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Doxycycline-doped collagen membranes accelerate in vitro osteoblast proliferation and differentiation.

Title: Doxycycline-doped collagen membranes accelerate *in vitro* osteoblast proliferation and differentiation.

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Abstract

*Objective. T*o evaluate the effect of doxycycline and dexamethasone-doped collagen membranes on the proliferation and differentiation of osteoblasts.

Background. Collagen barrier membranes are frequently used to promote bone regeneration, and to boost this biological activity their functionalization with antibacterial and immunomodulatory substances has been suggested.

Methods. The design included commercially available collagen membranes doped with doxycycline (Dox-Col-M) or dexamethasone (Dex-Col-M), as well as undoped membranes (Col-M) as controls, which were placed in contact with cultured MG63 osteoblast-like cells (ATCC, Manassas, VA, USA). Cell proliferation was assessed by MTT-assay and differentiation by measuring the alkaline phosphatase activity using spectrophotometry. Real-time quantitative polymerase chain reaction (RT-qPCR) was used to study the expression of the genes: Runx-2, OSX, ALP, OSC, OPG, RANKL, Col-I, BMP-2, BMP-7, TGF-β1, VEGF, TGF-βR1, TGF-βR2, and TGF-βR3. Scanning Electron Microscopy was used to study osteoblast morphology. Data was assessed using one-way ANOVA or Kruskal-Wallis tests, once their distribution normality was assessed by Kolmogorv–Smirnov tests ($p > 0.05$). Bonferroni for multiple comparisons were carried out ($p < 0.05$).

Results. Osteoblasts proliferation was significantly enhanced in the functionalized membranes as follows: (Col-M<Dex-Col-M<Dox-Col-M). Alkaline phosphatase activity was significantly higher on cultured osteoblasts on Dox-Col-M. Runx-2, OSX, ALP, OSC, BMP-2, BMP-7, TGF-β1, VEGF, TGF-βR1, TGF-βR2, and TGF-βR3 were overexpressed and RANKL was down-regulated in osteoblasts cultured on Dox-Col-M. The osteoblasts cultured in contact with the functionalized membranes demonstrated an elongated spindle-shaped morphology.

Conclusion. The functionalization of collagen membranes with Dox promoted an increase in the proliferation and differentiation of osteoblasts.

Keywords: bone regeneration, doxycycline, dexamethasone, membranes, osteoblasts.

1. INTRODUCTION

Regenerative therapies aimed to reconstruct the anatomy and function of oral tissues lost due to trauma or disease, have included different technologies and surgical approaches, although guided tissue regeneration (GTR) and guided bone regeneration (GBR)[1–3] are the ones most frequently used and with more predictable outcomes.[4,5] These interventions have in common the use of a membrane that acts as a physical barrier excluding the colonization of the defect by undesired cells and allowing those with capability to regenerate the lost or damaged tissues.[1] With the current understanding of the biological processes of wound healing and regeneration, membrane technology is evolving from a mere physical activity to a more active role combining the barrier effect with biological activity aiming to boost the regenerative process.[6]

One of the key factors in the biological activity of a barrier membrane for bone regeneration (GBR) is its bio-absorbability, since the membrane must maintain its physical integrity during the post operatory wound healing process to predictably achieve the desired regenerative outcomes. Although there is not an ideal resorption time, it is proven that the longer the membrane maintains its function, the regenerated bone will be more dense and mature.[6] Collagen-based membranes, are the most widely used membrane material due to their biocompatibility, bio-absorbability, good handling properties and its biological ability to attract and activate gingival fibroblast, periodontal ligament cells, and osteoblasts, what may lead to enhanced soft and hard tissue healing.[7,8,9] However, as main disadvantage, natural collagen membranes have faster resorption kinetics due to the released of collagenases and proteases by the host, which may be enhanced in presence of a pro-inflammatory phenotype or in unfavorable mechanical environment.[10] This disadvantage has been partially overcome by the utilization of different physical/chemical cross-linking processes.[11] Although cross-linking has improved collagen stability, toxic residues from this process have been reported to induce severe inflammation at the regeneration site.[11]

Furthermore, barrier membranes may be exposed during the healing period, mainly in presence of large osseous defects or when the soft tissue borders of the flap are not properly sutured or with excessive tension. Once exposed in the oral environment, the membranes will become contaminated, what will promote the proinflammatory environment during wound healing and hence, will hamper the desired regenerative outcomes. To counteract these unwanted effects of classical barrier membranes, a new generation of bioactive membranes has been developed where membrane materials are

functionalized with substances either with antimicrobial activity or with immune-modulatory effects, this promoting a pro-healing rather than a pro-inflammatory phenotype.[12-14]

As immune-modulating substances, dexamethasone has been proposed, both due to the immunomodulation effect as well as its intrinsic capacity to stimulate mesenchymal stem cells proliferation and differentiation to osteogenic lineages[15]. Dexamethasone is also a synthetic glucocorticoid that shares this combined effect, thus potentially enhancing bone regeneration. Different antimicrobial substances have also been used to functionalize barrier membranes to prevent bacterial contamination in case of membrane exposure to the oral environment.[16] Tetracyclines have been extensively used since they are highly biocompatible, they are chemically stable at body temperature, do not interfere with wound healing, have a broad-spectrum bactericidal activity and can easily be produced with slow release pharmaco-dynamics.[17] It has been previously reported how doxycycline may enhance the proliferation and differentiation of osteoblasts, as well as this potential in human bone marrow stem cells.[18,19,20] These functionalized membranes, however, have been scarcely tested both pre-clinically as well as in clinical studies.

It was, therefore, the objective of this preclinical *in vitro* investigation to use doxycycline and dexamethasone as bioactive substances to dope commercialized collagen-based membranes and to study their effect on the ability of osteoblasts to proliferate and differentiate.

2. MATERIALS AND METHODS

2.1. Collagen membranes functionalization

Commercially available natural collagen membranes obtained from bovine purified Achilles tendon type I (Symbios®, Dentsply Sirona GmbH, Konstanz, Germany) were trimmed into 7mm diameter discs and doped with doxycycline or dexamethasone. For this process, aqueous solutions of doxycycline hyclate (Dox) and dexamethasone (Dex) (0.2mg/mL and 0.0125mg/mL, respectively) were prepared and 15μL of each, were added to each membrane disc. Hence, three groups of membrane discs were obtained: 1) Undoped (Col-M), 2) Dox functionalized (Col-Dox-M) and 3) Dex functionalized (Col-Dex-M).

