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Multilevel biological responses following piezocision to accelerate orthodontic tooth movement: A study in rats

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ABSTRACT

Aim: The objective of the present study was to explore the alveolar bone tissue response and its dynamic at the tissue, cellular, and molecular levels following a piezocision procedure in a rat model.
Methods: Sixty rats were randomly allocated to either a control group (conventional orthodontic tooth movement) (TM) or a test group (piezocision-assisted orthodontic tooth movement) (TM+PS). Tissue, cellular, and molecular analyses were performed at 7, 28, and 42 days after the procedures. Orthodontic Tooth Movement (OTM) and Bone Volume Fraction (BVF) were evaluated using Nano-computed tomography. Moreover, histological parameters such as the number of osteoclasts were assessed, and the expression of cytokines involved in the bone turnover was investigated using quantitative reverse transcriptase polymerase chain reaction analysis.
Results: The OTM was 1.8 times faster in the TM+PS compared with the TM at day 42. A significant decrease in BVF was found in the TM+PS group compared with the TM group at day 7 and day 28, whereas no difference was observed at day 42. The number of osteoclasts was significantly higher in the TM+PS group compared with the TM group at day 7. No difference between the two groups was found in the number of osteoclasts involved in root resorption. RANKL and osteoprotegerin were significantly higher in the piezocision group than in the control group at day 7.
Conclusions: In these conditions, the efficacy of piezocision-assisted alveolar decortication to accelerate tooth movement was demonstrated, and the underlying biological responses at the tissue, cellular and molecular levels were emphasized.

1. Introduction

A recent systematic review, based on 22 prospective studies involving 1089 participants, emphasized that the mean duration of conventional orthodontic treatment in young patients is slightly less than 2 years [1]. In adult patients, orthodontic movement requires longer time because of the slower cell mobilization and tissue turnover compared with children and teenagers [2]. Moreover, long orthodontic treatment was shown to be directly related to adverse events such as root resorption, white spots, or patient noncompliance. In addition, from a patient perspective, the duration remains a limitation to start orthodontic treatment.

During the past decade, different relevant surgical methods have been described that accelerate orthodontic tooth movement (OTM), such as osteotomy procedures, dental distraction, and the most

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The Animal Research Reporting of In Vivo Experiments (ARRIVE) guidelines were carefully followed as well as national and European legislated guidelines.

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popular, corticotomies. Corticotomies require extensive flap elevation associated with significant reported postoperative pain levels [3]. More recently, less invasive alternatives have been proposed, such as piezopuncture [4], micro-osteoperforations [5,6] and piezocision [7–9] with the aim of reducing morbidity and postsurgical discomfort. The piezocision procedure consists of flapless corticotomies through vertical interproximal incisions using a piezoelectric device. According to a specific protocol [7,8], the procedure demonstrated its efficacy in accelerating OTM in different clinical conditions without adverse effects, except the presence of minor scars in some cases [7,8,10–14].

The biological process underlying this acceleration of the OTM is related to the increase of the Rapid Acceleratory Phenomenon (RAP) due to a bone injury [15]. The RAP is characterized by a decrease in bone density and an increase in bone turnover [16], leading to faster OTM and, according to some authors, the RAP is proportional to the extent of the surgical injuries [17] and fully reversible. The biological response following alveolar decortication using burs has been widely investigated through several preclinical models and demonstrated its efficiency to increase OTM, inducing a transient alveolar demineralization and activating cytokines involved in bone turnover [18–24]. However, the understanding of biological responses related to piezoelectric alveolar decortication remains limited [25]. Considering, for example, the new tools available, such as nanometer-scale X-ray computed tomography (CT) (Nano-CT), which generates accurate characterization and quantification of tissue microarchitecture [26], further investigations to highlight the biological responses following piezoelectric bone injuries are relevant.

The objective of the present study was to understand the alveolar bone tissue response for the first time in the same study at the tissue, cellular, and molecular levels, following a piezocision procedure in a rat model.

2. Materials and methods

2.1. Registration

All experimental procedures and protocols used in this study were reviewed and approved by the Institutional Animal Care and Use Ethics Committee of the University of Liège, Belgium. The Animal Research Reporting of In Vivo Experiments (ARRIVE) guidelines were carefully followed as well as national and European legislated guidelines (file number: 17–1985).

