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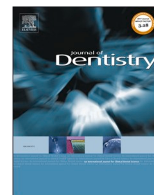

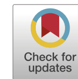

Doxycycline-doped membranes induced osteogenic gene expression on osteoblastic cells

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ABSTRACT

Objectives: To analyze how novel developed silicon dioxide composite membranes, functionalized with zinc or doxycycline, can modulate the expression of genes related to the osteogenic functional capacity of osteoblastic cells.

Methods: The composite nanofibers membranes were manufactured by using a novel polymeric blend and 20 nm silicon dioxide nanoparticles (SiO_2-NPs). To manufacture the membranes, 20 nm SiO_2-NPs were added to the polymer solution and the resulting suspension was processed by electrospinning. In a second step, the membranes were functionalized with zinc or doxycycline. Then, they were subjected to MG63 osteoblast-like cells culturing for 48 h. After this time, real-time quantitative polymerase chain reaction (RT-qPCR) was carried out to study the expression of Runx-2, OSX, ALP, OSC, OPG, RANKL, Col-I, BMP-2, BMP-7, TGF-β1, VEGF, TGF-βR1, TGF- βR2, and TGF-βR3. Mean comparisons were conducted by One-way ANOVA and Tukey tests ($p < 0.05$).

Results: In general, the blending of SiO_2-NPs in the tested non-resorbable polymeric scaffold improves the expression of osteogenic genes over the control membranes. Doxycycline doping of experimental scaffolds attained the best results, encountering up-regulation of BMP-2, ALP, OPG, TGFβ-1 and TGFβ-R1. Membranes with zinc induced a significant increase in the expression of Col-I, ALP and TGF β1. Both, zinc and doxycycline functionalized membranes enormously down-regulated the expression of RANKL.

Conclusions: Zinc and doxycycline doped membranes are bioactive inducing overexpression of several osteogenic gene markers.

Clinical significance: Doxycycline doped membranes may be a potential candidate for use in GBR procedures in several challenging pathologies, including periodontal diseases.

1. Introduction

The basic concepts of Guided Bone Regeneration (GBR) were firstly described by Nyman and Karring in the early 1980s [1]. They found that the cells that first populated a wound area, determined the linage of tissue that would ultimately occupy the space [2]. They also introduced the need to use a barrier membrane, preventing the undesired cells from entering the wound and permitting cells with the capacity to form the desired hard tissue to access the wound space [2].

Since 1980, the techniques and biomaterials used in GBR have been upgraded, enlarging the clinical scenarios in which GBR can be performed and allowing broadening the indications of dental implants to regions with an anatomy that is unfavorable for implant placing. Currently, the employed membranes can be resorbable or non-resorbable. Non-resorbable membranes are made of polytetrafluoroethylene (PTFE) and represent the gold-standard for clinicians, due to its

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higher predictability [3]. However, PTFE is associated with low adhesiveness for cells, total absence of ability to connect to bone tissue and osseointegration, and frequent infections due to the membrane exposition to the oral cavity and lack of antibacterial properties [4]. Therefore, higher rates of wound dehiscences and the necessity of a second surgery to retrieve the membrane after the healing period are associated to the use of these membranes [4,5]. For resorbable membranes, a lack of space maintenance is the drawback, mainly due to low resorption times [4,6,7].

That is why there is a need to develop a membrane for GBR which enable clinicians to accomplish, in a more predictable way, bone regenerative surgeries [8]. Recent developments in biomanufacturing are driving innovations in the technology for membranes designing. The major efforts in membranes design are being put into: i) the creation of nanostructured membranes mimicking the native tissue [9] and ii) the active participation of the membrane in the healing and regenerative process trough drug-delivery and cells interaction, away from being a passive barrier [10–12].