2.2. Doxycycline and dexamethasone liberation

Doxycycline and dexamethasone liberation was evaluated by soaking in PBS (pH 7.4) loaded scaffolds at 37 ºC for 24 h, 48 h, 7 d, 14d and 21 d. Doxycycline and dexamethasone concentration in supernatants were measured 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 to 500-nm). Calibration curves were created by plotting attained values against known concentrations. The quantities of released drugs in each well were determined using these calibration curves. The cumulative release rate of doxycycline and dexamethasone (%) were calculated with the following equation: (amount of drug liberated at each time point / total loading amount of drug in scaffolds) x 100%.[21]

2.3. Cell Culture

Culture cells from the human MG63 osteosarcoma cell line (ATCC, Manassas, VA, USA) were obtained in Dulbecco's modified Eagle medium (DMEM; Invitrogen Gibco Cell Culture Products, Carlsbad, CA, USA). After adding penicillin 100IU/mL (Lab Roger SA, Barcelona, Spain), amphotericin B 2.5mg/mL (Sigma, St. Louis, MO, USA), gentamicin 50mg/mL (Braum Medical SA, Jaen, Spain), 1% glutamine (Sigma), and 2% HEPES (Sigma) supplemented with 10% fetal bovine serum (FBS; Gibco, Paisley, UK), cultures were kept in a humidified atmosphere at 37 ºC with 95% air and 5% CO_2. Then the cells were detached from the flask using 0.05% trypsin (Sigma) and 0.02% ethylenediaminetetraacetic acid solution (EDTA; Sigma), and then rinsed and resuspended in culture medium with 10% FBS.[22]

2.4. Cell Proliferation Assay

The obtained osteoblasts were seeded at 1×10^4 cells/mL per well onto the functionalized collagen membranes, within a 24-well plate and cultured in a humid atmosphere of 95% air and 5% CO_2 at 37º C. After 48 h, cell proliferation was assayed by means of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) test. First, the media was replaced by phenol red-free Dulbecco's Modified Eagle Medium (DMEM) with MTT 0.5mg/mL (Sigma), incubated during 4h and the insoluble crystal deposits of formazan from the MTT cellular reduction were dissolved by adding dimethyl sulfoxide (Merck Biosciences, Darmstadt, Germany), and the resulting absorbance (expressed as

mean absorbance ± standard deviation (SD)) was measured with a spectrophotometer (Sunrise, Tecan, Männedorf, Switzerland) at 570nm.[23] At least three experiments were conducted for each type of membrane disc.

2.5. Alkaline Phosphatase Activity

Early osteoblast differentiation was indirectly assessed by the alkaline phosphatase (ALP) activity measured with a colorimetric assay (Diagnostic kit 104-LL, Sigma). The colour shift corresponds to the amount of ALP enzyme present in the culture, since the ALP enzyme mediates the conversion of the colourless substrate p-nitrophenyl phosphate to the yellow p-nitrophenol. Standards curves of p nitrophenol (0–250μM) were prepared in parallel using dilutions of a 1000μM stock solution.[18] In brief, cell cultures seeded onto the functionalized membranes within 24-well plate during 72 h, were lysed in 100μL of Triton X-100 and then with 1 M Tris pH 8.00 by ultrasonication for 4 min. Then, the suspension was mixed with a 7.6 mM p-nitrophenylphosphate solution at a proportion of 1:10 and incubated for 15 min at 37ºC. A substrate solution was prepared by merging an aqueous solution of 4mg/mL of 4-nitrophenyl phosphate disodium salt (Sigma) with an equal volume of 1.5 M alkaline buffer (Sigma). The reaction was stopped by adding 1mL 0.05N NaOH, and the final absorbance was measured with a spectrophotometer (Sunrise, Tecan, Männedorf, Switzerland) at 405nm. 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). All samples were conducted in triplicate.

2.6. Matrix Mineralization Evaluation

Mineralized deposition above the membranes was evaluated using Alizarin Red S method. MG63 cells were seeded at 5×10^4 cells/mL/well in a 24-well plate and cultured in osteogenic medium (DMEM supplemented with 5 mM β-glycerophosphate and 0.05 mM ascorbic acid) on the different membrane prototypes at 37°C in a humified atmosphere (95% air and 5% CO_2). After 15 and 21d of culture, the mineral deposition of the cells was evaluated. Ten percent (w/v) cetylpyridinium chloride was used to stop red calcium deposits for 15 minutes. Then, the absorbance of was measured with a spectrophotometer (BioTek ELx800) at a wavelength of 562nm.[24]

2.7. RNA extraction and real-time polymerase chain reaction (RT-PCR)

Cells messenger RNA (mRNa) was extracted using the Qiagen RNeasy extraction kit (Qiagen Inc., Hilden, Germany) and the mRNA amount measured by UV spectrophotometry at 260nm (Eppendorf AG, Hamburg, Germany). 1μg of mRNA from each group was brought to 40μL of total volume, reverse-transcribed to complementary DNA (cDNA) and amplified with iScriptTM cDNA Synthesis Kit (Bio-Rad laboratories, Hercules, CA, USA) by means of the polymerase chain reaction according to the manufacturer's instructions.[25] Then, the NCBI- nucleotide library and Primer3-design were used to design the primers to detect mRNA of the following genes: runt-related transcription factor 2 (Runx-2), osterix (OSX), alkaline phosphatase (ALP), 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 normalized using ubiquitin C (UBC), peptidylprolyl isomerase A (PPIA), and ribosomal protein S13 (RPS13) as housekeeping genes.[26,27] The primer sequences have been included in Table 1.

The RT-qPCR was conducted using the SsoFast[TM] 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 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.[18] The whole process was performed in triplicate.