2.2. Animals

Sixty healthy adult male OFA (Oncins France Strain A) rats provided by the Charles River Laboratories (Bar Harbor, ME) were included in the study (males; body weight 440–480 g). The animals were obtained and acclimatized in the animal housing for at least 6 days. The rats were kept in cages at a temperature of 22.5 ± 0.5°C, with a 12-hour:12-hour light-dark cycle and were fed with a special diet, a combination of a powdered diet (RM3; TechniLab, Someren, The Netherlands) and soft food (diet gel; ClearH20, Uden, The Netherlands) and water ad libitum.

2.3. Study design and sample size

The 60 animals were randomly allocated to one of two groups:

1) Tooth movement (TM) (n = 30; control group)
2) Tooth movement + Piezocision surgery (TM+P) (n = 30; test group)

The left side of each animal's maxilla served as the experimental side (TM or TM+P), whereas the right side did not receive any treatment and was used as a negative control for each rat, in order to consider interindividual variability. The animals were euthanized at 7, 28, and 42 days after the procedure. Ten samples per condition were available. Five samples were used for the tissue and cellular analyses (Nano-CT and histology), and the five others served for the molecular analysis (reverse transcriptase polymerase chain reaction [RT-PCR]).

Power calculation showed that a total of 60 rats were needed, specifically five rats at each of the three time points in both test and control groups, in order to obtain reliable estimates of Nano-CT and molecular parameters.

2.4. Experimental procedure

All procedures were performed under general anesthesia with an intraperitoneally administered combination of ketamine (8 mg/kg) and xylazine (5 mg/kg).

The orthodontic appliance as well as the piezocision procedure are explained and represented in Figure 1. In the piezocision group, the surgical procedure was performed the same day as the placement of the orthodontic appliance. After their recovery from general anesthesia, all rats were allowed to move freely in their cages.

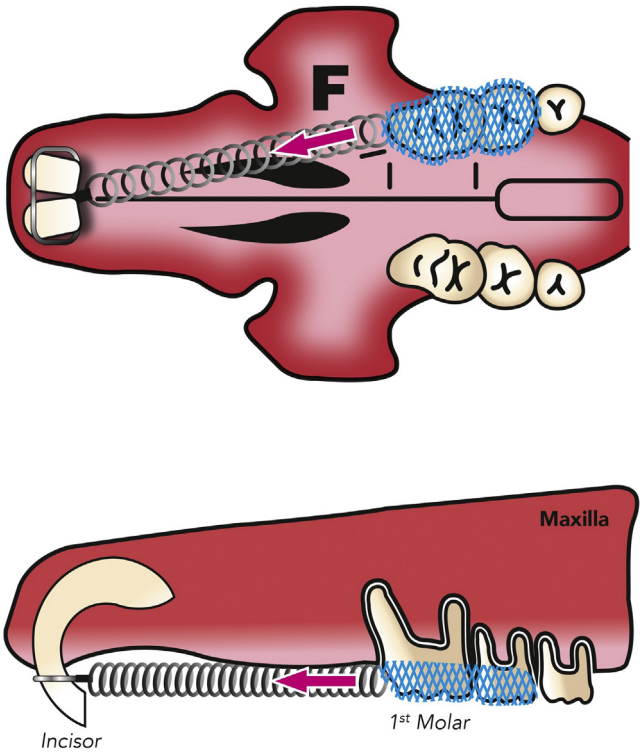

Fig. 1. The experimental orthodontic appliance in the rat model. The occlusal surface of the two first molars and the lateral surface of the incisors were etched with the Transbond Self Etching Primer (3M, Maplewood, MN). A metal ring (GAC International LLC, Islandia, NY) with a hook (3M) and a rectangular metal mesh (5 mm × 2 mm) were bonded to the teeth using a resin composite (Venus Flow, A2; Kulzer, Hanau, Germany). A mesial force was applied to the 2 molars using a 25-g Sentalloy stainless steel coil spring (GAC International LLC) according to previous study [25] and using the incisor as anchorage. The piezocision lines are represented by the black lines: incisions were made mesial and distal to the first molar as well as supracrestally in the edentulous space between the first molar and the incisor. Osteotomies (3 mm in length and 1.5 mm in depth) were subsequently performed using a Piezotome (Satelec, Acteon group, Merignac, France).

