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BONE STIMULATION THROUGH SONIC VIBRATION

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Due to the high demand for orthodontic treatment in adult patients, one of the great current challenges is to reduce the time of use of conventional devices, without causing damage to periodontal tissues. Thus, the control of force levels applied to brackets and orthodontic wires with friction reduction (FATHIMANI et al., 2015), combined with non-invasive approaches such as photobiomodulation (KAU et al., 2013), use of drugs (MCGORRAY et al., 2012) and low intensity laser (CRUZ et al., 2004) have been alternatives to accelerate orthodontic movement.

Since the mid-twentieth century, devices have emerged in dentistry that generate bidirectional linear sonic vibrations, known as piezoelectric systems. These devices were initially used for cavity preparation and dental wear, since the small low-frequency vibration of sonic devices results in less pain and less tissue damage (MESQUITA; LOBATO; MARTINS, 2006). However, in 2011, Jeremy Mao presented a device, the AcceleDent®, which applies the concept of using low-intensity sonic vibration force to accelerate tooth movement during orthodontic treatment. The appliance uses the application of pulsating forces to make teeth move faster through accelerated bone remodeling. This science has already been applied in other parts of the body, for example, to accelerate fracture repair and bone density in long bones.

The device devised by Mao is a removable device similar to a dental tray with a small motor that is maintained by a rechargeable battery through a docking station. The premise is simple, instead of just using constant pressure, the device applies very light vibrations to the teeth daily for 20 minutes of gentle pulsation through a silicone insert that is attached to the teeth as a protective plate.

The principle of orthodontic movement by the low-intensity sonic pulsation device is the same as that used by the biomechanical forms applied in the clinic, triggering an inflammatory reaction under the effect of pressure generated by the system, thus resulting in bone resorption and consequent tooth movement., 2013). This stimulus is characterized by the release of cytokines and chemical mediators that participate in the bone resorption and remodeling process, essential for tooth movement.

The primary stimulus, which corresponds to the exerted force, engenders the release of arachidonic acid by the plasma membrane, which is the precursor of prostaglandins. Prostaglandins increase vascular permeability by vasodilation and bind to specific receptors on inflammatory cells. The force applied in orthodontic movement then causes vascular compression and local inflammation, increasing the concentration of chemical mediators that induce chemotaxis and diapedesis of inflammatory cells (LEE, 1990). Besides this, during orthodontic movement, interleukins (IL)-1, and the tumor necrosis factor (TNF) are increased and stimulate bone resorption (BAŞARAN et al., 2006).

Thus, due to the scarcity of research that elucidates the role of low-intensity pulsed sonic devices in molecular and cellular events during osteogenesis and bone remodeling, the objective of this study was to evaluate, in vitro, the influence of sonic vibration force in culture of osteoblastic cells in the expression of IL-1 and IL-17.

The periodontal ligament is a structure that supports each dental element, connecting them to the alveolar bone. It is constituted by a network of collagen fibers arranged in parallel and that are inserted in the root cementum. (PROFFIT; FIELDS JUNIOR; SARVER, 2007). This structure of ligaments allows the normal movement of each dental

element. There is also in the periodontal ligament a network of blood vessels and nerve endings, responsible for the painful sensation, as well as undifferentiated mesenchymal cells, fibroblasts and osteoblasts (PROFFIT; FIELDS JUNIOR; SARVER, 2007).

According to Moyers (1991) the odontoblastic and osteoclastic cells are responsible for the remodeling of the bone and collagen matrix, thus remodeling the bone alveolus and the root cementum, in the face of natural forces. These forces are the result of forces transmitted through occlusion of the teeth, swallowing, speaking, chewing and breathing, these originating from the muscles. There are also forces coming from inside the teeth, such as eruption. Abnormal forces are constituted by tongue thrusting, digital suction, traumatic occlusal dysfunction, bruxism and forces induced for therapeutic purposes.

Heller and Nanda (1979) evaluated the effects of metabolic alteration of periodontal fibers on orthodontic movement in rats. The results suggested that the typical biological response to the application of orthodontic force may occur in the presence of a chemically and physically altered periodontium. Quantitatively collected data inferred that fiber tension in the alveoli may not be absolutely necessary to stimulate bone formation. Distortion of alveoli related to the applied force may be an important factor, initiating the bone response. However, the periodontal ligament fibers may play a passive role by transferring orthodontic force to the alveoli.

Amadei et al. (2006) described the factors that influence the physiology of bone remodeling and orthodontic movement, consisting of resorption by osteoclasts and the consequent formation by neoformation of a new bone matrix by osteoblasts. This interaction of osteoblasts and osteoclasts is crucial for renewal, both during bone development and during inflammatory processes of bone remodeling.

Osteoblasts are cells that synthesize bone matrix. These proliferate, mediated by transforming growth factor-beta (TGF-β), insulin-derived growth factor (IGF-I and II), fibroblastic growth factor (FGF) and platelet-derived growth factor (PDGF). These factors, in addition to helping bone formation, also prevent osteoblastic cell apoptosis. From these stimuli, cells secrete alkaline phosphatase, hyaluronic acid, chondroitin sulfate, osteopontin, osteonectin, bone sialoprotein, procollagenase, plasminogen activator and bone morphogenetic protein (BMP), among others (KESSEL, 2011).

During the inflammatory process, osteoblasts synthesize and secrete cytokines in the non-mineralized osteoid layer, which have the role of inducing and controlling osteoclast differentiation. The main factors responsible for the genesis of osteoclasts are osteoprotegerin (OPG), tumor necrosis factor activating receptor (NF-kB [RANK]), and the ligand cytokine RANK (RANKL), with the main role of inhibiting the apoptosis of these cells. Through the integrins present in the cell membrane, there is the recognition of the proteins of the extracellular bone matrix, forming focal adhesions, where the reabsorption gaps will be created. There is then stimulation of osteoclasts by calcitrol, parathyroid hormone (PTH), tumor necrosis factor (TNF), prostaglandin E2, in addition to interleukins (IL-) 1, 11 and 6 (BANDEIRA et al., 2000; LINS et al., 2007).

