CESPU
INSTITUTO UNIVERSITÁRIO
DE CIÊNCIAS DA SAÚDE

Optimization of the ultrasound protocol to increase the proliferation and viability of periodontium cells

Orientador: Professora Doutora Teresa Pinho
Co-orientador: Mestre Selma Pascoal

Rita Costa
Nº 22581

Gandra, March 2023

ACKNOWLEDGEMENTS

First of all, I would like to thank to my supervisors Professora Doutora Teresa Pinho and Doctor Selma Pascoal for their guidance, constant support and advice throughout my MSc research studies.

A special thank to Sofia Oliveira, who was patient and kind in guiding me in the laboratory and who was always ready to help me. The results of this work were only possible because of your availability and kindness in helping me.

To the University of Minho and Department of Biological Engineering, my sincere thank for providing the material and excellent people, which contributed for the good development of this work.

To my family and friends, I would like to thank for their unconditional support.

ABSTRACT

Orthodontic tooth movement relies on coordinated tissue resorption and formation in the surrounding bone and periodontal ligament. The resorption-formation sequence remodeling process of the bone and periodontal ligament is performed and controlled by the cellular activity. Understanding biology has had a great impact on clinical orthodontics, essentially how we can accelerate orthodontic movement and thus reduce treatment times. Surgical and non-surgical interventions are being tested to move teeth faster. Low-intensity pulsed ultrasound (LIPUS) is a non-invasive technique that accelerates tooth movement however there are few studies of its clinical application. This study, in addition to summarizing the effect of in vitro mechanical stimuli on the cells involved in orthodontic movement, also highlights the best ultrasound conditions to which the osteoblasts and fibroblasts must be submitted.

Design: human fetal osteoblast cell line (HFOB) and a human primary cell line (hPDLF) were cultured in vitro and subjected in a first phase to different ultrasound parameters (1 MHz and 1.5 MHz; 30mW/cm^2 and 60mW/cm^2 during 5 and 10 min). After observing the 3 timepoints (1h, 24h and 72h) the best conditions were daily stimulated up to 3 days.

Results: In our study, for both types of cells, the 1 MHz, 30 mW/cm2 for 5 min was the condition that provided the best metabolic activity.

Further cellular studies should be conducted to understand the effect of using ultrasound as promising therapy in accelerating orthodontic movement.

KEYWORDS: ("osteoblast" OR "bone" OR "bone cells" OR "bone growth" OR "bone remodeling" OR "bone differentiation" OR "osseodensification" OR "osteogenesis" OR ossification" OR "bone tissue" OR "bone apophyses" OR

"bone apophysis" OR "periodontal ligament" OR "periodontal fiber" OR "bone resorption" OR "biomechanical" OR "RANKL Ligand" OR "osteoclast" OR "hypoxia" OR "mechanobiology" OR "mechanotransduction" OR "mechano-cell") AND ("ultrasound" OR " sonication" OR "ultrasonic" OR "vibroacoustic" OR "vibration" OR "acoustic" OR "wave" OR "vibrotactile" OR "physioacoustic" OR "cyclic loading") AND ("orthodontic" OR "orthodontic movement" OR "tooth movement".

GENERAL INDEX

FIGURE INDEX

TABLE INDEX

ABBREVIATIONS

TRAP: Tartrate resistant acid phosphatase

RANKL: Receptor activator of nuclear factor-kappa β ligand

OPG: Osteoprotegerin

TGF-β: Transforming growth factor β

Cathepsin K: Cysteine protease

IL-1β/IL-4/IL-6/IL-8/IL-13/IL-17: Interleukin (cytokines)

hPDL: human periodontal ligament

hPLF: human fibroblasts

COLA1: Collagen type alpha 1

ALPL: Alkaline phosphatase

RUNX2: Runt-related transcription factor 2

FGF2: Fibroblast growth factor 2

CTGF: Connective tissue growth factor

ALP: Alkaline phosphatase

OCN: Osteocalcin

TNF- α: Tumor necrosis factor alfa

COX-2: Ciclo-oxigenase-2

PGE2: Prostaglandin 2

COL-1: Type 1 collagen

BSP: Bone sialoprotein

hPDL: human periodontal ligament fibroblasts

OPN: Osteopontin

MCP-1: Monocyte chemotactic protein 1

hPDLSCs: Human periodontal ligament stem cells

rpm: rotations per minute

1- INTRODUCTION

During orthodontic tooth movement, mechanical and biological processes take place. Immediately after the application of an external force, the cells are deformed and in response of the deformation, fibroblasts and osteoblasts in the periodontal ligament cells (PDL) as well as osteocytes in the bone are activated. Finally, a combination of PDL remodeling and localized apposition and resorption of alveolar bone enables the tooth to move. (1–4)

The amount of orthodontic tooth movement depends on the modeling and remodeling of the alveolar process and the rate is determined by the level of activity of bone cells-osteoclasts, osteoblasts and osteocytes, which under the control of mechanical and biochemical factors.(1,2) Receptor activator of nuclear factor-kappa β (RANKL) and Osteoprotegerin (OPG) are examples of these factors that are involved in the formation of osteoclasts and bone resorption as a consequence. Osteoblast proliferation, differentiation, survival and function are regulated by a number of extracellular factors including growth factors, cytokines, and hormones, by interactions with osteoclastic cells like TGF-β1, BMPs and others.(2,5)

Orthodontic treatment is usually associated as a long-term treatment, which can lead to several complications. For this reason, we need to investigate how we can accelerate orthodontic movement and the molecular mechanisms to identify the key factors that make the treatment more effective with the fewest side effects, including risk of caries, periodontal disease and

root resorption, shortest times with decrease pain and discomfort, and lowest costs to patients. (2,3,6)

A number of techniques affect the expression of these factors on osteoclast and osteoblast formation to activate bone remodeling and modeling, and the acceleration of the orthodontic tooth movement. These can be surgical (e.g., corticomy, osteostomy, piezoincision, surgery first) and non-surgical (e.g., vibration, cyclic forces, compressive force, laser irradiation, magnectic fields, electric current, photobiomodulation). (2,7)

Among the non-surgical approaches, high or low-frequency vibration is the most employed by commercially available devices, such as AcceleDent® and VPro5®, with well-defined protocols and effects. In contrast, few clinical studies report the application of ultrasound and shockwaves.(8)

Low-intensity pulsed ultrasound (LIPUS) uses acoustic waves and has been approved by The Food and Drug Administration (FDA, USA) as a non-invasive and safe therapeutic technique for treating delayed union and/or nonunion of bone fractures(9) and might as well accelerate orthodontically-induced tooth movement.(10,11) The LIPUS application stimulates cell differentiation by increasing the number of cells and consequently increases the production of responsible mediators involved in orthodontic movement.(12) Although there is biological evidence that LIPUS accelerates tooth movement, clinically it has to be better substantiated.

This dissertation has two main goals:

Objective 1: to carry out a systematic review on the effect of in vitro mechanical stimuli on cells involved in orthodontic movement.

Objective 2: to understand which ultrasound parameters influence the viability of osteoblasts and fibroblasts to improve the orthodontic movement in the laboratory setting.

2-INTEGRATIVE SYSTEMATIC REVIEW

METHODS

RESEARCH STRATEGY AND STUDY SELECTION

The systematic review was performed in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines. The articles included in this systematic review were selected according to the Population, Intervention, Comparison, Outcomes and Study design, following the PICOS strategy (Table 1).

The aim of this systematic review is to understand the effect of different types of mechanical stimuli on cellular activity involved in the orthodontic movement.

Table 1: PICOS strategy

Patient	Cells involved in the orthodontic movement (e.g., periodontal ligament fibroblasts, osteoblasts, osteocytes, osteoclasts)
Intervention	Different types of mechanical stimulation (e.g., vibration; compression, ultrasound; shockwaves, mechanical strain)
Comparison	Untreated cells
Outcome	Biochemical outcomes (e.g., cell proliferation, viability, cellular markers expression, inflammatory activity)
Study design	In vitro studies that used mechanical stimuli to promote orthodontic movement

The electronic databases PubMed, Scopus and Web of Science were searched to identify original in vitro studies that assessed the effects of mechanical stimuli on cellular behavior involved in orthodontic movement. Advanced searches were performed from databases inception up to January 25, 2022. The search strategy was conducted using the search key: ("osteoblast" OR "bone" OR "bone cells" OR "bone growth" OR "bone

remodeling" OR "bone differentiation" OR "osseodensification" OR "osteogenesis" OR ossification" OR "bone tissue" OR "bone apophyses" OR "bone apophysis" OR "periodontal ligament" OR "periodontal fiber" OR "bone resorption" OR "biomechanical" OR "RANKL Ligand" OR "osteoclast" OR "hypoxia" OR "mechanobiology" OR "mechanotransduction" OR "mechano-cell") AND ("ultrasound" OR " sonication" OR "ultrasonic" OR "vibroacoustic" OR "vibration" OR "acoustic" OR "wave" OR "vibrotactile" OR "physioacoustic" OR "cyclic loading") AND ("orthodontic" OR "orthodontic movement" OR "tooth movement"). The exclusion criteria were as follows: (1) review or meta-analysis, case studies or conference proceeding, (2) articles not in English, (3) combination of mechanical stimulation with surgical techniques or others stimulation method, (4) absence of mechanical stimulation on tooth movement, and (5) clinical and in vivo studies.

