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Effect of Corticopuncture (CP), Photobiomodulation (PBM) and the Combined Method on the Rate of Tooth Movement and Root Resorption: A Molecular, Histological and Micro-CT Study in Animals

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Effect of Corticopuncture (CP), Photobiomodulation (PBM) and the Combined Method on the Rate of Tooth Movement and Root Resorption: A Molecular, Histological and Micro-CT Study in Animals

A thesis submitted in partial satisfaction of the

requirements for the degree Master of Science

in Oral Biology

by

Martha Carolina Torres

2020

ABSTRACT OF THE THESIS

Effect of Corticopuncture (CP), Photobiomodulation (PBM) and the Combined Method on the Rate of Tooth Movement and Root Resorption: A Molecular, Histological and Micro-CT Study in Animals

by

Martha Carolina Torres

Master of Science in Oral Biology

University of California, Los Angeles, 2020

Professor Kang Ting, Chair

Introduction: Orthodontic treatment time on average lasts 24 months. The long duration of the treatment is one of the most frequent complaints of patients. Moreover, the risks associated include caries, root resorption, decalcification, periodontal disease, and others. For this reason, there is pressing need to develop new methods to accelerate orthodontic tooth movement. The Corticopuncture (CP), Photobiomodulation (PBM) and the combination of both methods have shown in prior studies significant results of increasing tooth displacement. Based on these data, we hypothesize that CP, PBM and CP+PBM can enhance alveolar bone remodeling, rate of tooth movement and minimize root resorption by stimulating

metabolic changes and cellular differentiation. I tested my hypothesis through four aims: (1) to assess the rate of tooth displacement on days 1,3 and 7; (2) to investigate the expression levels on days 1,3 and 7; (3) to analyze the changes on bone remodeling on days 1,3 and 7; and (4) to asses root resorption crater volume after 7 days. Methods: Orthodontic tooth movement was induced in 27 male Wistar rats. CP procedure included 3 perforations. GaAlAs diode laser was performed every other day for 7 days (810 nm, 100 mW, 15 s). Gingival tissue was collected and qPCR to isolate and quantify mRNA levels. The tooth displacements were measured directly from rat's mouth. Bone responses at the tension and compression sites and root resorption were evaluated from micro-CT images. Hemi-maxillae from all groups were dissected and prepared for histological and immunohistochemistry analysis. Results and conclusions: Our findings suggest that all (1) ATM groups showed increase tooth displacement compared to control after 7 days (30% PBM, 45% CP and 58% CP+PBM); (2) all ATM groups showed less RR volume compared to the control group; (4) PBM showed a more significant role in increasing bone formation and CP showed a more significant role in catabolic activity; (5) CP+PBM illustrated synergic effects; (6) higher expression of VEGF, Prx1 and GLUT1 found in all methods; (7) and that PBM can enhance tooth movement by stimulating the RANK/RANKL/OPG system.

The thesis of Martha Carolina Torres is approved.

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2020

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INTRODUCTION

Orthodontic tooth movement is a complex biological process that involves many different local responses in the PDL, alveolar bone, gingiva, and pulp[1]. Therefore, numerous cells, mediators, tissues and signaling pathways are related to tooth movement.[4]

Orthodontic tooth movement occurs as a result of a force applied to the tooth. The force applied produces alterations in vascularity, leading to the release of inflammatory mediators such as neurotransmitters, growth factors, cytokines and chemokines, which play an important role in remodeling the bone [2,3].

Orthodontic treatment time on average lasts 24 months. The long duration of the treatment is one of the most frequent complaints of patients. Moreover, the risks associated with longer treatment time include caries, root resorption, decalcification, periodontal disease, and others[4]. For this reason, the innovation of wires and brackets have greatly improved. However, technology modernization may be close to reach its limit, thus more recently, new methods to accelerate orthodontic tooth movement based on biological principals have been proposed, such as: pharmacological and biological agents (Cytokines[5], Prostaglandin E[6], Vitamin D[7]); device-assisted techniques (lower-level laser therapy[8-12], vibration[13]); surgical approaches (corticotomies[14,15]), and minimally-invasive surgical techniques such as corticision[16], piezopuncture[17] , microosteoperforation[18,19],and corticopuncture[20]. Although pharmacological approaches have demonstrated an acceleration of tooth movement, they also have produced adverse effects, such as severe root resorption[6]. For this reason, the trend nowadays is to find a non-invasive or less invasive method in order to avoid the complications and side effects of the biological molecules and drugs: Corticopuncture (CP) and photobiomodulation (PBM).

Previous studies have shown that the corticopuncture (CP) technique increases the bone remodeling response through the regional acceleratory phenomenon (RAP)[20,21]. This phenomenon occurs when a regional noxious stimulus affects the bone. This process generates normal healing process, increases the turnover of the alveolar bone and, consequently, encourages faster tooth movement. [22] The biologic principle of the RAP is used when a physical trauma performed surgically in the cortical portion of the alveolar bone[23]. Another method to accelerate tooth movement is the photobiomodulation (PBM), which stimulates bone resorption and bone deposition. The combination of both methods has shown significant cumulative effects to speed up the tooth movement[21].

The rationale behind these iatrogenic interventions is to activate the osteoclast differentiation at the site of orthodontic tooth movement by stimulating cytokine release locally. Inflammatory biomarkers such as tumor necrosis factor (TNF-alpha) has been found to induce osteoclast differentiation as well as regulation of bone remodeling[24]. And the role of receptor activator of nuclear factor- kappa (RANK), receptor activator of nuclear factor- kappa ligand (RANKL) and osteoprotegerin (OPG) mechanism has also been shown to be strongly related to the bone remodeling process[23,25].

RANKL is released by osteoblast cells and binds to RANK receptor on osteoclast cells to initiate differentiation of hematopoietic osteoclast precursors to the mature osteoclast cells. Meanwhile, OPG is released by osteoblastic cells and binds to RANKL receptor to inhibit the formation of osteoclast cells. Obviously, RANK-RANKL/OPG signaling pathway is one of the major mechanisms in regulating the osteoclastic activity in bone structure[27]. A previous study reported[11] that low-energy laser irradiation increased the rate of tooth movement via RANK and RANKL expression.

The balance between the level of osteoblast and osteoclast is important in terms of the bone remodeling process during tooth movement. In prior studies[28], TRAP as a bone resorption biomarker and Runx-2 as a bone formation biomarker have been investigated to gain a better insight into the velocity of tooth movement.

The process of osteoblasts and osteoclasts differentiation is controlled by signal transduction and complex gene transcription factors. Runt-related transcription factor 2 (RUNX2) and Osterix (OSX) are among the key factors of transcription for osteoblasts. RUNX2 is a multifunctional transcriptional factor and expressed in the whole process of osteoblast cell differentiation. It also regulates the expression of osteoprotegerin[41]. OSX is a derivative of RUNX2 in the transcriptional differentiation cascade of osteoblastogenesis whereas its expression is upregulated by the direct binding of RUNX2 to the responsive element in the OSX gene promoter. Based on prior studies[41] the authors suggested that RUNX2 and OSX may be involved in the early response of bone cells to mechanical signal.

Moreover, the expression of certain genes, such as the vascular endothelial growth factor (VEGF)[29], Peroxiredoxin 1 (Prx1) [30], glucose transporter 1 (GLUT1)[31] and type I collagen (COL1) [32] play a major role in the process of bone remodeling in tooth movement. They enhance the cellular metabolism due to their association with angiogenesis, glucose uptake, collagen synthesis, and oxidative stress.

OBJECTIVES AND SPECIFIC AIMS

The purpose of this study was to evaluate the effect of Corticopuncture technique (CP), Photomodulation (PBM) and the combination of both methods (CP+PBM) on the rate of tooth movement and root resorption in rats using histological, immunohistochemistry and micro-CT evaluation.

SPECIFIC AIMS

AIM 1: To determine the rate of tooth displacement in the control, CP, PBM and CP+PBM group after 1, 3 and 7 days of tooth movement in order to find which method(s) results in accelerating tooth movement.

AIM 2: To investigate the expression levels of VEGF, PRX1, GLUT1 and COL1 genes in the control, CP, PBM and CP+PBM group after 1,3 and 7 days of tooth movement aiming to understand the metabolism of the evaluated methods.

AIM 3: to analyze the changes on bone remodeling in the control, CP, PBM and CP+PBM group after 1, 3 and 7 days of tooth movement in order to study molecular, cellular and tissue response and changes.

AIM 4: To assess root resorption crater volume in the control, CP, PBM and CP+PBM group after 7 days of tooth movement with the purpose of finding whether the ATM methods has an effect on root structure.

MATERIALS AND METHODS

All experimental procedures followed the current norms of the National Council for Control of Animal Experimentation and were submitted to the approval of the Committee for Ethics in the Use of Animals of the São Leopoldo Mandic Research and Post-Graduation Center. The following study was conducted with a sample size of 27 male *Wistar* rats (*rattus norvergicus, albinus*) of 2-3 weeks of age with an average weight of 300g. Animals were housed in an environment with regular cycle of day and a standard 12-h light/dark and fed with standard powdered food and water ad libitum.

A randomized study group selection with split-mouth controlled experimentation was designed. Rats underwent orthodontic force application to both maxillary first molars against one mini-implant placed in the anterior palatal bone. Mini-implants were inserted on the right and left sides randomly for all groups. Corticopuncture and/or photobiomodulation (PBM) was performed only on the left side of the maxillae. The contralateral side, the right maxillae, did not received any stimulation and was used as control group. The maxillary left first molars were stimulated by photobiomodulation (PBM), corticopuncture (CP) and corticopuncture+photobiomodulation (CP+PBM) during the tooth movements; and right first molars were retracted without additional stimulation as the control group (Fig. 1).

The animals were placed under general anesthesia Ketamine Hydrochloride (Dopalen ®, Ceva, Paulínia, SP, Brazil), 50 mg / ml, intraperitoneally and immobilized with open mouth on a table specifically designed for this experiment. A tapered mini-implant (MI) with dimensions of 1.5mm diameter and 3mm total length (Peclab, Belo Horizonte, MG, Brazil) was inserted 1 mm distal to the upper incisors of the rats. Applied force was 50g using adapted Nickel Titanium coil

springs (Rocky Mountain Orthodontics, Denver, Colorado, USA) from MI to the first molars (Fig. 2). The magnitude of force for each spring was checked using a force gauge (Zeusan, Brazil).

Corticopuncture Technique

Corticopuncture was performed with three perforations: two on the palatal side of the left first molars and one mesial to the left first molars, using a manual driver. Self-drilling tapered miniscrews used for perforation had the following dimensions: 3mm total length (head to tip), 2mm thread and maximum diameter of 1.5 mm (0.7mm diameter at the tip).

Photobiomodulation

Punctual laser irradiation was performed for 15 seconds at the labial and palatal sides on days 0, 2, 4 and 6, a total of 4 irradiations. The laser used was a Gallium-Aluminum-Arsenide (GaAIAs) diode laser (λ = 810 nm, 100 mW output power, 600 μm fiber diameter) with an energy per point of 1.5J. The power of the laser beams was measured before each irradiation using a power meter (Lasercheck, MMOptics, São Carlos, Brazil). The right side had a metal barrier to avoid the indirect irradiation.