Bone resorption by osteoclasts occurs in several stages, including the recruitment and differentiation of hematopoietic precursors of the monocyte-macrophage lineage into pre-osteoclasts (mononuclear cells) and, successively, the fusion of pre-osteoclasts into multinucleated osteoclasts, for example:

mature osteoclasts, which are characterized by having a rim full of invaginations in their plasma membrane. The differentiation of osteoclasts from stem cells occurs by several molecules and proteins, including MCSF (macrophage colony-stimulating factor) (ROSS; PAWLINA, 2012).

According to Ross and Pawlina (2012), after recruitment, osteoclasts attach to the bone surface, triggering the synthesis and expression of RANKL by osteoblasts. Osteoblasts up-regulate osteoclast activity by secreting RANKL, the key activator of osteoclast differentiation, and down-regulate osteoprotegerin (OPG) expression, which inhibits RANKL. This ligand binds to the KB nuclear factor activating receptor (RANK), secreted by osteoclast precursors, which stimulates their differentiation into mature osteoclasts. Therefore, both RANK and RANKL, cell surface proteins, are essential regulators for the formation and performance of osteoclasts.

In orthodontics, bone apposition begins around 48 hours after tension is applied to the bone surface. Osteocytes participate in the osteogenesis process, being very sensitive and reactive to tensile forces applied orthodontically, and their cellular projections favor communication with surrounding osteocytes. Osteoblasts, which are in direct contact with osteocytes, respond to these signals by beginning to lay bone. Thus, when tension is applied to the periodontium, it induces an aseptic inflammatory reaction through several mediators (Figure 1). These mediators are called cytokines.

Cytokines are extracellular proteins that act directly in the inflammatory process and bone remodeling, bringing together the cells involved in this process, as well as activating the differentiation of mesenchymal cells. Research has shown that the use of ultrasound has the effect of activating pro-inflammatory mechanisms (CHERIAN et al., 2005; GENETOS et al., 2005; LI et al., 2005; MUKAI et al., 2005; IKEDA et al.., 2006; MADDI et al., 2006; TAKAYAMA et al., 2007; XU et al., 2007; REN; LI; LIN, 2010; ANGLE et al., 2011; AL-DAGHREER et al., 2012; GUSMÃO ; MARIOLANI; BELANGERO, 2012; XUE et al., 2013). Among the various pro-inflammatory cytokines, take, for example, the cytokines IL-1 and IL-17, the objects of this study.

IL-1 is a pro-inflammatory cytokine secreted mainly by monocytes-macrophages, and induces bone resorption by recruiting mature osteoclasts and by proliferating their precursors (DINARELLO, 1996). In vitro researches relate IL-1 with osteoclastic activation, since it is an inducing factor of increased production of M-CSF and PGE2, and inhibition of OPG production by osteoblasts (TANABE et al., 2005).

IL-17 is a cytokine produced by Th17 cells (subgroup of T lymphocytes, differentiated to produce IL-17), whose main role is to eliminate pathogens, being a potent inductor of inflammation. This cytokine can induce a wide variety of pro-inflammatory mediators (Figure 2) in several types of cells involved in tissue damage, including macrophages, by stimulating the production of MCSF, in addition to inducing the secretion of RANKL in osteoblasts, thus promoting the osteoclastogenesis (LEE, 2013).

Recent studies have related IL-17 as an important pro-inflammatory mediator. Xiong, Wei and Peng (2015) found that increased IL-17 expression stimulates the production of pro-inflammatory mediators IL-6 and IL-8 in fibroblastic cells of human dental pulp. Severino, Napimoga and de Lima Pereira (2011) demonstrated that there was a relationship between the increase in IL-17 and the production of other inflammatory cytokines (IL-6 and IL-8) in peri-implantitis.

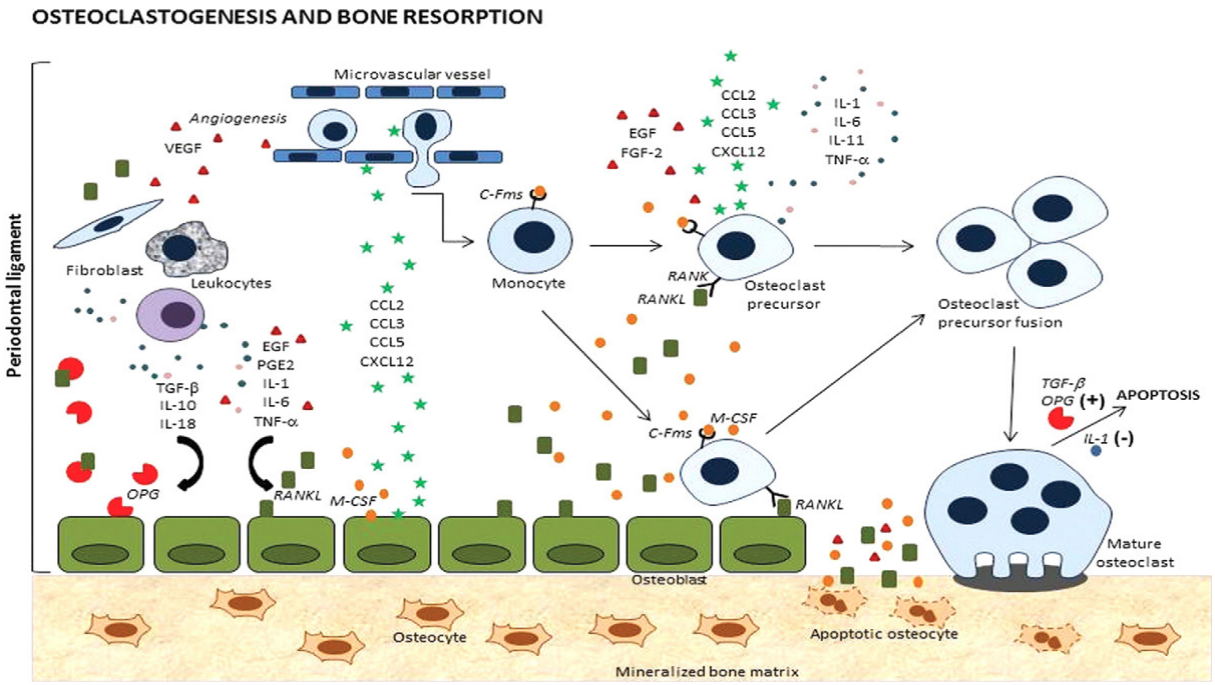


Figure 1 – Process of osteoclastogenesis and bone resorption.