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Postoperative care was provided as follows: adrenergic antagonist (Antisedan, 1 mg/kg) intramuscular injection following antibiotics (Baytril, 5 mg/kg), painkiller (Temgesic, 0.05 mg/kg), and anti-inflammatory (Rimadyl, 5 mg/kg) subcutaneous injections. The day after the surgery, antibiotics (Baytril, 5 mg/kg) were placed in the drinking water for 1 week.

At 7, 28, and 42 days, animals were euthanized (Fig. 2) by an overdose of pentobarbital and samples were collected before being fixed in 4% formaldehyde for 1 day at 4°C and then stored in sterile phosphate-buffered saline at 4°C.

2.5. Data collection

2.5.1. Tooth movement

Based on the Nano-CT images, OTM was measured in two dimensions in the two experimental groups: the distance between the distal surface of the second molar and the mesial surface of the third molar, and was defined as the interdental distance (ID) and expressed in pixels.

2.5.2. Three-dimensional (3D) tissue-level analysis (Nano-CT)

After the fixation process, the samples were scanned using a Nano-CT system called Phoenix NanoTom M (GE Measurement and Control Solutions, Wunstorf, Germany): Nano-CT analysis was performed on each specimen. The image acquisition conditions were as follows: voltage at 80 kV, current at 190 μA, applied filter of 0.25 mm, used a voxel size of 7.5 μm, took 1800 images over 360° scan, and used "fast scan" mode.

Reconstruction was performed using Phoenix datos|x CT software. All sample scans were reoriented using the DataViewer software (Bruker micro-CT, Kontich, Belgium). For 3D image visualization, regions of interest (ROI), including the roots of the first and second maxillary molars, were drawn using the CTAn software (Bruker micro-CT) for every 10 pictures. Volume of interest (VOI) was established by interpolation of all ROIs: The VOI was defined as the volume in-between the roots of the first and the second maxillary molars. A quantitative evaluation of the mineralized bone and the trabecula thickness, number, and separation were calculated by setting a threshold on the basis of their respective gray levels.

Then, the following parameters were assessed in each experimental and negative control group:

(1) Bone Volume Fraction (BVF) defined as the ratio of mineralized tissue (Bone Volume: BV) to the total Volume of the VOI (TV): BVF= BV/TV
(2) Trabecular Thickness (Tb. Th)
(3) Trabecular Number (Tb. N)
(4) Trabecular Separation (Tb. Sp)

2.5.3. Cellular level analysis (histology)

After Nano-CT scanning, the samples were decalcified in a 14% ethylenediaminetetraacetic acid solution for 5 days and then processed for standard paraffin embedding. Three transversal sections were obtained as follows: 0.45, 0.90, and 1.35 mm in the apico cervical direction from the apex and stained with hematoxylin and eosin.

Three parameters were assessed by a blinded examiner on the experimental side:

(1) Number of osteoclasts
    Osteoclasts were defined as follows: As described in previous studies [20,22], osteoclasts were morphologically identified as large multinucleated cells with cytoplasmic vesicles in close contact with alveolar bone.
(2) Root osteoclasts
    Root osteoclasts were defined as follows: osteoclasts involved in root resorption were identified by the same morphology as described previously but in close contact with the tooth roots.
(3) Blood vessels more than 300 μm in perimeter
    Blood vessels more than 300 μm in perimeter were defined as follows: Blood vessels were defined as a lumen lined by elongated endothelial cells, sometimes bordered by cuboidal or flattened smooth muscle cells and often facilitated by the presence of red blood cells in the lumen [20].

In the three sections, a count of these structures including their perimeters was performed. The results were expressed by the average of the three sections.