Tooth Movement Measurement

Tooth movement was assessed directly by measuring the distance between the cervical area on the mesial aspect of the first molar and the center of the MI head. The measurement was obtained by a compass while the rats were under general anesthesia. The distance recorded was transferred to a paper card and was measured with an electronic digital caliper (Mitutoyo Co., Miyazaki, Japan). To assess the first molar movement, the distance measured on day 0 was

subtracted from the distance on days 1, 3 and 7. All measurements were performed three times by the same investigator, and the average value was recorded in order to minimize the error.

qPCR Analysis

Gingival tissue adjacent to the cervical area of all maxillary first molars were collected and quantitative polymerase chain reaction (qPCR) was used to isolate and quantify mRNA levels. Total RNA was isolated from the full-thickness gingival tissue samples by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The amount of RNA was quantified using NanoDrop® ND-1000 spectrophotometer. For the reverse-transcription reaction, these samples were incubated with a cDNA reverse-transcription kit (Prime Script™ RT reagent kit, Takara, Japan) and kept on the ice before gene expression analysis. GAPDH gene was used as an internal control. The PCR primer sequences were as follows:

PRDX1 - F 5'-CATCCTGCTCCCAGCTTCAA -3'/R 5'-GCAATGATCTCCGTGGGACA -3'

GLUT1 - F 5'- GCTGTGGCTGGCTTCTCTAA-3'/R 5'- CCGGAAGCGATCTCATCGAA3'

VEGF- F 5'- CACGACAGAAGGGGAGCAGAAA-3'/R 5'-ACCGCATTAGGGGCACAC -3'

GAPDH- F 5'-CGCCTGCTTCACCACCTTCT-3'/R 5'-GACTGTGGATGGCCCCTCTG-3'.

Micro-CT Analysis

All the animals were euthanized on the 1st, 3rd and 7th day of the study by anesthetic overdose. The maxilla from each male Wistar rat was dissected, sectioned in hemiarches and were placed in 4% paraformaldehyde (PFA) for 48h and transferred to 70% ethanol afterwards. Samples were stored in cassettes.

All samples were scanned using micro-CT (Skyscan 1172; Bruker Micro-CT, Kontich, Belgium) at an image resolution of 7μm (70kV, amperage of 142 μA, power of 10 W through a 180o of rotation around the vertical axis, and a rotation step of at 0.4o). The 3D images were reconstructed from the raw image data using the NRecon software (SkyScan 1172, Belgium). After reconstruction of the upper first molars, images were first reoriented on each 3D plane using DataViewer software (SkyScan 1172, Belgium) to align the long axis of the distal root. Next, 3D morphometric analysis of the compression and tension sides of the mesial and distal buccal roots were performed using CT-Analyser software (Skyscan 1172, Belgium).

From the obtained three dimensional images we selected the following region of interest (ROI): two rectangular volumes of the alveolar bone next to the mesial and distal buccal roots (tension and compression sites) (Fig. 3). The dimensions of the rectangular volumes were of 200 μm width × 400 μm thickness × 700 μm height. From these two rectangles, the bone mineral density (BMD, g/cm^3), the bone volume and total volume fraction (BV/TV, %), trabecular thickness (Tb.Th, mm) and trabecular separation (Tb.Sp, mm) were measured and analyzed from both sites.

To obtain the root resorption craters, cross sectional views of the areas with root resorption lacunae were generated. Then, craters were outlined by drawing boundary of the lacunae individually (region of interest) in all cross-sectional micro-CT images (200 slices per root), and calculated the volume of each root resorption crater.

Histology Analysis

Hemi-maxillae from all groups were dissected and prepared for histological analysis. The specimens were dehydrated in a series of alcohol baths beginning with 50% and progressing to

100%. Thereafter, the samples were embedded in paraffin, and 4-µm sections were prepared, stained with hematoxylin and eosin dyes(H&E) and photographs of the specimens visualized through an optical microscope were taken using a digital camera (Pentax model K-S2, Tokyo, Japan). Alveolar bone surrounding both mesial and distal roots were histomorphometrically evaluated and the standardization of the histologic slices was done when the full length of the root from the cemento-enamel junction (CEJ) to the apex was observed. Histological descriptive analysis of alveolar bone at the tension and compression sides was performed.

Immunohistochemistry Analysis

For immunohistochemistry analysis, additional slices were prepared on days 1, 3 and 7. Sections of 5µm were mounted on glass slides previously silanized by a 10% solution of 3-aminopropytriethoxy-silane (Sigma Chemical CO, St Louis, Mo / USA) in absolute ethanol. They were deparaffinized in two xylol baths, two baths of 5 minutes at room temperature, and another one for 10 minutes. The cuts were then hydrated in decreasing concentrations of ethanol, from three passes in absolute ethanol (2 min / each) followed by 95% and 70% ethanol for 2 minutes each. These samples were incubated for 24 hours at room temperature with one of the following primary monoclonal or polyclonal antibodies (rabbit as host) anti-Osteoprotegerin (1: 100 Genetex, Irvine, USA - GTX55734); anti-RANKL (1: 100 Genetex, Irvine, USA - GTX32834); anti-TRAP (1: 500 Cloud-Clone, Katy, USA - PAA902Ra01); anti-RUNX2 (1: 100 Cloud-Clone, Katy, USA - PAB011Ra01), anti-Osterix (1: 100 Bioss, Woburn, USA - bs 1110R tr) and anti-TNF- α (1:100 Rheabiotech, Campinas, Brazil- IM-0406). For the secondary antibody, the biotylated anti-goat produced in rabbits was used. The immunohistochemistry reaction with a streptavidin/biotin system and final color reactions was performed by counter-staining with

Mayer- hematoxylin. Following Rogers et al.[33], the sections were scored as 1, 2, 3, or 4, indicating 0–20%, 20–40%, 40–60%, and > 60% positive staining, respectively.

Statistical Analysis

The Shapiro-Wilk test was applied prior to data analysis to assess the normality of each parameter. Considering the Gaussian distribution was obtained, two-way analysis of variance (2-way ANOVA) followed by the Tukey test were used to compare all the measurements. All tests were performed using GraphPad Prism (GraphPad Prism version 8.0.0 for MacOS, GraphPad Software, San Diego, California, USA). The significance level was established at an alpha of 0.05.

RESULTS

Results of Aim 1

Over time, the same pattern of movement was observed in all groups. Tooth movement was significant greater on all three ATM groups (30%CP, 45%PBM and 58% CP+PBM) on days 3 and 7 compared to control group ($p<0,05$). On day 3, CP+PBM group showed more tooth displacement than PBM and control groups($p<0.05$). On day 7, CP and CP+PBM groups had more tooth displacement than PBM group($p<0.05$) (Fig. 4).

Results of Aim 2

Regarding VEGF expression, PBM group showed higher expression on days 1 and 3 compared to other groups ($p<0.05$). By day 3, the combined group (CP+PBM) and CP group showed the peak of VEGF expression compared to the control group($p<0.05$). By day 7, all groups

expression of VEGF decreased significantly for all groups ($p<0.05$), although ATM groups still showing higher levels of VEGF compared to the control group ($p<0.05$). (Fig. 5).

Laser irradiation had a positive impact on the level of Prx1. PBM and CP+PBM groups showed higher expression on days 1 and 3 compared to CP and control groups ($p<0.05$). High expression was maintained for the PBM group until day 7 ($p<0.05$). Over time, the highest expression of Prx1 for ATM groups (PBM, CP and CP+PBM) was observed on day 3, with reduction on day 7 (Fig. 6).

All ATM groups showed higher expression of GLUT 1 compared to the control group on day 1. The highest expression of GLUT 1 was observed on day 3 for all groups, with PBM group showing the highest levels, followed by CP+PBM and CP groups ($p<0.05$). Nevertheless, all groups showed a decline in GLUT1 expression on day 7 (Fig. 7).

Results of Aim 3

Volumetric Analysis of Trabecular Bone: Compression Side

Bone Mineral Density (BMD)

BMD of the trabecular bone at the compression side of the distal buccal root was lower in the CP and in the PBM group compared to the control and CP+PBM on day 1($p<0.05$). However, on day 3, all ATM groups showed significantly lower BMD compared to the control group ($p<0.05$). Lower values of BMD illustrate that there is less bone in the compression side, indicating more osteoclastic activity, and thus more tooth movement. As expected, on day 7, the CP+PBM group less BMD compared to Control and PBM ($p<0.05$) but no difference to CP group (Fig. 8).

Over time, the PBM group, the CP group and the Control group progressively increased the BMD values except for the CP+PBM group, which increased on day 3 but the value decreased again on day 7 ($p<0.05$) (Fig. 8).

Bone Volume to Total Volume Ratio (BV/TV)

Regarding the bone volume to total volume ratio (BV/TV), CP and PBM groups showed lower values compared to control and CP+PBM groups on day 1($p<0.05$). On day 3, the PBM group was significantly lower than the ATM groups and control group. Furthermore, on day 7, the CP and the CP+LLLT groups showed lower values compared to the control and PBM group. These results might indicate that on day 7, the intervention groups showed less bone, more osteoclastic activity, and thus an increase of tooth movement. (Fig. 9)

Over time, the control group showed the highest increased of BV/TV compared to ATM groups. CP and CP+PBM groups showed an increase of BV/TV values on day 3, but they remained the same on day 7($p<0.05$). PBM group showed an increase of BV/TV on day 3, however, it decreased on day 7 ($p<0.05$). (Fig. 9)

Trabecular Thickness (Tb.Th.) and Trabecular Separation (Tb.Sp.)

On day 1, CP group showed the lowest trabecular thickness among the groups ($p<0.05$). On day 3, CP and CP+PBM showed significantly less trabecular thickness compared to the control and PBM groups. Furthermore, on day 7, all the ATM groups showed less trabecular thickness than the Control group($p<0.05$) except for PBM group that showed no statistically difference. Over time, the CP and PBM groups remained the same, while the CP+PBM and control groups progressively decrease. (Fig. 10)

Regarding the trabecular separation, on day 1 and 3, PBM group showed the highest trabecular separation among all groups ($p<0.05$). However, on day 7, it was the CP+PBM group that showed the highest value among all groups($p<0.05$). Over time, the CP and CP+PBM progressively increased, while PBM and control groups showed an increase on day 3, maintaining it till day 7. (Fig. 10)

Volumetric Analysis of Trabecular Bone: Tension Side

Bone Mineral Density (BMD)

BMD of the trabecular bone at the tension side of the mesial buccal root was higher on the CP+PBM and control group than the PBM and CP groups on day 1 ($p<0.05$) . On day 3, the PBM group showed a significant increase in BMD compared to the ATM groups and control group($p<0.05$). On day 7, CP+PBM showed a significant decreased in BMD compared to the rest of the groups ($p<0.05$). Over time, all the groups progressively increase the BMD values except for the CP+PBM group. (Fig. 11)

Bone Volume to Total Volume Ratio (BV/TV)

Regarding bone volume to total volume ratio (BV/TV) in the tension side, the PBM group showed the lowest value compared to control, followed by CP group on day 1($p<0.05$). No difference was found between CP+PBM and control groups on day 1. However, on day 3, the BV/TV value increased significantly in the PBM group compared to the ATM groups and control group($p<0.05$). On day 7, CP showed a significant decreased in BV/TV compared to the CP+PBM, PBM and control groups (Fig. 12). Analyzing these results, we can say that the PBM and CP+PBM groups played a more significant role in increasing the anabolic activity during the new bone

formation than CP on days 3 and 7. The corticopuncture technique may decrease BV/TV in all alveolar bone area around roots, not only compression but also tension side as observed on day 7 where we can have some of the CP effect.

