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Research Article

Gene Expression of the RANK/RANKL/OPG System on a Three-Dimensional Culture of Human Periodontal Fibroblasts Under Continuous Compression

Andrea Cristina Baptista Coelho de Faria*, Antonio Carlos Aloise and Lidya Masako Ferreira

Surgical Translational Graduate Program at Unifesp, Rua Botucatu, São Paulo- SP, Brazil

***Corresponding Author:** Andrea Cristina Baptista Coelho de Faria, Surgical Translational Graduate Program at Unifesp, Rua Botucatu, São Paulo- SP, Brazil.

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Abstract

Introduction: The cultivation of periodontal ligament fibroblasts, associated with the simulation of compression or tension on the cell, enables the analysis of changes in morphology and identification of molecules and protein expression. The system formed by the receptor activator of nuclear factor κB (RANK), receptor activator of nuclear factor κB ligand (RANKL), and osteoprotegerin (OPG) is directly linked to bone remodeling, in the formation and reabsorption of bone tissue.

Objective: To evaluate gene expression of RANK \ RANKL and OPG in a three-dimensional culture of human periodontal fibroblasts in chitosan and xanthan membranes.

Method: Immortalized human periodontal fibroblasts were used. Cells were randomized into two groups: control group (CG), with three-dimensional cultivation without application of load for 6h, and experimental group (EG1, EG2, EG3 and EG4), with three-dimensional cultivation with application of 4, 12, 24, and 48 g/cm^2 loads for 6h, respectively. Cell mRNA was extracted from all groups and evaluated through real-time polymerase chain reaction (RT-PCR) and TaqMan probe. Results: No RANK / RANKL expression was detected, however OPG expression occurred, which was significantly higher in EG2 compared to CG, EG1, EG3 and EG4.

Conclusion: The 12g/cm^2 load applied onto a three-dimensional culture of periodontal fibroblasts for 6 hours displayed greater OPG expression.

Keywords: RANK; RANKL; OPG; RT-PCR

Introduction

In recent years, techniques for culturing several types of cells *in vitro* have enabled the analysis of models which simulate certain situations and characteristics akin to those occurring *in vivo*. Among the advantages of cell culture are the fixed parameters, such as temperature control, oxygenation, osmolarity, pH and CO_2 control [28]. Cells that reside, proliferate, and differentiate in living organisms are arranged in a three-dimensional (3D) microenvironment known as the extracellular matrix (ECM). The ECM is a complex structure, formed by components synthesized and deposited on the outer surface of the cell (collagens, glycoproteins, and proteoglycans), which provide structural and functional integrity

for connective tissues and organs [4,6]. Several attempts have been made to establish a culture system that mimics *in vivo* interactions between cells and the ECM. To reconstitute the *in vivo* environment, cells must adhere and grow properly in three-dimensional (3D) scaffolds or substrates, thus facilitating cell-cell contact and expression of the expected phenotype [7,22]. Regarding physiology, shape, and environment, cells cultured in a 3D matrix represent more closely the natural structure and function of tissues *in vivo* [5,23,33].

Mechanotransduction is the process of producing a biochemical reaction from a mechanical stimulus. Biochemical chain reactions induced by mechanical stimuli act at cellular level and can cause, among other effects, inhibition of apoptosis, increased cell proliferation, and altered cell migration. For example, when chewing, the mechanical forces transferred onto the teeth are dissipated through the periodontal ligament. Histological studies of the tissue around the teeth, have shown that this force exerts tension associated with bone formation, as well as bone resorption inducing compression [21]. Research in the field of cell biology often aims to identify which molecules are present in this cell differentiation process while subjected to different types of loads due to tooth movement. High loads can reduce cell viability, causing apoptosis or significant tissue damage, whereas periodontal tissue cells can develop osteoblastic activity when subjected to mechanical stress [32]. Osteoclastogenesis is an indicator of such bone remodeling, responsible for cell differentiation, proliferation, and expression of the extracellular matrix [27].