Recently, it has been developed a new biomimetic membrane barrier based on the electrospun of a polymer mixture of $(MMA)_1$-co-$(HEMA)_1$ and $(MA)_3$-co-$(HEA)_2$. Nanofibrous scaffolds mimic closely the scale and morphology of the bone collagen matrix (fibers with diameters ranging from 50 to 500 nm) [8]. The composite membranes were formed with silica nanoparticles and functionalized with zinc or doxycycline. These membranes have previously been subjected to osteoblasts' culture. HOS TE85 human osteosarcoma cells were grown on the membranes for different timepoints; in which the membranes showed a clear enhancement of osteoblasts' viability and proliferation [8]. In different studies, this membranes have also produced an increase the rate of new bone formation in rabbit's skull models [13] and an inhibition of the biofilm formation *in vitro* [14] by virtue of zinc ions and doxycycline, respectively. The new composite membranes should combine both the mechanical properties of polymeric materials and the bioactivity of SiO_2-NPs [8].

The active participation of the novel membranes in the bone healing process and their ability to interact with osteoblasts trying to accelerate bone repair, away from acting as the mere physical barrier described by Nyman and Karring in the 1980s, needs to be further evaluated. For this reason, it is necessary to focus on specific factors influencing the signaling pathway of bone regeneration and osteoinduction, which can be studied using RT-qPCR. The deficiency or overexpression of the related target genes encoding osteogenic markers of interest at a specific time point of cells differentiation stages is crucial. It may provide information about cells differentiation potential and their role in matrix production and mineralization [12].

The objective of the present study was to analyze the modulation of the gene expression related to the osteogenic functional capacity of osteoblastic cells cultured on silicon dioxide composite membranes functionalized with zinc or doxycycline. The null hypothesis is that SiO_2-NPs and zinc or doxycycline addition on membranes does not affect the gene regulation of the cultured osteoblasts.

2. Material and methods

2.1. Preparation of nanostructured polymeric membranes

Nanostructured membranes were manufactured by NanomyP® (Granada, Spain) using a novel polymeric blend: $(MMA)_1$-co-$(HEMA)_1$/$(MA)_3$-co-$(HEA)_2$ 50/50 wt, doped with 5 % wt of SiO_2-NPs. The membranes were incubated during 2 h in a sodium carbonate buffer solution (333 mM; pH = 12.5) in order to activated them with carboxyl groups (HOOC-Si-M). This functionalization is possible due to the partial hydrolysis of ester bonds and the disposal of carboxyl groups on the surface of the artificial tissue [15]. Then, membranes were rinsed with distilled water and dried in a vacuum oven [16]. In a second step, the membranes were functionalized with zinc using the ability of carboxyl groups to complex divalent cations. Doxycycline (Dox), was immobilized on the membranes by acid-base interactions between amino groups of Dox and carboxyl groups present in the membranes. In order to achieve this, HOOC-Si-M were immersed under continuous shaking at room temperature and in aqueous solutions (pH = 7) of both 330 mg L^{-1} of $ZnCl_2$ and 800 mg L^{-1} of Dox [8]. Four different membranes were designed: 1) Non functionalized and SiO_2-NPs undoped membrane (HOOC-M), 2) SiO_2-NPs doped membrane (HOOC-Si-M), 3) SiO_2-NPs doped membrane functionalized with Zn (Zn−HOOC-Si-M) and 4) SiO_2-NPs doped membrane functionalized with Dox (Dox−HOOC-Si-M). The control group in our experiment (HOOC-M), acts as negative control, since it is the group where the independent variable being tested (zinc and doxycycline doping) cannot influence the results. This isolates the independent variable's effects on the experiment and rules out alternative explanations of the experimental results. The membranes were, then, placed at the bottom of 24-well plate (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ) and sterilized using an ultraviolet radiation sterilization desk (J.P. SELECTA, Barcelona, Spain).