Trabecular Thickness (Tb.Th.) and Trabecular Separation (Tb.Sp.)

On days 1 and 7, the CP+PBM group showed the highest trabecular thickness among all groups ($p<0.05$), indicating more bone formation. On day 3, the CP group showed the lowest trabecular thickness among all groups ($p<0.05$). Over time, the CP+PBM group increased progressively compared to the other groups. (Fig. 13)

Regarding the trabecular separation, CP and PBM showed increased Tb.Sp. values compared to the Control group. However, on day 3, all groups showed increased Tb.Sp values except for the PBM group ($p<0.05$). On day 7, the CP+PBM showed higher values of trabecular separation than PBM and Control groups ($p<0.05$). Among all the intervention groups, the CP+PBM group was the one that increased progressively over time ($p<0.05$). (Fig. 13)

Histology Results

On day 1, the irradiated groups (PBM and CP+PBM) showed a wider periodontal ligament (PDL) space. The CP group showed higher vascularity, and the combined group (CP+PBM) exhibited more osteoclasts activity (Fig 15). On day 3, all intervention groups showed higher number of osteoclasts, wider PDL space and more bone resorption compared to the control group. The CP group showed highest osteoclast activity and greater bone resorption (Fig. 15). On day 7, the PBM group showed a large area of resorbed bone with small Root Resorption (RR) close to the apex of the first molars. The CP group showed greater number of osteoclasts, bone resorption, disorganized PDL and a small area of RR can be noted. The CP+PBM group showed more bone

demineralization, wider PDL space and minor areas of RR compared to other ATM groups. The control group exhibited more extensive area of RR as well as larger area of alveolar bone surrounding the root and less bone demineralization can be noticed. (Fig. 15).

For the tension side, more extensive area of alveolar bone was seen in the PBM group with vast incremental lines (characteristic of a bone neoformation process), uniform frontal alveolar bone and more organized periodontal ligament on day 7. The CP groups and CP + PBM on day 7 showed the less amount of alveolar bone, but several more blood capillaries compared to the control group. Control group at the end of 7 days showed wide periodontal ligament space and irregularity at the alveolar bone edges immediately close to PDL (Fig. 16).

Immunohistochemistry Results

TRAP

On day 1, the expression of TRAP was no statistically different among the groups. However, on day 3, the PBM group and the CP+PBM group showed higher expression of TRAP compared to the control and CP group ($p<0.05$), indicating more osteoclastic activity in these groups. On day 7, the CP+PBM group showed the highest expression of TRAP compared to the other groups in the compression side ($p<0.05$). Over time, the expression of TRAP progressively increased in the CP+PBM group in the compression side ($p<0.05$) (Fig. 17).

For the tension side, the Control and PBM group showed higher osteoclastic activity than CP group on day 1 ($P<0.05$). Among the treatment groups, the CP group showed the highest expression of TRAP on Day 3. But, it was on day 7, that CP group showed the highest expression of TRAP among all groups ($p<0.05$). The CP group is the only group that showed a progressively increase over time, while the other groups remained the same ($p<0.05$) (Fig. 17)

RANKL

On the compression side, the CP+PBM group showed a significant increased expression of RANKL compared to control groups on days 1 and 3 ($p<0.05$). However, there was not a statistically difference among the groups on day 7. Over time, the PBM, CP and control groups increased the expression of RANKL from day 1 to 3, maintaining it until day 7. The CP+PBM group did not showed a significant increased over time, since a high level of RANKL expression was observed from day 1 and maintained until day 7 (Fig. 18)

For the tension side, all the ATM groups showed significant increased expression of RANKL than the control group on day 1 ($p<0.05$). Among the ATM groups, the CP+PBM showed higher expression of RANKL compare to the PBM group, but the CP showed no difference among these groups. On days 3 and 7, the CP+PBM groups showed higher expression of RANKL than control and CP groups ($p<0.05$). Among the ATM groups, CP+PBM showed a significant difference of RANKL expression compare to the CP group ($p<0.05$), however, there was no difference between PBM and these groups. (Fig. 18)

OPG

On day 1, PBM group showed the lowest expression of OPG among the groups, indicating that the PBM group had more osteoclastic activity not inhibited by the OPG markers ($p<0.05$). On day 3, the PBM group showed lower OPG expression compared to the control and the CP group ($p<0.05$). On day 7, CP, PBM and control groups showed lower OPG levels compared to the CP+PBM ($p<0.05$). Over time, there was not a statistically increased in CP and control groups,

however, the PBM and the CP+PBM groups showed a progressively increase of OPG over time ($p<0.05$). (Fig. 18)

On the other hand, in the tension side, the PBM group showed less expression of OPG compared to the other groups on day 1 ($p<0.05$). On day 3 and on day 7, the CP and CP+LLLT showed an increase of OPG expression compared to the other groups ($p<0.05$), indicating more inhibition of bone resorption and osteoclastogenesis. Over time, the PBM and CP+PBM groups showed a progressive increase of OPG expression in the tension side ($p<0.05$) (Fig. 18), indicating more osteoblastic production of OPG and thus more inhibition of osteoclastogenesis.

TNF-alpha

On day 1, the CP+PBM showed the highest expression of TNF-alpha compared to the ATM and control groups($p<0.05$), indicating more bone resorption. On day 3, all the ATM groups showed higher expression of TNF-alpha than the control group($p<0.05$). However, on day 7, only the PBM and CP+PBM maintained the increased expression of TNF-alpha ($p<0.05$). Over time, the PBM group showed a progressively increased expression of TNF-alpha from day 1 to day 7 ($p<0.05$). (Fig. 19)

For the tension side, the CP+PBM showed higher expression of TNF alpha compared to the CP and control groups on day 1($p<0.05$). On day 3, all the ATM groups showed higher levels of TNF-alpha compared to the control group($p<0.05$). On day 7, the CP and CP+PBM groups maintained the higher expression of TNF-alpha compared to control and PBM groups ($p<0.05$). Over time, all the groups showed a progressively increase of TNF-alpha except for CP+PBM that maintained the same levels throughout the entire timeline. (Fig. 19)

RUNX2

In the compression side, on day 1 and 3, there was no significant difference among the groups. On day 7, the CP+PBM showed higher levels of RUNX2 compared to the CP group ($p<0.05$), however, there was no statistically significance between the control and PBM group. Over time, only the CP+PBM group showed an increase of RUNX2 from day 3 to day 7 ($p<0.05$). (Fig. 20)

On the other hand, in the tension side, all groups showed higher expression levels of RUNX2 except for the CP group that showed lower RUNX2 levels on day 1 ($p<0.05$) On day 3, there was no statistically difference among the groups. However, on day 7, the CP showed again the lowest expression of RUNX2 among the groups($p<0.05$). Over time, the PBM and CP+PBM showed an increase of RUNX2 from day 3 to 7, indicating more stimulus for bone formation. (Fig. 20)

OSTERIX

On day 1, all ATM groups showed decreased expression of Osterix compared to the control group ($p<0.05$). On day 3, the PBM showed lower expression of Osterix compared to control and CP+PBM groups ($p<0.05$). In addition, on day 7, the CP group showed the lowest expression of Osterix among the other groups ($p<0.05$), indicating less stimulus for bone formation. (Fig 20)

For the tension side, on day 1, the CP+PBM group showed higher expression of Osterix among the ATM groups ($p<0.05$). However, on days 3 and 7, the PBM and CP+PBM showed higher levels of Osterix than the other groups, indicating more bone formation. There was a progressive increased of bone formation or Osterix expression from day 3 to day 7 on CP+PBM

and PBM groups, indicating more osteoblastic differentiation on these days and these groups. (Fig 20)

Results of Aim 4

Total Volume of Root Resorption Craters

Our findings showed that all the accelerated tooth movement groups showed less volume of root resorption compared to the control group on day 7 ($p<0.05$). Among the treated groups, the CP+PBM showed the lowest amount of root resorption ($p<0.05$). (Fig.14)

DISCUSSION

There is some evidence in the literature that photobiomodulation and corticotomy are promising approaches to accelerate orthodontic tooth movement.[34,35] Also, the association of both therapies have been used as a synergic approach to produce faster tooth movement.[21] However, the mechanism of the acceleration is still unknown.

As previously reported, in a long-term evaluation (14 to 21 days), combining CP and PBM improves tooth movement approximately 61-78%, as shown by Suzuki et al. [21] The present study had similar results, also showing robust bone response to forces in a short term (7 days). CP and CP+ PBM improved tooth movement just in one day after orthodontic activation, compared to control and PBM groups. Moreover, on 3 and 7days, all ATM groups showed significantly more tooth displacement than the control group, while CP+PBM and CP showing the largest tooth movement after 1 week.

In order to determine the dynamics of orthodontic tooth movement and bone response, we evaluated 3D morphometric methods including the bone mineral density, bone volume density,

and three-dimensional trabecular bone architecture obtained by Micro-CT analysis. In this study, the distal buccal root of maxillary first molar on the compression side and the mesial buccal root of the same tooth on the tension side were evaluated.

BMD and BV/TV values on the compression side were lower in treatment groups compared to control on day 3 showing the bone on the pressure side had been demineralized more rapidly in all ATM groups. On Day 7, CP and CP+PBM groups had the lowest bone density measurements (BMD and BV/TV) which illustrates that CP was more effective than PBM in promoting catabolic activity in the compression side; however, the effect of the combined approach (CP+PBM) was significant on lowering BMD and BV/TV. Our results confirmed previous studies that BMD and BV/TV were decreased significantly after 7 days of cortical bone manipulation when tooth movement was combined with alveolar decortication or corticopuncture[12,23]. In addition, our results demonstrate that combined application of corticopuncture and photobiomodulation can indeed increase the rate of orthodontic tooth movement by inducing more rapid bone remodeling on the compression side of the root. On tension side, although BMD value was similar in experimental groups initially, it was increased significantly on day 3 in PBM group. On day 7, CP+PBM showed a significant decrease compared to ATM and control groups, which shows the synergistic effect of corticopuncture and photobiomodulation on bone catabolism is not limited to the compression side and it leads to a decrease in the bone mineral density on both compression and tension sides of the root. Therefore, a decrease in BV/TV and BMD in both sides underlies regulatory processes that initiate accelerated tooth movement. Some studies[36] showed that the alveolar bone fraction (BV/TV) around the tooth was significantly decreased after orthodontic tooth movement in rat models with the decreases occurring on both the compression and tension sides. While others reported that the bone fraction

increased on the compression side and did not change significantly on the tension side one week following orthodontic tooth movement in rats[37]. One possible explanation was that the orthodontic force interferes with the activation-resorption-formation sequence, as it is not exclusively inducing resorption on compression side of a tooth and formation on the tension side. On the contrary, the mechanical disturbance and the RAP caused by orthodontic force and corticopuncture initially cause a highly active bone catabolism and osteoclastic resorption around all root surfaces reflected by the decreased bone fraction around the compression and tension sides of molar. The new bone formation would take place only at a later stage.