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
ALP	CCAACGTGGCTAAGAATGTCATC	TGGGCATTGGTGTTGTACGTC

Col-1	AGAACTGGTACATCAGCAAG	GAGTTTACAGGAAGCAGACA
OSC	CCATGAGAGCCCTCACACTCC	GGTCAGCCAACTCGTCACAGTC
OPG	ATGCAACACAGCACAACATA	GTTGCCGTTTTATCCTCTCT
RANKL	ATACCCTGATGAAAGGAGGA	GGGGCTCAATCTATATCTCG
UBC	TGGGATGCAAATCTTCGTGAAGACCCTGAC	ACCAAGTGCAGAGTGGACTCTTTCTGGATG
PPIA	CCATGGCAAATGCTGGACCCAACACAAATG	TCCTGAGCTACAGAAGGAATGATCTGGTGG
RPS13	GGTGTTGCACAAGTACGTTTTGTGACAGGC	TCATATTTCCAATTGGGAGGGAGGACTCGC

Table 1. Primer sequences for the amplification of osteoblasts' cDNA by real-time PCR.

2.8. Scanning Electron Microscopy (SEM)

Osteoblasts were seeded at 1×10^4 cells/mL onto the membranes discs and placed in the 24-well plate and then cultured in a humid atmosphere of 95% air and 5% CO_2 at 37ºC for 48 h. Then, 2 membranes of each experimental group were subject to critical point drying and covered with carbon. Cell morphology was evaluated with a scanning electron microscope (SEM) (GEMINI, Carl Zeiss SMT, Oberkochen, Germany).[18]

2.9. Statistical Analysis

Data were expressed as means ± standard deviation (SD) for all measured variables. After testing for the normality of the obtained distribution using Kolmogorov–Smirnov test, comparisons among experimental and control groups were conducted by one-way ANOVA for variables following a normal distribution and Kruskal-Wallis one-way ANOVA on ranks for non-parametric distributions. Then post-hoc Bonferroni test for multiple comparisons was applied. Significance was set at $p<0.05$.

3. RESULTS

3.1. Doxycycline and dexamethasone liberation

Cumulative liberation (%) of dexamethasone and doxycycline are displayed in Figure 1. Dexamethasone was released in two phases: i) a first burst during the initial 24 h (0.13µg/mL), where almost 70% of dexamethasone was released, ii) and a second phase from 48h to 14d with a slow release of dexamethasone. From 7 to 14d the whole amount of dexamethasone was released. For doxycycline, a slow and maintained liberation occurred during the evaluation period. After 24h only a 5% of the loaded amount was

liberated and after 21d, 90% of the loaded doxycycline was still remaining within the membrane.
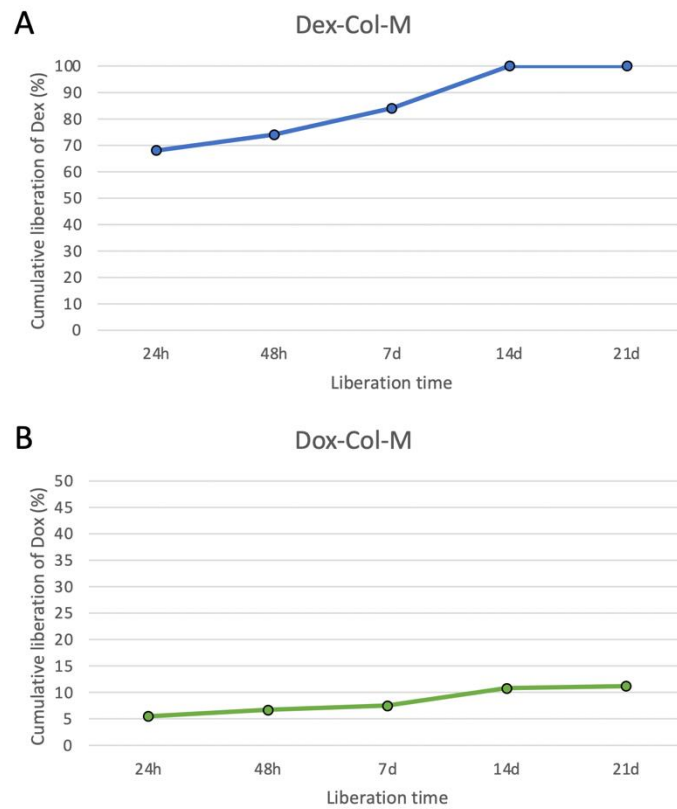
Figure 1. Cumulative liberation (%) of doxycycline and dexamethasone to phosphate buffered saline from experimental loaded collagen membranes measured at different time points.

3.2. Cell Proliferation Assay

The results of the MTT assay are presented in Figure 2. A significantly higher absorbance, indicating higher osteoblastic cell proliferation, was attained in the Dox-Col-M (0.44), when compared with the Dex-Col-M (0.32) and with the control group Col-M (0.23).
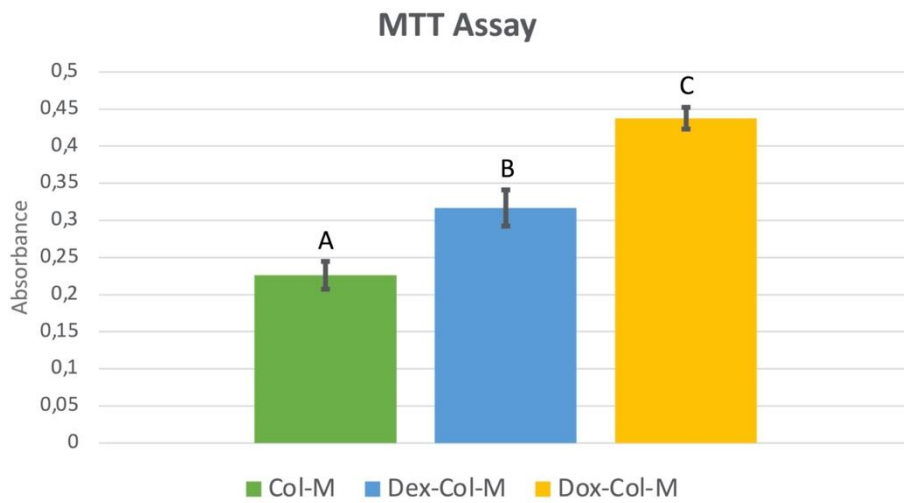
Figure 2. Absorbance mean values and standard deviations obtained after the MTT assay for the different doped membranes. Different letter indicates statistically significant differences between membranes after ANOVA and post-hoc Bonferroni comparisons ($p < 0.05$).