All records were exported to an Excel file (Microsoft® Office) and the duplicates were removed by the software filter and the manually verified. The title and abstract of identified articles were evaluated and the full texts of the selected articles were analyzed. All data related to study characteristics and outcomes were organized in the form of a table containing the information on first author, year of publication, stimulation type and parameters, cell type and the main findings.

RESULTS

SEARCH STRATEGY

The electronic search identified 5738 studies. After removing 239 duplicates, 5499 articles remained. The title and abstract were analyzed, being 273 selected for full-text reading. From those, 18 articles were selected and included in this review after checking the eligibility criteria. The selection process is explained by the PRISMA flowchart search in *Figure 1.*

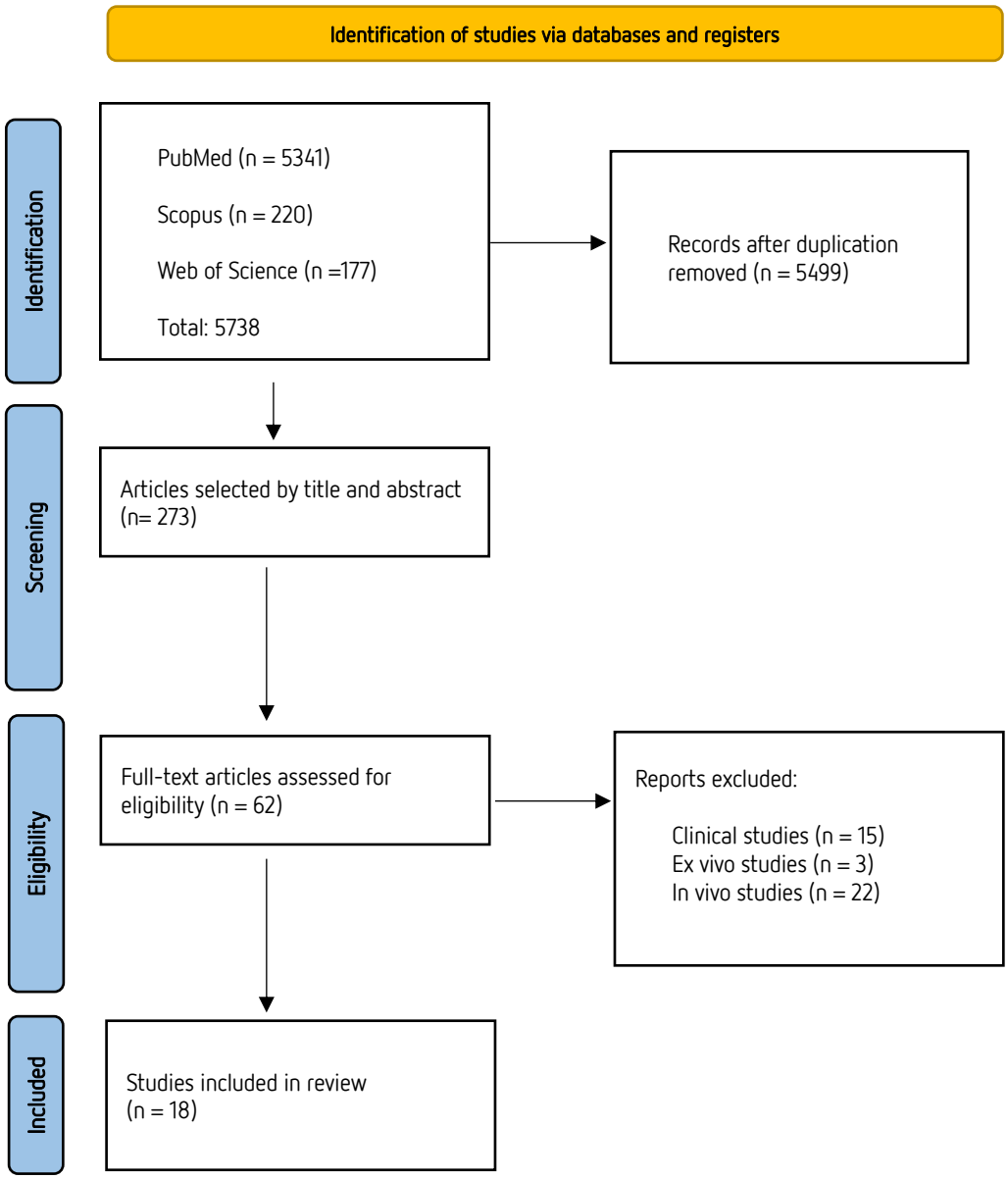

Figure 1: PRISMA flow diagram

IN VITRO STUDIES

STUDY CHARACTERISTICS

Of the 18 results, 12 used vibration, four ultrasound, only one used shockwave and other study used mechanical strain. Seven studies (39%, k=18) applied only vibration, while this therapy was combined with compressive forces in five studies (28%, k=18), with photobiomodulation in one study (6%, k=18) and with mechanical strain (6%, k=18). Three studies (17%, k=18) applied only ultrasound and only one study (6%, k=18) used ultrasound with compressive forces. Shockwave was applied alone in one study (6%, k=18). Table 1 reports the type of stimulation and its parameters used and on which type of cells it was applied.

Table 1- Study design and reported biomechanical outcomes for in vitro studies

First author, year	Type of Stimulation	Stimulation parameters	Cell type	Main Findings
Sakamoto, M *et al.* (2019)(3)	Vibration	0.5gF 48.3Hz 1min Once a day for 2 days	Osteocyte cell line MLO-Y4; Pre-osteoclast cell line RAW264.7	**Proliferation:** ↑ at 48h (RAW 264.7) **Protein quantification:** No effect on TRAP (RAW 264.7); ↑ staining (co-culture); ↑ RANKL 30min (MLO-Y4); No effect on OPG (MLO-Y4)
García-López S *et al.* (2019)(13)	Vibration	Vibration: 0.25N 30Hz 20 min one time	Mouse osteoblasts: Bone marrow-derived osteoclasts	**Proliferation:** ↑ Osteoblasts **Osteoblast markers:** ↑ TGF-β ; ↓ RANKL; ↑ OPG ; ↑ caspase 3/7 (mouse osteoblasts) **Protein quantification:** ↑ cathepsin K; ↑ TRAP ; ↑ RANK (bone marrow cells) **Inflammatory markers:** ↑ IL-4, IL-13, IL-17 (in both cell types)
Judex *et al.* (2018)(8)	Vibration	AcceleDent 0.24g 30Hz 20 min one time VPro5 0.41g 120Hz 5min one time	Human osteoblasts, hPDL, hPLF, and human osteoclasts	**Proliferation:** ↑ osteoblasts, ↑ hPLF, (significantly greater for VPro5); ↑ osteoclasts (no effects between both devices) **Osteoblast markers:** ↑ COLA1 and ↑ALPL (significantly greater for VPro5); ↑ RUNX2 (only statistically different for VPro5) **Protein quantification:** ↑ RANK (no effects between both devices) **Periodontal ligament fibroblasts markers:** ↑FGF2 and ↑ CTGF (significantly greater for VPro5); ↑ ALPL (only statistically different for VPro5)
Phusuntornsakul, et al. (2020)(14)	Vibration	0.3g 30Hz 20min one time	hPDL cells	**Inflammatory markers:** ↑ IL-6 and IL-8
Pravitharangul, et al. (2018)(15)	Vibration	0.49g 30, 60 Hz 30min once a day for 2 days	Osteoblast-like cell (from mandible and iliac crest)	**Osteoblast markers:** no effects on TGF-β in iliac cells; in mandibular cells ↓ TGF-β at 30 and 60Hz **Protein quantification:** ↓ RANKL in both cell types at 30 and 60 Hz; ↓ RANKL/OPG in iliac cells hIOBs and no effects in mandibular cells; No effects on biomarkers after ELISA assay; ↑ OPG in iliac cells; ↓ OPG in mandibular cells