Other bone morphometric values also showed the cumulative nature of heightened catabolic/anabolic activity in the ATM groups. In CP + PBM and CP groups trabecular thickness (Tb.Th.) decreased and trabecular separation (Tb.Sp.) increased significantly on day7 illustrating corticopouncture induces catabolic activity in trabecular bone adjacent to compression side. On tension side, both Tb.Th. and Tb.Sp. measurements were significantly higher on day 7 in CP+PBM group which indicates CP and PBM procedures applied together play a role in increasing the bone anabolic activity on the tension side of the root during tooth movement. The findings further suggest that the orthodontic tooth movement enhancement could be due to increased metabolic activity and rapid turnover of the alveolar bone when osteoclastic activity can be initiated earlier, and osteoblastic activity can be amplified by RAP during bone remodeling.

As per the histologic examination, on day 1, groups treated with photobiomodulation (PBM, CP+PBM) showed a wider PDL space whereas corticopuncture treated groups (CP, CP+PBM) had higher vascularity and number of osteoclasts. The increased bone resorption activity observed in all intervention groups on day 3. On day 7, the control group exhibited more extensive areas of root resorption craters (RR) as well as larger area of alveolar bone surrounding

the root, less bone demineralization and narrower PDL space compared to ATM groups. While in CP+PBM group, more bone demineralization, wider PDL space and minor areas of RR were noticed compared to CP, PBM, and control groups. This can be attributed to RAP because it stimulates cell-mediated responses around the tooth and provides a favorable microenvironment for tissue remodeling. Previously, it has been shown that micro-osteoperforation can increase the expression of cytokines and chemokines responsible for stimulating the differentiation of osteoclasts in bone remodeling and thereby enhancing the rate of the bone turnover and tooth movement. Tsai et al.[38] (2016) studied the effects of corticotomy and microperforations and concluded that both minimally invasive interventions increased bone remodeling and there were no significant differences between them. Kim et al.[16] (2009) assessed the effects of corticision on paradental remodeling and showed that corticision could activate catabolic remodeling represented by extensive direct resorption of bundle bone and more rapid removal of necrotic tissue. Our data further support this idea and provide more evidence that Corticopuncture and photobiomodulation procedures have a cumulative effect by activating catabolic remodeling in the direction of tooth movement.

It has been shown that pro-inflammatory cytokines play an important role in the regulation of osteoclastic alveolar bone resorption as well as odontoclastic root resorption. Expression of Cytokines are upregulated in response to inflammatory triggers; thus, they contribute to the differentiation, chemotaxis and activation of osteoclasts and their precursors. Active osteoclasts, inflammatory macrophages, and their mononuclear precursors exhibit a high content of a specific enzyme, tartrate-resistant acid phosphatase (TRAP), which is thought to participate in or signal active bone resorption. Thereby, TRAP+ staining has been a frequently used method for defining osteoclastic activity. In our study, TRAP-positive cells were detected in all groups studied. In

compression side, there was no difference among the groups on day 1. However, On Days 3 and 7, TRAP+ cells were more abundant in PBM and CP+PBM groups showing higher level of osteoclastic activity in groups treated with photobiomodulation. On tension side, the control and PBM showed higher number of TRAP+ cells while on days 3 and 7, the CP group had the highest osteoclastic activity compared to other groups. Our findings are in agreement with previous studies. Suzuki et al[39] (2016) showed that photobiomodulation influenced bone resorption by increasing the number of TRAP+ osteoclasts and the RANKL expression at the compression side in rats.

One of the major proinflammatory cytokines released during orthodontic tooth movement is Tumor necrosis factor-alpha. TNF-alpha is produced by inflammatory cells such as activated monocytes and macrophages and by local cells such as osteoblasts, fibroblasts, and endothelial cells. TNF-alpha stimulates osteoclastic bone resorption and it is an important factor for osteoclast differentiation and initiating bone resorption during orthodontic tooth movement. Our results illustrated that in both compression and tension sides, the level of TNF-alpha expression was increased from day 1 to day 7 in all ATM groups compared to the control. Furthermore, the CP and CP+PBM groups maintained higher expression of TNF-alpha compared to other two groups. Higher levels of expression of cytokines and their receptors are important, since it has been shown that inflammatory cytokines play an important role in recruitment of osteoclasts and activation of the bone remodeling mechanism. Alikhani et al.[18] (2013) reported that the level of specific cytokines including TNF-alpha was elevated during orthodontic tooth movement and adding microosteoperforations could increase the expression of cytokines significantly leading to higher osteoclast activation and a higher rate of tooth movement. Teixeira et al.[5] (2010) also reported that shallow perforations of the cortical plate increased the levels of cytokine expression in response

to orthodontic forces without changing their pattern of expression. Our findings confirm previous studies and further support the idea of increased expression of proinflammatory cytokines are the key factor in the role of corticopuncture in accelerated tooth movement.

Alveolar bone remodeling is a biologic process that involves an interaction of osteoclastic bone resorption with osteoblastic bone formation. Initiation of bone resorption involves recruitment of new osteoclasts and activation of existing osteoclasts. In addition, in both processes the OPG/ RANK/ RANKL signal pathway plays a pivotal role. RANKL (Receptor Activator of Nuclear Factor KappaB Ligand) is a proinflammatory cytokine produced by osteoblasts, and it is activated by lymphocyte T as a regulating factor, fusion, and osteoclastogenesis. Osteoprotegerin (OPG) is a secreted tumor necrosis factor (TNF) receptor member produced by osteoblastic cells and competes with RANK for RANKL binding. RANKL and OPG coordinate to regulate the differentiation and activation of osteoclasts through RANK receptors. It was found that RANKL/OPG ratio was significantly higher during orthodontic tooth movement, thereby, the signaling and regulation of the expression of RANKL and OPG seem to play a critical role in bone remodeling during orthodontic tooth movement.

As of our findings show, in the compression side the level of RANKL expression increased in all groups, however, we observed a significantly higher levels of RANKL in PBM and CP+PBM on day 3. On the tension side RANKL expression increased more in ATM groups compared to the control on days 1 and 7. Comparing ATM groups, on day 3 PBM and CP+PBM groups, and, on day 7, CP and CP+PBM groups showed higher expression of RANKL. In terms of OPG expression, on the compression side the OPG concentration was increased significantly in PBM on day 7 while other groups showed insignificant changes in OPG expression from day 1 to day 7. The reason for the constant level of OPG expression throughout experiment on the compression

side may be to prevent excessive increase in RANKL/OPG ratio in order to limit the resorption process due to increased osteoclastic activity. On the tension side, the CP and CP+PBM groups showed higher concentrations of OPG on days 3 and 7, while PBM had the lowest level of expression on day 1 and 3 compared to other groups. Baloul et al[23] (2011) studied the mechanism of action and morphologic changes in the alveolar bone in response to selective alveolar decortication–facilitated tooth movement. Their findings suggested that selective alveolar decortication might increase the expression of OPG and provide a unique pattern of interaction in the alveolar bone in which the coupling of RANKL and OPG is more rapid and simultaneous compared with conventional tooth movement, where an increase in RANKL was associated with decreased OPG. Fujita et al.[11](2008) studied the effect of low-energy laser on rate of tooth movement. They concluded that the amount of tooth movement was significantly greater in the laser group, and cells that were positive immunoreactions to antibodies of RANKL and RANK were significantly increased in the irradiation group compared with the non-irradiation group. On the contrary, they found that the expression of OPG was not changed during the experiment. Altan et al.[40] (2012) evaluated the effects of low-level laser therapy on orthodontic tooth movement and they also found that the OPG levels were not changed during the experiment. In agreement with current evidence, our findings suggest that photobiomodulation can enhance tooth movement by stimulating the RANK/RANKL/OPG system, reflecting the differentiation level of osteoclasts and essential for bone remodeling.

The process of osteoblasts and osteoclasts differentiation is controlled by signal transduction and complex gene transcription factors. Runt-related transcription factor 2 (RUNX2) and Osterix (OSX) are among the key factors of transcription for osteoblasts. RUNX2 is a multifunctional transcriptional factor and expressed in the whole process of osteoblast cell

differentiation. It also regulates the expression of osteoprotegerin. OSX is a derivative of RUNX2 in the transcriptional differentiation cascade of osteoblastogenesis whereas its expression is upregulated by the direct binding of RUNX2 to the responsive element in the OSX gene promoter. Based on our findings, on the compression side only CP+PBM group showed a significant increase of RUNX2 expression over the 7-day experiment period, while other groups showed insignificant fluctuations in the expression of RUNX2. In the tension side, the PBM and CP+PBM group expressed higher level of RUNX2 on day 7 of the experiment compared to day 1, and the CP group showed the lowest expression of RUNX2. With regard to OSX expression, on the compression side the PBM and CP+PBM group showed higher level of OSX on day 7 compared with day 1, while other groups did not show significant changes throughout the experiment. On day 7, the CP group showed less OSX expression compared to other groups. On the tension side, again the PBM and CP+PBM groups showed higher levels of OSX than the other groups which indicates more bone formation in photobiomodulation and combination groups. Han et al.[41] (2015) showed that the orthodontic force upregulated the expression of Runx2 and OSX over the 7-day experiment period and the upregulation increased with increase in experimental time. Based on these findings the authors suggested that RUNX2 and OSX may be involved in the early response of bone cells to mechanical signal.

As per the gene expression, the VEGF expression increased by bone trauma attracts osteoprogenitor cells and endothelial cells, creating an environment rich in growth factors and cells[42]. Furthermore, VEGF is a proangiogenic cytokine that has been shown to promote osteoclast differentiation and survival[43], therefore, it may potentially influence the process of bone remodeling during tooth movement. As expected and described earlier in other studies [44,45], PBM

and CP have a positive effect on angiogenesis[46], improving the VEGF gene expression after irradiation in PBM and CP+PBM groups.

PBM group presented a 300% increase of VEGF expression compared to other groups after 1 day, and PBM and CP+PBM groups had 3-fold increase in gene expression after 3 days, which seems to be the peak of VEGF expression in our experiment. The expression was higher only by about 50% in all experimental groups compared to control after 7 day. Similar results were found by Zaniboni et al.[46], using western blot analysis after 7 days of orthodontic force; however, they found no significant difference on VEGF levels after 2 weeks.[46]Since PCR method analyses gene expression and western blot method evaluate protein expression, this could explain the different results.

VEGF was positively expressed during the entire period of tooth movement in all groups. Although higher VEGF expression was found in the PBM group in the first day, CP and CP+PBM groups showed no difference on VEGF levels compared to control. Previous study has indicated that a bone trauma results in the partial interruption of blood flow and weak VEGF expression in the following week. [47] On days 3 and 7, VEGF expression was higher in all intervention groups, whereas day 3 representing the peak of VEGF expression for both CP and CP+PBM groups.