Source: Andrade Junior, Taddei and Souza, 2012. p. 259.

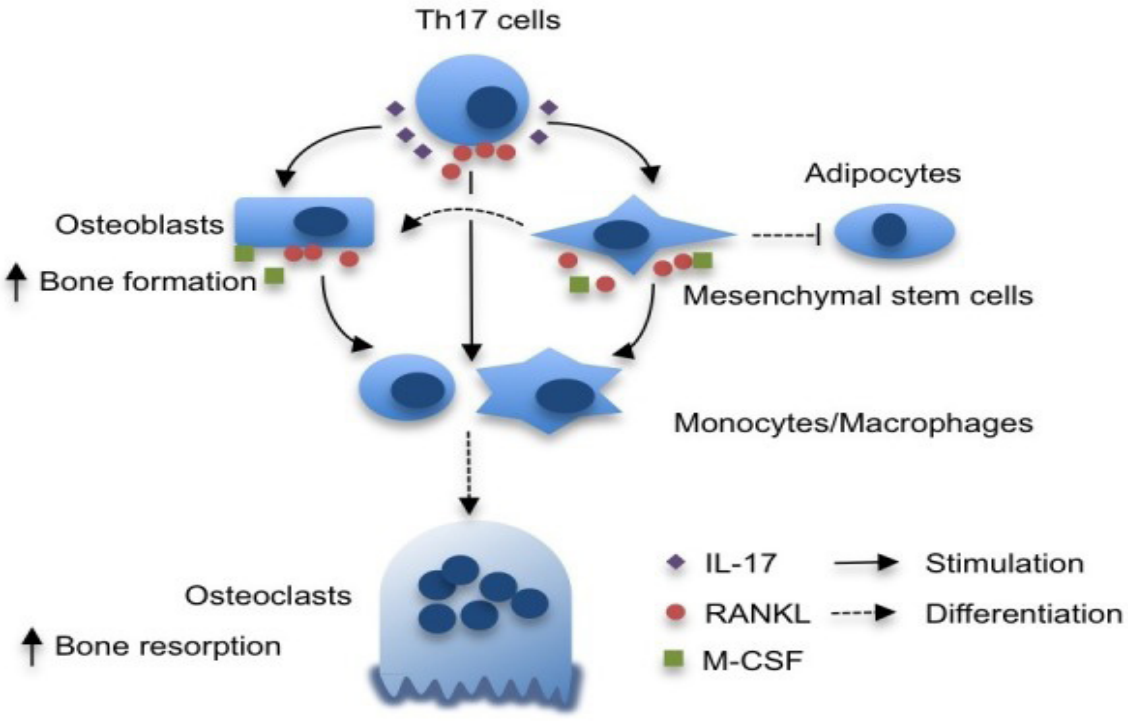


Figure 2 – Action of IL-17 on osteoclastogenesis.

Source: Lee, 2013. p. 475.

The published works related to the AcceleDent® device are clinical case reports, but there is a vast scientific material with good results regarding the use of sonic and ultrasonic vibration in the areas of physiotherapy and orthopedics with the use of the LIPUS® device.

In dentistry, the application of LIPUS® (low intensity pulsed ultrasound) in clinical therapy is clinically proven in bone remodeling and periodontal tissues. It is a physical force – mechanical vibration – that has a direct effect, both in vivo and in vitro, on osteogenic cells when applied at low frequency and low pulse. Espinndula (2014) carried out a bibliographical review in the Scielo, Lilacs, Medline and Bireme databases, published in the period from 1965 to 2014, noting that the literature recognizes that orthodontic treatment brings some limitations, such as the time required for its completion, the patient discomfort and root resorption. Electrotherapies and ultrasound show in the literature that they have a great influence on bone metabolism and are already used in the field of physiotherapy with excellent results. The author concluded that the authors obtained good results in orthodontic movement and in the recovery of teeth that suffered root resorption.

Reher et al. (1997) carried out a controlled study to evaluate the effects of ultrasound (SATA®, USA) at different intensities for 5 days on rat calvaria and tissue culture in order to analyze the following parameters: synthesis of collagen and non-collagenous proteins (NCP), as well as temperature changes associated with the application of this device. It was found that when ultrasound was used at a power of 0.1 W/ cm2, pulsed, at a frequency of 1:4.3 MHz for 5 min, it significantly stimulated bone formation, represented by an increase in collagen synthesis and NCP. However, pulsed ultrasound with higher doses (1.0-2.0 W/ cm2) significantly inhibited the synthesis of these proteins.

Angle et al. (2011) tested in cultures of mouse stem cells, the effects of LIPUS® at 2.15 and 30 mW/cm² in the primary (cell activation), intermediate (differentiation into osteogenic cells) and final (biological mineralization) stages of differentiation osteogenic. Compared to the control groups, after 5 days of using LIPUS®, cells treated at 2.15 and 30 mW/cm² had an increase of 79%, 147% and 209%, respectively, in alkaline phosphatase activity, a primary indicator of osteoblastic differentiation, demonstrating that the lower the ultrasound intensity, the greater the effect on osteogenic differentiation.

Mukai et al. (2005) applied low-intensity ultrasound (LIPUS®) to a culture of chondrocytes to analyze the action of the device. Northern analysis revealed that the device maintained higher levels of collagen type II expression, as well as alkaline phosphatase activity. In addition, TGF-β expression levels remained high throughout the ultrasound application phase.

The effects of the low-intensity pulsed ultrasound device have also been reported to increase the proliferation and protein synthesis activity of various other cell types. Ren, Li and Lin (2010) measured the length of neurites after treatment with LIPUS® to define the effectiveness of stimulation on neurons and then examined the activity of GSK-3β to study the intracellular mechanism of cell proliferation. LIPUS® was applied to cultures of neurons originating from rat primary cortices for 5 minutes, every day with average intensities of 10 mW/cm2, pulse width of 200 microseconds, repetition rate of 1.5 kHz, and a frequency operating time of 1 MHz. The neurons were evaluated after 3, 7, 10 days of treatments and, at each moment, a semi-

quantitative rtPCR analysis was performed. The results showed a change in the neurite extension morphology when using pulsed ultrasound, there was also a notable reduction of proteins including p-Akt, p-GSK-3β, and p-CRMP-2, observed at 7 and 10 days and of GSK -3β, mainly on the seventh day. The authors concluded that the ultrasound device used can reduce neuritis and, therefore, its use to control them is possible.