2.2. Cell culture

The human MG63 osteosarcoma cell line was purchased from the American Type Culture Collection (Manassas, VA). This cell line is commonly used as an osteoblast model because it shares the same characteristics with primary human osteoblasts. MG63 osteoblast-like cells have no interspecies differences with primary human osteoblasts, have a shorter isolation time and there is unlimited accessibility [12]. The MG63 cell line was maintained as described by Díaz Rodríguez et al. [17], in Dulbecco's modified Eagle medium (DMEM; Invitrogen Gibco Cell Culture Products, Carlsbad, CA) with penicillin 100 IU/mL (Lab Roger SA, Barcelona, Spain), gentamicin 50 mg/mL (Braum Medical SA, Jaen, Spain), amphotericin B 2.5 mg/mL (Sigma), 1% glutamine (Sigma), and 2% HEPES (Sigma) supplemented with 10 % of fetal bovine serum (FBS; Gibco, Paisley, UK). Cultures were kept in a humidified atmosphere at 37 °C of 95 % air and 5% CO2. Cells were detached from the flask using 0.05 % trypsin (Sigma) and 0.02 % ethylenediaminetetraacetic acid (EDTA; Sigma) solution. After it, they were rinsed and resuspended in complete culture medium with 10 % FBS as described by Manzano-Moreno et al. [18]. Osteoblasts were seeded at 2×10^4 cells/mL per membrane, which had previously been placed into a 24-well plate. They were then cultured at 37 °C in a humidified atmosphere of 95 % air and 5% CO_2. Before seeding, the cell density had been adjusted by means of a Neubauer chamber in order to achieve the previously mentioned cell density [19,20].

2.3. RNA extraction and cDNA synthesis (reverse transcription)

After 48 h of culturing osteoblasts with the experimental membranes, the cells were detached using 0,05 %trypsin-EDTA solution (Sigma). A silicate gel technique was used to extract the mRNA, provided by Qiagen RNeasy extraction kit (Qiagen Inc., Hilden, Germany). UV spectrophotometry at 260 nm (Eppendorf AG, Hamburg, Germany) was used to calculate the amount of extracted mRNA from the cells and to determine the contamination with proteins using the 260/280 ratio. After this, 1 μg of mRNA of the osteoblasts cultured on each type of membrane was brought to 40 μL of total volume, reverse-transcribed to cDNA and amplified by PCR with iScriptTM cDNA Synthesis Kit (Bio-Rad laboratories, Hercules, CA, USA) according to the manufacturer's instructions [21].

2.4. Real-time polymerase chain reaction (RT-PCR)

Primers were design using NCBI- nucleotide library and Primer3-design to spot mRNA of Runx-2, OSX, ALP, OSC, OPG, RANKL, Col-I, BMP-2, BMP-7, TGF-β1, VEGF, TGF-βR1, TGF- βR2, and TGF-βR3 (Table 1).

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

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

Final results were normalized using ubiquitin C (UBC), peptidyl-prolyl isomerase A (PPIA), and ribosomal protein S13 (RPS13) as stable housekeeping genes [22,23].

The SsoFast™ EvaGreen® Supermix Kit (Bio-Rad laboratories) was used following the manufacturer's recommendations in order to conduct the quantitative RT-PCR (q-RT-PCR). The cDNA samples were placed in 96-well microplates and an IQ5-Cycler (Bio-Rad laboratories) was used to amplify the genetic information. Over 40 cycles were performed, with a specific annealing temperature ranging from 60 to 65 °C and an elongation temperature of 72 °C. PCR reactions were performed in a total volume of 20 μL, of which 5 μL were obtained from the cDNA samples and 2 μL from the specific primer. Standard curves for each targeted gene were constructed by plotting Ct values against log cDNA dilution. Afterwards, nonspecific PCR products and primer dimers were ruled out creating a melting profile and carrying out an agarose gel electrophoresis. The values of mRNA concentration for each gene was expressed as the proportion of ng of mRNA per average ng of housekeeping mRNA [24]. This process was performed in triplicate. Cells were seeded in three independent membranes for each of the four experimental groups (12 samples), and 5 replicates were obtained from each well for PCR analysis ($n = 60$).

2.5. Statistical analysis

Mean and standard deviations are expressed in ng mRNA/ng HK. Mean comparisons were conducted by One-way ANOVA and Tukey tests. Significance was set at $p < 0.05$. Five independent experiments were performed. Data were expressed as means ± standard deviation (SD).

