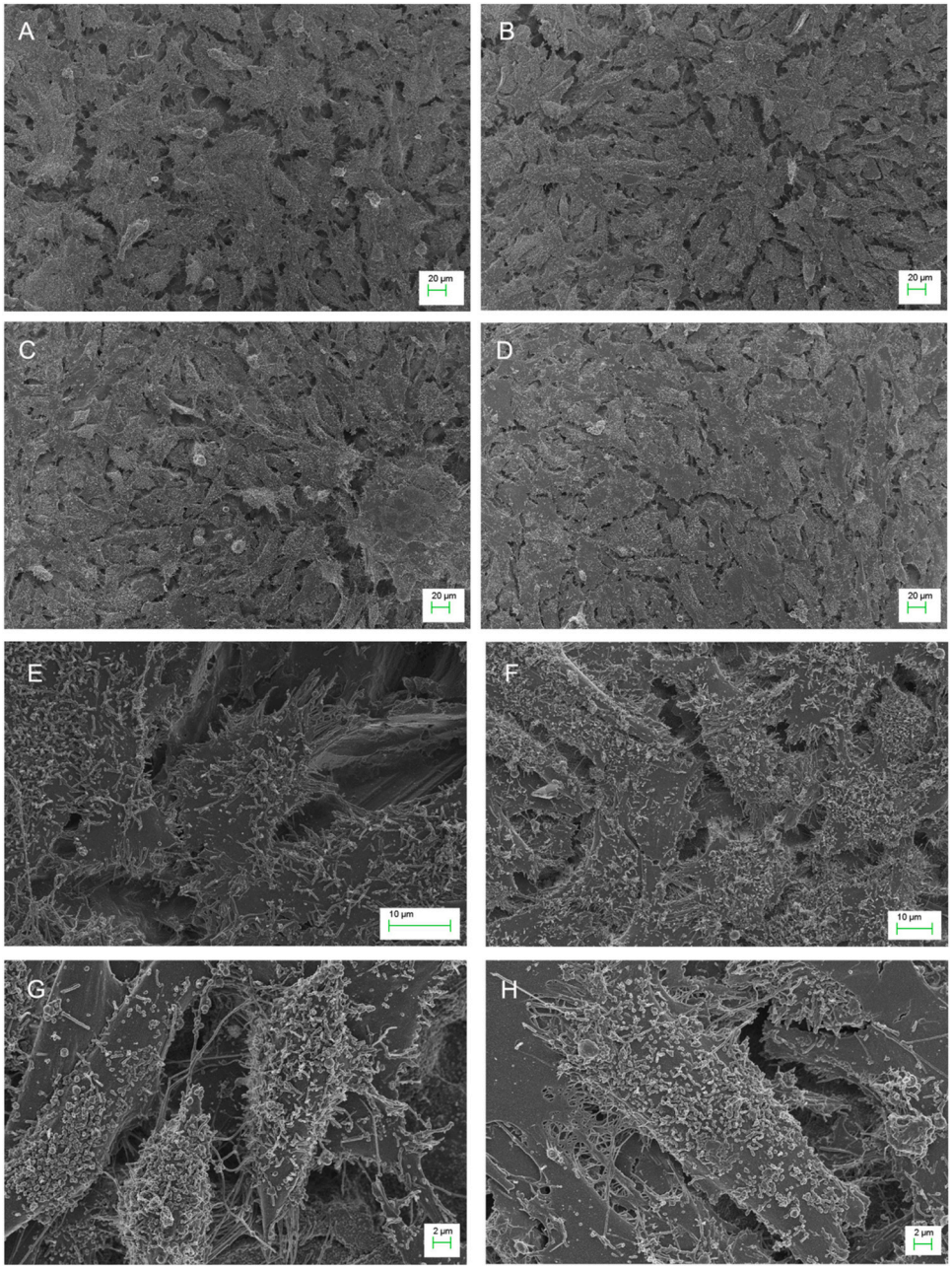


**Fig. 4.** SEM images of the osteoblasts cultured on the experimental titanium discs for 48 h. (A, E) Osteoblasts seeded on titanium are flat and polygonal shaped with multiple cytoplasmatic connections. (B, F) Osteoblasts cultured on Ti-NPs are clearly visible, covering the complete surface. (C, G) Osteoblasts grown on Ti-DoxNPs are fusiform and extracellular substance production is clearly noticed. (D, H) Osteoblasts proliferation on Ti-DexNPs discs are spindle shaped, they constitute a continuous cell layer and they also have abundant extracellular substance deposits. Magnifications are 500x and 5,000x.

