Effect of gingival fibroblasts and ultrasound on dogs' root resorption during orthodontic treatment

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ABSTRACT

Objectives: To investigate the effect of using osteogenic induced gingival fibroblasts (OIGFs) and low intensity pulsed ultrasound (LIPUS) on root resorption lacunae volume and cementum thickness in beagle dogs that received orthodontic tooth movement.

Materials and Methods: Seven beagle dogs were used, from which gingival cells (GCs) were obtained and were induced osteogenically to produce OIGFs. Each third and fourth premolar was randomly assigned to one of the five groups, namely, LIPUS, OIGFs, bone morphogenetic protein-2 (BMP-2), OIGFs + LIPUS, and control. All groups received 4 weeks of bodily tooth movement, then LIPUS-treated groups received LIPUS for 20 min/day for 4 weeks, and OIGFs groups received an injection of OIGFs near the root apex. Microcomputed tomography analysis was used to calculate root resorption lacunae volume and histomorphometric analysis was performed to measure the cementum thickness of each root at 3 root levels on compression and tension sides.

Results: There was no significant difference in resorption volume between the treatment groups. OIGFs + LIPUS increased cementum thickness ($P > 0.05$) in third premolars near the apex, and LIPUS increased cementum thickness ($P > 0.05$) in fourth premolars near the apex. Furthermore, BMP2 increased cementum thickness at the coronal third at the compression side.

Conclusion: OIGFs, LIPUS, and BMP-2 can be potential treatments for orthodontically induced root resorption, however, improvements in experimental design and treatment parameters are required to further investigate these repair modalities.

Key words: Dental cementum, gingival fibroblasts, root resorption, ultrasound

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INTRODUCTION

A favorable tooth crown-to-root ratio is important to support a tooth. Severe root resorption and/or resorbed alveolar bone adversely affect this ratio by shortening the root that is invested in the alveolar bone.[1] A report on orthodontically induced tooth root resorption (OIRR) revealed that 40% adults had at least one tooth with 2.5 mm of resorption.[2] OIRR can take place within 35 days of orthodontic treatment even with forces as light as 50 g.[3]

OIRR is a pathological process resulting in cementum and dentin loss.[1] The original root contours cannot be reconstructed

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after this type of root resorption has occurred.[4,5] OIRR is characterized by a decreased cementum thickness.[6] Low intensity pulsed ultrasound (LIPUS) can prevent the progression of OIRR if discovered early or during orthodontic treatment,[7] but no treatment is available to repair severe OIRR. A new technique is needed to be developed to repair the lost root parts after OIRR.

An interest in using stem cells for regenerating dental tissues has risen recently.[8-10] Human PDL stem cells are capable of repairing PDL defects in mice and rats,[11] and in dogs.[12] PDL fibroblast-like cells can prevent root resorption and induce cementum formation in dogs.[13] PDL cells can be differentiated into osteogenic, adipogenic,[13,14] and neural phenotypes.[15] Current techniques that use stem cells in root repair have achieved preliminary successes. However, they suffer from drawbacks such as donor-site morbidity. Better sources of stem/pluripotent cells are needed for PDL tissue repair and OIRR treatments.

Gingival cells/fibroblasts (GCs/GFs) show promise in dental repair due to their accessibility. GCs can enhance vascularization and improve attached gingiva,[16] inhibit osteoclast activity,[17] have neural differentiation potential,[18] and be induced into osteogenic cells.[19]

Other techniques concerning repair of dental tissues involve the use of a variety of growth factors such as bone morphogenetic protein-2 (BMP-2). Previous studies showed an increase in cementum formation in root defects after application of BMP-2,[20] and that BMP-2 plays a role in increasing alkaline phosphatase activity, leading to an increased mineralization activity of cells.[21]

LIPUS is acoustic pressure waves transmitted through living tissues. LIPUS can enhance PDL cell differentiation into cementoblast-like cells,[22] increase cellular proliferation and induce osteogenic differentiation in GFs,[19,23] and enhance repair of OIRR during orthodontic treatment in dogs.[24]

The aim of this study was to analyze the possible effect of osteogenic-induced gingival fibroblasts (OIGFs) and LIPUS on cementum in beagle dogs undergoing orthodontic treatment. It was hypothesized that OIGFs and LIPUS would help repair roots damaged by OIRR, and that the repair effects of OIGFs would be complementary to those of LIPUS when OIGFs and LIPUS are applied in combination. It was also hypothesized that BMP-2 would help repair root resorption and that this effect would be comparable to those of LIPUS and OIGFs.