Trauma or Orthodontic force could induce bone remodeling due to inflammatory response to these stimuli into bone.[48] The inflammatory process starts when oxidative stress or overproduction of reactive oxygen species (ROS) is generated inside the bone cells and induces apoptosis of osteoblast and osteocytes localized in the matrix of the affected tissue, favoring osteoclastogenesis. Prx1 is an antioxidative enzyme and have the function of balancing the levels of Reactive Oxygen Species (ROS) inside the cell during these processes.[49] For Prx1expression, one of the mechanisms that could explain PBM effects is the oxidative stress model[50-52].

Metabolism modulation occurs when low doses of oxidative stress, i.e Reactive Oxygen Species – ROS, is generated in mitochondria, for example by light irradiation, starting a biochemical cascade inside the cells that results in an increase of ATP[53]; however, high levels of oxidative stress inside the cells could be detrimental to the biologic function and the function of Prx1 enzyme is to control these levels. The effects of oxidative stress were observed for PBM and CP+PBM groups after 1 and 3 days, resulting in an increased expression of Prx1, an anti-oxidant enzyme, and could aid to explain the higher tooth movement for these groups. Corticopuncture also increased Prx1 levels, but only with significant difference on day 3.

GLUT1 is one of the most important glucose transporter enzymes and plays key roles in regulating periodontal ligament cell's functions, including mediating bone remodeling through increased RANKL expression.[31,54] Increase of GLUT1 levels could also be attributed to the hypoxic stress created by physical strain in PDL, where enhanced RANKL expression could be observed, promoting osteoclast differentiation and activation[31]. In this experiment, increased levels of GLUT1 was observed on days 1 and 3 in all experimental groups. GLUT1's peak level occurred on day 3, whereas higher expression was found on PBM, CP+PBM and CP groups. For PBM, CP and CP+PBM groups, higher levels of GLUT1 could explain the higher metabolic cell activity in these groups and consequently more tooth movement, since increased glycolysis process should result in more intracellular ATP production and more energy for metabolic activation. [55]

CONCLUSION

Based on our results, all the ATM groups have indeed shown increased tooth displacement compared to the control after 7 days. Despite the fact that corticopuncture and photobiomodulation could independently accelerate tooth movement and reduce the volume of root resorption, the CP+PBM group showed the highest amount of tooth displacement and the least amount of root amount of root resorption after 7 days. Based in this study, we have concluded that higher expressions of VEGF, Prx1 and GLUT1 found in all methods may have stimulated tooth movement portraying as a pro-inflammatory effect at the PDL and alveolar bone.

CP seemed to have a more significant role in bone catabolism and PBM has shown a more significant role in bone formation, however, the combination of these two techniques has illustrated a synergic effect, and this combined approach could lead to reduction in the number of corticopuncture procedures and/or the number of PBM sessions, achieving similar or even improved bone remodeling response. This therapeutic approach may improve the orthodontic outcome in humans.

STUDY LIMITATIONS

Although our study showed strong evidence for the efficiency of Accelerated Tooth Movement (CP, PBM and CP+PBM), some limitations should be considered. One of the limitations of this study was that the short-term (7-day) treatment period chosen in this study may not cover the time of maximum regional acceleratory phenomenon (RAP) in rats. [19]. Another important limitation is the fact that the study was non-random, the interventions including corticopuncture and/or photobiomodulation were performed only on the left side of the maxillae, and the contralateral side, the right maxillae, did not received any stimulation and was used as

control, it would have been better if one side of the maxilla could be randomly allocated to ATM interventions and the other side to the control to randomly assign samples to groups.

FUTURE DIRECTIONS

Prior to applying this method in clinical setting, future studies should recruit more samples to increase the power of the study. Additionally, future studies should consider further time points to more thoroughly evaluate the effects of ATM methods, specially at the tension side. Also, additional studies need to be conducted to identify the optimal number of perforations and the best protocol regarding dose and time for laser irradiation in ATM methods.

Knowing that the ATM methods showed promising results, it would be appealing to establish human clinical studies. In order to start doing human trials, further studies would be necessary to elucidate the kinetics of tooth movement associated with corticopuncture and PBM, as well as to establish the optimal clinical protocols for CP and PBM procedures for orthodontic treatment in humans. Reducing the orthodontic treatment time definitely help fight and combat the risk of caries, periodontal disease, root resorption, decalcification and other risks associated with long orthodontic treatments. Establishing clinical protocols to speed up the treatment of the patients would definitely increase the patient satisfaction and compliance.

FIGURES

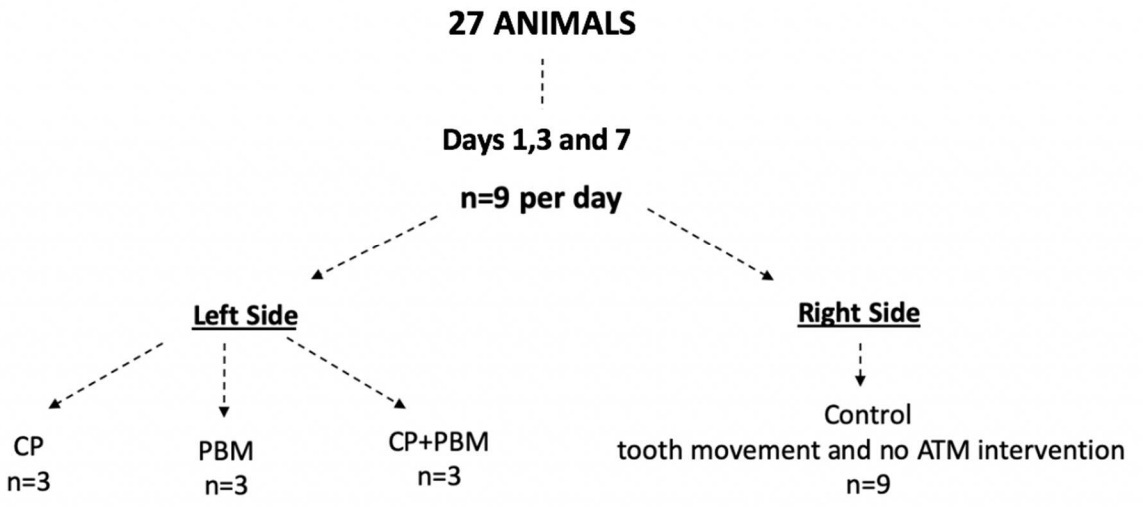

Fig 1. Flowchart showing the sample distribution for the split-mouth design of this study.

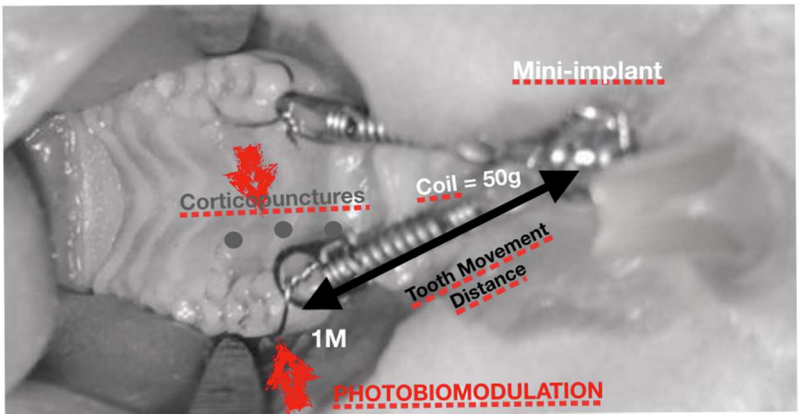

Fig 2. Representative drawing of the design of the orthodontic appliance in palate. A tapered 1.5x3 mini-implant was inserted 1 mm distal to the upper incisors. Applied force was 50g using Nickel Titanium coil springs from mini-implant to the first molars.

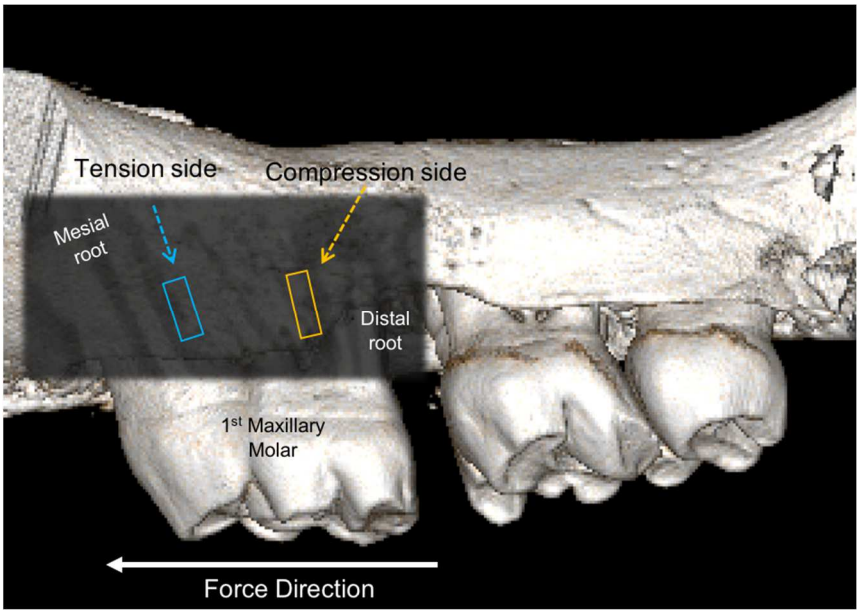

Fig 3. Micro-CT images showing the rectangles delimiting the volume of alveolar bone evaluated.

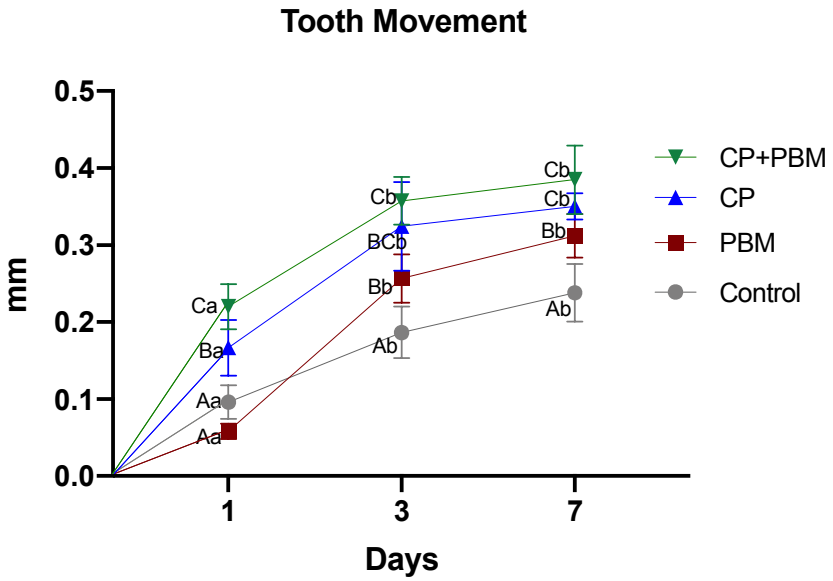

Figure 4. The pattern of the tooth movement in all groups. All ATM groups showed increased tooth displacement compared to control after 7 days. Different capital letters indicate comparison between groups at each time point (Control x PBM x CP x CP+PBM) and lowercase letters indicate comparison for each group over time (days 1 x 3 x 7). Significant level $p<0.05$.