3. Results

Results from qPCR analysis are displayed in Fig. 1. In general, the results shown that the blending of silica NPs in the tested non-resorbable polymeric scaffold improves the expression of osteogenic genes involved in bone formation and regeneration over the control HOOC-M group. The up-regulation of osteogenic markers in osteoblasts seeded at silica blended nanofibers was observed when analyzing the expression of BMP-2, BMP-7 and OSC ($P < 0.05$, $P < 0.05$ and $P < 0.001$) (3.1, 1.7, and 4-fold change increases, respectively). The expression of genes encoding TGFβ-1, TGFβ-R1 and VEGF in cultured osteoblasts on Si−HOOC-M were found to exhibit a significant overexpression when compared to the control group ($P < 0.008$, $P < 0.001$, $P < 0.009$) (1.2, 2 and 2.2, fold increases, respectively). We did not find changes in the expression of TGFβ-R2 or TGFβ-R3 when silica blending of nanofibers was achieved ($P > 0.6$, $P > 0.29$), neither when analyzing the osteogenic markers OSX, Col 1, ALP or OPG ($P > 0.41$, $P = 1$, $P > 0.98$, $P > 0.97$). RANKL encoding osteoclastogenic factors was down-regulated (1.6 times lower) in osteoblasts seeded on HOOC-Si-M if compared to the control HOOC-M ($P < 0.05$). Runx2 also exhibited down-regulation (1.5-fold change decrease) in the membranes blended with silica NPs if compared to control group ($P < 0.02$).

After, doxycycline doping of experimental scaffolds, several osteogenic markers were up-regulated, as BMP-2, ALP and OPG respect to the HOOC-Si-M ($P < 0.001$); if these values are compared to the control group the fold change increases were 7, 3.5 and 3.2, respectively ($P < 0.001$, $P < 0.001$, $P < 0.001$). TGFβ-1 and TGFβ-R1 were also overexpressed respect to HOOC-Si-M ($P < 0.001$), and respect to the control HOOC-M values were 1.8 and 3.3 times higher, respectively ($P < 0.001$). RANKL was down-regulated about 8.7 times respect to HOOC-Si-M ($P < 0.007$) and represents a 13.9-fold change decrease, if compared to HOOC-M ($P < 0.001$). Col-1 expression was down-regulated, it was about 900 times lower, in the presence of doxycycline ($P < 0.05$) if compared to HOOC-M. No influence of doxycycline was encountered when considering the expression of TGFβ-R2, TGFβ-R3, VEGF, OSC, or BMP7 if compared to HOOC-Si-M ($P > 0.99$, $P > 0.26$, $P > 0.99$, $P > 0.93$, $P > 0.73$, respectively).

When osteoblasts were cultured on zinc-doped membranes, an over-representation of genes encoding Col 1 (2.4-fold increase) was identified if compared to control or HOOC-Si-M groups ($P < 0.02$). ALP was also increased by Zn−HOOC-Si-M if compared with HOOC-Si-M (1.8-fold increase and $P < 0.05$). TGFβ1 values were 1.4 times higher than control ones ($P < 0.001$) and 1.2 greater than values attained with HOOC-Si-M ($P < 0.02$). Down-regulation of VEGF, OSC and RANKL gene expression was found; mean values were about 4.3, 4.6 and 5.2 times lower than those values from the control group, respectively ($P < 0.001$, $P < 0.001$, $P < 0.02$).

The resultant OPG/RANKL ratios were expressed in Fig. 2. They indicate that the highest bone-building osteoblasts activity was achieved when cells were cultured on Dox−HOOC-Si-M; if compared to the control or HOOC-Si-M groups, a 28 and 14.5-fold changes increase was evidenced, respectively ($P < 0.001$). When analyzing the HOOC-Si-*Zn*-M group, the ratio was also different from HOOC-Si-M and HOOC-M; the OPG/RANKL ratio was 7.3 and 14.2 times higher, respectively ($P < 0.05$).

The genes TGFβ-R2 and OSX were not differentially regulated in osteoblasts seeded on silica blended scaffolds, neither in those cultured on zinc nor doxycycline doped membranes (ANOVA P values were 0.44 and 0.23, respectively).