Al-Daghreer et al. (2012) also investigated the effect of the same low-intensity pulsed ultrasound used by Ren, Li and Lin (2010), however they applied it to human dentin-pulp complexes in an in vitro model. LIPUS® was applied using a 3.9 cm2 transducer, which produces an incident intensity of 30 mW/cm2. After 5 days, the tissue was collected for histomorphometric analysis and investigation of the expression of genes of interest (Collagen I, DMP1, DSPP, TGF-β1, RANKL and OPG) through real-time PCR. Histomorphometric analyzes showed that the odontoblastic cell count was higher in the application groups (5, 10 and 15 min, respectively) than in the control group. The predentin thickness was greater in the single application group (10, 5 and 15 min) respectively than in the daily application group and the control groups, however, they were not significantly different from each other. Real-time PCR showed no statistically significant difference between groups in the expression of collagen I, DMP1, TGF-β1, DSPP, RANKL and OPG. The authors of this work concluded that the reactional response of the dentin-pulp complex was greater when using a single application of LIPUS® in times of 5, 10 and 15 minutes.

Ikeda et al. (2006), within this context, suggested that LIPUS® accelerates osteoblastic differentiation of mesenchymal cells through MAPK phosphorylation. Takayama et al. (2007) demonstrated that the formation of mineralized nodules and their calcium content increase considerably after the 14th day of application of LIPUS®, concluding its direct effect on osteogenic cells.

Man et al. (2012), in turn, investigated the effects of osteoblast migration under low-intensity pulsed ultrasound frequency – 1 MHz in cultures of MC3T3-E1 from rat calvaria for 30 minutes. They concluded that the in vitro application of ultrasound accelerated osteoblastic proliferation and migration.

The mechanism of mechanically vibration-induced osteogenesis is not fully understood, according to Gusmão, Mariolani and Belangero (2012). In response to the mechanical stimulus, there is induction of PGE-2 synthesis culminating in the influx of calcium into the intracellular space. This pro-inflammatory cytokine is responsible for 50-90% of the osteogenesis inducing mechanism, therefore being the most important prostaglandin in the mechanotransducer mechanism, being both related to the process of bone neoformation and reabsorption. This association has been mainly related to the role of calcium, since its intracellular concentration, when increased by the mechanical stimulus, determines the synthesis of PGE-2 (CHERIAN et al., 2005; GENETOS et al., 2005; LI et al.., 2005; XU et al., 2007).

Suzuki et al. (2009) applied 1.5 MHz of LIPUS® in mouse cell culture for 7 days. Their results showed that the expression of bone morphogenetic proteins (BMP-2, -4 and -7) increased significantly in relation to the control group (without LIPUS application).

Xue et al. (2013) detected in in vivo and in vitro assays that, from the 5th day of application of LIPUS® in mouse tissues, both the distance of the orthodontic movement and the levels of BMP-2 and the expression of RANKL increased significantly if compared to the control group.

Maddi et al. (2006) verified in a culture of human osteoblasts that the use of therapeutic ultrasound can increase bone regeneration by altering the OPG/RANKL ratio. Therefore, the literature demonstrates that there is a direct relationship between the use of low-intensity pulsed ultrasound and osteoclastogenesis.

The sonic device AcceleDent® (Ortho Accel Technologics. Inc. Texas, USA) was used in the work, which uses the application of pulsating forces (soft pulsed), described as accelerators of tooth movement and bone remodeling (Ortho Guide AcceleDent®).

The device is similar to a retainer with a small motor on the handle of a simple dental tray used in the office (Figure 3). The manufacturer recommends its daily use for 20 minutes. The activator and mouthpiece assembly is lightweight (71 g), comfortable, and can be used hands-free and during other everyday activities (Figure 5). A charging base and travel case are included (Figure 4).

Figure 3 – AcceleDent®.

Source: Ortho Guide AcceleDent®, 2016.

Figure 4 – Device base.

Source: Ortho Guide AcceleDent®, 2016.

The components of AcceleDent® are:

a) activator – this small extraoral component is what generates the vibratory force, of approximately 0.25N (25 g), at a frequency of 30 Hz;

b) mouthpiece – this piece connects to the activator, in which the patient occludes so that the vibratory force derived from the activator is transferred to the dentition. They are supplied in two sizes in order to meet the anatomical dimensions of the patients' dental arches. For each size, there are three types of formats according to the type of malocclusion (anterior open bite, anterior deep bite and normal flat occlusion);

c) charging base – for recharging the device and providing information on the use of the device through a SmartDisplay LCD Screen.;

d) travel case – allows the device to be transported easily and safely.

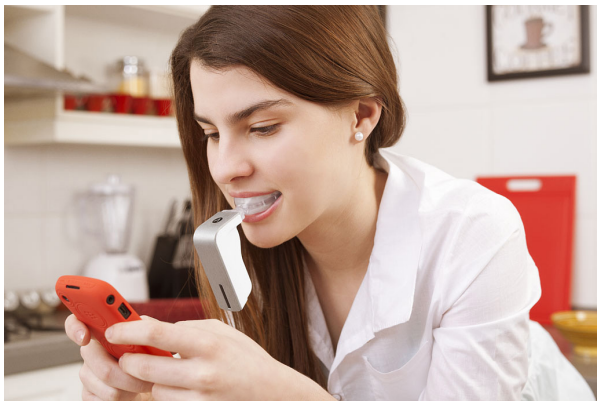


Figure 5 – Clinical application of AcceleDent®.
Source: Acceledent Ortho Accel Technologies, 2016.

CELL CULTURE

The mouse pre-osteoblast cell line (MC3T3-E1) was obtained from the ATCC (American Type Culture Collection, ATCC, USA). Pre-osteoblastic cells were cultured in Essential Minimal medium, alpha modification (α-MEM) supplemented with 10% fetal bovine serum (Cultilab®, Campinas, SP, Brazil) and 1% antibiotic-antimycotic solution (Sigma, St. Louis, Missouri, USA).