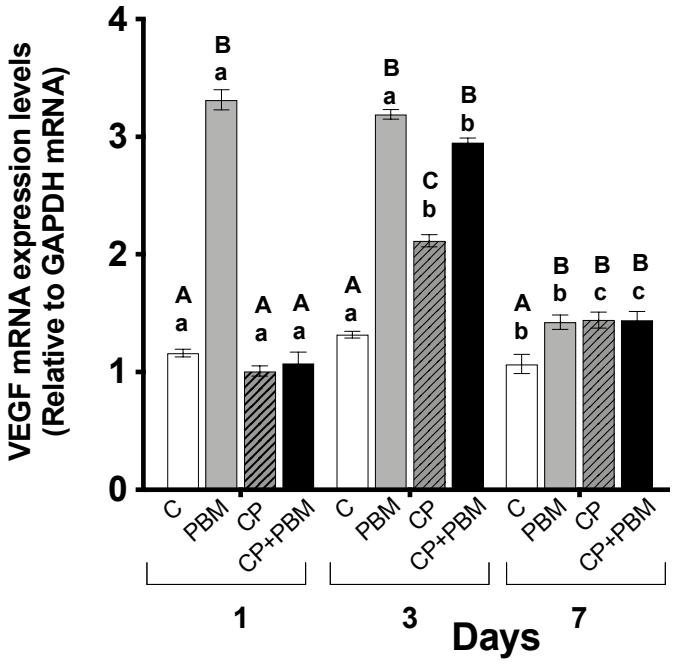

Figure 5. Graphical representation of VEGF expression on days 1,3 and 7 in ATM and control groups. Different capital letters indicate comparison between groups at each time point (Control x PBM x CP x CP+PBM) and lowercase letters indicate comparison for each group over time (days 1 x 3 x 7). Significant level $p<0.05$. Same letters indicate no significant difference.

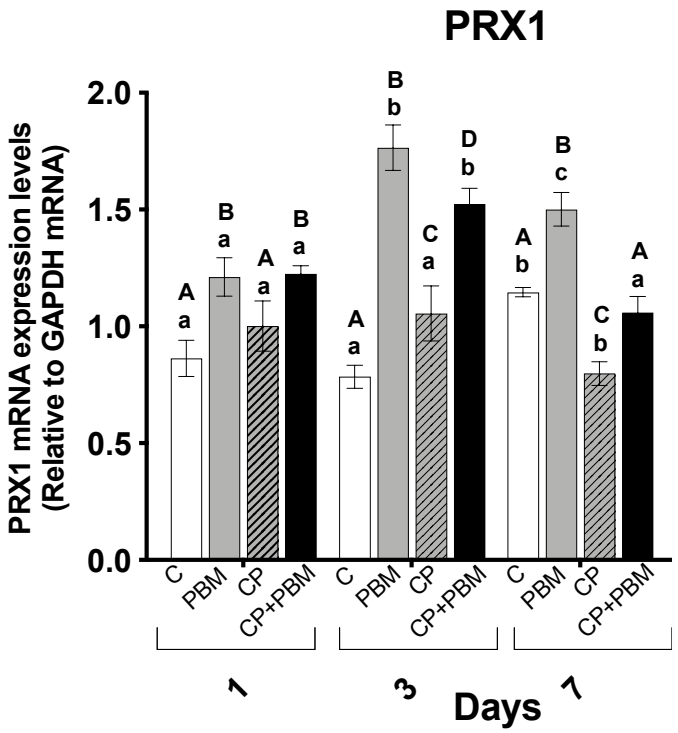

Figure 6. Graphical representation of Prx1expression on days 1,3 and 7 in ATM and control groups. Different capital letters indicate comparison between groups at each time point (Control x PBM x CP x CP+PBM) and lowercase letters indicate comparison for each group over time (days 1 x 3 x 7). Significant level $p<0.05$. Same letters indicate no significant difference.

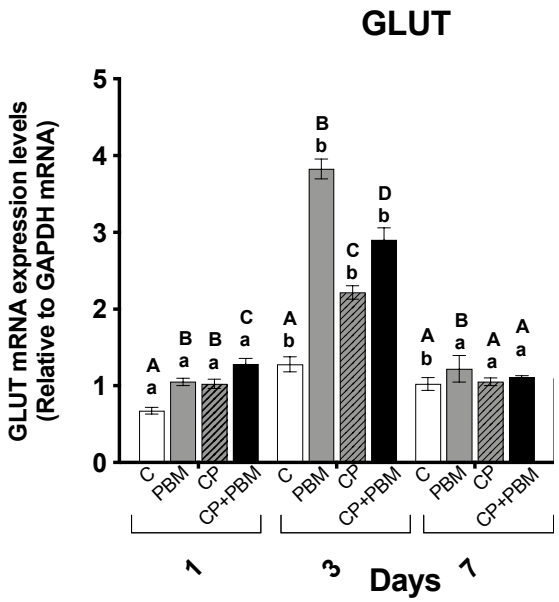

Figure 7. Graphical representation of GLUT expression on days 1,3 and 7 in ATM and control groups. Different capital letters indicate comparison between groups at each time point (Control x PBM x CP x CP+PBM) and lowercase letters indicate comparison for each group over time (days 1 x 3 x 7). Significant level $p<0.05$. Same letters indicate no significant difference.

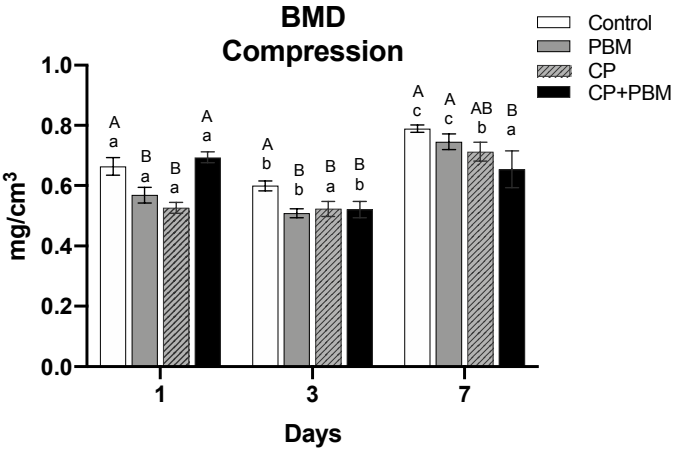

Figure 8. Bone Mineral Density (BMD) results in the compression side over time for intervention and control groups. Error bar indicates the standard deviation. Different capital letters indicate comparison between groups at each time point (Control x PBM x CP x CP+PBM) and lowercase letters indicate comparison for each group over time (days 1 x 3 x 7). Significant level $p<0.05$. Same letters indicate no significant difference.

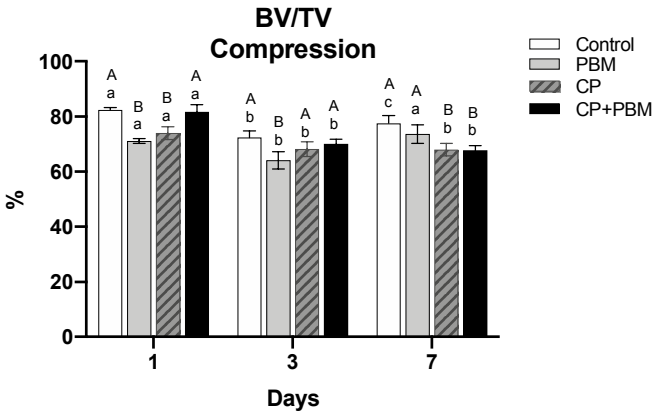

Figure 9. Bone volume to total volume ratio (BV/TV) results in the compression side over time for interventation and control groups. Error bar indicates the standard deviation. Different capital letters indicate comparison between groups at each time point (Control x PBM x CP x CP+PBM) and lowercase letters indicate comparison for each group over time (days 1 x 3 x 7). Significant level $p<0.05$. Same letters indicate no significant difference.

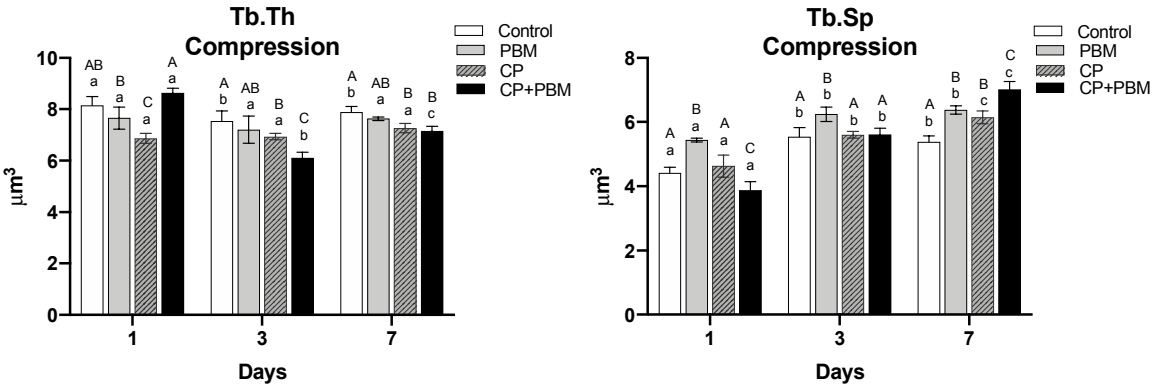

Figure 10. Trabecular thickness (Tb.Th.) and Trabecular separation (TB.Sp.) results in the compression side over time for ATM and control groups. Error bar indicates the standard deviation. Different capital letters indicate comparison between groups at each time point (Control x PBM x CP x CP+PBM) and lowercase letters indicate comparison for each group over time (days 1 x 3 x 7). Significant level $p<0.05$. Same letters indicate no significant difference.

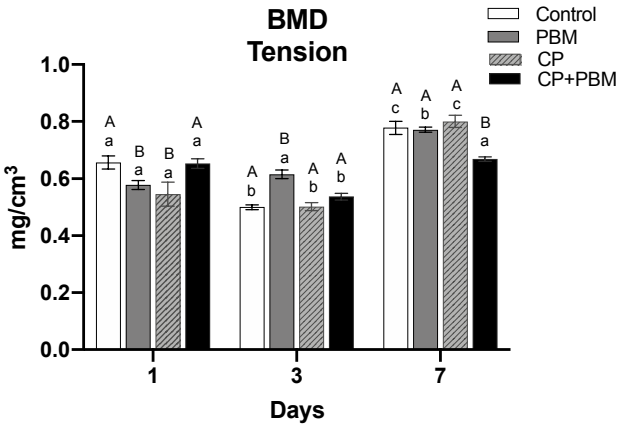

Figure 11. Bone Mineral Density (BMD) results in the tension side over time for intervention and control groups. Error bar indicates the standard deviation. Different capital letters indicate comparison between groups at each time point (Control x PBM x CP x CP+PBM) and lowercase letters indicate comparison for each group over time (days 1 x 3 x 7). Significant level $p<0.05$. Same letters indicate no significant difference.