3.3. Alkaline Phosphatase (ALP) Activity

Mean and standard deviations of alkaline phosphatase expressed as international units (IU) of ALP per mg of total proteins are presented in Figure 3. Statistically significant differences were found between Dox-Col-M (0.95IU) and the other two groups (0.11IU for Dex-Col-M and Col-M).
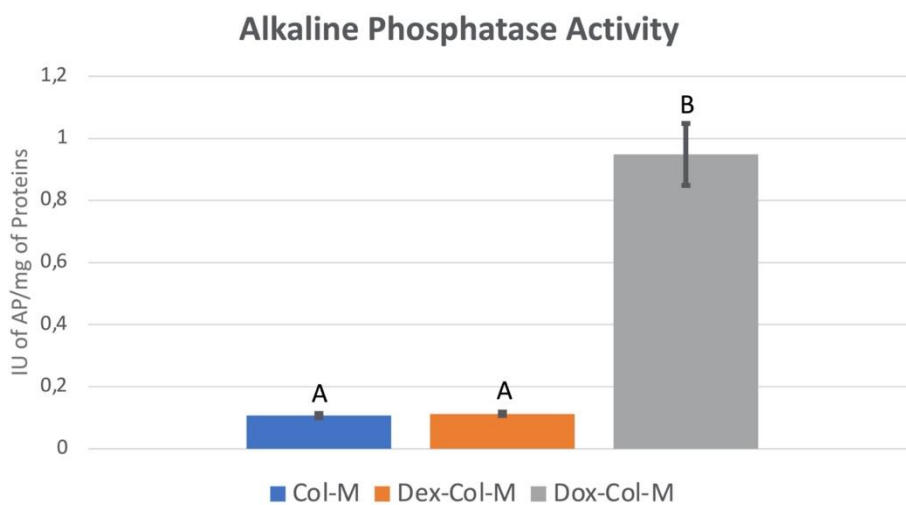
Figure 3. Mean and standard deviation of international units of ALP per mg of proteins values obtained with the different membranes. Distinct letter indicates significant difference between membranes after ANOVA and post-hoc Bonferroni comparisons ($p<0.05$).

3.4. Mineralization Assay

Means and standard deviations of the attained absorbance for the different membranes at 15 and 21d are displayed in figure 4. After 15d of culture, Dox-Col-M displayed the highest absorbance, when compared to Col-M and Dex-Col-M ($p=0.03$ and 0.02, respectively), with a mean absorbance of 1.51. These differences were more evident after 21 d of culture, when Dox-Col-M stilled attained the highest mean absorbance (2.76) compared to Col-M (2.06) and Dex-Col-M (1.95). These differences were statistically significant, obtaining p values beneath 0.001 for both comparisons. While after 15 d there were no differences regarding the absorbance of the groups Col-M and Dex-Col-M, after 21 d osteoblasts cultured on Col-M attained a higher mineralization than those cultured on Dex-Col-M ($p=0.009$).
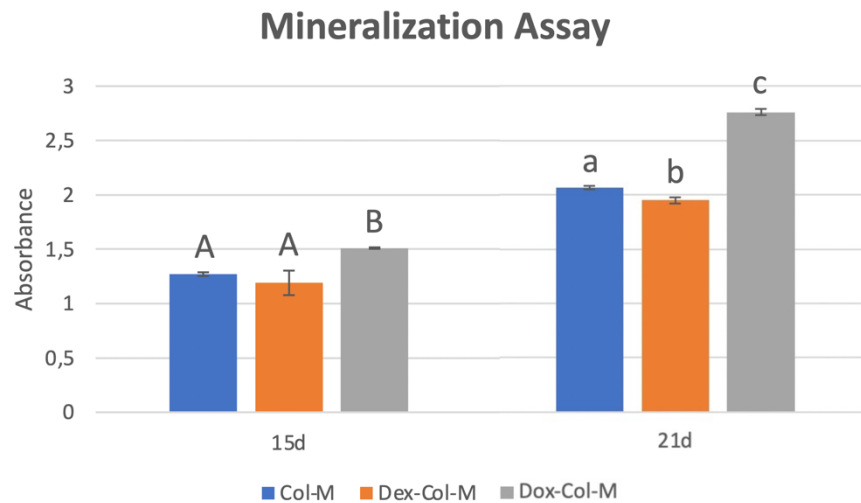

Figure 4. Mean and standard deviation of absorbance obtained with the different membranes using the Alizarin Red S method. ANOVA and post-hoc Bonferroni comparisons ($p<0.05$) were employed. Distinct capital letter indicates significant difference between membranes in the 15d group and distinct lower case letter indicates significant difference between membranes in the 21d group.

3.5. Real-time Quantitative Polymerase Chain Reaction

Results from the RT-qPCR analysis are displayed in Figure 5. Doxycycline doped membranes caused a significant up-regulation in the expression of osteogenic genes, compared with the other two groups. Specifically, Dox-Col-M significantly up-regulated TGF-β1, TGF-βR1, TGF-βR2, and TGF-βR3 compared to Col-M ($p<0.001$). Dex-Col-M, only demonstrated significant upregulation of TGF-βR3 when compared to Col-M ($p<0.001$).

The expression of ALP and Runx-2 were largely increased by Dox-Col-M (3.3-fold, $p=0.03$ and 3.8-fold increase, $p<0.001$; respectively). Both genes were not altered by Dex-Col-M; in both cases using Col-M as reference. With a similar pattern, OSX and OSC were overexpressed in the presence of Dox-Col-M (2.4-fold increase, $p=0.002$ and 3.3-fold change, $p=0.003$).