					Inflammatory markers: ↓ IL-1B at 30 or 60 Hz, with lowest levels at 30Hz for iliac cells; ↑ IL-6 at 30 or 60 Hz, with highest levels at 60 Hz for iliac cells; No effects on biomarkers after ELISA assay
Pravitharangul, et al. (2019)(16)	Vibration	0.49g 30, 60, 90, 120 Hz 30min one time for 2 days		Osteoblast-like cell (from mandible and iliac crest)	**Protein quantification:** no effects on RUNX2 for both cells; ↑ ALP in mandibular cells
Chintavalakorn, et al. (2016)(17)	Vibration and photobiomodulation	680nm 0 to 50 Hz 3 min once for 42 days		Pre-osteoblastic cells: MC3T3 cells	**Proliferation:** ↑ cell number migration (histology analysis) **Protein quantification:** ↑ Calcium deposition; **:** ↑ ALP for mechanical and combination groups (PCR) ; No effects on RUNX2, OPN and OCN
Benjakul et al. (2017)(6)	Vibration and compressive forces	Vibration 0,3g 30Hz 20min one time for 2 days	Compressive Forces 1.5 g/cm2 48h	hPDL cells	**Proliferation:** ↓ viability for groups with only compression and compression + vibration compared to without stimulation and only vibration. **Protein quantification:** ↑ RANKL only compression and compression + vibration (PCR) and on all treated groups (ELISA) compared to control; No effect on OPG; ↑ RANKL/OPG ratio for vibration + compression groups compared to only compression; ↓ RUNX for compression and compression + vibration groups.
Benjakul, et al. (2019)(18)	Vibration and compressive forces	Vibration 0,3g 30Hz 20min one time for 2 days	Compressive Forces 1.5 g/cm2 48h	hPDL cells	**Protein quantification:** ↑ RANKL in all treated groups; ↓ OPG in compression group only (PCR, ELISA) and in Vibration + compression group (ELISA).
Benjakul, et al. (2020)(19)	Vibration and compressive forces	Vibration 0.3g 30 or 60Hz 20min one time for 3 days	Compressive Forces 2 g/cm2 48h	hPDL cells	**Inflammatory markers:** ↑ IL-1B and TNF- α in compression group and in compression + vibration group (ELISA, PCR); ↑ IL-1β and ↑ TNF-α in compression + vibration at 30Hz compared to compression group only.
Chatmahamongkol et al. (2019)(20)	Vibration and compressive forces	Vibration 0.49g 60Hz	Compressive Forces 2 g/cm2	Alveolar bone osteoblasts	**Protein quantification:** No differences detected on RANKL; ↓ OPG in Compression group and Compression + Vibration groups at: 12, 24 and 48 h

		30min one time for 2 days	48h		(PCR) and 24, 48 h (ELISA); ↑ Vibration group than control group at: 12, 24 and 48 h (PCR) and 24, 48 h (ELISA). **Inflammatory markers:** ↑ IL-1B in Compression group and Compression + Vibration group at: 12, 24 and 48 h (PCR) and 24, 48 h (ELISA); ↑ IL-6 in Compression group and Compression + Vibration group at: 12, 24 and 48 h (PCR) and 48h (ELISA).
Phusuntornsakul, et al. (2018)(21)	Vibration and compressive forces	Vibration 0.3g 30Hz 20min one time	Compressive Forces 1.5 g/cm2 T: NR	hPDL cells	**Proliferation:** No effects among groups. **Protein quantification:** ↑ RANKL at 48 and 72 h for all treated groups; No effects on OPG; ↑ RANKL/OPG RATIO after vibration (PCR). **Inflammatory markers:** ↑ IL-6, IL-8 after mechanical vibration at 24 h (PCR), and from 24 h to 72 h (ELISA). **Periodontal ligament fibroblasts markers:** COX-2 inibition: ↓ IL-6, IL-8 for treated groups; PGE2 endogenous: ↑ IL-6, IL-8(ELISA).
Feres et al. (2016)(10)	Ultrasound	1.5MHz 30 mW/cm2 10 and 20 min daily for one week Pulse mode 200ms		Osteoclast-like cell: RAW 264.7 cells	**Protein quantification:** Negative control (without adding RANKL): ↓ RANKL intensity Positive control (RANKL + No LIPUS) Vs Group 1 (RANKL + 10 min LIPUS): No differences. Group 2 (RANKL + 20 min LIPUS): ↑ significantly RANKL Intensity.
Inubushi, et al. (2008)(11)	Ultrasound	1 MHz 30 mW/cm2 15 min daily for 6 days Pulse mode		hPDL cells	**Proliferation:** No effect on cell viability. **Periodontal ligament fibroblasts markers:** ↑ ALP, ↑ COL-1, ↑ RUNX-2; No effects on BSP, OCN, and OPN.
Xue, et al. (2013)(22)	Ultrasounds	1.5MHz 30mW/cm2 20 min daily for 14 days Pulsed		hPDL cells	**Proliferation:** No effect after LIPUS
Inubushi, et al. (2013)(12)	Ultrasound + compressive force	1 MHz 30 mW/cm2 15 min	Compressive Forces 7 kPa T: NR	Cementoblast cell line: OCCM-30 and MC3T3-E1 cells	**Protein quantification:** ↑ RANKL in MC3T3-E1 at 4 and 12 h and in OCCM-30 at 12 h, after Ultrasound; ↑ in both cell types after compression; no effects on OPG in MC3T3-E1 and ↑ in OCCM-30 at 12 h after ultrasound; ↓ in both cell types after compression.

		one time Pulse mode		
Cai, et al. (2016)(23)	Shockwave	3 Hz 0.05; 0.10; 0.19 j/cm2 Pulsed Impulses number 100, 300, 500 times	hPDLF cells	**Proliferation:** No significant difference **Inflammatory markers:** - ↓ IL-6 with ↑ shock pulses (300 and 500 times) and ↓ MCP-1, TNF-a at **1h** (PCR) - ↓ IL-6, IL-8 in all treated groups; MCP-1 no significant differences, ↑ TNF-a with ↑ Chock pulses and ↑ energy densities; (PCR); IL-8 no significant differences (ELISA) **at 2h;** - ↑ IL-6 in groups with ↑ shock pulses and ↑ energy densities, ↑ IL-8 in groups with ↑ shock pulses (500 times), TNF-a and MCP-1 no significant differences; (PCR) IL-6 no significant differences, ↑ IL-8 in the group receiving the most shock pulses (500 times); (ELISA) **at 4h;** - ↑ IL-6 in all treated groups than control (PCR, ELISA); TNF-a No differences (PCR); ↑ IL-8 in the group receiving the most shock pulses (500 times) (ELISA) **at 8h;** - ↑ IL-6 in all treated groups than control (PCR); TNF-a ↓ that in control (PCR); ↑ IL-8 in the group receiving the most shock pulses (500 times) (ELISA **at 24h.**
Liu, et al. (2004)(24)	Mechanical strain	3 cycles/min 10 min Pulsed	MG-63 human osteosarcoma cell line	**Protein Quantification:** ↑ OPN at 1h and 4h after mechanical strain (Western Blot and SDS-page)

Legend: TRAP: Tartrate resistant acid phosphatase; RANKL: Receptor activator of nuclear factor-kappa β ligand;OPG: Osteoprotegerin;TGF-β: Transforming growth factor β; Cathepsin K: Cysteine protease; IL-1β/IL-4/IL-6/IL-8/IL-13/IL-17: Interleukin (cytokines); hPDL: human periodontal ligament; hPLF: human fibroblasts; COLA1: Collagen type alpha 1; ALPL: Alkaline phosphatase ; RUNX2: Runt-related transcription factor 2 ; FGF2: Fibroblast growth factor 2 ; CTGF: Connective tissue growth factor ; ALP: Alkaline phosphatase; OCN: Osteocalcin; TNF- α: Tumor necrosis factor alfa; COX-2: Ciclo-oxigenase-2; PGE2: Prostaglandin 2; COL-1: Type 1 collagen ; BSP: Bone sialoprotein ; hPDL: human periodontal ligament fibroblasts; OPN: Osteopontin ; MCP-1: Monocyte chemotactic protein 1 ; hPDLSCs: Human periodontal ligament stem cells; rpm : rotations per minute

CELL TYPES

All studies used cell types that are sensitive to perceiving and responding to mechanical signals and are intimately involved in faciliting tooth movement (Table 1). Most of the in vitro studies explored fibroblasts (50%, k=18) and osteoblasts (39%, k=18). Osteoclasts (22%, k=18), osteocytes (6%, k=18) and cementoblasts (6%, k=18) were also used to determine the effects of different stimulus on cells expression and differentiation.

Some studies have cultured more than one type of cells. Primary cells were used in 13 studies (72%, k=18), obtained from human sources in 11 of those studies. The remaining five studies (28%, k=18) used cell lines, being most of them obtained from animal sources.

STIMULATION PARAMETERS

The most reported vibration parameters were frequency (Hz), magnitude (gf or g) and time of stimulus (min) (Table 1). Most of the cells were exposed to 30Hz (range, 30-120Hz), at 0.3g (range, 0.24-0.49g) for 20min (range, 1-30*min*). To mimic the acceleration of healing process and bone calcification, one study investigated the effect of the combined mechanical and emitted light at wavelength 680 *nm* stimulation. Similarly, others studies evaluated the vibration effect in cells continuously compressed using a glass cylinder containing acrylic mass with a total force of 1.5 g/cm^2 or 2 g/cm^2 for 48*h*.