Molecules involved in this bone remodeling are osteoprotegerin (OPG), receptor activator of nuclear factor κB (RANK), and receptor activator of nuclear factor κB ligand (RANKL). The RANKL/OPG/RANK is an important signaling path, which plays a key role in bone resorption and can be modified by osteoblasts, cementoblasts and osteoclasts [13,20]. RANKL facilitates bone resorption through osteoclasts and activates bone maturation [34]. Osteoblasts also produce OPG, which acts as a receptor for RANKL and subsequently inhibits bone resorption.

Studies that evaluated cellular events in osteoclastogenesis, used polymerase chain reaction (PCR) as an instrument for precise analysis. This technique has revolutionized research in molecular biology, as it enables the obtention of enough genetic material to detect and analyze the targeted sequence under study [18,27].

The purpose of the three-dimensional substrate culture system is to promote cell propagation by using 3D matrix scaffolds. This study model involving mechanotransduction, through continuous compression of cells, enables the analysis of changes in their morphology and identification of molecules and protein expression (Figure 1).

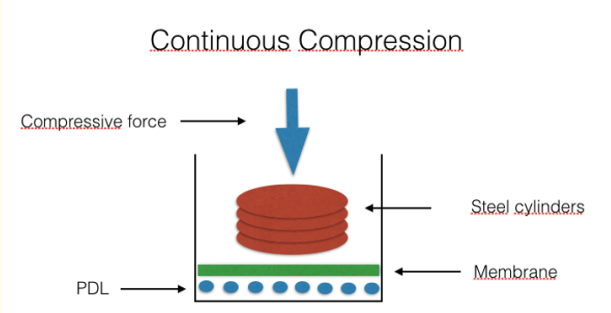

Figure 1: Schematic of the continuous compression forces applied onto chitosan and xanthan membranes, representing the three-dimensional model used.

Materials and Methods

Materials

Chitosan-xanthan scaffolds were prepared using chitosan at an 82% degree of deacetylation (Sigma-Aldrich Co; Saint Louis, MO, USA), xanthan gum (Sigma-Aldrich Co.; Saint Louis, MO, USA, with pyruvic acid content higher than 1.5%), *Pluronic*® F127 (Sigma-Aldrich Co.; Saint Louis, MO, USA), and water, purified in a Milli-Q® system (Millipore). All other reagents used were of analytical grade.

Scaffold preparation

Dense and porous Ch-X matrices were prepared as per adaptations of the procedures described by Bellini., *et al*. [15], at a Ch:X mass ratio of 1:1. The Ch-X polyelectrolyte complex was prepared using 1% (w/v) solution of chitosan (in aqueous acetic acid solution, 1% v/v) and xanthan gum (aqueous solution). Using a peristaltic pump (TE-BP-01), 200 mL of chitosan solution at a flow rate of 9 mL/min was added into a jacketed stainless steel reactor (internal diameter of 10 cm and height of 20 cm) containing 200 mL of xanthan gum solution. The temperature was kept at 37°C,

in a TE-393-80L oven (TECNAL), throughout the process, and the mixture stirred at 1000 rpm, with a mechanical stirrer equipped with a marine-type propeller (2.5 cm radius). After the addition of the chitosan solution, the system was agitated at 1000 rpm for 10 minutes. To obtain porous matrices of *Pluronic*®, F127 was added to the xanthan solution prior to the addition of chitosan. After that, the mixture was transferred to two 15 cm polystyrene Petri dishes and the solvent was evaporated in an oven with air circulation at 37°C for 24 or 48h, for dense or porous formulations, respectively. After that, the scaffolds were washed with water for 30 min (200 mL, twice), 10 mM HEPES buffer for 20 minutes (150 mL, twice) and water for another 15 minutes. Final drying was conducted at room temperature for dense formulations and at 37°C for 24h for porous formulations. For the biological tests, the scaffolds were sterilized with ethylene oxide (EO) via Oxyfume-30 exposure (30% EO and 70% carbon dioxide) for 8h at 40°C and relative humidity of 30 - 80% at Acecil Central de Esterilização Comércio e Indústria (Campinas, SP, Brazil).