MATERIALS AND METHODS

Animals and Gingival Cells

Seven beagle dogs (aged 19 months ± 8 days) were used in the study. The rights of the animals were protected, and the experimental procedure was approved by the Animal Care Committee at the University of Alberta. GCs were isolated and induced for osteogenic differentiation as described before.[19] In short, interdental papilla from each dog was excised, cut, and dispersed on slides, placed in culture plates, and incubated. The cells that became confluent (2–3 weeks) were removed from the plates and were transferred into flasks.[25] The cultured GFs (cells) were transferred to 48-well plates (2.5×10^3 cells/well), treated with osteogenic medium (basic medium, 10 mM β-glycerophosphate, 50 mg/L ascorbic acid, and 0.1 μM dexamethasone), and received LIPUS treatment for 20 min/day for 4 weeks according to the described protocol[7] to produce OIGFs. LIPUS was applied with 30 mW/cm^2 intensity pulsed at 1.5 MHz and repeated at a frequency of 1 kHz (SmileSonica Inc., Edmonton, AB, Canada).

Orthodontic Tooth Movement and Treatment Groups

Bodily orthodontic tooth movement was performed to move 3rd premolars mesially and 4th premolars slightly distally for 4 weeks using 100 cN force (RMO, Denver, CO, USA), as described before.[26-28] Premolars were randomly assigned to one of five treatment groups [Table 1]. Treatments were performed after 4 weeks of tooth movement. OIGFs groups were injected transosseously (Dentsply™ X-Tip Intraosseous Anesthetic Delivery System, Pennsylvania) with 0.5 mL of OIGFs (in DMEM) from each dog into the same dog through the buccal plate of bone near the apex using a 30-gauge needle. The concentration of cells was 2×10^5 cells/mL of DMEM, and the viability of OIGFs after passing through the needle was confirmed prior to the actual injection. LIPUS treatments were applied for 20 min/day for a total of 4 weeks.[7] BMP-2 (Julius-Maximilians-Universität Würzburg) was conjugated in poly-D, L-lactic acid-polyethylene glycol (PLA-PEG) polymer (25 mg of PLA-PEG polymer mixed with 10 μL of BMP-2 in buffered solution per injection) was injected through the buccal plate of the bone near the apex of each corresponding tooth. This procedure has been described by Saito *et al*.[29] BMP-2 was used as a positive control. After 4 weeks, the animals were euthanized with ketamine HCl overdose injected intravenously according to the approved standard operating procedures.

Histology and Histomorphometric Analysis

Tissue blocks were dissected and stored in 4% paraformaldehyde. After the samples were air dried for 30 minutes, microcomputed tomography scanning was performed using SkyScan® 1076 MicroCT scanner and associated software (Version 2.6.0). Reconstructed images were created, and resorption lacunae volumes were measured using CTAn software (SkyScan®)

Table 1: Number of premolars included in each treatment group

Treatment Groups	Sample Size (Number of premolars)
Control	6
LIPUS	5
OIGF	6
BMP2	5
OIGF+LIPUS	6

according to the method described before [Figure 1].[24] The total resorption lacunae volume was calculated for each tooth root in each treatment group and then compared. Tissue blocks were prepared for histomorphometric analysis by demineralizing in 10% formic acid and decalcifying in ethylenediaminetetraacetic acid (EDTA), then cutting serial sections (7 μm) in the buccolingual plane through mesiodistal extension and staining with hematoxylin and eosin. Slides were analyzed by light microscopy (Leica Qwin 500 image analyzer computer system [England]) at 40× magnification and photos were then produced. MetaMorph Software (Molecular Devices LLC, California) was used to measure cementum thickness on compression and tension sides at 3 root levels, namely, coronal (level 1), middle (level 2), and apical (level 3) [Figure 2]. Sample photomicrographs and micro-CT images from a tooth root from each treatment group and the control group were produced [Figure 3]. The assessor was blinded during all data collection.