For therapeutic ultrasound, frequency (MHz), intensity (mW/cm^2) and time (min) were provided. All studies applied ultrasound in pulse mode at 1 or 1.5 MHz, at 30 mW/cm^2 for 10-20min.

The impacts of the shock waves therapy on the viability of cells were analyzed using a frequency of 3Hz, with different energy densities (0.05,0.10 and 0.19 mJ/mm^2) and different impulses (100,300 and 500 times) was analyzed.

In another study, the mechanical strain was generated by a vacuum chamber, which exerted a frequency of 0.5 % (5000 με) of 3 cycles/minute for 10 min in pulse mode.

BIOCHEMICAL OUTCOMES

All studies analyzed biochemical outcomes (Table 1) such as cells proliferation and the expression of osteoblasts, osteoclasts, fibroblasts and inflammatory markers.

Vibration

In studies exploring vibration therapy, the stimulus effect was investigated on the cell proliferation of osteocytes, osteoclasts, osteoblasts and fibroblasts. Cell proliferation and gene expression are indicators of tissue turnover and remodeling, essential processes to increase orthodontic movement.

Osteocytes

The receptor activator of nuclear factor kappa-B ligand (RANKL) expression significantly increased following intervention and, consequently, the ratio of the differentiation factor, receptor activator NF-kappa B ligand (RANKL) and its decoy receptor, osteoprotegerin (OPG) (RANKL/OPG), even though the level of OPG was not altered in osteocytes.(3)

Osteoclasts

After vibration, the cell proliferation of osteoclasts was significantly increased.(3) Longer vibration times, such as 3 days, improved osteoclasts activity.(8,13) Osteoclasts were positively stained for tartrate resistant acid phosphatase (TRAP).(13) Also, the cytokines (IL-4,IL-13 and Il-17)(13), cathepsin K, an enzyme that in humans is encoded by the CTSK gene(13) and RANK(8) were significantly upregulated.

Osteoblasts

The soluble factors for proliferation and differentiation, TGF- β and PCNA, were upregulated during the stimulation, as well as the IL-4, IL-13, IL-17 and caspase 3/7 expression. RANKL downregulated, while OPG expression enhanced in osteoblasts.(13) Another study showed an increase of collagen type alpha 1 (COLA1), alkaline phosphatase (ALP) and Runt-related transcription factor 2 (RUNX2) in stimulated cells, which are indicators of osteoblasts activity and differentiation.(8) Two similar studies exposed models of mature osteoblasts from mandible and long bones to various vibration frequencies and observed an increase of OPG and decreased in RANKL

expression and RANKL/OPG ratio in iliac cells.(15) However, ALP activity was higher in mandibular cells.(16)

Vibration and photobiomodulation

The vibration isolated or combinated with photobiomodulation was applied to pre-osteoblastic cells (MC3T3). ALP levels were upregulated as well as proliferation and calcification with deeper cell migration.(17) However, these effects may depend on different light stimulation parameters.

Vibration and compressive forces

Human periodontal ligament

Vibration in compressed human periodontal ligament cells enhanced the levels of ciclo-oxigenase-2 (COX-2), prostaglandin 2(PGE_2), tumor necrosis factor alfa (TNF-α), IL-1β, IL-6 and IL8. The RANKL expression was increased, which resulted in higher RANKL/OPG ratio.(6,18,19,21) Although the application of mechanical vibration combined with compressive force on human alveolar bone osteoblasts upregulated the IL-1β and IL-6 expression, the OPG was inhibited and had no effects were detected in the RANKL expression.(20)

Ultrasound

The effect of ultrasound was studied on proliferation and differentiation of osteoclasts, cementoblasts and osteoblasts.

Osteoclasts

The application of ultrasound significantly increased the osteoclasts activity and the RANKL intensity when daily applied and for longer exposure times (20min per day).(10)

Human periodontal ligament fibroblasts

Ultrasound exposure had no significant effect on the proliferation. However, not only increased the expression levels of early cementoblastic differentiation (Col-1, ALP and $RUNX_2$), but also the collagen synthesis and ALP activity.(11) Another study showed that low-intensity pulsed ultrasound for up to 14 days had no effect on cell viability and, after this stimulation, the number of bone morphogenic protein 2 (BMP-2) positive cells were increased via RUNX2 regulation. The RANKL expression and the number of osteoclasts were significantly higher, specifically on day 7.(22)

Ultrasound and compressive forces

Cementoblasts and osteoblasts were subjected to compression force by the Biopress system® (Flexercell International, USA) and the authors observed in both type of cells an increase in the RANKL and a decrease of OPG expression. Nevertheless, ultrasound application, prior to compressive force,

inhibited the up-regulation of RANKL in cementoblasts, whereas the RANKL expression was unaffected in osteoblasts.(12)

Extracorporeal shock wave therapy

Extracorporeal shock wave therapy (ESWT) did not cause detrimental effect on hPDLF viability or proliferation even when using different energy intensities and pulses. Shockwaves upregulated the IL-6 and IL-8 and down-regulated the TNF-α, both in a dose-dependent manner in hPDLF cells.(23)

Mechanical strain

A vacuum which generated a homogeneous and biaxial strain was applied to the MG-63 human osteosarcoma cell line. This cell line was distributed on the concave side of the half-balls shaped silicon rubber membranes and the secretion of osteopontin (OPN) was significantly increased after 1 hour.(24)

3-EXPERIMENTAL SECTION

Ultrasound therapy has been utilized in dentistry as a safe method to accelerate of bone regeneration and to prevent tooth root resorption, which are critical for successful and long-term orthodontic treatment results. To better understand its bio-stimulatory effects and stimulation conditions, we have studied the effect of different ultrasound stimulation parameters applied to osteoblasts and periodontal ligament fibroblasts using a customized stimulation device developed by the Department of Mechanical Engineering Department (DEM) at University of Minho (UM).

METHODS

CELL CULTURE AND SEEDING

A human fetal osteoblast cell line (HFOB; American Type Culture Collection – ATCC, USA) was cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM-F12) without phenol red (PAN- Biotech GmbH, Germany) containing 10 % fetal bovine serum (FBS; PAN- Biotech GmbH, Germany) and 0,3 mg/ml geneticin (G418; PAN- Biotech GmbH, Germany) (Figure 3.1.1). The hFOBs was used between 12-14 passages.

A human primary cell line (hPDLF; Innoprot®- Spain), was cultured in two flasks with DMEM-F12 containing stable glutamine and 1.2 g/L $NaHCO_3$ supplemented with 10 % FBS and 1% penicillin-streptomycin (Figure 3.1.2); all reagents from PAN-Biotech GmbH, GERMANY. Primary hPDLF cells at 3rd passage was used.

The cells were maintained at 37°C in a humidified atmosphere of 5 % CO_2 and the medium was changed twice a week. After confluence of 90 %, the cells were detached from culture flasks with 0,25 % trypsin/EDTA (Biowest, France). The hFOB cells were seeded at $2x10^4$cells/well and the hPDLF at $3x10^4$ cells/well in 12-well plates. The plates were pre-incubated for 72h at 37°C and 5 % of CO_2 before starting the stimulation. Cells were observed under an inverted microscope at 10x magnification (Figure 3.1.3 and 3.1.4).

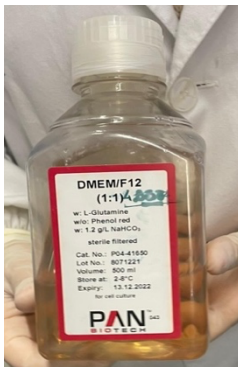

Figure 3.1 1- Culture medium used in osteoblats

Figure 3.1.2- Culture medium used in fibroblasts

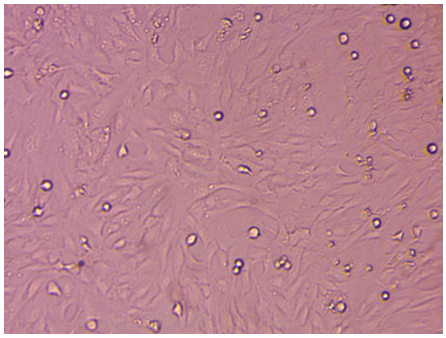

Figure 3.1.3 - Microscopic image (10x) of osteoblast cell culture

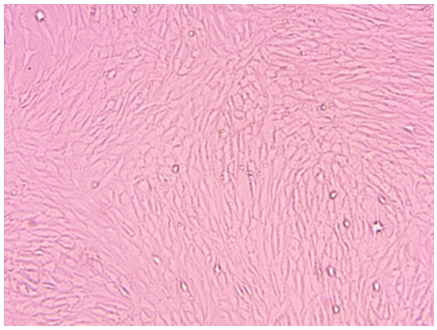

Figure 3.1.4 - Microscopic image (10x) of fibroblast cell culture

i. Remove the cells that we previously have cultured.

ii. Cells were observed under an inverted microscope (Kern®, Germany) to verify the confluence. The greater the space filled by the cells on the surface of the flask, the greater confluence. Cells were trypsinized when they reached 80-90% of confluence.