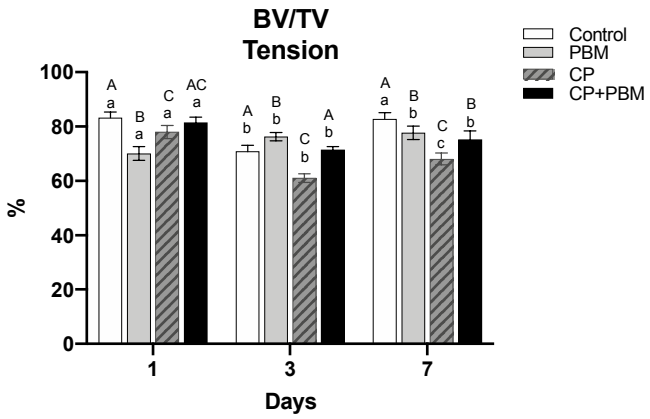

Figure 12. Bone volume to total volume ratio (BV/TV) results in the tension side over time for interventation and control groups. Error bar indicates the standard deviation. Different capital letters indicate comparison between groups at each time point (Control x PBM x CP x CP+PBM) and lowercase letters indicate comparison for each group over time (days 1 x 3 x 7). Significant level $p<0.05$. Same letters indicate no significant difference.

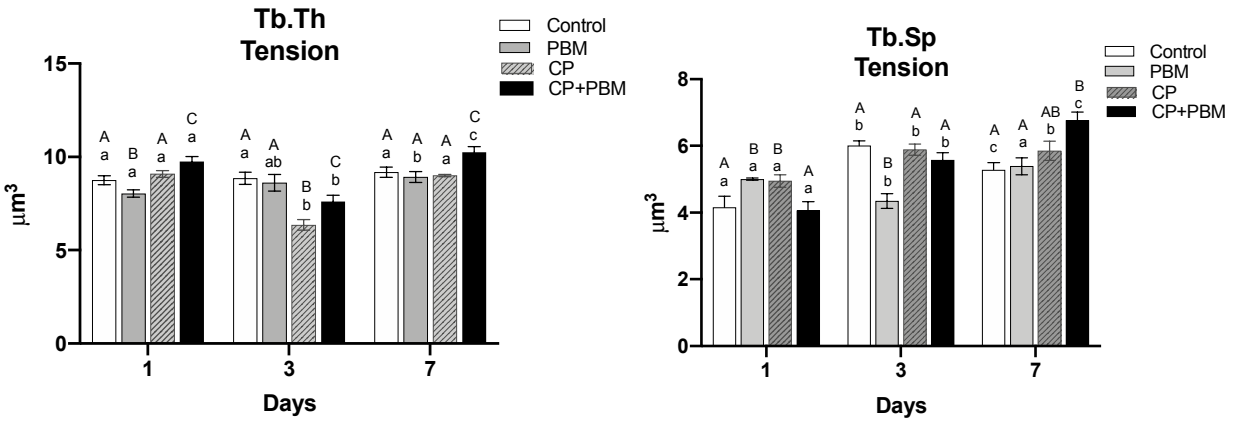

Figure 13. Trabecular thickness (Tb.Th) and Trabecular separation (TB.Sp.) results in the tension side over time for ATM and control groups. Error bar indicates the standard deviation. Different capital letters indicate comparison between groups at each time point (Control x PBM x CP x CP+PBM) and lowercase letters indicate comparison for each group over time (days 1 x 3 x 7). Significant level $p<0.05$. Same letters indicate no significant difference.

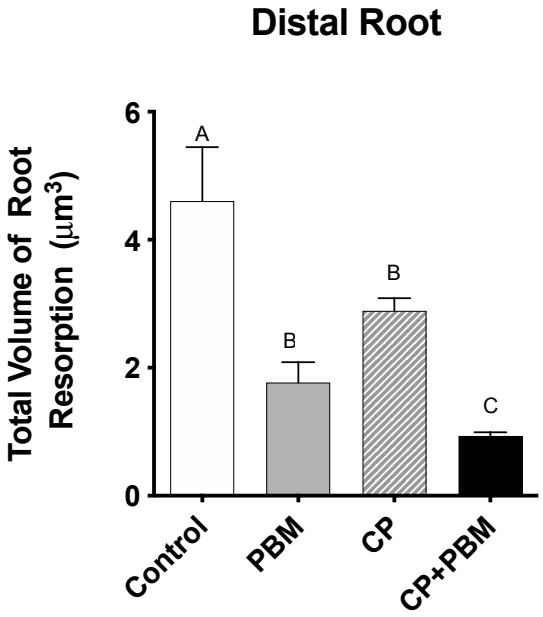

Figure 14. Total volume of root resorption lacunaes for ATM groups and control group.Different capital letters indicate comparison between groups at each time point (Control x PBM x CP x CP+PBM) and lowercase letters indicate comparison for each group over time (days 1 x 3 x 7). Significant level $p<0.05$. Same letters indicate no significant difference.

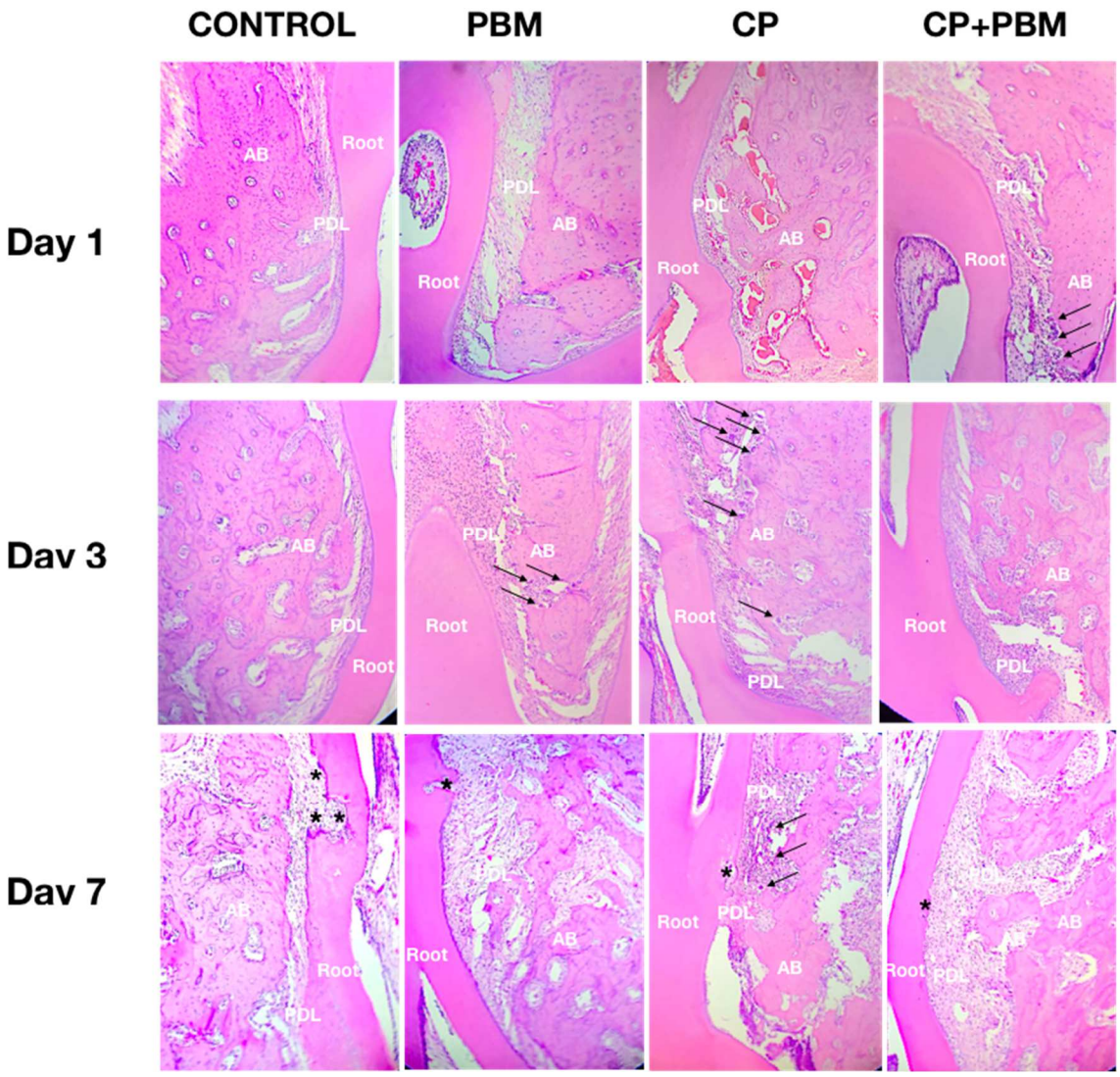

Figure 15.H&E stained sections of the compression side for all groups on days 1, 3 and 7.

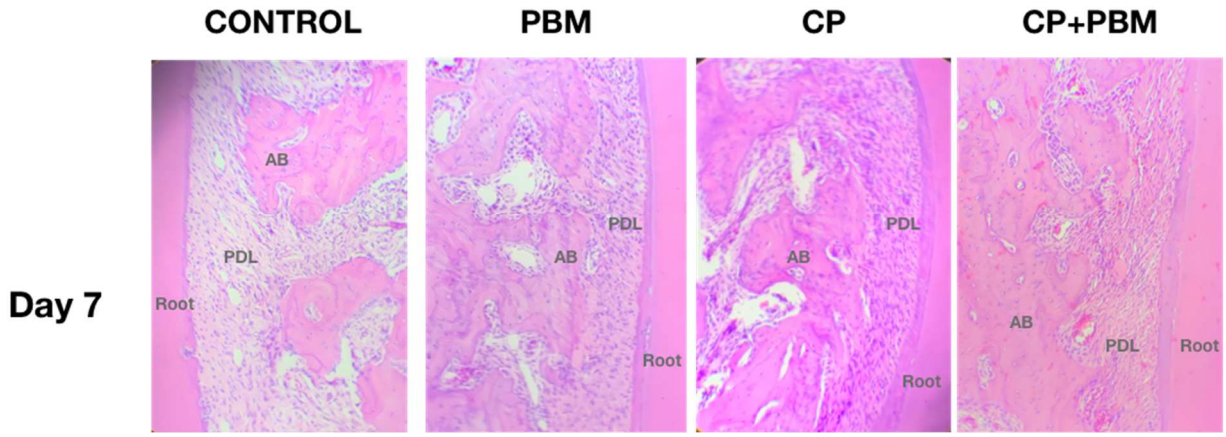

Figure 16.H&E stained sections of the tension side for all groups on day 7.