being below 500 nm [21]. They have been found to be non-cytotoxic in previous in vitro experiments using fibroblasts [11] or stem cells derived from human bone marrow [7]. They are non-resorbable and do not elute any chemicals which may be harmful to cells [11]. This initial proliferation and early adhesion of cells to titanium covered by NPs, which is also evidenced by SEM (Fig. 4) is essential, as it may determine the final fate of osteoblasts on the implant surface. In this study, it is also evidenced by SEM analysis that the osteoblasts were firmly attached to the NPs-covered Ti surfaces, and after 48 h they were completely spread around the disks (Fig. 4). Osteoblasts cultured on Ti-DoxNPs and Ti-DexNPs did also show production of abundant extracellular matrix and exhibited some mineral deposits (Fig. 4 G and 4 H). Therefore, it may be hypothesized that these NPs covering the Ti surfaces may enhance the ability of osteoblasts to secrete matrix proteins and deposit bone mineral for osteogenesis [22]. It is also worth noting that the overall results from RT-qPCR analysis evidenced that the incorporation of doxycycline and dexamethasone to the experimental NPs-covered Ti surfaces produced, in the cultured osteoblasts, an over-expression of the main tested osteogenic proliferation-related genes. Dox-NPs and Dex-NPs upregulated TGF-β1 TGF-βR1 and TGF-βR2. TGF-βR3 was the only gen that was not upregulated in any case (Fig. 3). These growth factors are known to participate in the activation of the MAPK cascade [22,23]. The MAPK signaling pathway is involved in controlling cell growth, development and division, by influencing nuclear transcription factors [23,24]. In addition, the MAPK signaling pathway plays a pivotal role in the activation and signaling of other various osteogenic growth factors [23,25].

When evaluating AP activity, significant differences were found between osteoblasts cultured on Ti-DoxNPs and the other experimental groups (Fig. 2). Our results are in accordance with a previous in vitro study in which doxycycline appears to enhance osteoblasts maturation and differentiation, rather than proliferation [24]. It seems that doxycycline exert activity through the Wnt/β-catenin pathway, which regulates and accelerates bone regeneration [26,27]. The activation of Wnt pathways has the potential to improve bone healing processes. During the bone repair activity, the expression of many Wnt ligands and receptors is upregulated [26,28], being this point specially important in peri-implantitis treatment. The presence of Dkk-1, an inhibitor of Wnt signaling, results in osteogenesis inhibition. In a previous work, it was evidenced that doxycycline inhibited the Dkk-1 pathway while activating Wnt signaling, which is pivotal for osteoblast differentiation [26,29]. Thus, doxycycline, by acting on Wnt pathway activation, may accelerate bone regeneration as a part of a healing process [30].

Osteoblast differentiation is a complex process. It includes two main steps: i) osteogenic commitment, mainly characterized by the upregulation of a transcription factor, Runx2 [31], and ii) osteogenic differentiation, in which the expression of specific osteogenic proteins and transcription factors such as AP, OPG and OSX is produced [32,33,34]. In this study, the expression Runx-2 was up-regulated when DoxNPs and DexNPs were present (3-fold and 4-fold times, respectively) (Fig. 3). This effect has been previously encountered after dexamethasone [35] and doxycycline administration [30]. Moreover, the expression of specific osteogenic proteins (AP, OSX and OPG) was also higher on osteoblasts cultured on Ti-DoxNPs and Ti-DexNPs (Fig. 3). This finding, points out the participation of the tested drug-loaded NPs, at both steps of the process of osteoblasts differentiation. The canonical Wnt signaling is an important pathway controlling osteoblast differentiation and it has been advocated to be promoted by dexamethasone [35] and doxycycline [30]. However, the exact mechanisms underlying these effects have not been proved at the present experimental design, and it may be considered as a study limitation.

OSC expression was only significantly up-regulated in the group of DexNPs (Fig. 3), as dexamethasone has been shown to increase the transcription of osteocalcin, promoting osteogenic differentiation, and activating the function of osteoblasts [23].