Statistical Analysis

Normal distribution of data was assessed using Kolmogorov–Smirnov tests of normality and box-plots. Cementum thickness data that was not normally distributed was transformed using the natural logarithm (x + 1) [i.e. Ln (thickness + 1)] in order to obtain normal distribution. Root resorption volume data that was not normally distributed was also transformed using the natural logarithm (x + 1) [i.e., Ln (volume + 1)] because some tooth roots contained no resorption. After data transformation, significant differences ($P < 0.05$) were calculated using analysis of variance (ANOVA) with least squares difference (LSD) post-hoc tests for normally distributed data and using Kruskal–Wallis test with Tukey post-hoc tests for data that remained non-normally distributed after transformation. Levene's test of variance was performed to determine differences in cementum thickness within populations of third and fourth premolars in the control group in order to provide a substitute for baseline measurements when determining the effect of treatment on tooth roots. To consider the possibility of cross-contamination of LIPUS treatment on tooth roots treated with OIGFs and roots treated with BMP-2 because of their location immediately beside LIPUS-treated roots, cementum thicknesses and root resorption lacunae volumes of possibly cross-contaminated roots were compared with tooth roots that were not located immediate to LIPUS-treated roots. Data that was normally distributed was statistically analyzed using independent *t*-tests and data that was not normally distributed was analyzed using Mann–Whitney U tests. Intraclass correlation coefficients (ICCs) were calculated for cementum thickness in each treatment group and for root resorption volume by randomly re-measuring five samples and five roots, respectively, at least 8 weeks after the original measurements.

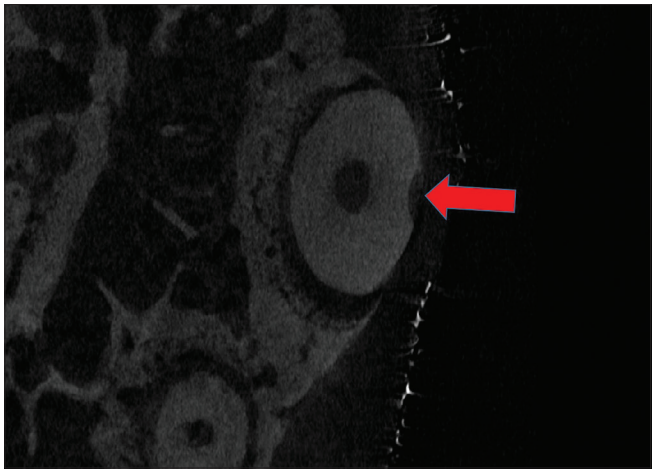

Figure 1: Microcomputed tomography analysis showing root resorption lacunae (red arrow)

RESULTS

Figure 4 shows the comparison of the root resorption volumes of each tooth root in each treatment group. Figure 5 shows the comparison of cementum thickness between groups. Levene's test of variance shows that there was no significant difference within populations of third and fourth premolars in the control group [Table 2]. Comparison of root resorption volumes and cementum thicknesses between teeth, which were expected to have some sort of cross contamination between them, are presented in Figures 6-8. ICCs were calculated [Table 3] and were at least 0.80 (strong agreement).