Cell culture

Immortalized human periodontal ligament fibroblasts purchased from Lonza Walkersville, Inc. (Walkersville, USA), identified by the company as Clonetics™ Human Fibroblast Cell System, were used. All cell culture procedures were performed under a laminar flow hood (Vertical Laminar Flow, Pachane, Piracicaba, Brazil), and all maintenance and sterility protocols of materials and solutions used followed. In the beginning of the culture process, a DHFL-AS sowing density of 5 x 10^5 cells, in 75 cm^2 culture flasks was used (Corning, MO, USA), in a controlled environment of 37°C and 5% CO_2 (Revco-Elite II, Rio de Janeiro, Brazil). The culture medium used was SCBM™ (Lonza Walkersville, Inc. Walkersville, USA). For cell expansion, the SCBM™ culture medium was supplemented with SFB (Gibco, Gibco Industries Inc., USA), Recombinant Human Insulin, in 0.5 ml vials, Gentamicin Sulfate and Amphotericin-B, in 0.5 ml vials.

After supplementing the medium, the cells were expanded to 80% confluence in culture flasks, subcultures were performed in culture flasks to which 0.25% trypsin was used with 0.02% EDTA (Sigma Chemical Co., Saint Louis, MO, USA).

Application of continuous static compression

To mimic the pressure conditions during orthodontic movement, the following *in vitro* experiment was performed on a three-dimensional matrix, according to the method described by Nakajima., *et al.* [25]. Putting it briefly, the cells were seeded on a membrane of Chitosan and Xanthan already conformed to the bottom of 6-well plates (Figure 2).

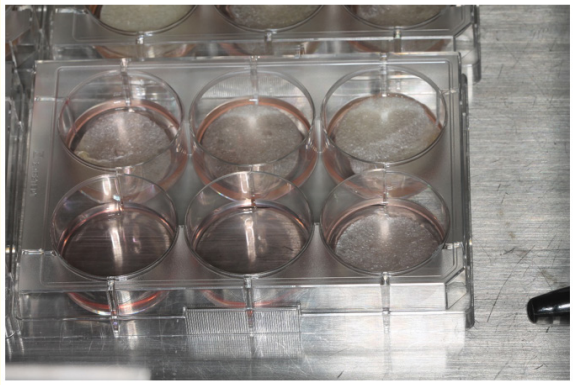

Figure 2: Periodontal ligament cells under cultivation on Chitosan and Xanthan membranes adapted to the bottom of the six-well plate.

The cells on the Chitosan and Xanthan membrane were pre-incubated in culture medium containing complete Alpha-MEM for six hours, before compression was applied. After that, weights (27 mm stainless steel cylinders) were applied onto the membranes with a compression force of 4.0 g/cm^2, 12 g/cm^2, 24 g/cm^2, 48 g/cm^2 for six hours, as shown in the model below (Figure 3 and table 1).

Gene expression evaluation

TaqMan Gene Expression Assays RT-PCR probe system was used to assess the presence of the target genes through PCR.

PCR amplification was performed in a thermocycler (QuantStudio™ 5 Digital ThermoFisher Scientific CA, USA). The advantage of real-time PCR is the possibility of accurately quantifying nucleic acids, with higher reproducibility, as it determines values during the exponential phase of the reaction.

RNA extraction

RNA extraction and purification

Total RNA was extracted with the PureLink®RNA Mini Kit Invitrogen kit (Carlsbad, Ca. 92008. USA), as per manufacturer's

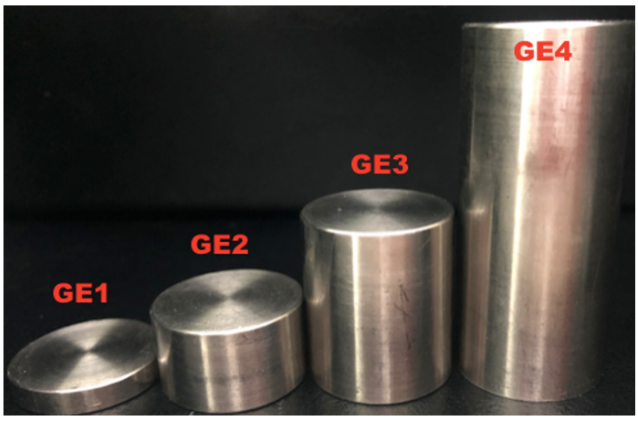

Figure 3: Static compression model used in experimental groups.