2.5.4. Molecular Analysis (RT-PCR)

Samples were collected with their surrounding soft tissue, cut in small pieces, and frozen in liquid nitrogen. Then samples were transferred to 1 mL of TRIzol (Life Technologies, Carlsbad, CA) containing a Stainless Steel Bead (Qiagen, Hilden, Germany) and disrupted using a TissueLyzer (Qiagen) followed by RNA extraction with Direct-Zol (Zymo Research, Irvine, CA) according to the manufacturer's protocol. The RNA quantity was controlled with a NanoDrop. Complementary DNA (cDNA) was synthesized with a RevertAid H Minus First Strand cDNA Synthesis Kit (ThermoScientific, Waltham, MA). SYBR Green quantitative PCR was performed in duplicate (ABI 7900 HT; Applied Biosystems, Foster City, CA) to determine

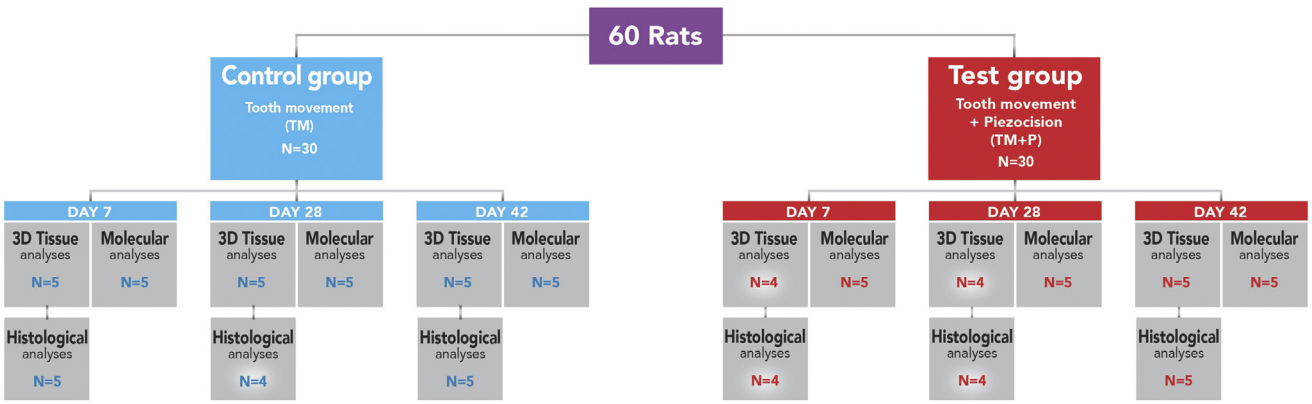

Fig. 2. Study design and flowchart. Orthodontic appliances broke in two rats at day 7 and at day 28 in the test group, and they were excluded from the tissue and cellular analyses. An additional rat was excluded at day 28 in the control group because of a technical problem with the histology. Therefore, five rats were available for each analysis in each group and time point, except in five conditions where only four were available.

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messenger RNA relative expression levels for target genes normalized to a reference gene (β-actin). Primer sequences, designed using the Primer 3 online program, are listed in Table 1. The obtained values were analyzed using the Qbase software (Biogazelle, Zwijnaarde, Belgium).

After this procedure of RNA extraction followed by the preparation of cDNA and the quantitative PCR, the following inflammatory cytokines and genes involved in bone formation and resorption were analyzed on the experimental side with specific primers for rat genes: RANK L, interleukin (IL)6, osteoprotegerin (OPG), tartrate-resistant acid phosphatase, and bone morphogenetic protein (BMP)2.

2.6. Statistical analysis

Results were expressed as a mean and standard deviation or as median and interquartile range (IQR). Study end points were analyzed by mixed effects models in relation to three experimental factors: time (both linear and quadratic effects), side (equipped vs. nonequipped), and treatment (TM vs. TM + piezocision). Interactions between factors were also investigated. For BV/TV (%), a logit transform was applied to normalize its distribution. To reduce interindividual variability, values observed on the nonequipped side (negative control) were subtracted from those observed on the equipped side (experimental side). Then, differences were modeled with respect to time and treatment only. Results were considered significant at the 5% critical level ($P \leq 0.05$). All calculations were made with SAS version 3.4 (SAS Institute, Cary, NC) and R version 3.2.2.