All procedures were performed in a laminar flow hood to maintain the sterility of the materials and substances used for cell culture. Cells were cultured in 24-well plates at an initial density of 110 cells/mm2 and after 24 hours of cell culture, the culture medium was changed and 20 min were used. of daily application of the sonic force of the AcceleDent® device (MUKAI et al., 2005) in direct contact with the polystyrene plates and evaluated its effects after 3, 7 and 10 days. As a control group, osteoblastic cells were not subjected to any treatment. The cells were kept in an oven at 37ºC, in a humid atmosphere containing 95% air and 5% carbon dioxide. The culture medium was changed every 3 days and the culture progression was evaluated by phase microscopy.

CELL PROLIFERATION ASSAY

For the evaluation of cell proliferation, the method of vital exclusion by Trypan blue was used after 3, 7 and 10 days of cell cultures subjected to sonic vibration.

Cells were enzymatically removed from the plates, and the cell precipitate resulting from centrifugation was suspended in 1 ml of medium. 10 µL of the cell suspension were removed and 10 µL of Trypan blue were added to it, and 1 µL of this solution was placed in a hemocytometer (Neubauer-Fisher Scientific chamber, Pittsburgh, PA, USA) and taken phase inverted microscope (Nikon, Eclipse TS100) for cell counting and observation.

The total number of cells present in each well at different analysis times was obtained through the following mathematical equation:

$$\text{Total number of cells} = \frac{\text{Number of cells counted X Volume. Initial X Dilution: X } 10^4}{\text{Number of squares used for counting}}$$

CELL VIABILITY ASSAY

Cell cultures were tested for cell viability using the MTT assay. This assay evaluates the ability of metabolically active cells to reduce MTT, converting yellow tetrazolium salts (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazole bromide) to purple formazan crystals and, therefore, on the ability of viable cells to cleave the tetrazole ring present in MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazole bromide) by the action of dehydrogenase enzymes present in active mitochondria, forming formazan crystals.

After 3, 7 and 10 days after the application of sonic vibration, 10 µL of the MTT solution (5 mg/mL - Sigma, USA) diluted in serum-free DMEM culture medium were added to the treated cultures, and these were incubated

for a period of 3 hours at 37°C. After the total incubation period, 100 µL of 10% DMSO (dimethylsulfoxide) solution were added.

After crystal solubilization, quantification was performed in an ELX800 microplate reader (Epoch Biotek Instruments, Inc.) at 590 nm, obtaining optical density (OD) measurements.

IMMUNOENZYMATIC ASSAY FOR QUANTIFICATION OF IL-1 AND IL-17 (ELISA)

The quantification of IL-1 and IL-17 secreted by osteoblastic cells subjected to sonic vibration was evaluated by Elisa. For this, the supernatant was aspirated and centrifuged at 5000g for 15 min at a temperature of 4°C. Aliquots of each sample were evaluated by enzyme immunoassay (ELISA) to determine the levels of proteins to be analyzed according to the manufacturer's recommendations (R&D Systems, USA). After that, 100 µl of detection antibody was added to all wells and incubated for 1 hour at room temperature. The plates were washed with a buffer solution (0.05% Tween 20 in PBS) and 100µl of the streptavidin-peroxidase conjugate was added to the plates for incubation for 30 minutes at room temperature. The plates were again washed and a substrate (tetramethylbenzidine) was added in the amount of 100µl for each well, and incubated for 15 minutes protected from light.

The reaction was completed by adding 50µl 2N sulfuric acid (H2SO4) to the substrate solution present in each well, and the color was measured in a spectrophotometer (Epoch, Biotek, Winooski, VT, USA) at a wavelength of 450nm. The total amount of IL-1 and IL-17 was determined in picograms (pg/ml).

The collected data were subjected to two-way analysis of variance and Tukey's tests. For statistical calculations, the SPSS 20 program (SPSS Inc., Chicago, IL, USA) was used, with a significance level of 5% ($\alpha = 0.05$).

The data relating to the cell proliferation assay under the different conditions studied are shown in Table 1 and Graphic 1. After 3 and 7 days, no difference was observed in cell proliferation when using AcceleDent® **in** relation to the control, however after 10 days, proliferation was significantly higher in the group using sonic force, when compared to the control.

CELL VIABILITY ASSAY

The data relating to the cell viability test under the different conditions studied are shown in Table 2 and Graphic 2. No difference was observed in cell viability ($p>0.05$) when using AcceleDent® when compared to the control, at any of the times evaluated.

IMMUNOENZYMATIC ASSAY QUANTIFICATION IL-1 AND IL-17 (ELISA)

Data related to IL-1 and IL-17 secretion in osteoblastic cells subjected or not to sonic force are shown in Table 3 and Graphics 3 and 4.

With regard to interleukin IL-1, it was observed at all times of culture, greater secretion of IL-1 when using sonic force compared to control ($p = 0.022$), with no difference for the different times of culture ($p = 0.348$) for each condition studied (table 3 and Graphic 3).

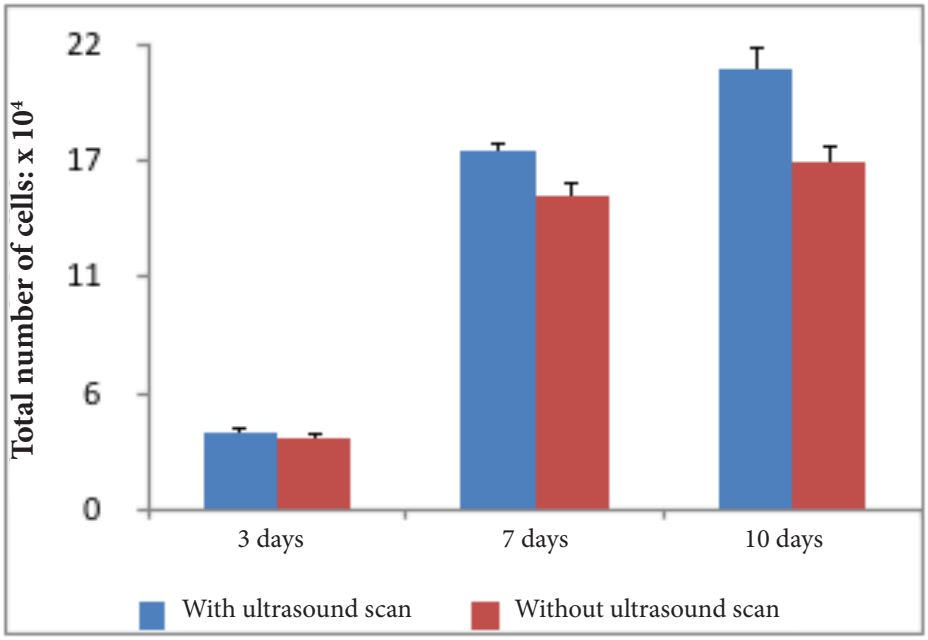
For interleukin IL-17, it was observed that after 7 and 10 days, the concentration of IL-17 was significantly higher when using sonic force compared to control ($p<0.05$). In addition, when using sonic force, the IL-17 concentration was significantly lower at time 3 days compared to times 7 and 10 days, which did not differ significantly from each other, as shown in Table 3 and Graphic 4.