Weight	Height	Diameter	Load
22g	0,5 cm	2,7 cm	4 g/cm^2
68g	1,22 cm	2,7 cm	12 g/cm^2
138g	3,0 cm	2,7 cm	24 g/cm^2
274g	6,1 cm	2,7 cm	48 g/cm^2

Table 1: Weight, height, diameter, and total load of stainless steel cylinders used in the compression model.

instructions. After treatment, the culture medium was collected, and the cells, as well as the chitosan and xanthan membrane were washed with Phosphate-buffered saline solution (PBS) once. PBS was removed and 300 µL of lysis buffer plus 1% β-mercaptoethanol added directly onto the plate. The cells were homogenized with the pipette and transferred to a 1.5 mL conical tube, where they were diluted in 70% ethanol (v/v) and applied into a silica microcolumn with guanidine isothiocyanate (which retains the total RNA), and centrifuged at 12,000g, for 15 seconds, at room temperature and the eluate discarded. Then, the microcolumn was washed with 700 µL of wash buffer I (Wash Buffer I) and washed twice with 500 µL of wash buffer II (Wash Buffer II). Each washing step was followed by a 15-second centrifugation at 10,000g after 2 minutes of centrifugation at 12,000g, the purified RNA adhered to the microcolumn was eluted in 20 µL with ultrapure water, free of RNAses and DNAses.

RNA samples were decontaminated of the remaining genomic DNA using DNase I, Amplification Grade (Invitrogen). To do so, 16 µL of samples were incubated for 15 minutes at room temperature with 2 µL of DNase I Buffer and 2 µL of DNase I, Amplification Grade (1 unit/µL).

Total RNA was quantified using a NanoDrop spectrophotometer (Thermo Scientific, USA) to determine the concentration and degree of purity of the RNA for the synthesis of complementary DNA (cDNA) (Figure 4).

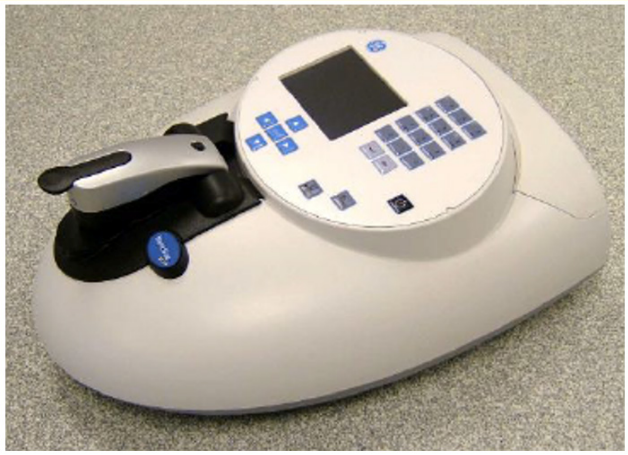

Figure 4: NanoDrop spectrophotometer to determine RNA concentration and purity.

Synthesis of complementary DNA (cDNA)

cDNA was synthesized with SuperScript™ III First-Strand Synthesis SuperMix (Invitrogen, USA).

Real-time polymerase chain reaction

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as endogenous control of the RT-PCR reaction.

The RT-PCR reaction was performed using TaqMan™ Fast Advanced Master Mix (Applied Biosystems™). To evaluate the efficiency of TaqMan™ Gene Expression Assays (Applied Biosystems™), different concentrations of cDNA (25 ng, 12.5 ng, 6.25 ng, 3.12 ng and 1.56 ng) were used for each TaqMan, in the same concentration (1 µL), in triplicates. Cts and initial cDNA concentrations were