3. Results

3.1. Animals

All rats remained healthy and had a slight body weight increase over the 42 days of experimentation. Numbers analyzed are detailed in Figure 2.

3.2. Tooth movement

The Intermolar Distance (ID) increased significantly over time ($P = 0.0014$) in both groups. However, a significant difference between the two groups was observed only at 42 days ($P = 0.034$): OTM was 1.8 times faster for the piezocision-assisted procedure than for the conventional one (14.9 ± 5.5 pixels vs. 26.8 ± 6.9 pixels). Details are presented in Figure 3A.

Table 1
Primer sequences (5′–3′) for the reference and the tested genes designed with the Primer3 online software

Genes	Primer sequence 5′–3′
β-Actin Forward	CGT CTT CCC CTC CAT CGT G
β-Actin Reverse	AGG ATG CCT CTC TTG CTC TG
RunX2 Forward	GGC CCT GGT GTT TAA ATG GT
RunX2 Reverse	ACG CCA TAG TCC CTC CTT TT
Rank-L Forward	CCC ATC GGG TTC CCA TAA AGT C
Rank-L Reverse	GCC TGA AGC AAA TGT TGG CGT A
OPG Forward	GTC CCT TGC CCT GAC TAC TCT
OPG Reverse	GAC ATC TTT TGC AAA CCG TGT
BMP-2 Forward	GAA GCC AGG TGT CTC CAA GAG
BMP-2 Reverse	GTG GAT GTC CTT TAC CGT CGT
TRAP Forward	TGC ATG ACG CCA ATG ACA A
TRAP Reverse	GAG GGC ACG GTC AGA GAA C
IL6 Forward	GCC CTT CAG GAA CAG CTA TGA
IL6 Reverse	TGT CAA CAA CAT CAG TCC CAA GA
IL1β Forward	ACT CAT TGT GGC TGT GGA GA
IL1β Reverse	TAG CAG GTC GTC ATC ATC CC
TNFα Forward	CAG CAA CTC CAG AAC ACC CT
TNFα Reverse	GCC AGT GTA TGA GAG GGA CG

BMP, bone morphogenetic protein; IL, interleukin; OPG, osteoprotegerin; TNF, tumor necrosis factor; TRAP, tartrate-resistant acid phosphatase.

3.3. 3D tissue-level outcomes

BVF and Trabecular Number (Tb. Nb) were significantly lower in the piezocision group compared with the control group at day 7 ($P = 0.014$; $P = 0.014$) and day 28 ($P = 0.05$; $P = 0.014$), whereas no difference was observed at day 42. The trabecular separation (Tb. Sp) was significantly higher in the piezocision group compared with the control group at day 7 ($P = 0.014$) and day 28 ($P = 0.028$), whereas no difference was observed at day 42. Only the trabecular thickness (Tb. Th) showed no difference between the two groups at each time point. All details are presented in Figure 3B.

3.4. Cellular level outcomes

The number of osteoclasts was higher in the piezocision group than in the control group at day 7 ($P = 0.014$), whereas at day 28 and 42 no difference was observed between the groups. However, no difference was found in the number of osteoclasts involved in root resorption between the test and the control group. For the number and the size of blood vessels, no differences were found between the two groups. Details about the cellular outcomes are displayed in Figure 4.

3.5. Molecular outcomes (quantitative RT-PCR)

Relative expressions of RANKL and OPG were significantly higher in the piezocision group than in the control group at day 7 ($P = 0.046$; $P = 0.027$). IL6 was also higher in the piezocision group at each time point, although not significant. At 28 and 42 days, nonsignificant differences were observed for RANKL, OPG, and IL6. Moreover, tartrate-resistant acid phosphatase and BMP2 displayed similar values for each time point.

4. Discussion

In the present preclinical study, the increase in OTM after 42 days was significant (1.8 times higher) in the piezocision group compared with the control group, and these results were associated with higher bone demineralization based on tissue and cellular outcomes (BVF and osteoclast number) at 7 and 28 days. Despite being less consistent, molecular expression of some cytokines involved in the osteoclastogenesis process were found to be greater in the piezocision group 7 days after the procedures.