DISCUSSION

To the best of our knowledge, this is the first study to evaluate the effect of a transosseous injection of OIGF and LIPUS on

Table 2: Levene's test of equality of error variances for cementum thickness in control group

Levene's test of equality of error variances				
Dependent Variable: thickness				**Significant**
Tooth	***F***	**df1**	**df2**	
3	1.292	5	38	0.288
4	2.163	5	66	0.069

Tests the null hypothesis that the error variance of the dependent variable is equal across groups

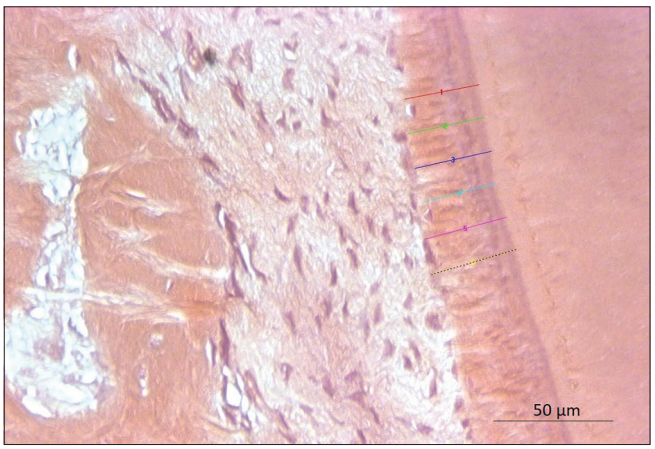

Figure 2: Photomicrograph demonstrating cementum thickness measurement

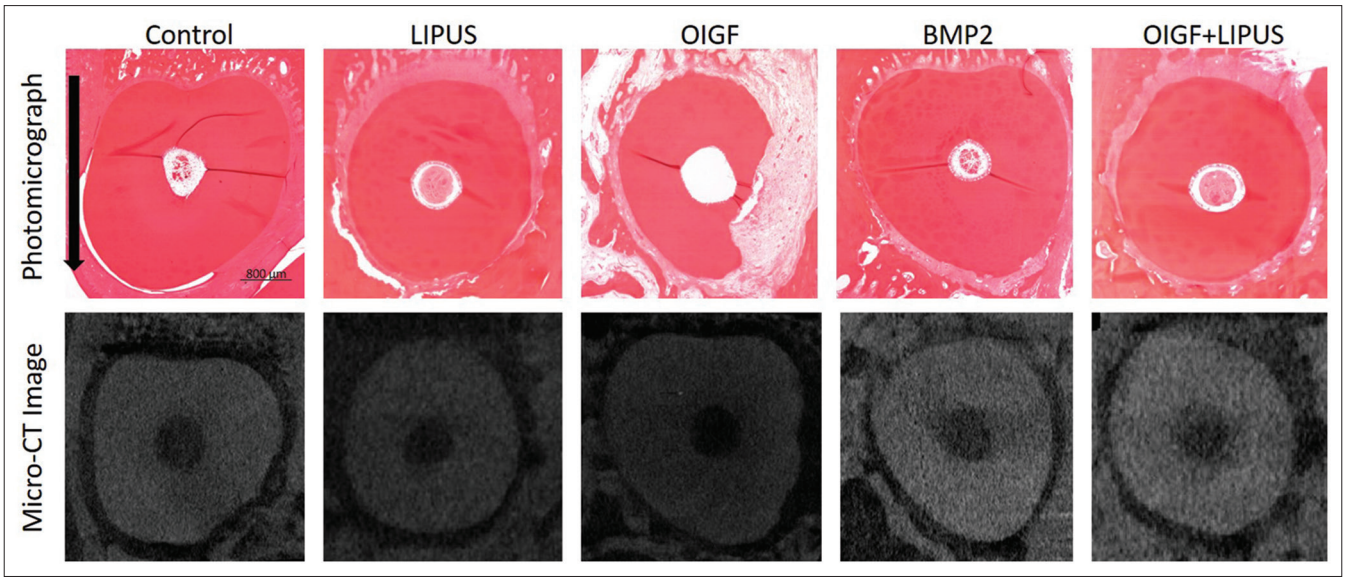

Figure 3: Sample photomicrographs and their corresponding micro-CT images of a tooth root from each treatment group. Black arrow indicates the direction of tooth movement