used to develop an efficiency curve, where higher concentrations should proportionally generate lower Ct values. After the efficiency curves were obtained, 6.25 ng was determined to be the optimum concentration. For each reaction, 6.25 ng cDNA, 5 µL of TaqMan™ Fast Advanced Master Mix (Applied Biosystems™), 1 µL of TaqMan and RNAse-free water were added to a final volume of 10 µL. All reactions were performed in triplicate. After the reaction was prepared, QuantStudio™ 5 Real-Time PCR (Applied Biosystems™) was used. The amplification cycle consisted of 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, 60°C for 1 minute, and 72°C for 30 seconds. The dissociation curve was made with 1 cycle of 95°C for 1 minute, 60°C for 30 seconds, and 95°C for 30 seconds. The number of cycles that pass the Ct threshold, in which all samples can be compared, was determined from the amplification curves generated. The relative quantification method was used with these values, through which the results obtained from the analysis of the genes in question were compared with the reference gene - GADPH. This way, arbitrary levels of mRNA were expressed as a difference of "n" times in relation to the gauge (control group). Relative genes expression was calculated using the 2-ΔΔCt method, where: Ct: indicates the number of the cycle in which the amount of the amplified gene in question reaches a fixed threshold; the lower the numerical value of Ct, the faster the number of targeted copies was achieved:

- ΔCt: Normalization in relation to the constitutive expression gene, obtained from the difference between the Ct of the gene in question and the Ct of the reference gene (in this case, GAPDH) for each sample;
- ΔΔCt: Control group ΔCt average - ΔCt of each gene for each sample.

Statistical analysis

All data were analyzed through one-way analysis of variance (ANOVA), Tukey test corrected. Values of $p < 0.05$ were considered significant and marked with *.

Results

To evaluate gene expression of periodontal ligament fibroblasts a compression model was developed, consisting of 27 mm-diameter stainless steel cylinders placed on the center of each culture plate well containing a chitosan and xanthan membrane. Polymerase Chain Reaction (PCR) was used to evaluate the presence of target genes, using TaqMan Gene Expression Assays RT-PCR.

The TaqMan Gene Expression Assays RT-PCR method displayed no gene expression of the nuclear factor κB ligand receptor activator (RANKL), as shown in table 2.

Groups	RANKL Relative Expression
CG	0,03 ± 0,01
EG1	0,04 ± 0,01
EG2	0,09 ± 0,02
EG3	0,08 ± 0,01
EG4	0,03 ± 0,01
Value of p	0,8

Table 2: Mean and standard deviation of the values of the triplicates of RANKL expression. Values in relative expression (VER) obtained via RT-PC.

CG (control group); EG1 (experimental group 1- 4g/cm^2); EG2 (experimental group 2- 12 g/cm^2); EG3 (experimental group 3- 24 g/cm^2); EG4 (experimental group 4- 48 g/cm)^2. RANKL ANOVA test with $p < 0.05$.

The TaqMan Gene Expression Assays RT-PCR method displayed no gene expression of the receptor activator of nuclear factor κB (RANK), as shown in table 3.

Groups	RANK Relative expression
CG	0,02 ± 0,01
EG1	0,07 ± 0,02
EG 2	0,03 ± 0,01
EG 3	0,02 ± 0,01
EG 4	0,03 ± 0,01
value of p	0,8

Table 3: Mean and standard deviation of the values of the triplicates of RANK expression. Values in relative expression (VER) obtained via RT-PC.

CG (control group); EG1 (experimental group 1- 4 g/cm^2); EG2 (experimental group 2- 12 g/cm^2); EG3 (experimental group 3- 24 g/cm^2); EG4 (experimental group 4- 48 g/cm^2). RANK ANOVA test with $p < 0.05$.

After reading the gene expression in RT-PCR, the analysis of osteoprotegerin expression data was conducted whose results are shown in table 4.

Groups	OPG Relative expression
CG	0,5 ± 0,1
EG1	0,6 ± 0,1
EG2	1.5 ± 0,2
EG3	0.8 ± 0,1
EG4	0,6 ± 0,1
Value of p	0,03*

Table 4: Average and standard deviation values of the triplicates of the osteoprotegetin (OPG) gene expression. Values in relative expression (VER) obtained via RT-PCR.

CG (control group); EG1 (experimental group 1- 4 g/cm^2); EG2 (experimental group 2- 12 g/cm^2); EG3 (experimental group 3- 24 g/cm^2); EG4 (experimental group 4- 48 g/cm^2). OPG ANOVA test with $p < 0.05$ marked with*.