BMPs are proteins that belong to the TGF-β superfamily, and several effects have been demonstrated on osteoblasts proliferation, differentiation and general functioning. At the present research, BMP-2 and BMP-7 were both up-regulated by DoxNPs and DexNPs (Fig. 3), which may also be a signal of enhanced osteogenesis [36]. Therefore, it may be advocated an osteogenic effect induced in response to these bone morphogenic proteins stimulation (the BMP-Smads pathways) which is further sustained by the Wnt signalling [35,37]. Taking this into account, it seems that DoxNPs and DexNPs may be able to act in three of the main molecular pathways involved in osteogenesis: i) the BMP-Smads, ii) the Wnt/β-catenin and iii) the mitogen-activated protein kinase (MAPK). It is known that these several pathways coordinate and interact with one another to promote the proliferation and differentiation of osteoblasts [22].

The receptor activator of NF-kB ligand (RANKL) and the osteoprotegerin (OPG) are a system of molecules that regulate bone remodeling, stimulating and inhibiting osteoclast differentiation, respectively. It has also been established their participation in the complex set of molecular interactions leading to bone loss in the peri-implantitis process [38]. Local deregulation of the RANKL-OPG interplay, in favour of the pro-osteoclastic component, may lead to bone resorption at the alveolar site [39] and play an important role in the onset and severity of the peri-implant diseases [40]. Osteoblasts grown in the presence of DoxNPs attained a marked OPG overexpression and a down-regulation of the RANKL. The OPG/RANKL ratio was about 300-fold increase respect to the control group. DexNPs did not produce OPG up-regulation, which is in accordance with previous reports about glucocorticoids action on osteoblast differentiation [39,41]. However, a marked lowering effect in RANKL expression was encountered (Fig. 3). It may seem to be contradictory, but it has been evidenced that not all glucocorticoids are stimulators of RANKL gene expression. These drugs have the ability to generate glucocorticoid receptor ligands that regulate their own cellular effect. This permits an anti-inflammatory activity without inhibiting OPG production and/or without stimulating or even by lowering RANKL production [39,41].

In the light of the present results, and lacking further research, it could be hypothesized that the application of these NPs on the titanium surface of dental implants may promote peri-implant bone formation. It could be considered as an effective local strategy to promote osteogenic differentiation by a proper localized drug-delivery system. It should be considered that other substances as growth factors including bone morphogenetic proteins and transforming growth factor-β have been previously proposed to favour the differentiation into bone cells or to stimulate bone cells proliferation in this same clinical scenario. However, they posse high cost, difficult dosage and rapid degradation, limiting their clinical use. Therefore, there is a clear need to expand alternative high capacity osteogenic inducers [42]. Doxycycline and dexamethasone have been previously proposed to induce osteogenic differentiation of bone marrow-derived mesenchymal stem cells in vitro [7,43] and to significantly enhance bone-related gene expression and the formation of mineralized nodules [44]. Doxycycline loaded NPs seem to have a major osteogenic effect than those functionalized with dexamethasone. Moreover, they have been shown to have a potent antibacterial and anti-biofilm formation properties [6]. However, the benefit of DexNPs as immunomodulator agents remains to be ascertained.

The novelty of this investigation should be highlighted, due to the thorough evaluation of the effect of these experimental drug-loaded NPs on osteoblastic cells, including proliferation, differentiation, mineralization and genes expression. DexNPs and DoxNPs have been shown to be able to induce osteoblasts differentiation and to modulate the expression of the main molecules implicated in osteogenesis. Therefore, these NPs may constitute a potential new tool for multiple potential in-chair treatments. However, as it is an in vitro study, it should be interpreted with caution and next steps including preclinical in vivo experimental research is needed.

Another limitation of the present study is that the experimental design does not include mechanistic assays. However, it should be taken into account that osteogenic differentiation is a complex process involving numerous signaling cascades; and mechanistic assays, which could facilitate the understanding of a particular action, are usually unable to ascertain the complex biological and regulatory processes, requiring expression of the multiple implicated steps. It is recognized that new experiments for discovering unknown interactions of doxycycline and dexamethasone with target genes and biochemical pathways are needed in future investigations.