In relation to bone morphogenetic proteins, Dox-Col-M produced a positive up-regulation of BMP-2 and BMP-7 (3 and 4.9-fold change, respectively and $p<0.001$ for both comparisons). Both doped membranes, Dox-Col-M and Dex-Col-M showed a marked down-regulation of the expression of RANKL (11.84 and 5.13-fold change respectively, $p<0.001$ in both comparisons).
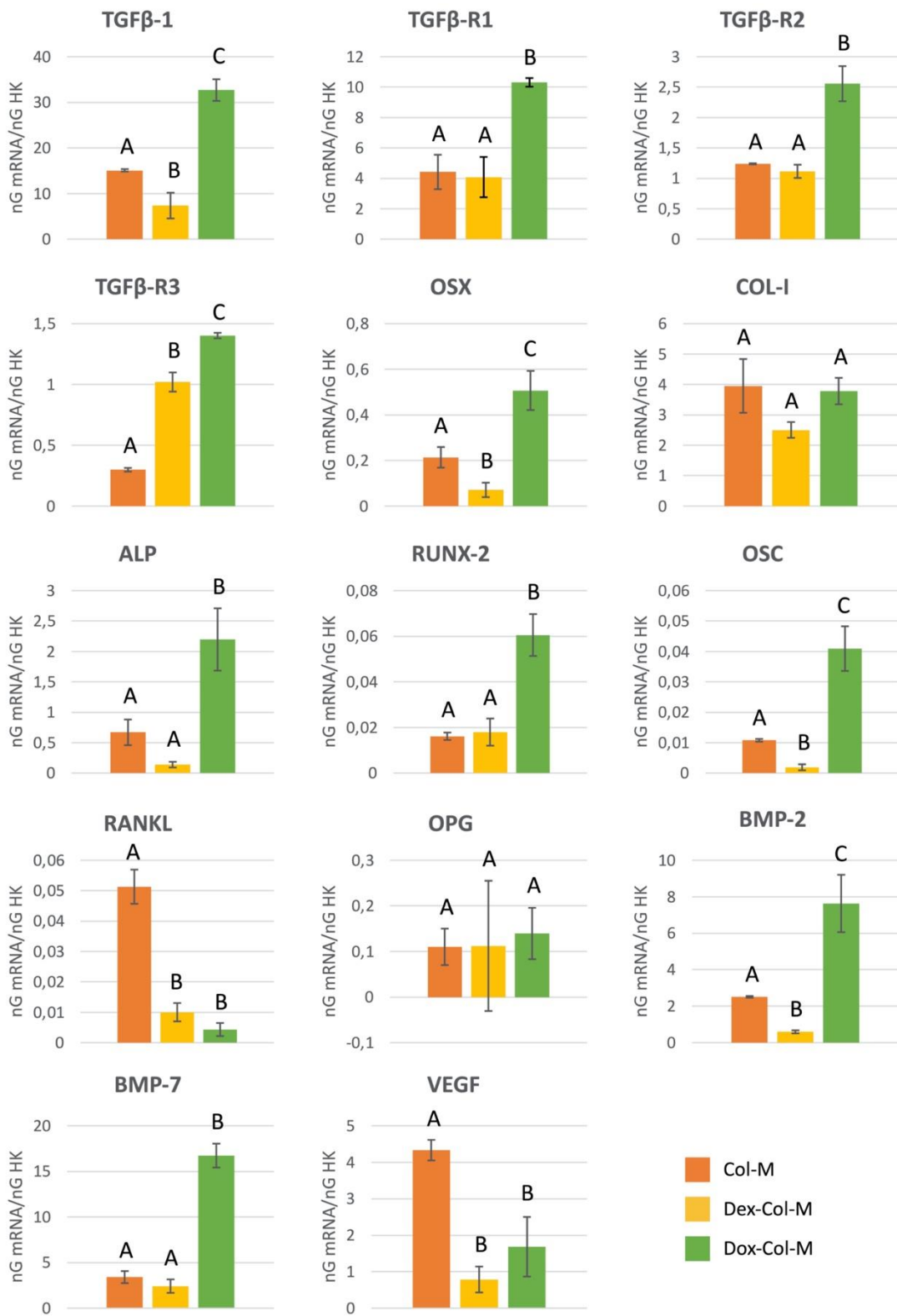
Figure 5. Quantitative real-time PCR gene expression analysis of TGFβ-1, TGFβ-R1, TGFβ-R2, TGFβ-R3, VEGF, BMP2, BMP7, OSC, RANKL, OPG, OSX, Col-I, ALP, Runx-2 established for

cultured osteoblasts seeded on the several experimental membranes, after 48 h. Results were expressed as mean and standard deviation and presented in ng mRNA/ng HK. Different letters indicate significant difference after ANOVA and Bonferroni multiple comparisons ($p \leq 0.05$). Col-M: collagen undoped membranes. Dox-Col-M: doxycycline functionalized collagen membranes. Dex-Col-M: dexamethasone functionalized collagen membranes.