- analyzed in phase contrast
- using a 10x objective lens

iii. Thaw trypsin/EDTA (Biowest, France) and warm phosphate buffered saline (PBS) and culture medium to 37°C (Figure 3.1.5 and 3.1.6). Trypsin is used to detach cells while PBS is used to wash cells.

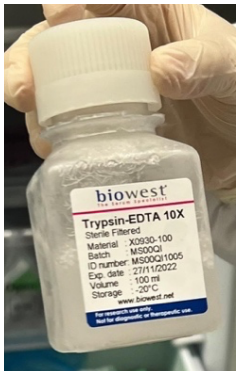

Figure 3.1.5 - Trypsin-EDTA

Figure 3.1.6 - Warming PBS and culture medium

iv. Remove the culture medium from the flask.

v. Wash cells twice with PBS. PBS is a buffer solution that prevents cells from lysis. The presence of culture medium compromises the action of trypsin.

- T75: 10 mL PBS

vi. Add trypsin.

- T75: 2 mL trypsin/EDTA

- 5min at 37°C and 5% of CO_2

vii. To neutralize the action of trypsin after 5 minutes we add culture medium (4x the volume of the medium).

- 2 mL trypsin/EDTA → 8 mL culture medium
- We resuspend and wash the walls where the cells were attached
- Aspirate the entire middle of the flask and put it in a falcon

viii. Centrifuge at 300 G for 5 min (Figure 3.1.7).

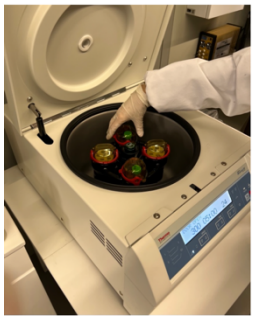

Figure 3.1.7 - Placing the falcon in the centrifuge

ix. Carefully remove the falcon from the centrifuge;

x. Aspirate the supernatant and resuspend the *pellet*. The supernatant consists essentially of culture medium and dead cells, while the pellet is composed of cells deposited at the bottom of the tube (Figure 3.1.8).

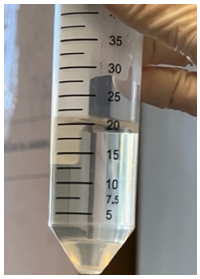

Figure 3.1.8 - Pellet deposited at the bottom of the tube

xi. Place 20 µL of 0.4% trypan blue solution in an *Eppendorf.* Trypan Blue Solution, 0.4% (Figure 3.1.9), is used as a cell stain to assess cell viability using the dye exclusion test. The dye exclusion test is based upon the concept that viable cells do not take up trypan blue but dead cells are permeable and take up the dye.

Figure 3.1.9 - Trypan-Blue 0.4% solution

xii. Resuspend the *pellet* in a 2 mL of culture medium.

xiii. We take 20 µL of the cell suspension and add it to *eppendorf* with trypan blue. We resuspend again.

xiv. Count the cells under the microscope.

- using a Neubauer chamber or a haemocytometry

- with the cover-slip in place, we transfer 10 µL of trypan blue-cell suspension mixture to both chambers of the hemocytometer (Figure 3.1.10)

- count 4 corner squares of both chambers (Figure 3.1.11) and the cell concentration was calculated according to the equation:

$$\textbf{Cell Concentration} = \frac{\textbf{Nº cells x 10.000}}{\textbf{4}}$$

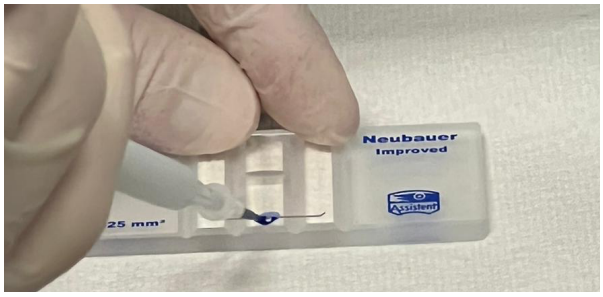

Figure 3.1.10 - Place the trypan blue-cell suspension mixture in hemocytometer

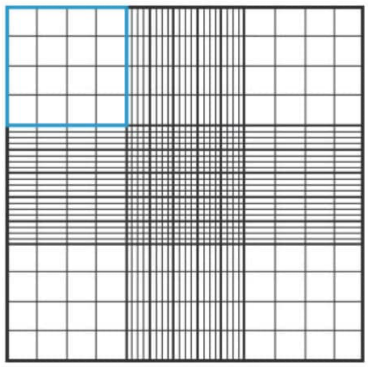

Figure 3.1.11 - Haemocytometry

ULTRASOUND STIMULATION

The stimulation of the cells was performed in two phases to evaluate the effect of ultrasound on cells behavior. In a first phase, cells were stimulated once with different frequencies (1 MHz and 1.5 MHz), power densities (30 $mW/cm^2$and 60 mW/cm^2) and duration (5 and 10 min) and were observed following 1h, 24h and 72h to identify the stimulation condition that induced the higher metabolic activities. The most promising stimulation conditions were selected and daily applied in the second phase up to 3 days.

MTT ASSAY

MTT cell proliferation assay (Celltiter 96®, Promega®, USA) is based on the cellular conversion of a tetrazolium salt into a formazan product that is easily detected using a 96-well plate reader (Figure 3.1.12). The assay is performed by the addition of a premixed optimized dye solution to cultured cells. During a 4-hour incubation, living cells convert the tetrazolium component of the dye solution into a formazan product. The solubilization solution is then added to the cells to solubilize the formazan product, and the absorbance at 570nm is recorded using a 96-well plate reader. To exclude the effect of cell culture medium, in each assay, a blank well was prepared with only culture medium without cells. Afterwards, its absorbance was subtracted to the absorbance obtained from the cultured wells.

The cell viability was analyzed after 1h, 24h and 72h of stimulation. MTT reagent was added to each well (150µL) where cells were previously cultured with culture medium. The cells and blank were incubated for 4h at 37°C in a humidified atmosphere of 5 % CO_2.

Figure 3.1 12- Microplate reader

RESULTS

For each timepoint and type of cells, the data obtained by the 96-well plate reader was organized in the form of graphs represented in the figures 3.2.1, 3.2.2 and 3.2.3. In these graphs, each condition is represented by a color.

OSTEOBLASTS

After 1h of stimulation, when compared to the control (untreated cells) all groups of cells presented higher metabolic activity with the exception of experimental groups with stimulation parameters of 1.5 MHz, 30 mW/cm^2 for 5 and 10 min (black and purple, respectively). The stimulated cells with the conditions 1 MHz, 30 mW/cm^2 for 5 min (yellow); 1 MHz, 60 mW/cm^2 for 10 min (green) and 1.5 MHz, 60 mW/cm^2 for 5 min (red) showed higher cell viability (Figure 3.2.1).

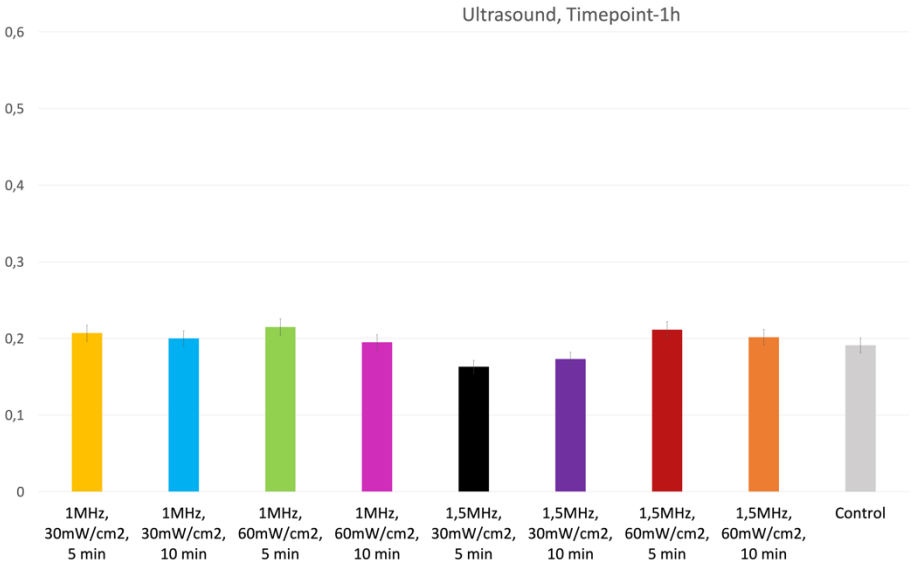

Figure 3.2.1- Results of osteoblasts first timepoint (1h)

After 24h of stimulation, all experimental cell groups exhibited improved viability results compared to the control group, being the stimulation

parameters 1 MHz, 30 mW/cm² for 5 and for 10 min (yellow and blue, respectively) and 1 MHz, 60 mW/cm² for 5 min (green) more effective in enhancing the osteoblasts viability (Figure 3.2.2).