## Acknowledgements

Grant PID2020–114694RB-I00 funded by MCIN/AEI 10.13039/501100011033. M.T.-O. is fellow FPU of Ministry of Universities [grant FPU20/00450]. Authors want to thank Klockner S.A. for financial support and for providing the titanium discs.

## References

[1] Berglundh T, Armitage G, Araujo MG, Avila-Ortiz G, Blanco J, Camargo PM, et al. Peri-implant diseases and conditions: consensus report of workgroup 4 of the 2017 world workshop on the classification of periodontal and peri-implant diseases and conditions. J Clin Periodo 2018;45:S286–91. https://doi.org/10.1111/jcpe.12957

[2] Ivanovski S, Bartold PM, Huang YS. The role of foreign body response in peri-implantitis: what is the evidence? Periodontol 2000 2022;90:176–85. https://doi.org/10.1111/prd.12456

[3] Yuan P, Qiu X, Liu T, Tian R, Bai Y, Liu S, et al. Substrate-independent polymer coating with stimuli-responsive dexamethasone release for on-demand fibrosis inhibition. J Mater Chem B 2020;8:7777–84. https://doi.org/10.1039/d0tb01127d

[4] Lee CT, Huang YW, Zhu L, Weltman R. Prevalences of peri-implantitis and peri-implant mucositis: systematic review and meta-analysis. J Dent 2017;62:1–12. https://doi.org/10.1016/j.jdent.2017.04.011
[5] Toledano M, Osorio MT, Vallecillo-Rivas M, Toledano-Osorio M, Rodríguez-Archilla A, Toledano R, et al. Efficacy of local antibiotic therapy in the treatment of peri-implantitis: a systematic review and meta-analysis. J Dent 2021;113:103790https://doi.org/10.1016/j.jdent.2021.103790
[6] Bueno J, Virto L, Toledano-Osorio M, Figuero E, Toledano M, Medina-Castillo AL, et al. Antibacterial effect of functionalized polymeric nanoparticles on titanium surfaces using an in vitro subgingival biofilm model. Polym (Basel) 2022;14:358. https://doi.org/10.3390/polym14030358
[7] Toledano-Osorio M, López-García S, Osorio R, Toledano M, García-Bernal D, Sánchez-Bautista S, et al. Dexamethasone and doxycycline doped nanoparticles increase the differentiation potential of human bone marrow stem cells. Pharmaceutics 2022;14:1865. https://doi.org/10.3390/pharmaceutics14091865
[8] Wu C, Miron R, Sculean A, Kaskel S, Doert T, Schulze R, et al. Proliferation, differentiation and gene expression of osteoblasts in boron-containing associated with dexamethasone deliver from mesoporous bioactive glass scaffolds. Biomaterials 2011;32:7068–78. https://doi.org/10.1016/j.biomaterials.2011.06.009
[9] Ghaffari-Bohlouli P, Zahedi P, Shahrousvand M. Enhanced osteogenesis using poly (l-lactide-co-d, l-lactide)/poly (acrylic acid) nanofibrous scaffolds in presence of dexamethasone-loaded molecularly imprinted polymer nanoparticles. Int J Biol Macromol 2020;165:2363–77. https://doi.org/10.1016/j.ijbiomac.2020.10.078
[10] Toledano-Osorio M, Manzano-Moreno FJ, Toledano M, Osorio R, Medina-Castillo AL, Costela-Ruiz VJ, et al. Doxycycline-doped membranes induced osteogenic gene expression on osteoblastic cells. J Dent 2021:103676https://doi.org/10.1016/j.jdent.2021.103676
[11] Osorio R, Alfonso-Rodríguez CA, Medina-Castillo AL, Alaminos M, Toledano M. Bioactive Polymeric Nanoparticles for Periodontal Therapy. PLoS One 2016;11:e0166217https://doi.org/10.1371/journal.pone.0166217
[12] Medina-Castillo AL. Thermodynamic principles of precipitation polymerization and role of fractal nanostructures in the particle size control. Macromolecules 2020;53:5687–700. https://doi.org/10.1021/acs.macromol.0c00973