3.6. Scanning Electron Microscopy

Selected SEM images are presented in Figures 6 and 7. In Figure 6, a representative image from each of the three groups is depicted. In the control Col-M group (Figure 6A) a scarce number of cells is present. In the Dex-Col-M group (Figure 6B) a higher number of osteoblasts can be observed on the membranes, while in the Dox-Col-M (Figure 6C), the osteoblasts are even more evident. Figure 7 presents SEM images at higher magnification where rounded osteoblasts emitting cytoplasmatic extensions and forming interconnected clusters of cells can be observed in the control group (Col-M) (Figure 7A, B). In the Dex-Col-M group, the predominant cell morphology is elongated rather than round and cell interconnections and interaction with the substrate can also be encountered (Figure 7C, D). In the Dox-Col-M group, only elongated and spindle-shaped osteoblasts are evidenced. The inter-cellular connections are more apparent and cells grow on different layers, establishing a 3D matrix (Figure 7E, F).
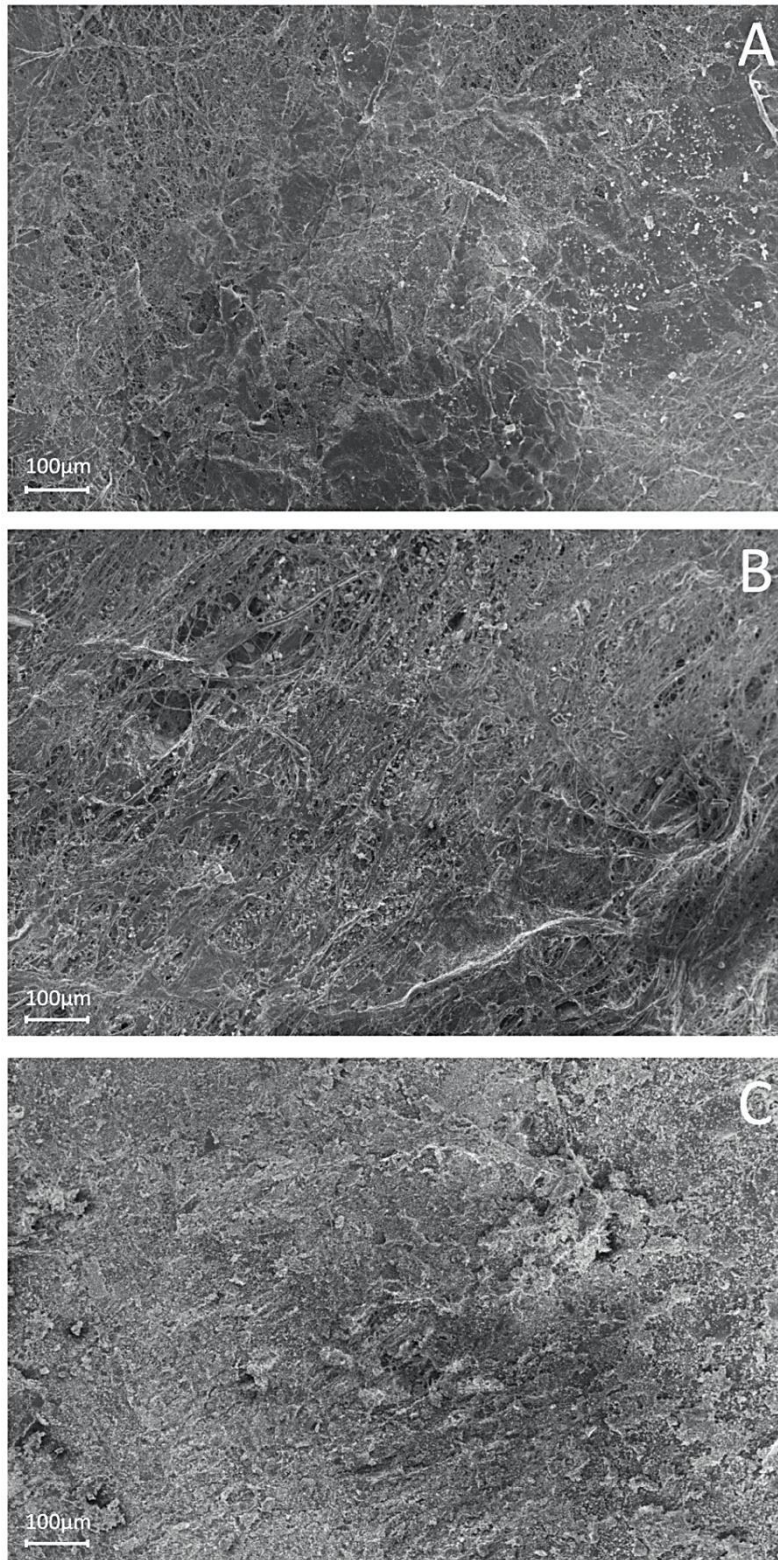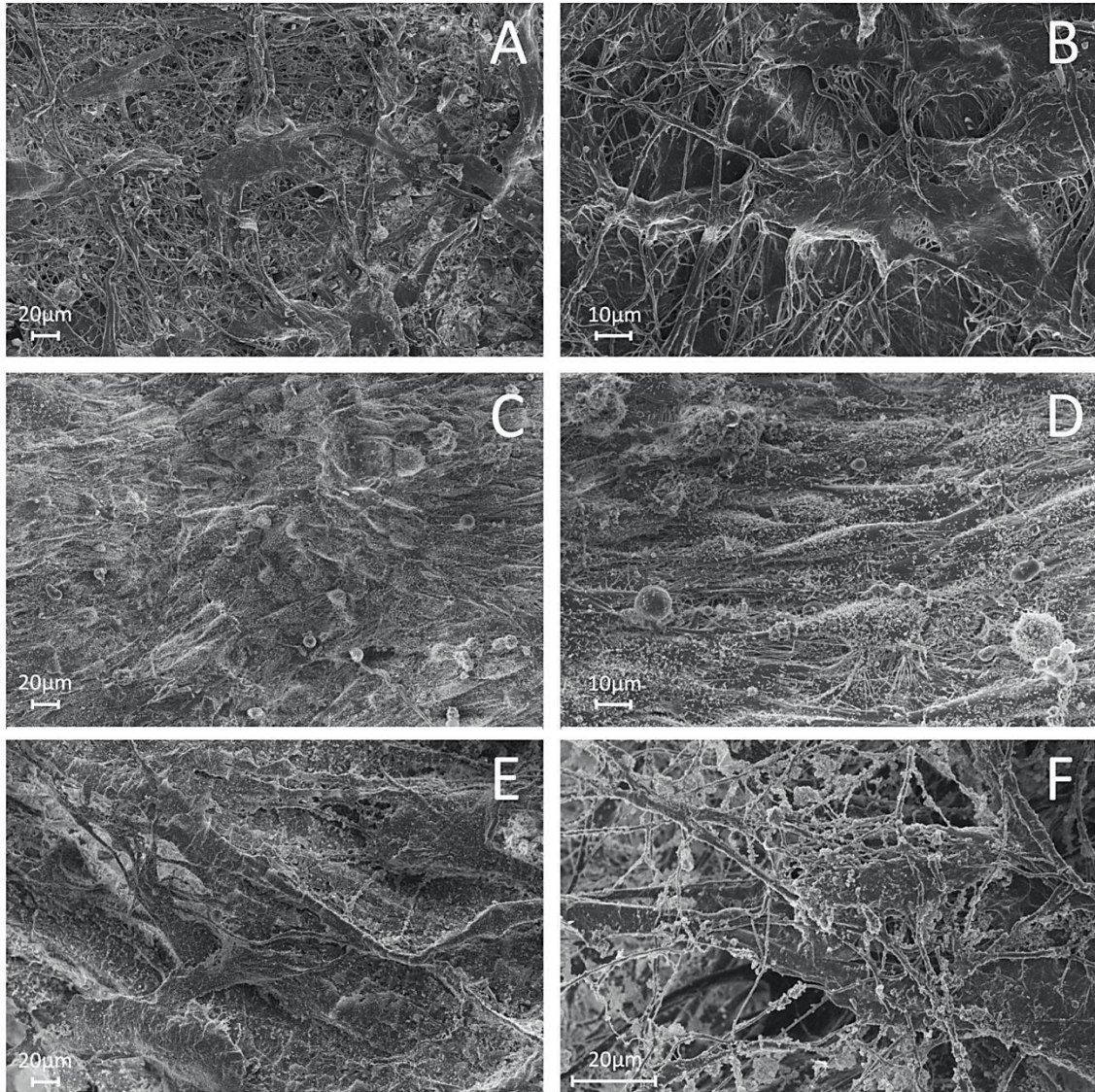
Figure 6. Low magnification (200x) SEM images of the experimental membranes. Osteoblasts are observed on the three images. (A) On Col-M, osteoblasts are flat and no extracellular substance is observable. (B) On Dex-Col-M, osteoblasts are more abundant and some of them are spindle-

shaped, some material secretion is evidenced. (C) On Dox-Col-M, collagen is not even visible as osteoblasts are covering the complete surface. Active extracellular substance can be noticed.