Condition	Time (days)		
	3	7	10
Sonic force	3,64X10⁴ **Aa** (0,346X10⁴)	17X10⁴ **Ab** (0,357X10⁴)	20X10⁴ **Bb** (0,103X10⁴)
Control	3,37X10⁴ **Aa** (0,263X10⁴)	14,9X10⁴ **Ab** (0,584X10⁴)	16,5X10⁴ **Ab** (0,787X10⁴)

Caption: Standard deviations in parentheses. Means followed by distinct capital letters indicate a significant difference between conditions with and without sonic force. Means followed by distinct lowercase letters indicate a significant difference between culture teams, considering individually whether or not sonic force was used (horizontal lines).

Table 1 – Means and standard deviations of osteoblastic cell proliferation, as a function of the culture team, with and without application of sonic force.

Source: Own authorship.



Legend: Vertical lines over columns indicate standard deviations.

Graphic 1– Mean values of osteoblastic cell proliferation as a function of the culture team, with and without application of sonic force.

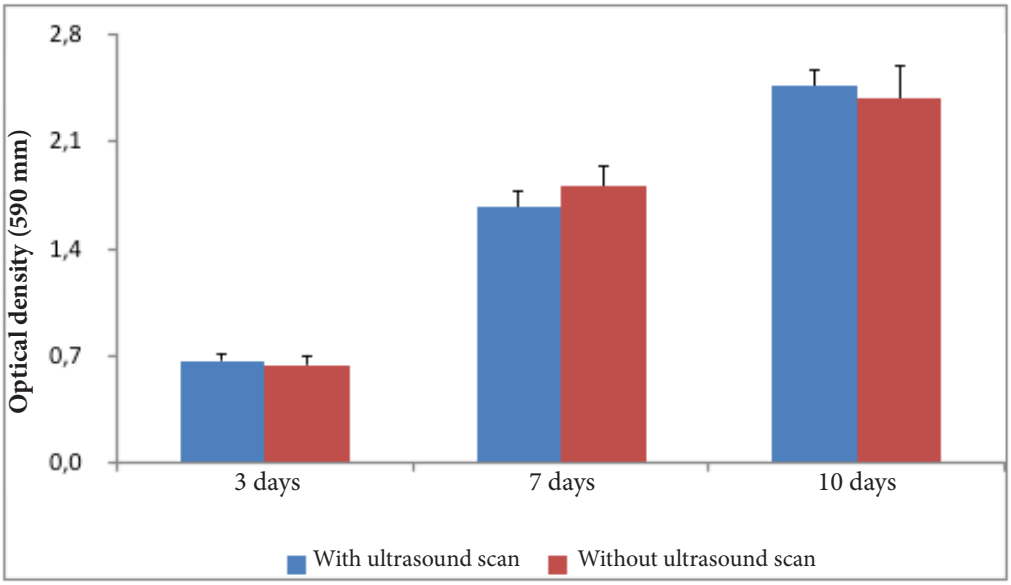
Source: Own authorship.

Condition	Time (days)		
	3	7	10
Sonic force	0,67**A, a** (0,06)	1,67**A,b** (0,12)	2,46**A,c** (0,11)
Control	0,64**A, a** (0,07)	1,81**A,b** (0,14)	2,38**A,c** (0,23)

Caption: Standard deviations in parentheses. Means followed by distinct capital letters indicate a significant difference between conditions with and without sonic force. Means followed by distinct lowercase letters indicate a significant difference between culture time, individually considering the use or not of sonic force (horizontal lines).

Table 2 – Means and standard deviations of osteoblastic cell viability, as a function of the culture team, with and without application of sonic force.

Source: Own authorship.



Legend: Vertical lines over columns indicate standard deviations.

Graphic 2 – Mean values of osteoblastic cell viability as a function of culture time, with and without application of sonic force.

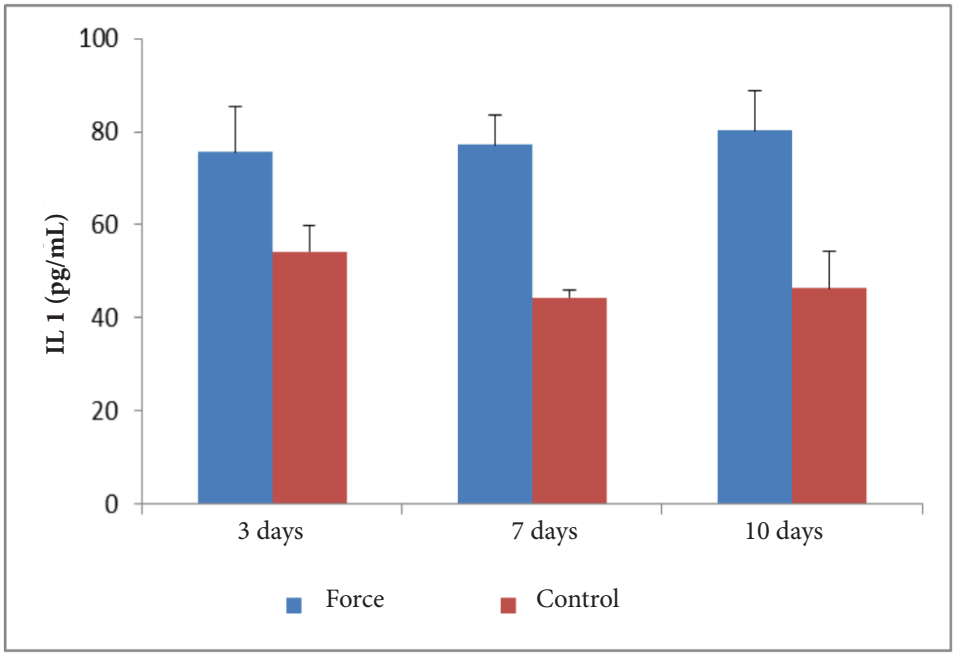
Source: Own authorship.