Although some aspects of the histological tissue response following piezocision have already been reported [25], this multilevel report, using 3D analyses of the mineralized tissues and molecular analyses, offers a better understanding of the underlying biological phenomenon induced by localized piezoelectric alveolar decortication.

In animal models, some authors have emphasized faster OTM using alveolar decortication, as initially described by Wilcko et al. [27]. Indeed, several studies compared corticotomy-assisted space closure following premolar extraction versus conventional OTM in dogs and humans [28]. Space closure was found to be two to four times faster with corticotomies and the positive effect on OTM appeared early after the procedure [21–24,28,29]. Moreover, the increase of the RAP phenomenon was observed from 1 week and the induced cellular activity involved in the bone turnover decreased significantly after 6 months [24]. Although the model was different, the present study also showed an increase of the cell activity and a localized bone demineralization from 1 week after the piezocision; however, the increase in OTM was not yet observed

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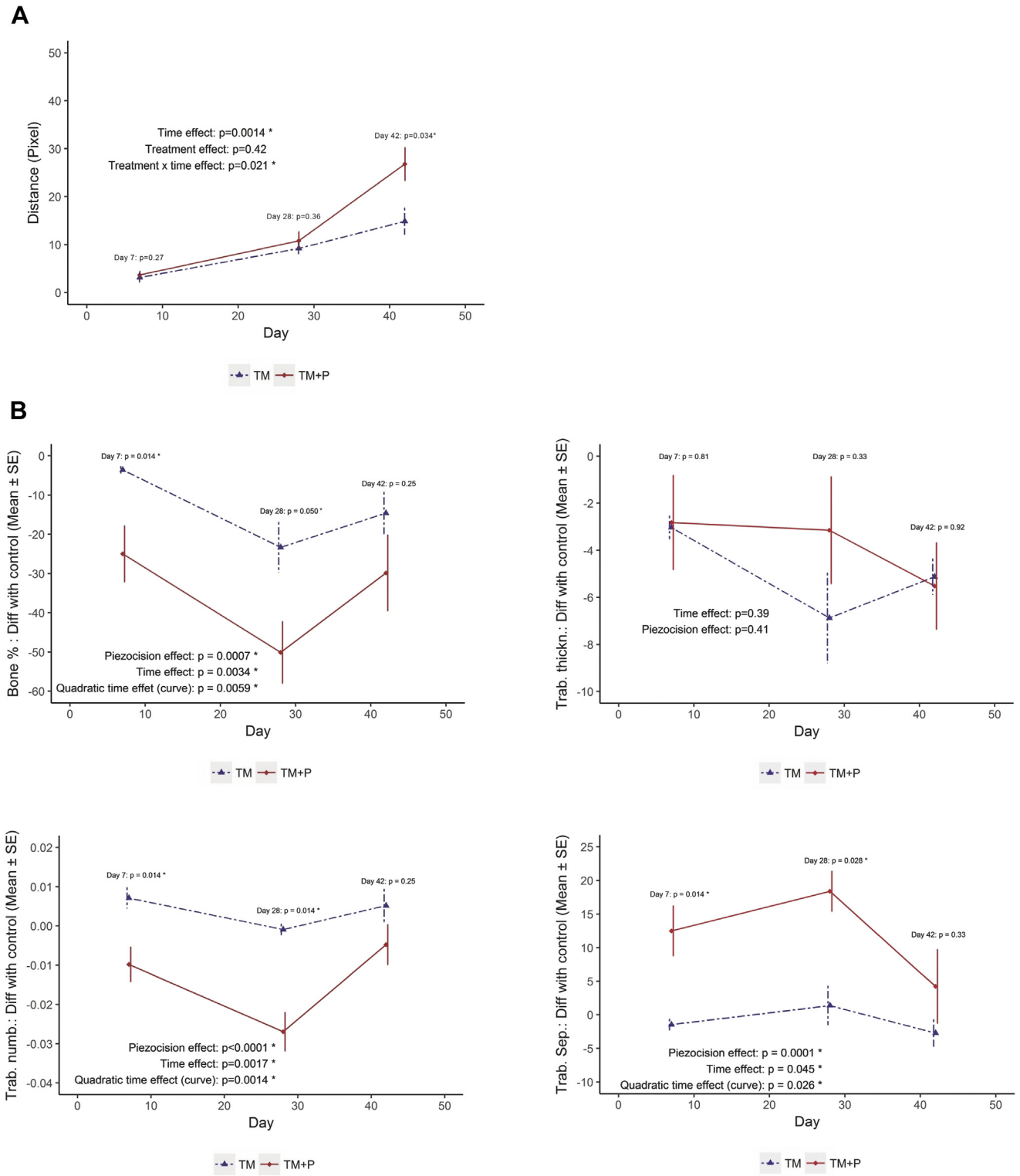