[13] Toledano-Osorio M, Babu JP, Osorio R, Medina-Castillo AL, García-Godoy F, Toledano M. Modified polymeric nanoparticles exert in vitro antimicrobial activity against oral bacteria. Materials 2018;11:1013. https://doi.org/10.3390/ma11061013
[14] Toledano-Osorio M, Manzano-Moreno FJ, Toledano M, Medina-Castillo AL, Costela-Ruiz VJ, Ruiz C, et al. Doxycycline-doped polymeric membranes induced growth, differentiation and expression of antigenic phenotype markers of osteoblasts. Polym (Basel) 2021;13:1063. https://doi.org/10.3390/polym13071063
[15] Boyce BF, Xing L. Biology of RANK, RANKL, and osteoprotegerin. Arthritis Res Ther 2007;9:S1. https://doi.org/10.1186/ar2165
[16] Schmidt C, Kaspar D, Sarkar MR, Claes LE, Ignatius AA. A scanning electron microscopy study of human osteoblast morphology on five orthopedic metals. J Biomed Mater Res 2002;63:252–61. https://doi.org/10.1002/jbm.10185
[17] Souza JCM, Sordi MB, Kanazawa M, Ravindran S, Henriques B, Silva FS, et al. Nano-scale modification of titanium implant surfaces to enhance osseointegration. Acta Biomater 2019;94:112–31. https://doi.org/10.1016/j.actbio.2019.05.045
[18] Czekanska EM, Stoddart MJ, Richards RG, Hayes JS. In search of an osteoblast cell model for in vitro research. Eur Cell Mater 2012;24:1–17. https://doi.org/10.22203/ecm.v024a01
[19] Toledano-Osorio M, Manzano-Moreno FJ, Ruiz C, Toledano M, Osorio R. Testing active membranes for bone regeneration: a review. J Dent 2021;105:103580https://doi.org/10.1016/j.jdent.2021.103580
[20] Esposito M, Coulthard P, Thomsen P, Worthington HV. The role of implant surface modifications, shape and material on the success of osseointegrated dental implants. A Cochrane systematic review. Eur J Prosthodont Restor Dent 2005;13:15–31.
[21] Vert M, Doi Y, Hellwich K-H, Hess M, Hodge P, Kubisa P, et al. Terminology for biorelated polymers and applications (IUPAC Recommendations 2012). Pure Appl Chem 2012;84:377–410. doi: 10.1351/PAC-REC-10-12-04.
[22] Shen MJ, Wang GG, Wang YZ, Xie J, Ding X. Nell-1 enhances osteogenic differentiation of pre-osteoblasts on titanium surfaces via the MAPK-ERK signaling pathway. Cell Physiol Biochem 2018;50:1522–34. https://doi.org/10.1159/000494651
[23] Xiao G, Jiang D, Gopalakrishnan R, Franceschi RT. Fibroblast growth factor 2 induction of the osteocalcin gene requires MAPK activity and phosphorylation of the osteoblast transcription factor, Cbfa1/Runx2. J Biol Chem 2002;277:36181–7. https://doi.org/10.1074/jbc.M206057200
[24] Seger R, Krebs EG. The MAPK signaling cascade. FASEB J 1995;9:726–35.
[25] Lee KS, Hong SH, Bae SC. Both the Smad and p38 MAPK pathways play a crucial role in Runx2 expression following induction by transforming growth factor-beta and bone morphogenetic protein. Oncogene 2002;21:7156–63. https://doi.org/10.1038/sj.onc.1205937
[26] Gomes PS, Resende M, Fernandes MH. Doxycycline restores the impaired osteogenic commitment of diabetic-derived bone marrow mesenchymal stromal cells by increasing the canonical WNT signaling. Mol Cell Endocrinol 2020;518:110975https://doi.org/10.1016/j.mce.2020.110975
[27] Secreto FJ, Hoeppner LH, Westendorf JJ. Wnt signaling during fracture repair. Curr Osteoporos Rep 2009;7:64–9. https://doi.org/10.1007/s11914-009-0012-5
[28] Xu H, Duan J, Ning D, Li J, Liu R, Yang R, et al. Role of Wnt signaling in fracture healing. BMB Rep 2014;47:666–72. https://doi.org/10.5483/bmbrep.2014.47.12.193
[29] Leucht P, Helms JA. Wnt signaling: an emerging target for bone regeneration. J Am Acad Orthop Surg 2015;23:67–8. https://doi.org/10.5435/JAAOS-23-01-67