Discussion

The periodontal ligament is a highly differentiated tissue, with fast turnover and high remodeling rates. Even though periodontal fibroblasts are predominant, other cells can also be found in this tissue, such as undifferentiated and epithelial mesenchymal cells. Not only are periodontal ligament cells responsible for maintaining the periodontal ligament, but also the alveolar bone, and root cementum [19]. The regenerative potential of the periodontal ligament is linked to the functions of periodontal fibroblasts [16]. Such cells are the most abundant in the periodontal ligament and known to modulate alveolar bone remodeling processes through the production of enzymes and active cytokines, including RANK (Receptor Activator of Nuclear Factor Kappa), RANKL (Ligand) and OPG (Osteoprotegerin), Nishijima., *et al.* [26]; Kook., *et al*. [16], when subjected to mechanical or physiological stress.

Osteoprotegerin (OPG) is the soluble attraction receptor of RANKL which can suppress the link between RANK and RANKL and block its effects on osteoclastogenesis and bone formation, whereas soluble RANKL is released from the plasma membrane by a disintegrin metalloprotease converter-TNF-α, with soluble RANKL and membrane-bound RANKL regulating osteoclastogenesis and bone formation [10,16,18,26]. Due to the difficulty in performing *in vivo* experiments, Kang., *et al*. [12], Nakajima., *et al.* [25] and Nishijima., *et al*. [26] used compressive models on periodontal fibroblasts to investigate the mechanism by which mechanical signals are transduced into biological signals, which regulate bone homeostasis via periodontal ligament.

Most cells in a native *in vivo* environment are integrated into a 3D gel, hydrated, flexible and porous, surrounded by other cells, called extracellular matrix (ECM). In this study, to reconstruct a model that more closely represents the natural structure and functions of tissues *in vivo*, a three-dimensional (3D) cellular compression model was developed to assess *in vitro* cellular events that occur *in vivo* when cells are subjected to a compressive force. Heckler., *et al*. [8] developed a three-dimensional (3D) model; in which collagen gels seeded with human periodontal ligament fibroblasts displayed cell proliferation, viability, and the appearance of a possible contractile phenotype, replicating the restricted condition of the human periodontal ligament *in vivo.*

To replicate the *in vivo* environment, cells must adhere and grow properly in three-dimensional (3D) scaffolds or substrates, thus facilitating cell-cell contact and expression of the expected phenotype [7,22]. Cells cultured on a 3D matrix represent the natural structure and function of tissues *in vivo* more closely with respect to the physiology, shape, and environment of the cell [5,23,33].

Mechanotransduction can be defined as the process of producing a biochemical reaction from a mechanical stimulus. Biochemical chain reactions induced by mechanical stimuli act at cellular level and can inhibit apoptosis, increase cell proliferation, alter cell migration, among other effects. For example, while chewing, the mechanical forces transferred onto the teeth are dissipated through the periodontal ligament. Histological studies have shown that while submitted to this force the tissue around the teeth display tension associated with bone formation, and compression on the other side, thus inducing bone resorption [21]. Research in the field of cell biology often seeks to identify which molecules are present during this process of cell differentiation when subjected to different types of loads during tooth movement. High loads can reduce cell viability, causing apoptosis or significant tissue damage, whereas cells in periodontal tissue can develop osteoblastic activity when subjected to mechanical stress [32]. An indicator of

this remodeling is the expression of osteoclastogenesis markers: osteoprotegerin (OPG), Receptor Activator of Nuclear kB (RANK) and Receptor Activator of Nuclear kB ligand (RANKL) responsible for cell differentiation, proliferation, and the expression of the extracellular matrix [27].

The RANK/RANKL/OPG system plays a key role in bone resorption and can be modified by osteoblasts, fibroblasts, and osteoclasts [13,20]. RANKL facilitates bone resorption through osteoclasts and activates bone maturation [34]. Osteoblasts and fibroblasts also express OPG, a balancing protein, which acts as a receptor for RANKL by inhibiting bone resorption.