Condition		Time (days)		
		3	7	10
IL-1	Sonic force	75,66 **Aa** (9,60)	77,21 **Aa** (6,26)	80,30 **Aa** (8,51)
	Control	54,13 **Ba** (5,82)	44,39 **Ba** (1,42)	46,39 **Ba** (8,00)
IL -17	Sonic force	49,19 **Aa** (4,07)	77,68 **Bb** (3,82)	79,21 **Bb** (10,14)
	Control	49,45 **Aa** (7,44)	32,57 **Aa** (7,31)	47,01 **Aa** (6,37)

Caption: Standard deviations in parentheses. Means followed by distinct capital letters indicate a significant difference between conditions with and without sonic force. Means followed by distinct lowercase letters indicate a significant difference between culture time, individually considering the use or not of sonic force (horizontal lines).

Table 3 - Means and standard deviations of the concentration of interleukins IL-1 and IL-17 when using or not sonic force, depending on the culture time.

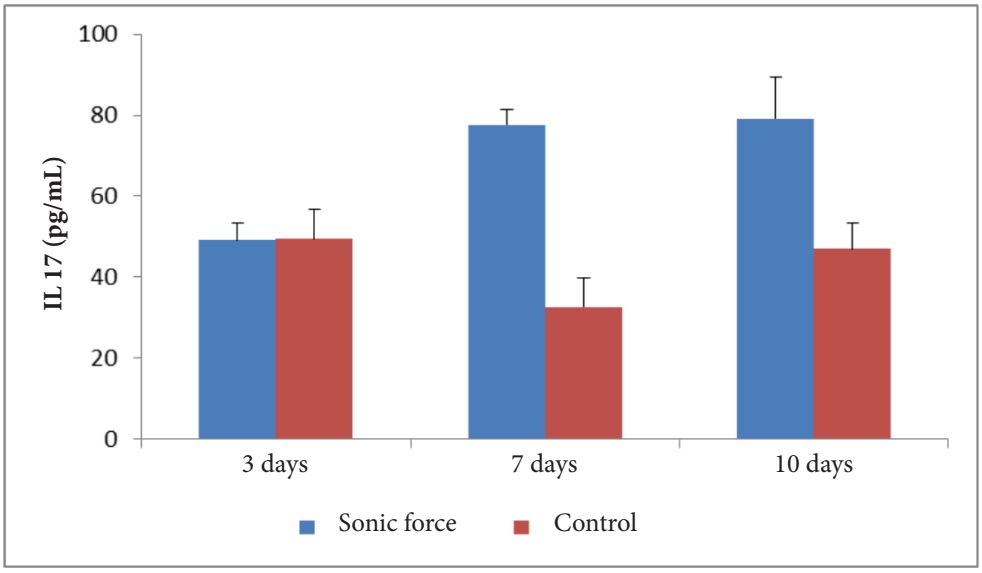
Source: Own authorship.



Graphic 3 –Column diagram of mean values of IL-1 concentration after using or not using sonic force, as a function of culture time.

Legend: Vertical lines over columns indicate standard deviations.

Source: Own authorship



Graphic 4 –Column diagram of mean values of IL-17 concentration after the use or not of sonic force, as a function of culture time.

Legend: Vertical lines over columns indicate standard deviations.

Source: Own authorship.

The application of ultrasonic forces as an exogenous physical force directly associated with bone remodeling and, consequently, with orthodontic movement (MOYERS, 1991) is a mechanism used for at least half a century. However, the action of pro-inflammatory biochemical mediators has not yet been fully elucidated, given the complexity of the histological, cellular and molecular processes that trigger osteogenesis (KESSEL, 2011). In this context, the correlation between the biophysical action of ultrasonic and sonic devices, mainly regarding time and pulse intensity factors and the biochemical expression of pro-inflammatory and osteogenic inducers and modulators, has been investigated using the LIPUS® device, in the which cytokines demonstrate to play an essential role in this process (CHERIAN et al., 2005; GUSMÃO; MARIOLANI; BELANGERO, 2005; LI et al., 2005; MUKAI et al., 2005; IKEDA et al., 2006; MADDI et al.., 2006; TAKAYAMA et al., 2007; XU et al., 2007; SUZUKI et al., 2009; REN; LI; LIN, 2010; ANGLE et al., 2011; AL-DAGHREER et al., 2012; ANDRADE JUNIOR; TADDEI; SOUZA, 2012, GENETOS et al., 2012; MAN et al., 2012; XUE et al., 2013). According to these studies, both in vitro and in vivo, the longer the tissue is exposed to low-intensity pulsed ultrasonic forces, the greater the expression of pro-inflammatory mediators.

While the above studies demonstrate that LIPUS® is related to bone remodeling, through the expression of pro-inflammatory mediators, the AcceleDent® device is still experimental at its current stage, being marketed as a device for accelerating tooth movement in order to reduce the duration of orthodontic treatment. Currently, there are studies being carried out in order to prove the manufacturer's argument. Kau (2011) performed a radiographic study of tooth morphology (3D tomography) in patients treated with the AcceleDent® and concluded that its use, related to orthodontic movement, did not cause root resorption, which suggests that the use of the device would not cause

additional damage to periodontal tissue. The device follows the same biomechanical principle as the LIPUS® and emits low-intensity sonic pulses, adapted to a mouth mold for exclusive dental use. It can be suggested that Acceledent®, like LIPUS®, also has the role of increasing pro-inflammatory factors in bone remodeling.

Thus, the present in vitro work evaluated the influence of the sonic vibration force of the Acceledent®, in culture of osteoblastic cells, on the parameters of cell proliferation and viability. Furthermore, it set out to analyze the effect on the secretion of some inflammatory mediators, IL-1 and IL-17 involved in bone neoformation.