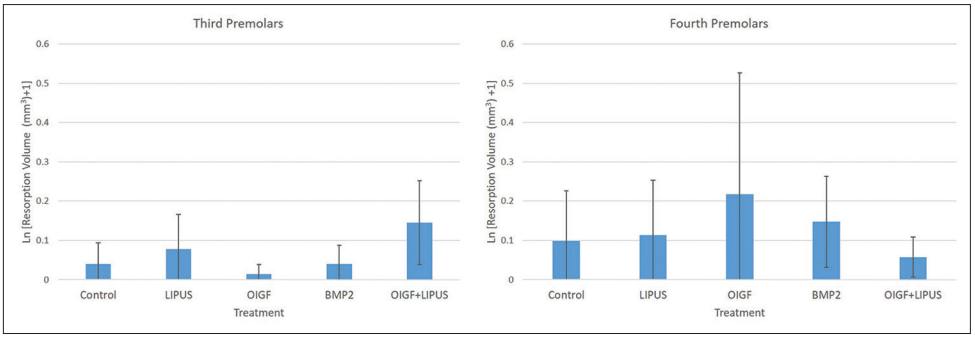

Figure 4: Root resorption lacunae volume (mm^3) (mean ± SD) on third and fourth premolars of each treatment group

Table 3: Intraclass correlation coefficients for resorption lacunae volume and cementum thickness for each group

	Cementum Thickness					Resorption Volume
	Control	LIPUS	OIGF	BMP2	OIGF+LIPUS	
ICC	0.993	0.999	0.984	0.911	0.997	0.8

LIPUS – Low intensity pulsed ultrasound; OIGF – Osteogenic induced gingival fibroblasts; BMP2 – Bone morphogenetic protein-2

orthodontically induced root resorption in beagle dogs. The present study tested the hypothesis that an intraosseous injection of OIGFs and application of LIPUS for 4 weeks can enhance OIRR repair by decreasing root resorption volume and by increasing cementum thickness, which may be interpreted as regaining resorbed root volume. In orthodontics, a complication of tooth movement is root resorption, also known as apical root resorption, which is an injury resulting from pressure applied to tooth roots during orthodontic treatment. This continuous orthodontic pressure stimulates the activity of resorbing cells, known as osteoclasts, and increases the possibility of shortening the tooth root.[30] Although it is important to analyze the whole tooth root when considering root resorption, focus should be placed on damage to the apical third of the root, since resorbing of dental cementum in this location leads to this root shortening.[30] However, the present study employed bodily tooth movement with the intention of homogeneously distributing orthodontic force along the tooth root. Although this type of tooth movement is better at uniformly applying pressure in a more diffuse and less concentrated manner, there will always be some degree of tipping movement, which tends to concentrate forces on apical and cervical regions and is more associated with apical root resorption.[31] Therefore, it is expected in the present study that more root resorption would have resulted near the apex, but not to the extent as it would have been if tipping movement was used.

This study first measured root resorption lacunae on tooth roots in every treatment group. The sums of resorption lacunae on each root were then used in comparing root resorption volumes in each treatment group in order to determine the possible effect of treatment on reducing this volume. Figure 4 shows that the OIGF group had the greatest root resorption volume in fourth premolars but the least root resorption volume in third premolars. The OIGF + LIPUS group had the least lacunae