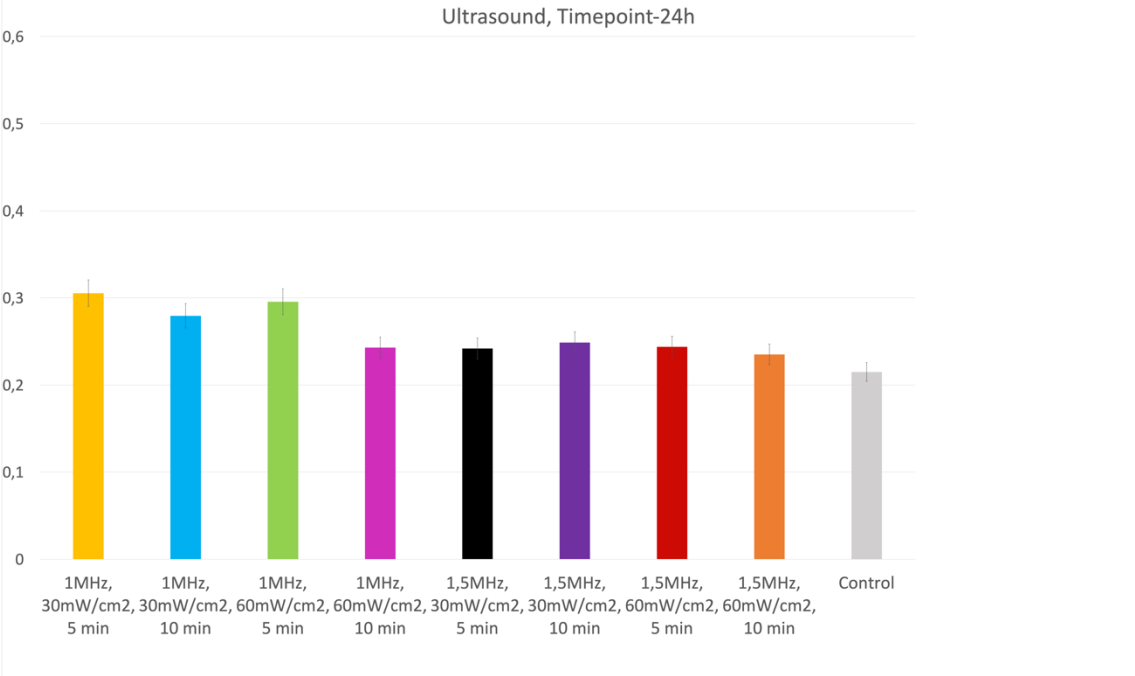

Figure 3.2.2- Results of osteoblasts second timepoint (24h)

The experimental conditions 1 MHz, 30 mW/cm² for 5 and/or 10 min (yellow and blue, respectively) increased the cell viability, after 72h of stimulation, which may demonstrate that these parameters are constant over time. In the other hand, we observed a significant increase for the experimental condition 1 MHz, 60 mW/cm² for 10 min (pink) at 72h compared to the others timepoints (Figure 3.2.3).

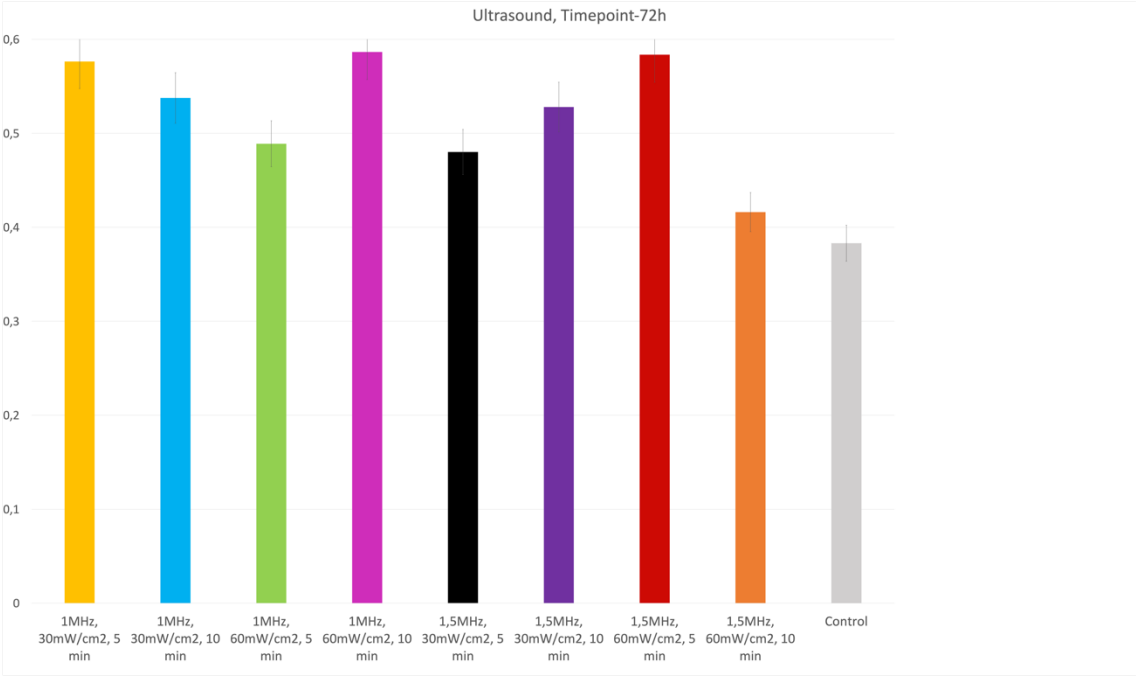

Figure 3.2.3- Results of osteoblasts third timepoint (72h)

Analyzing the behavior of cells under these different experimental conditions, ultrasound application may enhance the osteoblasts proliferation without negatively affecting their viability over time, regardless of the stimulation parameters used.

We selected the 1 MHz, 30 mW/cm^2 for 5 min (yellow); 1 MHz, 60 mW/cm^2 for 5 min (green) and 1 MHz, 60 mW/cm^2 for 10 min (pink) as the best stimulation conditions for the 2^{nd} phase study.

FIBROBLASTS

After 1h of stimulation, only the experimental groups with the parameters 1 MHz, 60 mW/cm^2 for 5 min (green) and 1.5 MHz, 60mW/cm^2 for 5 min (red) showed improved metabolic activity compared to the control group (Figure 3.2.4). These groups, after 24h of stimulation, maintained a higher cell viability compared to the control. However, it is possible to verify an improvement of the conditions 1 MHz, 30 mW/cm^2 for 10 min (blue) and the 1.5 MHz, 60 mW/cm^2 for 10 min (orange) in relation to the first timepoint (Figure 3.2.5).

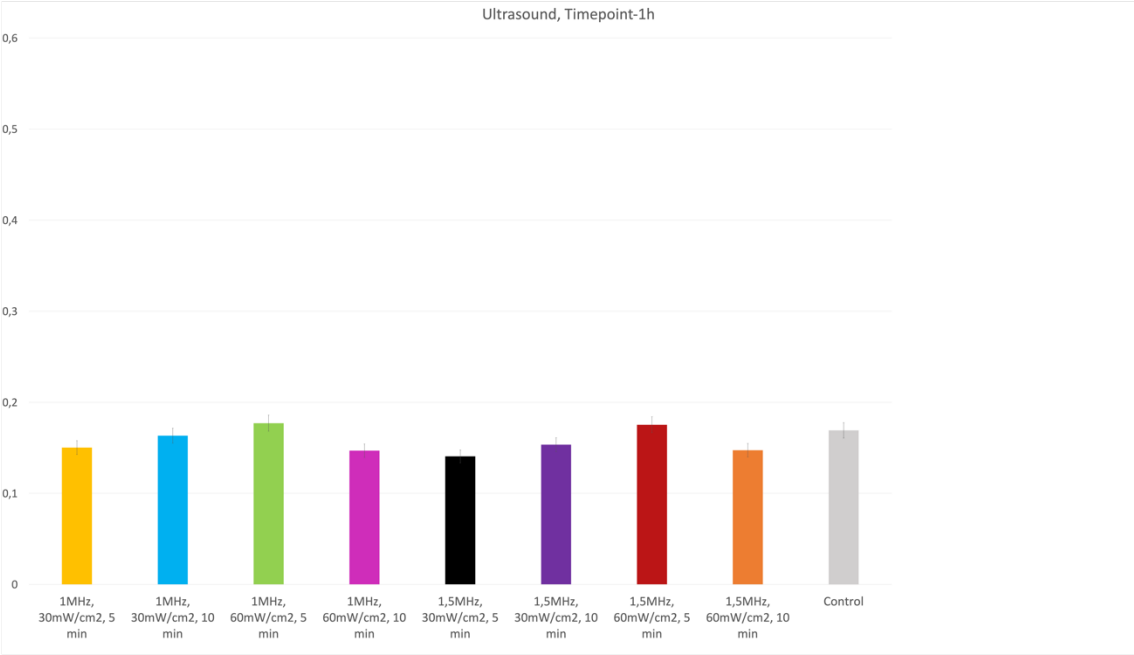

Figure 3.2.4- Results of fibroblasts first timepoint (1h)