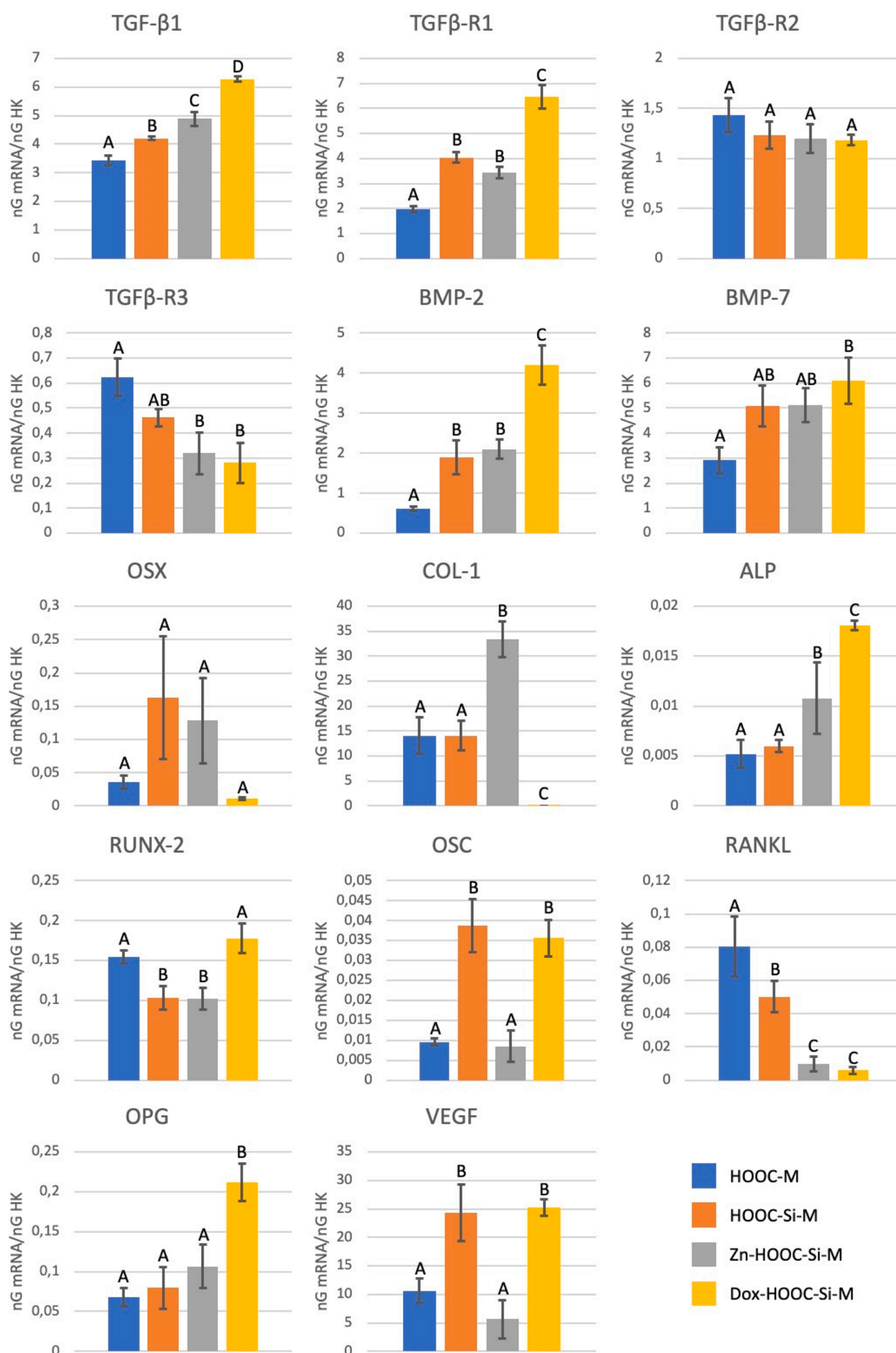

Fig. 1. Quantitative real-time PCR gene expression analysis of TGFβ-1, TGFβ-R1, TGFβ-R2, TGFβ-R3, VEGF, BMP2, BMP7, OSC, RANKL, OPG, OSX, Col-I, ALP, OPG, Runx-2 established for cultured osteoblasts seeded on the several experimental membranes, after 48 h. Results were expressed as mean and standard deviation and presented in ng mRNA/ng HK. Different letters indicate significant difference after One-way ANOVA and Tukey multiple comparisons ($p \leq 0.05$). HOOC-M: Non-functionalized membrane. HOOC-Si-M: SiO_2 nanoparticles-doped membrane. Zn−HOOC-Si-M: SiO_2 nanoparticles-doped membrane functionalized with Zn. Dox−HOOC-Si-M: SiO_2 nanoparticles-doped membrane functionalized with Doxycycline.

4. Discussion

A new approach to addressing difficult tissue regeneration problems in the oral cavity is to engineer new tissue by using selective molecules doped on polymer scaffolds. The objective is to develop membranes with suitable properties which would mimic the natural extracellular matrix and able to induce the proliferation and differentiation of osteoblastic cells. Previous research has demonstrated that osteoblast-specific factors are important at several stages of bone regeneration. Osteogenic markers may be determined by RT-qPCR and are a valuable and sensitive tool to examine osteogenic *in vitro* differentiation [24]. RT- qPCR is considered as a powerful technique capable of accurately quantitating mRNA expression levels over a large dynamic range. This makes RT-qPCR the most widely used method for studying quantitative gene expression [25]. In the present study, it has been tested the capacity that the novel nanostructured polymeric membranes loaded with silica NPs possess to modulate the expression of different osteoblast's genes, which are related to the functional capacity of this population and, therefore, with the bone regeneration process.