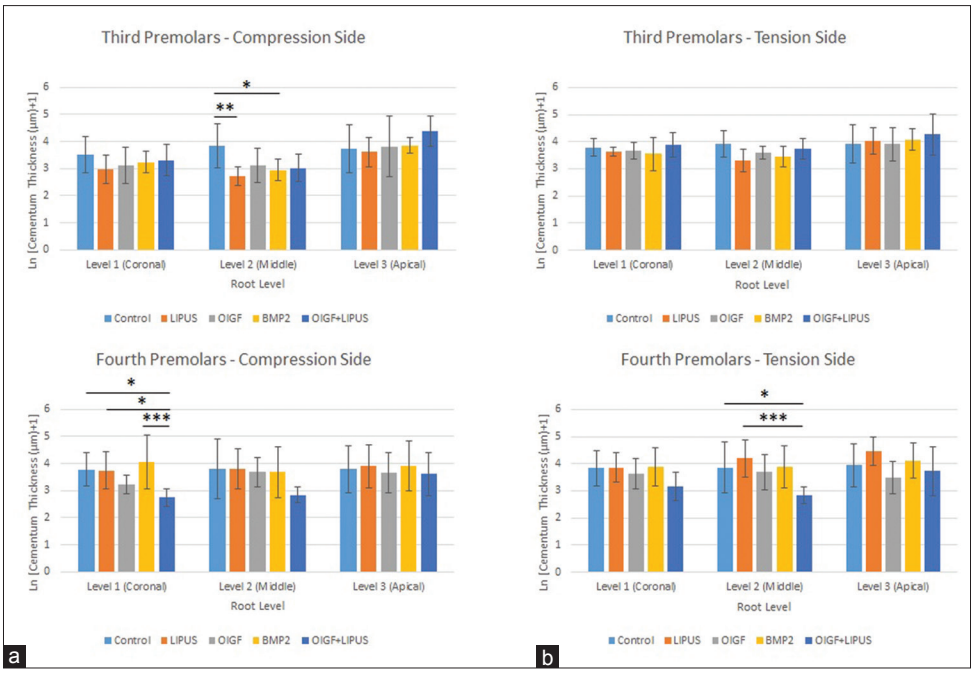

Figure 5: Cementum thickness (μm) for each group in third and fourth premolars on compression side (a) and tension side (b) of the root at three root levels (1 = coronal, 2 = middle, 3 = apical). * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.005$

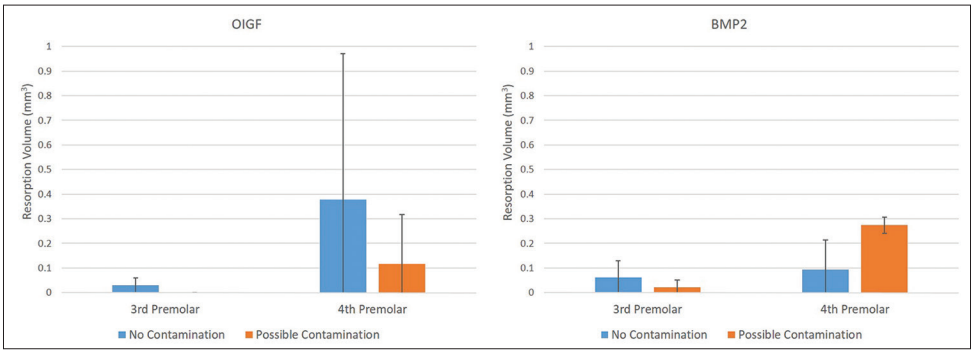

Figure 6: Root resorption volume (mm^3) of third and fourth premolars in OIGFs and BMP2 groups that contain possible cross-contamination from LIPUS treatment and groups that do not have possible cross-contamination from LIPUS

volume in fourth premolars but the least root resorption volume in third premolars. However, each of these groups was not significantly different from one another.

Because this study did not include pretreatment measurements of cementum thickness, the Levene's test of equal variance was performed to determine differences within each population of third and fourth premolars in the control group. Table 2 shows that there was no difference within populations of third and fourth premolars in the control group ($P > 0.05$). This conclusion can be used in place of baseline measurements to analyze the effect of treatment on tooth roots.

Figure 5 shows that, although on third premolars cementum thickness was greatest in the control group ($P < 0.05$) at the middle level on both compression and tension sides, the OIGF + LIPUS group had the greatest cementum thickness compared to the other groups at the apical level on both sides of the root. However, this group was not significantly different from the other treatment groups.