Figure 7. High magnification (600x and 2000x) SEM images of the experimental membranes. (A, B) On Col-M, flat osteoblasts with multiple cytoplasmatic connections are visible. (C, D) On Dex-Col-M osteoblasts are clearly visible, covering the collagen mesh. Many of them are spindle shaped, extracellular substance production is clearly noticed. (E, F) On Dox-Col-M, collagen is not observable. Osteoblasts are fusiform and big in size. They are constituting a three-dimensional layer and have abundant extracellular substance deposits.

4. DISCUSSION

This preclinical *in vitro* investigation aimed to assess the effect of doping natural collagen membranes with doxycycline and dexamethasone on the proliferation and differentiation of cultured osteoblasts. We have used MG-63 osteoblast-like cell model, which together with primary human osteoblasts are the most widely used cell lines to study osteoblast activity.[18,19,28] We selected MG63 osteoblast-like cells, since they share the main characteristics with primary human osteoblasts, but they need shorter isolation time and have unlimited accessibly.[28,29] To study the effect of the different membrane discs on the seeded cells we used different tests to measure their proliferation and differentiation.

Specific drug concentrations need to be determined for each activity and cell type. The dosages used in this investigation were based on previous studies on the in vitro effect of doxycycline and dexamethasone. For doxycycline, a dosage of 1μg/mL (0.2μg of antibiotic added twice a week) was tested for bone marrow cells.[30-31] In the present study, since doxycycline was going to be liberated from collagen in a relatively slow manner, a total amount of 3μg of doxycycline were loaded in each collagen specimen, at the initial stage of the study. In the case of dexamethasone, the loaded amount was 15μL of a 3×10^{-5}M solution, also based in a previous study where a dexamethasone loaded scaffold was tested using human bone marrow-derived mesenchymal stem cells.[32] It was also considered that concentrations within the range from 1×10^{-7} to 1×10^{-6}M were similar to those of the physiological level of glucocorticoids involved in the regulation of bone remodeling.[33]

It is of interest to highlight the results from the drugs release dynamics, since dexamethasone was released in two phases, with a first burst of release during the initial 24h, what may be due to the dilution of the adsorbed and not trapped molecules onto the collagen fibers.[32] Then, between 48h and 14d, a slow release of the dexamethasone residues within the collagen scaffolds was produced. After 14d, the total amount of loaded dexamethasone was liberated. In contrast, doxycycline maintained a slow-release pattern, what may be speculated it was due to its dependence on collagen degradation.[32]

Proliferation was studied by the MTT assay and by assessing the expression of proliferation-related genes by RT-qPCR (e.g., TGF-β1, TGFβ-R1, TGFβ-R2 or TGFβ-R3). Osteoblasts' differentiation was also quantitatively assayed by ALP activity and by measuring the expression of differentiation-related genes (e.g., ALP, OSC, Runx-2 or

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OSX). Finally, we used SEM to indirectly evaluate osteoblasts cell-to-cell interactions and their relation with the substrate, since previous studies have associated changes in osteoblast cell morphology and its proliferation and differentiation rates.[18,28,34,35]

With the MTT assay we measured the ability of the osteoblasts to proliferate by replication (cellular growth rate by quantifying daughter cell population) when cultured on the different disc membranes[28,36] (Figure 2). Since collagen is considered the *gold standard* scaffold for osteoblast proliferation, the present study demonstrated that osteoblasts cultured on the Dox-Col-M and the Dex-Col-M, attained higher mean proliferation values compared to those cultured on the Col-M. In fact, the proliferation of osteoblasts cultured on the Dox-Col-M practically doubled the one in the control group. Previous investigations have also reported the ability of tetracyclines to enhance osteoblast's proliferative capacity.[19,37] This effect was substantiated in the present investigation by the significant up-regulation of the proliferative-related genes demonstrated by RT-qPCR in the Dox-Col-M (Figure 5). Other investigations have also reported the effect of Dox-doped membranes on the expression of TGF-β1 and TGFβ-R1 in cultured osteoblasts.[19] The TGF-β superfamily are a set of proteins that enhance migration, proliferation, and differentiation of different kinds of cells, including osteoblasts[38] and at the same time they enhance matrix production and reduce RANKL synthesis by osteoblasts. It is therefore plausible that the overexpression of these genes indirectly favors bone regeneration by inhibiting osteoclasts activation via RANK[39] band by enhancing osteoblast proliferation and differentiation.[40] However, in the Dex-Col-M, only the TGFβ-R3 genes were up-regulated, while the rest of the osteoblastic proliferation-related genes were under-expressed, compared with the Dox-Col-M group. These results are in agreement with those reported by Walsh *et al.,*[41] who used primary human osteoblasts to evaluate the effect of dexamethasone, although other investigations have also shown up-regulation of osteoblastic proliferation genes when in presence of this glucocorticoid.[42,43] This discrepancy may be due to the use of cells derived from fetal rodents instead of osteoblasts from human origin, which may have marked functional and metabolic differences.[28,41]

We have indirectly assessed the effect of the different membrane discs on osteoblast differentiation by measuring ALP activity. It was demonstrated in this investigation that osteoblasts cultured on the Dox-Col-M had significantly higher ALP production of when compared with the other two groups ($p<0.001$). These results are in agreement with previous studies reporting an increase of ALP production in osteoblasts