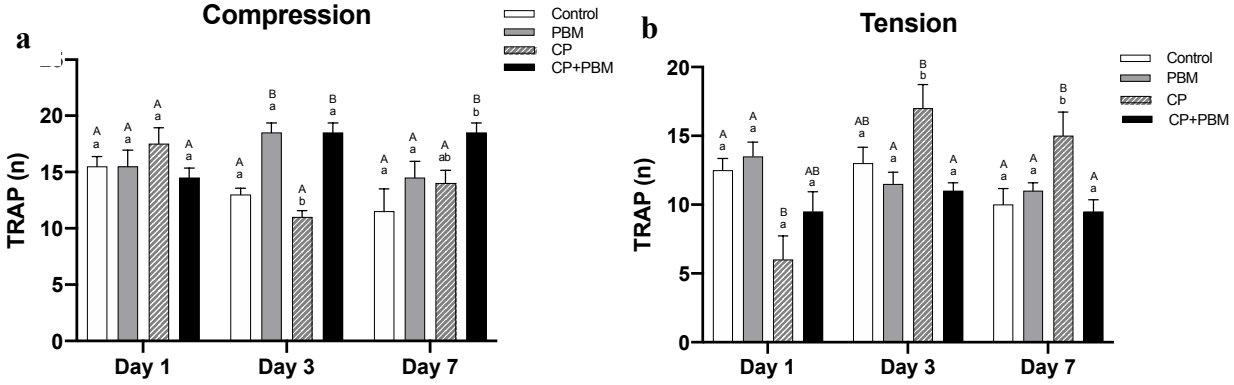

Figure 17. Number of TRAP positive osteoclasts observed in the alveolar bone in the compression (a) and tension (b) sides on days 1,3 and 7. Different capital letters indicate comparison between groups at each time point (Control x PBM x CP x CP+PBM) and lowercase letters indicate comparison for each group over time (days 1 x 3 x 7). Significant level $p<0.05$. Same letters indicate no significant difference.

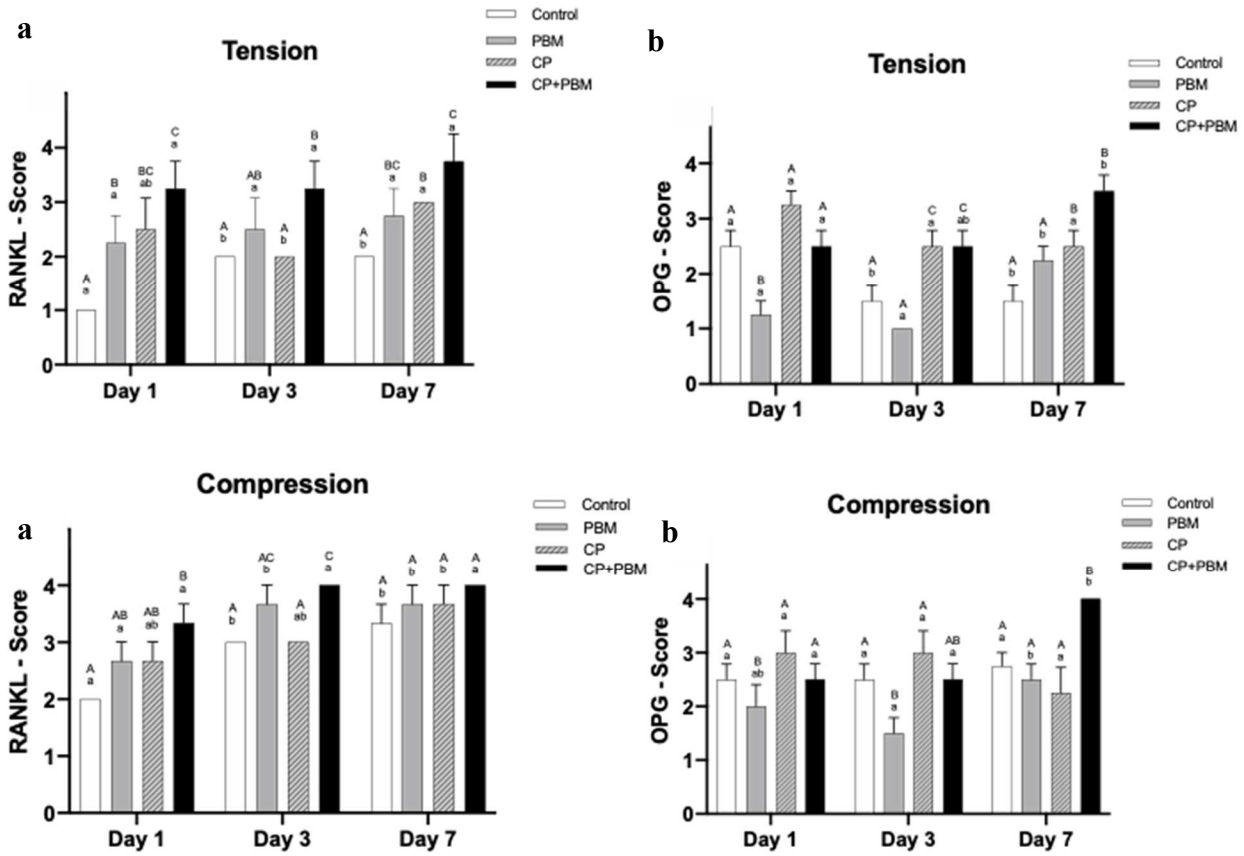

Figure 18. RANKL(a) and OPG (b) expression levels observed in the alveolar bone in the tension and compression sides on days 1,3 and 7. Different capital letters indicate comparison between groups at each time point (Control x PBM x CP x CP+PBM) and lowercase letters indicate comparison for each group over time (days 1 x 3 x 7). Significant level $p<0.05$. Same letters indicate no significant difference.

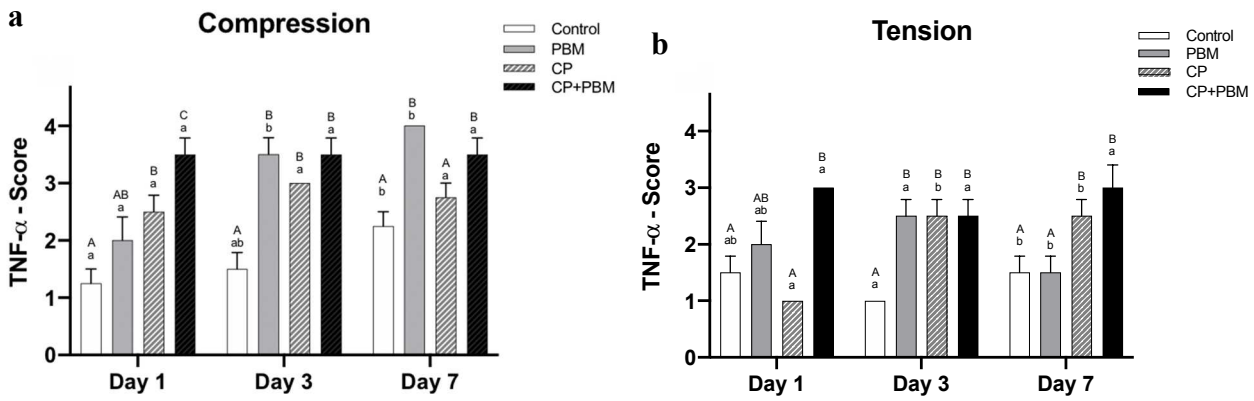

Figure 19. TNF alpha expression levels observed in the alveolar bone in the tension (a) and compression (b) sides on days 1,3 and 7. Different capital letters indicate comparison between groups at each time point (Control x PBM x CP x CP+PBM) and lowercase letters indicate comparison for each group over time (days 1 x 3 x 7). Significant level $p<0.05$. Same letters indicate no significant difference.

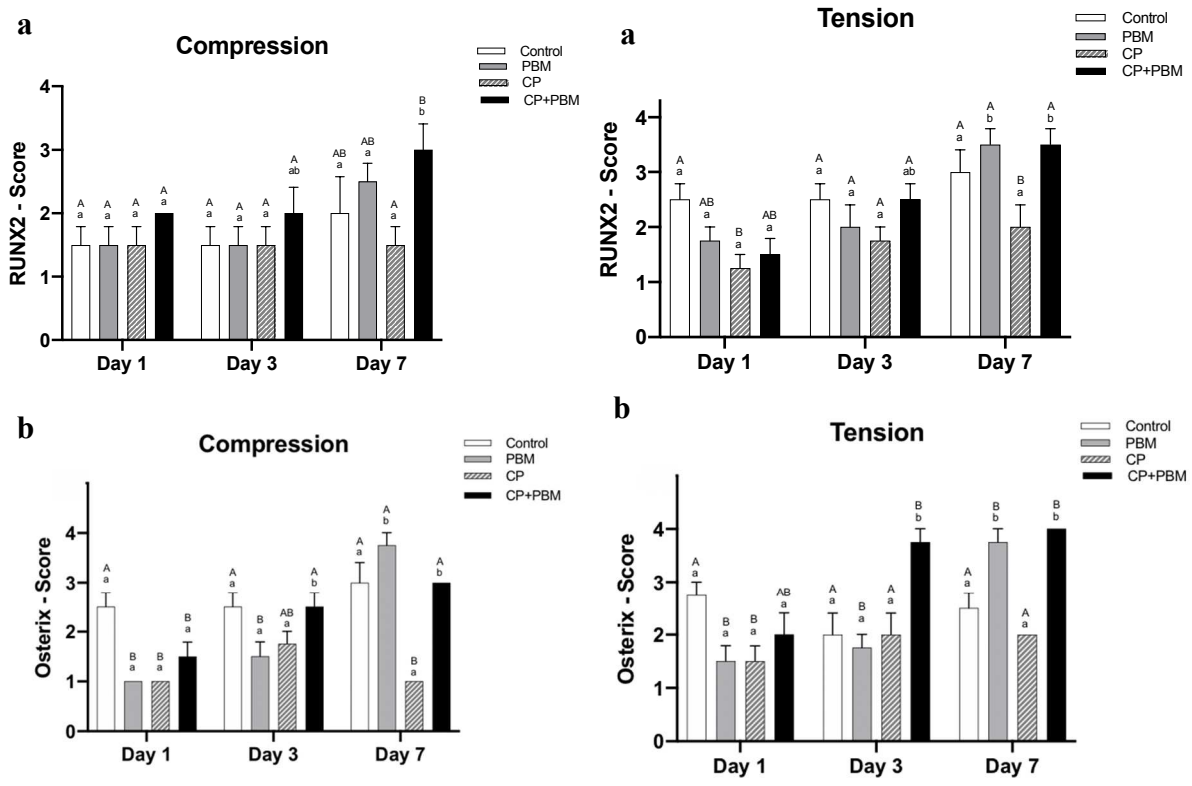

Figure 20. RUNX2(a) and Osteorix(b) expression levels observed in the alveolar bone in the tension and compression sides on days 1,3 and 7. Different capital letters indicate comparison between groups at each time point (Control x PBM x CP x CP+PBM) and lowercase letters indicate comparison for each group over time (days 1 x 3 x 7). Significant level $p<0.05$. Same letters indicate no significant difference.

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