[30] Issack PS, Helfet DL, Lane JM. Role of Wnt signaling in bone remodeling and repair. HSS J 2008;4:66–70. https://doi.org/10.1007/s11420-007-9072-1
[31] Xu J, Li Z, Hou Y, Fang W. Potential mechanisms underlying the Runx2 induced osteogenesis of bone marrow mesenchymal stem cells. Am J Transl Res 2015;7:2527–35.
[32] Klejna K, Naumnik B, Gasowska K, Mysliwiec M. OPG/RANK/RANKL signaling system and its significance in nephrology. Folia Histochem Cytobiol 2009;47:199–206. https://doi.org/10.2478/v10042-009-0035-x
[33] Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR, et al. The novel zinc finger containing transcription factor osterix is required for osteoblast differentiation and bone formation. Cell 2002;108:17–29. https://doi.org/10.1016/s0092-8674(01)00622-5
[34] Uchihashi K, Aoki S, Matsunobu A, Toda S. Osteoblast migration into type I collagen gel and differentiation to osteocyte-like cells within a self-produced mineralized matrix: a novel system for analyzing differentiation from osteoblast to osteocyte. Bone 2013;52:102–10. https://doi.org/10.1016/j.bone.2012.09.001
[35] Hamidouche Z, Hay E, Vaudin P, Charbord P, Schule R, Marie PJ, et al. FHL2 mediates dexamethasone-induced mesenchymal cell differentiation into osteoblasts by activating Wnt/-catenin signaling-dependent Runx2 expression. FASEB J 2008;22:3813–22. https://doi.org/10.1096/fj.08-106302
[36] Groeneveld EH, Burger EH. Bone morphogenetic proteins in human bone regeneration. Eur J Endocrinol 2000;142:9–21. https://doi.org/10.1530/eje.0.1420009
[37] Gregory CA, Gunn WG, Reyes E, Smolarz AJ, Munoz J, Spees JL, et al. How Wnt signaling affects bone repair by mesenchymal stem cells from the bone marrow. Ann N Y Acad Sci 2005;1049:97–106. https://doi.org/10.1196/annals.1334.010
[38] Duarte PM, de Mendonça AC, Máximo MB, Santos VR, Bastos MF, Nociti FH. Effect of anti-infective mechanical therapy on clinical parameters and cytokine levels in human peri-implant diseases. J Periodo 2009;80:234–43. https://doi.org/10.1902/jop.2009.070672
[39] Bostanci N, Ilgenli T, Emingil G, Afacan B, Han B, Toz H, et al. Gingival crevicular fluid levels of RANKL and OPG in periodontal diseases: implications of their relative ratio. J Clin Periodo 2007;34:370–6. https://doi.org/10.1111/j.1600-051X.2007.01061.x
[40] Duarte PM, de Mendonça AC, Máximo MB, Santos VR, Bastos MF, Nociti Júnior FH. Differential cytokine expressions affect the severity of peri-implant disease. Clin Oral Implants Res 2009;20:514–20. https://doi.org/10.1111/j.1600-0501.2008.01680.x
[41] Humphrey EL, Williams JH, Davie MW, Marshall MJ. Effects of dissociated glucocorticoids on OPG and RANKL in osteoblastic cells. Bone 2006;38:652–61. https://doi.org/10.1016/j.bone.2005.10.004
[42] Vadaye Kheiry E, Parivar K, Baharara J, Fazly Bazzaz BS, Iranbakhsh AR. The osteogenesis of bacterial cellulose scaffold loaded with fisetin. Iran J Basic Med Sci 2018;21:965–71. https://doi.org/10.22038/IJBMS.2018.25465.6296
[43] Xiao T, Fan L, Liu R, Huang X, Wang S, Xiao L, et al. Fabrication of dexamethasone-loaded dual-metal-organic frameworks on polyetheretherketone implants with bacteriostasis and angiogenesis properties for promoting bone regeneration. ACS Appl Mater Interfaces 2021;13:50836–50. https://doi.org/10.1021/acsami.1c18088
[44] Toledano-Osorio M, de Luna-Bertos E, Toledano M, Manzano-Moreno FJ, García-Recio E, Ruiz C, et al. Doxycycline-doped collagen membranes accelerate in vitro osteoblast proliferation and differentiation. J Periodontal Res 2022. https://doi.org/10.1111/jre.13091