During this experiment, the intention was to move third premolars mesially and fourth premolars distally. However, fourth premolars were prevented from moving distally as much as expected due to the first molars being distal and more proximal to the fourth premolars than the second premolars were to the third premolars. Because of this, fourth premolars would have decreased compression and tension sides compared to the third premolars, and it is reasonable to expect different results from treatments on fourth premolars. Figure 4 shows that OIGF + LIPUS groups on the compression side of fourth premolar roots resulted in the thinnest cementum at each root level; however, this difference was not statistically significant at the middle level and near the apex. LIPUS treatment appeared to have greater effect on increasing cementum thickness on the tension side of fourth premolar

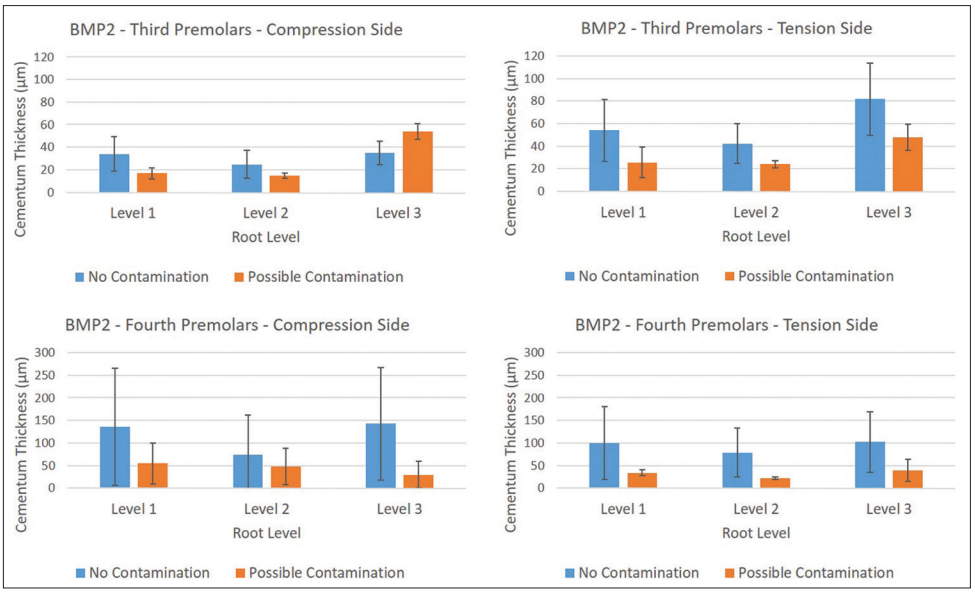

Figure 7: Cementum thickness (µm) of third and fourth premolars in the BMP2 group that contain possible cross-contamination from LIPUS treatment and groups that do not have possible cross-contamination from LIPUS