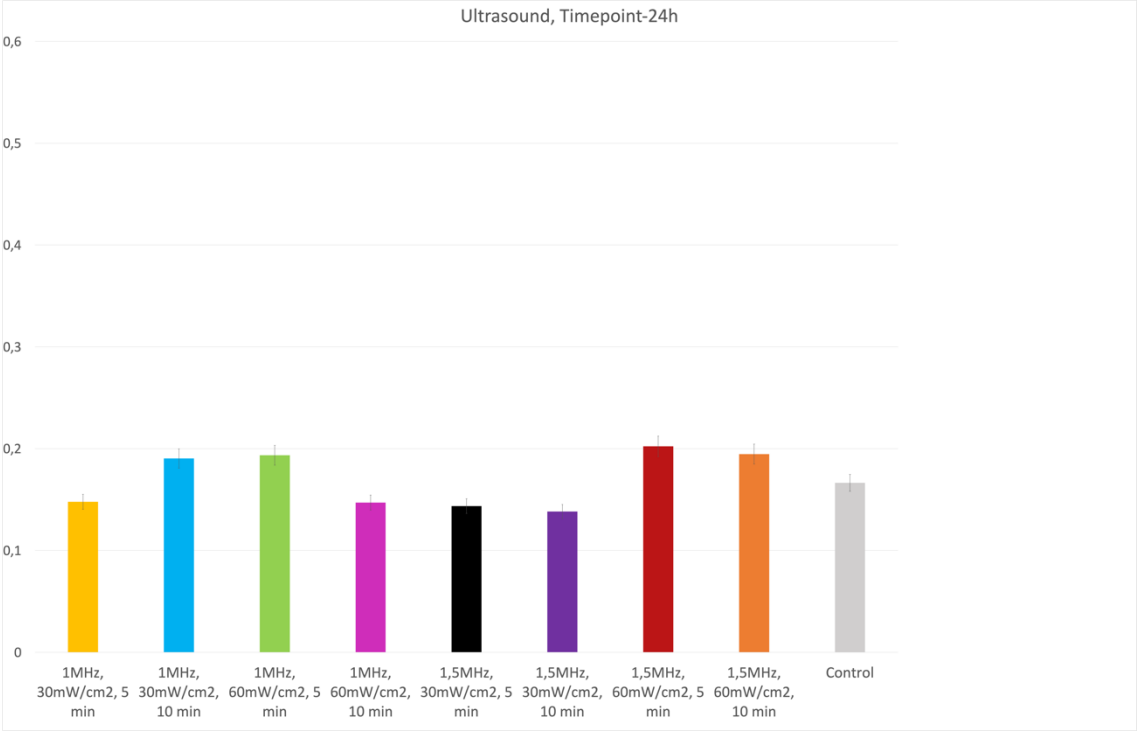

Figure 3.2.5- Results of fibroblasts second timpoint (24h)

After 72h of stimulation, the experimental condition 1.5 MHz, 60 mW/cm^2 for 5 min (red) improved the cell viability when compared to the same stimulation parameters but for 10 min (green). Also, it is evident, the increase of the activity of stimulated cells in the condition 1.5 MHz, 60 mW/cm^2 for 10 min (orange). The condition 1 MHz, 30 mW/cm^2 for 5 min (yellow) induced higher fibroblasts activity after 72h of stimulation (Figure 3.2.6).

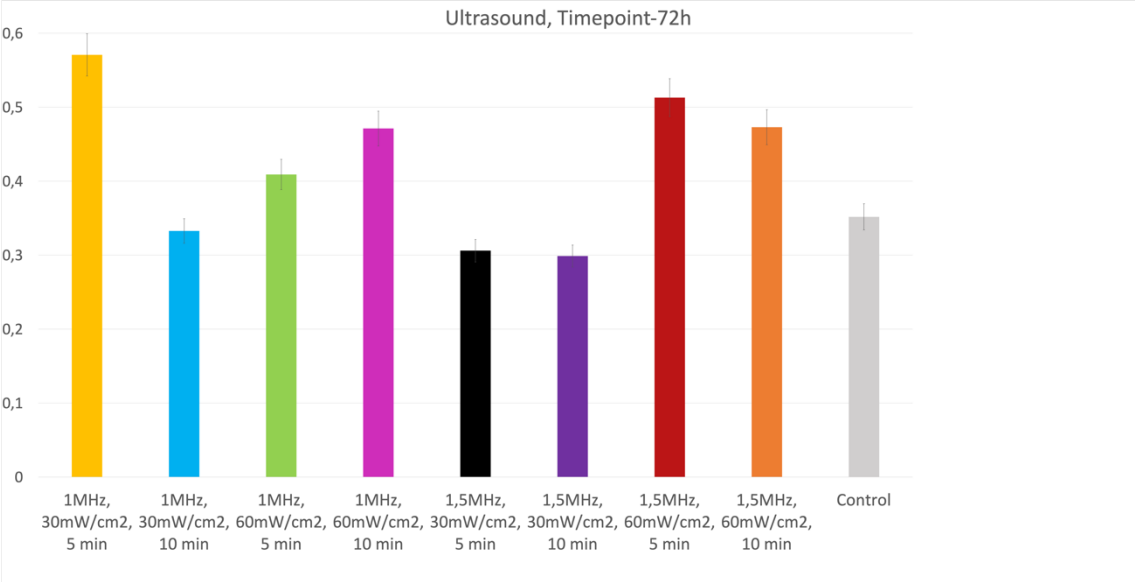

Figure 3.2.6- Results of fibroblasts third timepoint (72h)

We selected the 1 MHz, 30 mW/cm^2 for 5 min (yellow), 1.5 MHz, 60 mW/cm^2 for 5 min (red) and 1.5 MHz, 60 mW/cm^2 for 10 min (orange) as the best stimulation conditions for the 2nd phase study. In fibroblasts, we were able to verify that most experimental conditions promoted cell proliferation over time but not all, contrary to what happened in osteoblasts. However, none experimental condition compromised the viability of the cells.

4- DISCUSSION

In this systematic review, the main findings are that the application of different types of stimulation in *in vitro* does not negatively affect the cells viability and promote proliferation and differentiation, which suggests an accelerated process of bone remodeling.

Bone cells activation is crucial for elevated modeling and remodeling required for accelerated tooth movement.

All vibration studies reported increased cell activity in all cell types. Vibration increases the bone mass by osteocytes as a chief mechanosensory cells in the bone. Osteocyte damage induced by vibration also resulted in increased production of RANKL, an essential factor for osteoclastogenesis, and, consequently, increasing osteoclast formation. Thus, vibration could enhance osteoclastogenesis, although it was only explored in one *in vitro* study that used osteocytes have found.(3) On the other hand, OPG was increased, binding as a decoy receptor of RANKL and decreased the RANKL/OPG ratio in osteoblasts.(13,15,16)

Osteoblasts, osteoclasts and fibroblasts upregulated cytokines that suggests inflammatory response.(6,13,18,19,21) Compressive forces promote the expression of COX-2, the chief enzyme responsible for the majority of prostaglandin (PG) production, in PDL and osteoblastic cells. In PDL cells, RANKL was increased while had no effect on osteoblastic cells.(6,18,19,21)

After ultrasound stimulation, RANKL production was enhanced in osteoclasts, whereas RANKL expression decreased in cementoblasts or were unaffected in osteoblasts.(9–12,22) The altering of RANKL/OPG expression ratio resulted in inhibition of cementoclastogenesis and, consequently, reduced the number of cementoclasts located along the root surface. Therefore, the application of ultrasound was able to repair and minimize

orthodontically-induced tooth root resorption.(12) Another study has shown the increasing osteoclast number and the RANKL expression on pressure side, as well as the BMP-2 positive hPDL cells on the tension surface side. BMP-2, as the osteoclast differentiation factor, is crucial for osteoblast proliferation and this can accelerate bone remodeling.(22)

The effect of shock wave treatment can induce inflammatory reaction on periodontal ligament fibroblasts (PDLF) by the expression of inflammatory cytokines. The effect of cytokines expression on bone remodeling is important, since they can stimulate osteoclast differentiation and the speed of tooth movement depends on the efficiency of alveolar bone remodeling. The authors demonstrated that had a dose-dependent stimulatory effect on IL-6, IL8 and a TNF-α expression.(23)

OPN is a crucial factor in regulating bone remodeling in responses to mechanical stresses by promoting osteoclastogenesis and osteoclast activity. In mechanical strain study, the authors observed an early response of osteoblast-like cells. OPN was significantly secreted by the strained cells which implies an accelerated orthodontic tooth movement.(24)

Comparing the different types of therapy, vibration is the most documented and has several biological evidences. However, the application of ultrasound, although less studied, can enhance bone remodeling. The results of our study can contribute not only to increase the knowledge about the effect of ultrasound on orthodontic movement but also to select the best ultrasound parameters used. Future research with different parameters of ultrasound in tooth movement should be carried out.