Two of the more greatly influenced genes by Dox−HOOC-Si and Zn−HOOC-Si membranes, were RANKL and OPG (Fig. 1). RANKL is produced by osteoblasts and stimulates osteoclasts via its receptor RANK, which is a membrane-bound protein present in osteoclasts and their precursors [24]. This interaction between RANKL and RANK can be

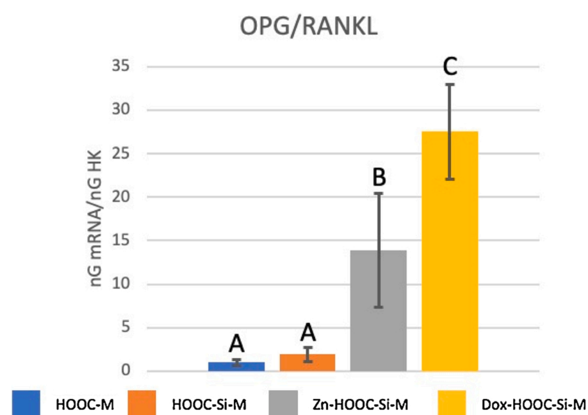

Fig. 2. OPG/RANKL ratio. Different letters indicate significant difference after One-way ANOVA and Tukey multiple comparisons ($p \leq 0.05$). HOOC-M: Non-functionalized membrane. HOOC-Si-M: SiO_2 nanoparticles-doped membrane. Zn−HOOC-Si-M: SiO_2 nanoparticles-doped membrane functionalized with Zn. Dox−HOOC-Si-M: SiO_2 nanoparticles-doped membrane functionalized with Doxycycline.

inhibited by OPG, a soluble protein also produced by osteoblasts which is associated to osteoblasts bone forming activity [26,27]. These underlying cellular mechanisms that mediate the coordinated activity between actions of bone-building osteoblasts and bone-resorbing osteoclasts, are critical for bone remodeling [26]. In our study, RANKL was substantially down-regulated in the presence of Dox and Zn, while OPG was up-regulated in these same groups, leading to a quite positive OPG/RANKL ratio [26]. Due to these results, osteoclasts would presumably be inhibited via RANKL down-regulating and OPG up-regulating. These cellular pathways would produce a positive balance in bone formation [28]. In the present study, attained OPG/RANKL ratios indicate that the highest bone-building osteoblasts activity may be attained by cells cultured on Dox−HOOC-Si membranes; a 28-fold increase was evidenced if compared to HOOC-M (Fig. 2). Similar results were previously found, demonstrating the ability of doxycycline to activate WNT-1b and neutralize Dkk-1. It results in an increase in osteoblast numbers and a decrease in osteoclast cells, also evidenced by a high OPG/RANKL ratio [29,30]. Tetracycline has been shown to significantly repress the RANKL-induced mRNA expression of the MMP-9 target genes in a dose-dependent manner, and produces inhibitory regulation of osteoclast-specific genes. It has been shown to inhibit osteoclast differentiation by modulating MMP-9 mediated proteolysis [31].