The results showed that there was an increase in the proliferation of osteoblastic cells with the application of AcceleDent® in the parameters used and 20 minutes of daily application as recommended by the manufacturer, after 10 days of evaluation, when compared to the untreated group. These findings were reported in some in vitro studies that demonstrate greater cell proliferation, as well as stimulation of osteogenesis when ultrasonic force is applied (MADDI et al., 2006; SUZUKI et al., 2009; MAN et al., 2012, XUE, et al., 2013). Indirectly, this may also be related to the anti-apoptotic effect described for low-intensity pulsatile devices such as the LIPUS® (APPLEFORD et al., 2007).

Corroborating the study by Suzuki et al. (2009) in which they used LIPUS® daily for 20 min with an intensity of 30mW/cm2 (1.5MHz) for 14 days, there was no increase in the viability of osteoblastic cells, although in the present work Acceledent® was used, which is a device which uses low-intensity pulsatile sonic force (30Hz), it is important to highlight that, in addition to the culture conditions and cell type used, the intensity of the forces used can interfere with this cell parameter. This would be related not only to the increase in temperature that can occur when directly applied to a cell culture monolayer, but also to the experimental model itself, which, unlike in vivo, cells are in contact with vascular tissue (AL-DAGHREER et al., 2012).

Furthermore, the effect of applying Acceledent® on the synthesis of some inflammatory mediators was evaluated. The results showed an increase in IL-1 and IL-17 secreted by osteoblastic cells, mainly evidenced after 7 and 10 days, after application of sonic force when compared to the control group.

Some studies show the potential of vibratory force to modulate the synthesis of pro-inflammatory mediators (CHERIAN et al., 2005; GENETOS et al., 2005; LI et al., 2005; MUKAI, et al., 2005; IKEDA et al.., 2006; MADDI et al., 2006; TAKAYAMA et al., 2007; XU et al., 2007; REN; LI; LIN, 2010; ANGLE et al., 2011; AL-DAGHREER et al., 2012; GUSMÃO ; MARIOLANI; BELANGERO, 2012; XUE et al., 2013). During bone remodeling, IL-1 and IL-17 have been related to osteoclastic activation, modulating the RANK-RANKL-OPG pathway mediated by M-CSF activation (TANABE et al., 2005; LEE, 2013). Thus, the increase in cytokines evidenced in this in vitro study by osteoblastic cells implies a potential role for Acceledent®, in the parameters used, in the activation of osteoclastogenesis.

Low-intensity vibratory forces have been widely used for interventions that imply accelerating the growth of bone tissue such as repairing bone fractures, where mechanical tensions received by bone cells are translated into biochemical events (WANG; THAMPATTY, 2006). It is described that LIPUS® exerts an extracellular mechanical force that, when in contact with the plasmatic membrane, emits electrical and/or biochemical intracellular signals. Previous

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studies also indicate that LIPUS® accelerates the differentiation of osteoprogenitor cells into osteogenic lineage cells via activation of phosphorylation of mitosis-activating protein kinase (MAPK) (IKEDA et al., 2006), increased expression of cyclooxygenase-2 (COX -2), prostaglandin-E2 (PGE-2) (ANGLE et al., 2011), modulating the synthesis of OPG/RANK-L in the bone microenvironment (MADDI et al., 2006) and stimulating the production of BMPs (SUZUKI et al., 2009).

While most studies demonstrate the effectiveness of using LIPUS® in accelerating bone remodeling and, consequently, orthodontic movement, through the expression of pro-inflammatory mediators, the AcceleDent® appliance is still experimental at its current stage, being marketed as a tooth movement acceleration device in order to improve orthodontic treatment. Currently, there are studies being carried out that corroborate the manufacturer's data. Kau et al. (2013) performed a radiographic study of tooth morphology using 3D tomography in patients treated with daily use of AcceleDent® and concluded that its use did not cause root resorption, thus suggesting that the use of the device would not cause additional damage to the periodontal tissue. The AcceleDent® device follows the same biomechanical principle as the LIPUS® and emits low-intensity sonic force pulses, adapted to a mouth mold for exclusive dental use.

Clinically these results suggest a potential role for Acceledent® in accelerating bone turnover. These results may imply the use of this equipment as a treatment proposal to accelerate and modulate orthodontic movement, given the stimulation of bone remodeling, shown by the increase in the present study of cytokines that stimulate bone resorption, and therefore bone repair.

The results presented and discussed above allow the following conclusions that the pre-osteoblastic cells submitted to the sonic forces of the AcceleDent® presented after 10 days of evaluation, cell proliferation significantly superior to the control group; the application of sonic force did not affect cell viability; the expression of pro-inflammatory cytokines IL-1 and IL-17 was significantly higher compared to the control group, specifically at 3 days for IL-1 and 7 days for IL-17 when using sonic force.

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OPINION OF THE ETHICS COMMITTEE

São Leopoldo Mandic

Faculdade de Odontologia

Centro de Pesquisas Odontológicas

Certificado de Cumprimento de Princípios Éticos

C E R T I F I C O que, após analisar o projeto de pesquisa

Título ***Estudo in vitro da influência da vibração ultrassônica em osteoblastos humanos***

Pesquisador principal: José Ricardo Mariano

Orientador: Elizabeth Ferreira Martinez

Data Avaliação: 10/12/2012 **Nº Protocolo:** 2012/0489

o Comitê de Ética em Pesquisa (CEP) da Faculdade de Odontologia e Centro de Pesquisas Odontológicas São Leopoldo Mandic considerou que o projeto está de acordo com as diretrizes para a proteção do sujeito de pesquisa, estabelecidas pela Resolução nº 196/96, do Conselho Nacional de Saúde, do Ministério da Saúde.

Campinas, SP, Brasil, **segunda-feira, 10 de dezembro de 2012**

CERTIFICATION OF COMPLIANCE WITH ETHICAL PRINCIPLES

I hereby, certify that upon analysis of the Research Project,

Title: *Study in vitro of the influence of ultrassonic vibration in human osteoblats*

Main Researcher(Author): José Ricardo Mariano

Advisor: Elizabeth Ferreira Martinez

the Committee of Ethics for Research of São Leopoldo Mandic School of Dentistry and Research Center, has considered the mentioned project to be in accordance to the guidelines of protection to the subject of the research, established by the Regulation number 196/96, from the National Health Council of the Brazilian Health Ministry.

Profa. Dra. Fernanda Lopes da Cunha
Presidente do Comitê de Ética em Pesquisa

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