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in contact with doxycycline.[18,44] Although the underlying pathway remains unclear, it has been suggested that the well-established inhibitory effect of tetracyclines over the matrix metalloproteinases[30,45] may support collagen stabilization and thus osteoblast differentiation. In fact, tetracyclines have shown to increase collagen synthesis.[46] This effect has been corroborated in clinical studies on the effect of low dose doxycycline topical antibiotics, demonstrated significant probing pocket depth reductions when compared to placebo.[47,48]

The effect of Dox-Col-M on the differentiation genes of cultured osteoblasts was also clear demonstrating a significant up-regulation of their expression, compared with the effect of Dex-Col-M and Col-M. Runx-2 (formerly called Cbfa1), a member of the runt homology domain transcription factor family, plays an important role in osteoblast differentiation[49] and together with ALP are the most frequently used markers of early osteoblasts differentiation.[19,49] These results, therefore, corroborate the effect of functionalizing the collagen membranes with doxycycline in the early osteoblastic differentiation stages. The effect of doxycycline on osteoblast differentiation is also supported by the expression patterns of OSC, since OSC is a late marker of differentiation and it is synthesized by mature osteoblasts just before and during matrix mineralization.[50] In the present study OSC was over-expressed in the Dox-Col-M group compared to Dex-Col-M and to Col-M by 21.5 and 3.8 times, respectively ($p<0.001$ in both cases). Since the levels of OSC in the Col-M group and specially, in the Dex-Col-M were very low, it could be argued that the culture time was not long enough to allow for differentiation of mature osteoblasts, since other studies culturing MG-63 osteoblast-like cells for 72h were not able to detect OSC.[50]

The genes encoding for BMP-2 and BMP-7 were also up-regulated by the Dox-Col-M. This is relevant since BMPs plays an important role in osteoblastic differentiation, bone formation/remodeling and overall osteo-induction.[51,52] It has been previously described that BMP-2 may induce the expression of ALP and other osteoblastic markers,[19,53] which is totally in accordance with the results obtained in our study, since the expression of BMPs and ALP followed the same pattern (Figure 5). Since both TGF-β and BMPs genes were up-regulated in the present study, it can be argued that this is due to the activation of Smad or MAPKs cascade, common pathway for both TGF-β and BMPs.[19,54]

One of the main mediators involved in bone homeostasis is RANKL. This marker is a protein synthesized by osteoblasts and its precursors which activates and stimulates

osteoclasts via its membrane-bound protein receptor RANK.[19,39] Thus, the down-regulation of the genes encoding for this protein would result in reduction of osteoclast activation and the subsequent reduction of bone resorption. The down-regulation of this gene was clearly manifested when the osteoblasts were seeded on Dex-Col-M and Dox-Col, compared to Col-M by 5.13 and 11.84 times, respectively ($p < 0.001$ for both comparisons).

It was found that VEGF expression was significantly reduced by Dex-Col-M and Dox-Col-M. It may be due to the inherent anti-inflammatory effect of both dexamethasone and doxycycline,[18,55] leading to a decrease in the production of cytokines and angiogenic factors by the cells.[56]

Using the Alizarin Red Assay, the obtained results by ALP and RT-qPCR were further confirmed. Dox-Col-M, attained higher values of mineralization that Col-M and Dex-Col-M, at both time points. Conversely, Dex-Col-M, after 7d, obtained similar values of mineralization than Col-M and lesser than when the analysis was carried out after 21 d, what could be explained by the dexamethasone release dynamics. The majority of the initially loaded glucocorticoid was liberated between 48h and 7d (Figure 4), thereafter losing its potential effect. However, with doxycycline an 11% of the originally loaded amount was liberated after 21d (Figure 4), thus demonstrating its long-lasting effect on the osteoblastic cells.

The effect of the functionalized membranes on the cultured osteoblasts was also studied morphologically using SEM. Osteoblastic cells were visible on the three groups, although more evident in the Dex-Col-M and even more in the Dox-Col-M group (Figure 6). These results corroborate previous investigations associating cell morphology and the metabolic/differentiation state of osteoblasts.[18,34] Rounded-shape osteoblasts, as the ones observed on Col-M group (Figure 7A, B), have been reduced mitotic activity, thus demonstrating a lower differentiation state and cell activity.[34,35] Conversely, spindle and fusiform-shape cells have been associated with higher proliferation and differentiation states. These morphologies could be clearly identified in the osteoblasts cultured on the membranes doped with Dox and Dex (Figure 7C-F). Furthermore, the osteoblasts grown on the Dox-Col-M group seem to form a three-dimensional cellular network (Figure 7F), which has been associated earlier *in vitro* osteoblast differentiation Schmidt *et al*.,[57] what also corroborates the results from the gene expression analysis, previously reported.

The results from this study, however, should be interpreted with caution due to the preclinical *in vitro* nature of this investigation and the use of MG-63 osteosarcoma

cell line, which despite sharing similar metabolic characteristics with primary osteoblasts and having been widely used in basic research, it is a tumoral cell line that may have alternative patterns of proliferation and differentiation.[28] It should also be remarked that result from cellular in vitro investigation should be extrapolated with cautiousness, since the in vivo wound healing milieu is not present, and this may somehow influence the clinical outcomes. It is however, a relevant investigation since different from previous investigations,[58] we achieved to functionalize commercialized collagen-based membranes with doxycycline and dexamethasone, demonstrating a clear effect on the seeded cultured osteoblasts. The novelty of this investigation resides in the thorough evaluation of the effect of these two substances doped on GBR membranes on osteoblastic cells, including metabolic, differentiation, mineralization and genes expression studies.

Next steps should include *in vitro* antibacterial assays, ideally using a subgingival biofilm model, as the one previously reported by our research group.[59] Furthermore, subsequent preclinical *in vivo* experimental studies are needed before clinical use.[28]

A limitation of the present study is the lack of mechanistic assays. However, although these assays enable the understanding of particular mechanism of action, they are usually unable to depict the complex biological and regulatory processes requiring multiple gene expression and regulation. Nevertheless, new experiments for discovering unknown interactions of doxycycline with target genes and biochemical pathways are needed in future investigations.

In conclusion, this *in vitro* investigation has demonstrated that functionalizing natural collagen GBR membranes with doxycycline significantly enhanced the proliferation and differentiation patterns of cultured osteoblasts, what may open clear possibilities for attaining bioactive GBR membranes, which should be further studied in appropriately designed preclinical *in vivo* and clinical investigations.

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