Other genes associated with functional mechanisms relevant to bone healing, as TGFβ-1and TGFβ-R1, were also differentially overexpressed (Fig. 1). TGF-β1 is involved in the control of proliferation, migration, differentiation and survival of different kinds of cells. It plays a crucial role on angiogenesis and inflammation. TGF-β1 is able to regulate the development and maintenance of bone [32]. TGF-β superfamily members play a main role in bone tissue repair and remodeling [33]. It is also known to upgrade matrix production and enhance osteoblast differentiation while reducing the ability of osteoblasts to secrete RANKL. Therefore, TGF-β1 could indirectly limits further osteoclast formation [24]. The addition of silica NPs as well as incorporating Zn and Dox to the membrane's formulation, have also shown to increase the expression of TGF-β1; thus, improving the bone formation potential of these novel membranes.

BMP-2 is also an important gene in osteogenic induction [34]. It plays a major role in bone formation/remodeling, development and in osteoblast differentiation [35]. It should be considered that the expression of BMP-2 in osteoblasts seeded on HOOC-Si-M and Dox−HOOC-Si membranes groups was significantly higher than that in the other scaffolds (Fig. 1). It has been previously described that BMP-2 may induce the expression of ALP and other osteoblastic markers [24,36,37]. This finding is also reflected in the results of our study, in which the overexpression of BMP-2 in the HOOC-Si-M and Dox−HOOC-Si membranes can also be observed in the expression of ALP, being Dox−HOOC-Si-M the one with the highest expression of both genes.

The overexpression of TGFβ and BMPs has been associated with an increase in bone formation. This cellular mechanism can be explained in part by virtue of the activation of Smad or MAPKs cascade, which are common pathways for both TGFβ and BMPs [38].

ALP is an enzyme involved in bone tissue mineralization. It increases when mineralization is well progressed during osteoblastic differentiation [39]. ALP is usually classified as an early marker of osteogenic cell differentiation [40]. ALP was significantly upregulated in both Zn−HOOC-Si-M and Dox−HOOC-Si-M (Fig. 1).

It is important to stress that Col-I expression was down-regulated (about 900 times) in the presence of doxycycline (Fig. 1). Collagen type-I is the predominant component of the bone extracellular matrix during osteoblast maturation. Collagen production has an important role in biomineralization. However, Col-I is expressed in high levels at the end of the proliferative osteoblasts state and during the period of matrix deposition [41]. In accordance with this, Col-I is expressed during the proliferative phase being, like ALP, an initial marker of osteoblast differentiation [40]. Both Col-I and ALP are observed in the first stages of osteoblast differentiation and persist in early and mature osteoblasts [24,42]. Then, it may be that Col-I overexpression may only be detected at a certain time of osteoblasts proliferation. At the present study, it was also encountered an upregulation of Col-I at osteoblasts cultures seeded on the Zn−HOOC-Si-M, indicating major activity on un-mineralized matrix deposition. It has previously been shown this effect on osteoblasts proliferating on poly-ethilen-glycol electrospun fibrous composites loaded with Zn_2SiO_4 bioceramic nanoparticles [43].

Runx-2 is as a key transcription factor associated with osteogenic differentiation [44], but exhibited down-regulation (1.5-fold change decrease) in the membranes blended with silica NPs; the same effect was found for silica NPs in bioengineered scaffolds [26]. The osteogenic markers Runx-2, Col-I and ALP and osteonectin, are major bone-related genes during osteogenic differentiation of stem cells. In osteoblasts cultures, it is established that Runx-2 (formerly called Cbfa1), a member of the runt homology domain transcription factor family, plays a crucial role in osteoblast development [43]. These genes are early osteogenic markers, indicating differentiation into mature osteoblasts; attained decreases could be due to the temporal changes in its mRNA expression during osteogenesis [40,45,46].