Fig. 3. (A) Mean (±SE) cumulative orthodontic tooth movement in both control and test groups. (B) Tissue-level outcomes. Evolution of bone volume fraction (BVF), trabecular number, trabecular separation, and trabecular thickness as measured by Nano-CT for both control (orthodontic tooth movement only) and test groups (orthodontic tooth movement assisted by piezocision). Data plotted are mean (±SE) of experimental values after subtraction of values measured on control negative side in each rat.

at 28 days, a significant difference in OTM was observed only at 42 days.

The impact of alveolar decortication performed with round burs was also investigated in a rat model [18,19]. Baloul at al. [18] already observed an acceleration of the OTM from 3 and 7 days after the corticotomies, and the positive effect was no longer observed after 28 days. At the tissue level, a strong decrease of BVF was found in the alveolar decortication + OTM group at 7 days, whereas it was restored to baseline values at day 28. Furthermore, in a rat model investigating alveolar decortication in a flapless approach, although

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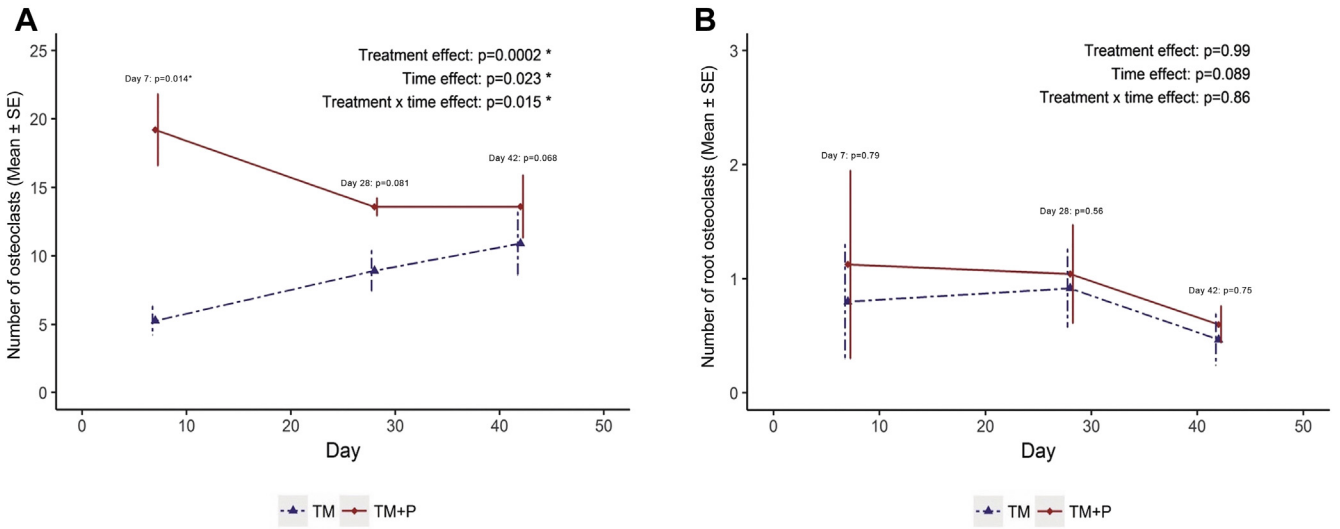

Fig. 4. Cellular outcomes. (A) Graphical presentation of mean (±SE) number of osteoclasts and (B) mean (±SE) osteoclasts involved in the root resorption in both control (orthodontic tooth movement only) and test groups (orthodontic tooth movement assisted by piezocision).

the results above 14 days were not investigated, the authors report increased OTM, demineralization, and osteoclast numbers at 7 and 14 days in the test group [19].