Ultrasonic stimulation of fibroblastic and osteoblastic cells appears to be promising. In the present study, we continuously stimulated human osteoblasts and fibroblasts, that play an important role for bone remodeling, and investigated which ultrasound parameters should be used. Previous in vitro ultrasound studies used cell lines obtained from animal sources, while our study only used human-derived cells which increases the potential of this therapeutic application in clinics. According to previous studies, none stimulation parameters compromised the cellular viability as well as the proliferation and suggested that ultrasound stimulation accelerates bone remodeling. This is in agreement with ours findings since we reported no negative effect on cells, following stimulation.(10–12,22) In our experiment, we showed that the best conditions of stimulation were 1 MHz, 30 mW/cm^2 for 5 min, 1 MHz and 60 mW/cm^2 for 5 min and for 10 min on osteoblasts and 1 MHz, 30 mW/cm^2 for 5 min, 1.5 MHz, 60 mW/cm^2 for 5 min and 1.5 MHz, 60 mW/cm^2 for 10 min on fibroblasts, since these stimulation parameters promoted the best metabolic activity results .Also, previous studies applied ultrasound for 10, 15 or 20 min in a pulsed mode and, in our experiment, it was demonstrated that when continuous ultrasound stimulation was applied for shorter times (5 min and 10 min per day), the findings are also equally good, exposing the cells to less stimulation.

5-CONCLUSION

Although vibration is the most used and studied type of accelerating orthodontic movement, the use and the effect of ultrasound and shock waves therapy in cells has also been positively described, although further studies are needed.

Following the results of our study, in both types of cells the ultrasound parameters 1 MHz, 30 mW/cm2 for 5 min improved the greatest effectiveness on cells viability and, therefore, could be potential stimulation condition. Thus, exposing the cells to ultrasound stimulation for a shorter time also causes a positive cells response without compromising the viability.

In the future, in order to understand the effect of using ultrasound as a promising therapy in accelerating orthodontic movement, more cellular assays are needed to analyze the expression of specific markers of bone remodeling (e.g RANKL, OPG) during orthodontic tooth movement.

BIBLIOGRAPHY

1. Henneman S, Hoff JWV den, Maltha JC. Mechanobiology of tooth movement. Eur J Orthodont. 2008;30(3):299–306.

2. Huang H, Williams RC, Kyrkanides S. Accelerated orthodontic tooth movement: molecular mechanisms. Am J Orthod Dentofac Orthop Official Publ Am Assoc Orthod Const Soc Am Board Orthod. 2014;146(5):620–32.

3. Sakamoto M, Fukunaga T, Sasaki K, Seiryu M, Yoshizawa M, Takeshita N, et al. Vibration enhances osteoclastogenesis by inducing RANKL expression via NF-κB signaling in osteocytes. Bone. 2019;123:56–66.

4. Proffit WR, Fields HW, Sarver DM. Ortodontia Contemporânea. 5. ed. Elsevier, editor. Rio de Janeiro; 2013. 768 p.

5. Asiry MA. Biological aspects of orthodontic tooth movement: A review of literature. Saudi J Biol Sci. 2018;25(6):1027–32.

6. Benjakul S, Jitpukdeebodintra S, Leethanakul C. Effects of low magnitude high frequency mechanical vibration combined with compressive force on human periodontal ligament cells in vitro. Eur J Orthodont. 2017;40(4):356–63.

7. Li Y, Jacox LA, Little SH, Ko C. Orthodontic tooth movement: The biology and clinical implications. Kaohsiung J Medical Sci. 2018;34(4):207–14.

8. Judex S, Pongkitwitoon S. Differential Efficacy of 2 Vibrating Orthodontic Devices to Alter the Cellular Response in Osteoblasts, Fibroblasts, and Osteoclasts. Dose-response. 2018;16(3):1559325818792112.

9. Zhu H, Cai X, Lin T, Shi Z, Yan S. Low‐intensity Pulsed Ultrasound Enhances Bone Repair in a Rabbit Model of Steroid‐associated Osteonecrosis. Clin Orthop Relat R. 2015;473(5):1830–9.

10. Feres MFN, Kucharski C, Diar-Bakirly S, El-Bialy T. Effect of low-intensity pulsed ultrasound on the activity of osteoclasts: An in vitro study. Arch Oral Biol. 2016;70:73–8.

11. Inubushi T, Tanaka E, Rego EB, Kitagawa M, Kawazoe A, Ohta A, et al. Effects of Ultrasound on the Proliferation and Differentiation of Cementoblast Lineage Cells. J Periodontol. 2008;79(10):1984–90.

12. Inubushi T, Tanaka E, Rego EB, Ohtani J, Kawazoe A, Tanne K, et al. Ultrasound stimulation attenuates resorption of tooth root induced by experimental force application. Bone. 2013;53(2):497–506.

13. García-López S, Villanueva RE, Massó-Rojas F, Páez-Arenas A, Meikle MC. Micro-vibrations at 30 Hz on bone cells cultivated in vitro produce soluble factors for osteoclast inhibition and osteoblast activity. Arch Oral Biol. 2020;110:104594.

14. Phusuntornsakul P, Jitpukdeebodintra S, Pavasant P, Leethanakul C. Vibration activates the actin/NF-κB axis and upregulates IL-6 and IL-8 expression in human periodontal ligament cells. Cell Biol Int. 2020;44(2):661–70.

15. Pravitharangul A, Suttapreyasri S, Leethanakul C. Iliac and mandible osteoblasts exhibit varied responses to LMHF vibration. Cell Biol Int. 2018;42(10):1349–57.

16. Pravitharangul A, Suttapreyasri S, Leethanakul C. Mandible and iliac osteoblasts exhibit different Wnt signaling responses to LMHF vibration. J Oral Biology Craniofacial Res. 2019;9(4):355–9.

17. Chintavalakorn R, Khantachawana A, Viravaidya-Pasuwat K, Santiwong P, Surarit R. In vitro effects of mechanical stimulation and photobiomodulation on osteoblastic cell function: A proof of concept study. Pediatric Dent J. 2017;27(1):29–41.

18. Benjakul S, Leethanakul C, Jitpukdeebodintra S. Low magnitude high frequency vibration induces RANKL via cyclooxygenase pathway in human periodontal ligament cells in vitro. J Oral Biology Craniofacial Res. 2019;9(3):251–5.

19. Benjakul S, Unat B, Thammanichanon P, Leethanakul C. Vibration synergistically enhances IL-1β and TNF-α in compressed human periodontal ligament cells in the frequency-dependent manner. J Oral Biology Craniofacial Res. 2020;10(4):412–6.

20. Chatmahamongkol C, Pravitharangul A, Suttapreyasri S, Leethanakul C. The effect of compressive force combined with mechanical vibration on human alveolar bone osteoblasts. J Oral Biology Craniofacial Res. 2019;9(1):81–5.

21. Phusuntornsakul P, Jitpukdeebodintra S, Pavasant P, Leethanakul C. Vibration enhances PGE2, IL-6, and IL-8 expression in compressed hPDL cells via cyclooxygenase pathway. J Periodontol. 2018;89(9):1131–41.

22. Xue H, Zheng J, Cui Z, Bai X, Li G, Zhang C, et al. Low-Intensity Pulsed Ultrasound Accelerates Tooth Movement via Activation of the BMP-2 Signaling Pathway. Plos One. 2013;8(7):e68926.

23. Cai Z, Falkensammer F, Andrukhov O, Chen J, Mittermayr R, Rausch-Fan X. Effects of Shock Waves on Expression of IL-6, IL-8, MCP-1, and TNF-α Expression by Human Periodontal Ligament Fibroblasts: An In Vitro Study. Medical Sci Monit Int Medical J Exp Clin Res. 2016;22:914–21.

24. Liu D, Vandahl BB, Birkelund S, Nielsen LB, Melsen B. Secretion of osteopontin from MG-63 cells under a physiological level of mechanical strain in vitro—a [35S] incorporation approach. Eur J Orthodont. 2004;26(2):143–9.