Present research was performed with MG63 osteosarcome cell line. It may be considered as a study limitation, as per the review by Czekanska et al., 2013 [47], cell lines such as the MG63 demonstrate some distinct similarities with primary human osteoblasts, and should not be used to replace primary cell studies. Therefore, future studies with primary human osteoblasts would be carried out in order to further permit comparisons between both cell types' behavior, when in contact with the tested membranes. Research including alkaline phosphatase activity, antigenic phenotype, phagocytic activity, immunofluorescence staining and Alizarin Red staining should also be performed as they would allow understanding about cell function and behavior under the influence of newly developed membranes.

Our results show that these novel membranes may have crucial implications for tissue engineering strategies, as it has been demonstrated that their modification with silica nanoparticles and doxycycline generated bioactive signaling. HOOC-Si-Dox membranes can effectively direct gene expression and differentiation of osteoblasts and probably of other progenitor cell populations, facilitating bone regeneration. HOOC-Si-Dox membranes do also posse a demonstrated antibacterial effect on periodontal pathogenic biofilms [14]. Therefore, they may be potential candidates for use in bone repair, at those pathologies having an infectious etiologic component (i.e. periodontal disease or peri-implantitis). Doxycycline has also been probed to produce

decreases in inflammatory infiltrates in bone [29], which may also be advantageous in these reparative process.

The main limitation of the present study is the lack of mechanistic assays. However, when these assays are attempted [48], although they contribute to understand a particular mechanism of action, they hamper the multi-regulatory processes present in these complex biological processes of genes expression regulation, and results have not clear application. However, by identifying the over or down-regulation of the targeted genes, the results may be successfully translated to the behavior that the new biomaterials would have in the clinic. The obtained results, pose an insight of integrated or holistic response rather than focusing on individual events. Nevertheless, challenging new experiments for discovering unknown interactions of doxycycline with target genes and molecular pathways descriptions are pendant for future research. Efforts to understand the predictability and translation of these assays to humans will also be performed in the near future.

Zinc and doxycycline have been previously shown to produce beneficial effects in some specific osteoblasts bone-related genes expression [29,30,43]. However, in the present study, a big step-forward is produced, as it has been shown for the first time that appropriately doped GBR membranes may act as carrier of these proliferation and differentiation factors. Novel developed membranes will potentially produce bone repair by the principle of osteoinduction, being active biomaterials in the regenerative process.

5. Conclusions

In the present study, it has been demonstrated that silica loading may offer beneficial effects to experimental membranes; an upregulation of several osteogenic markers and a highly favorable OPG/RANKL ratio were encountered. Moreover, additional doxycycline doping facilitates overexpression of BMP-2, ALP, OPG, TGFβ-1and TGFβ-R1 target genes. Dox-HOOC-Si membranes may be a potential candidate for use in GBR procedures in several challenging pathologies, including periodontal diseases and peri-implantitis.

CRediT authorship contribution statement

Manuel Toledano-Osorio: Investigation, Methodology, Conceptualization, Formal analysis, Data curation, Writing - original draft, Writing - review & editing. **Francisco Javier Manzano-Moreno:** Investigation, Methodology, Conceptualization, Formal analysis, Data curation, Writing - review & editing. **Manuel Toledano:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Writing - review & editing. **Raquel Osorio:** Investigation, Methodology, Conceptualization, Formal analysis, Data curation, Funding acquisition, Supervision, Writing - original draft, Writing - review & editing. **Antonio L. Medina-Castillo:** Methodology, Visualization, Writing - review & editing. **Víctor J. Costela-Ruiz:** Methodology, Visualization, Writing - review & editing. **Concepción Ruiz:** Conceptualization, Formal analysis, Investigation, Methodology, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare no conflict of interest.

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