The collagen framework has been used in tissue engineering because it has biocompatibility, biodegradability, and low antigenicity [1,17]. However, certain properties of three-dimensional collagen frameworks have limited their use due to their mild immunogenicity, lack of mechanical resistance and stability to hydration, in addition to the high cost of pure collagen [29]. Different types of natural or synthetic polymeric materials have been used in the production of substrates, or frameworks as structural models to direct cell growth. Properties such as biocompatibility, mechanical resistance, low toxicity, fluid absorption capacity, pore size and shape, are substantially important in the selection of the raw materials used to produce substrates or frameworks [2,3].

In the present study, the porous chitosan and xanthan membrane was developed as a three-dimensional (3D) scaffold or substrate, corroborating the work of Uygun., *et al*. [30], who reported membrane thickness as an important factor to achieve greater cell proliferation and differentiation. Varoni., *et al.* [31] who evaluated periodontal regeneration in nude mice, using porous and dense chitosan membranes, observed high biocompatibility, cell growth and vascularization within the porous membrane, suggesting a promising candidate for periodontal regeneration.

Li., *et al.* [18], used a (3D) model comprised of poly-L-glycolic polymer and poly-L-lactic acid matrix seeded with periodontal ligament cells subjected to compression force. The relationship between OPG and RANKL was significantly decreased for 3 and 6 hours, whereas, OPG expression decreased after 3 hours, increased for 6 hours, and was significantly expressive after 12 hours.

Mitsuhashiet., *et al.* [24] observed that recombinant protein 70 (HSP70) can modulate the expression of TNFx and RANKL mRNA in periodontal ligament cells during compression, depending on time and magnitude. Li., *et al*. [18] evaluated a three-dimensional model with periodontal ligament tissue using a thin sheet of lactic-co-glycolic acid as a framework. After applying the compression force of 25 g/cm^2 for 6, 24 and 72h, an increase in RANKL expression was observed for 6 and 24h, and a decrease in OPG expression. A decrease in RANKL expression and increase in OPG expression were observed, as the time increased to 72h. However, when Nishijima., *et al*. [26] and Nakagima., *et al.* [25] used compressive models in monolayer (2D) human periodontal fibroblast cultures, an increase in RANKL expression and a decrease in OPG expression were observed, under compression force of 4 g/cm^2 for a period of 48h. In those two studies, the results showed that the compression force magnitude and elapsed time are directly linked to increase in RANKL production. On the other hand, when Kanzaki*., et al*. [11] evaluated RT-PCR expression profiles of micro RNAs in periodontal fibroblasts submitted to tension strength and compression, they observed that the expression of certain micro RNAs increased regardless of time under stress, whereas other micro RNAs increased their expression only at specific times. This is in line with Kanzaki., *et al*. [10], Nishijima., *et al*. [26], Kook., *et al.* [15] and Kook., *et al.* [16] who suggested that periodontal ligament fibroblasts under stress increase the expression of RANKL and decrease the expression of OPG, thus facilitating bone resorption during tooth movement. There is evidence that cells in 2D and 3D cultures can display different levels of gene expression, suggesting a link to the extracellular matrix which can influence cellular responses to stress [10,14,15].

In this study, OPG expression was found in all groups evaluated, however, a statistical difference was only found in the values for EG2, which used a load of 12 g/cm^2. The results of this study in relation to EG2 were similar to those found by Jainru., *et al.* [9] who used a load of 25 g/cm^2 for 3, 6 and 12 hours, obtaining a lower expression of OPG after three hours, and a greater expression of OPG after six hours of compression, thus concluding that long-term static compression would increase the expression of OPG. In the present study, there was a decrease in the expression of OPG in EG4, submitted to a load of 48 g/cm^2. Work by Li., *et al*. [18] showed an increase in the expression of RANKL after six hours of mechanical compression (25 g/cm^2). The present study, however, when using the model proposed, found no expression of RANKL, but of OPG.

OPG is a gene that responds differently to mechanical stimuli in 3D cultures, when compared to 2D, which suggests that the bound to the extracellular matrix may influence cellular responses.

Conclusion

The load of 12 g/cm^2 for 6 hours in a three-dimensional culture of periodontal fibroblasts showed greater expression of OPG.

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