Therefore, it seems that the biological response of surgically facilitated OTM using a round bur and piezoelectric device are both associated with an increased bone turnover, a transient localized alveolar bone demineralization, and accelerated tooth movement. However, the phenomenon seems to be slightly different with piezoelectric surgery. Although the RAP induced by piezocision was present early after the procedure, the OTM acceleration was delayed compared with alveolar decortication using burs. This observation was also described by Dibart et al. [25], showing higher tooth movement with piezocision only after 28 days. Therefore, following piezocision surgery it seems that the process of bone demineralization is the first to take place, and this demineralization subsequently induces significantly faster tooth displacement. In fact, the positive effect of the piezocision procedure on the acceleration of OTM, mentioned in several clinical studies [7,10–13], appears to be delayed until the overall biological response has been established.

However, the bone demineralization seemed to last longer when the decortication was performed with the piezoelectric device as the initial BVF was restored only at 42 days in this study and at 56 in the Dibart et al. study [25].

Concerning the role of key osteoclastic regulators (RANKL, promoting osteoclastogenesis and OPG, inhibiting osteoclast differentiation) in the piezocision group, a higher expression of the couple cytokines involved in bone remodeling was found at 7 days. This is in accordance with the findings of Baloul et al. [18], suggesting that alveolar decortication diverts the biological response to conventional tooth movement.

By contrast, the surgically assisted OTM involving bone incision using a reinforced surgical blade in a flapless approach, namely corticisions, showed rather inconsistent outcomes in preclinical settings. Most of the studies have not emphasized accelerated OTM or significant bone demineralization when applying this technique [30]. Only Tsai et al. [31] have described a positive effect of the corticision 2 weeks after the procedure. These inconsistencies suggest that the efficacy of surgically facilitated orthodontic movement is related to the level of bone injury, itself influencing the proportion of RAP effect, as previously suggested by some authors [17].

These findings from animal experiments corroborate the clinical observations summarized in a recent systematic review exploring the effectiveness of corticotomy (more invasive) versus piezocision (less invasive) on canine retraction. A significant increase in the rate of tooth movement was found in both groups compared with the conventional method, but the movement acceleration was four times faster with the corticotomies and only twice as fast with the piezocision [32]. As it is less invasive, the patients could eventually be subjected twice to the piezocision procedure as the surgically induced augmentation of the RAP effect was shown to be limited in time.

In addition, signs of root resorptions (number of root osteoclasts) was low and similar in both the test and control groups of this study. Although this aspect is usally poorly investigated in preclinical studies, two other studies found these same findings when using surgically assisted orthodontics [29,31]. Therefore, the piezocision technique does not seem to induce root resorptions despite the accelerated OTM, as already suggested in clinical trials [7].

Finally, this in vivo study contributes to the understanding of the multilevel biological responses following a piezocision-assisted orthodontic movement. Postoperative care, such as anti-inflammatory subcutaneous injection, was provided to both groups, according to the procedures and protocols of the Institutional Animal Care and Use Ethics Committee to decrease animal postoperative discomfort and eventually pain. Thus, it had no influence on the comparison of the biological response between the two groups, even if the inflammatory response may have been equally underestimated in both groups. Furthermore, the rat model, although widely used to study surgically assisted OTM, exhibits some limitations and its comparison to clinical practice remains difficult, as the bone turnover in rats is different from that of humans as well as the kinetic of the OTM [33]. However, the anabolic and catabolic phases of bone remodeling are comparable to humans [34].

5. Conclusion

Within the limit of the present study, the efficacy of piezocision-assisted alveolar decortication in accelerating tooth movement was demonstrated and the underlying biological responses at the tissue, cellular, and molecular levels were highlighted. Increased 3D bone demineralization (Nano-CT) and osteoclast recruitment were observed in the test group compared with a conventional

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orthodontic approach. Moreover, an increased bone remodeling emphasized at the molecular level by the coupled RANKL-OPG expression was demonstrated for the first time using piezocision-assisted alveolar decortication. These biological phenomena were shown to be transient and completely reversible.

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