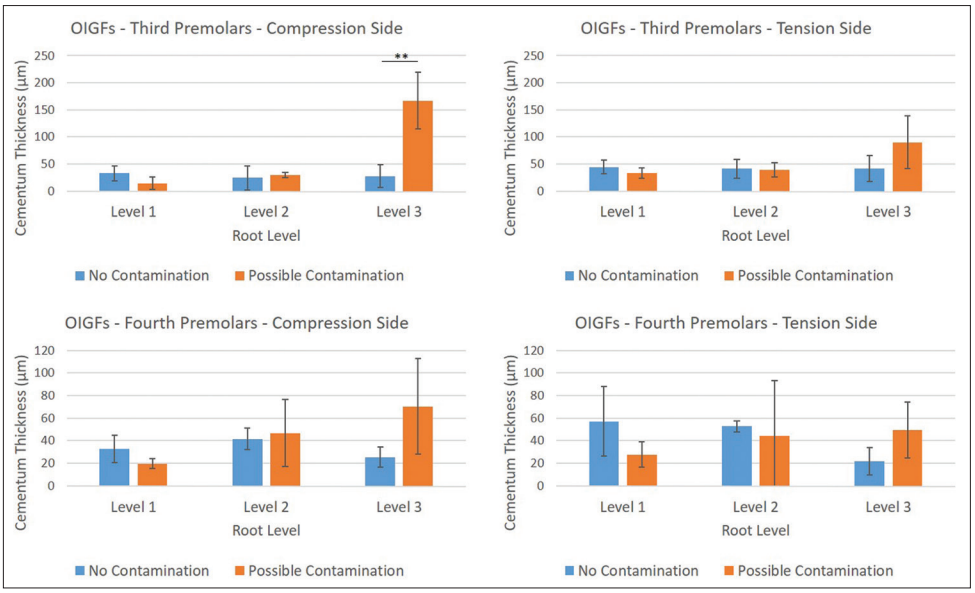

Figure 8: Cementum thickness (µm) of third and fourth premolars in the OIGFs group that contain possible cross-contamination from LIPUS treatment and groups that do not have possible cross-contamination from LIPUS. ** = $P < 0.01$

roots; however, this difference was only significant compared to the OIGF + LIPUS group at the middle root level.

It is interesting to note that BMP-2 increased cementum thickness at each root level on both sides of the fourth premolar root in comparison to the OIGF + LIPUS group. Our results are in agreement with a previous study that showed that the application of BMP-2 to tooth root defects resulted in cementum-like tissue formation compared to control root defects.[20] Upon analysis of the results, it was speculated that some tooth roots treated with BMP-2 may have been cross-contaminated by treatment with LIPUS because some LIPUS-treated roots were located immediately beside BMP-2-treated roots. To investigate this speculation, root resorption volume and cementum thickness (at each root level on compression and tension sides) were compared among BMP-2-treated roots located immediate to LIPUS-treated roots and BMP-2-treated roots not beside roots receiving LIPUS treatment. Figure 6 shows that there was no significant difference in resorption volume between third premolars with the possibility of cross-contamination and third premolars without cross-contamination, and between fourth premolars with the possibility of cross-contamination and fourth premolars without cross-contamination. Figure 7 shows that BMP-2-treated tooth roots with possible cross-contamination of LIPUS had thinner cementum at almost all root levels compared to those

roots without cross-contamination, however, no statistically significant differences were calculated. Sant'Anna *et al*.[32] investigated *in vitro* the effect of LIPUS applied in combination with BMP-2 treatment on the expression of genes associated with osteogenesis in rat stromal cells. They found that there was no additive or synergistic effect of the combination of these two treatments. Because in the present study, the exposure of LIPUS treatment on BMP-2-treated tooth roots was only through possible cross-contamination, it may be possible that this LIPUS exposure was at a lower intensity compared to direct application as in LIPUS- and OIGF + LIPUS-treated roots due to slight dissipation of LIPUS through tissue. Direct application of LIPUS to BMP-2-treated roots at a higher intensity or using more optimal levels may lead to a synergistic effect, but it may be possible that in the present study this continual and decreased exposure may have had a negative effect of BMP-2 on cementum thickness. It is suspected that, if possible cross-contamination of LIPUS on BMP-2-treated roots was absent, the overall effect of this treatment on cementum thickness may have been significantly greater than what is demonstrated in the present study.

Similarly, it was also suspected that LIPUS cross-contaminated OIGF-treated tooth roots. The possibility of cross-contamination of LIPUS on these tooth roots appears to have had a positive effect on OIGF treatment on resorption volume, but these differences are statistically significant ($P < 0.01$) [Figure 8]. This figure demonstrates that possibly-contaminated OIGF-treated roots had greater cementum thickness at the apical third of the root on the both sides of third and fourth premolars with a significant difference calculated on the compression side of third premolars. El-Bialy *et al*.[33] investigated the difference between human and dogs' gingival mesenchymal cells *in vitro*. This study demonstrated that canine gingival mesenchymal cells (CGMCs) responded differently than human gingival mesenchymal cells (HGMCs) when both types of cells were grown in osteogenic medium and received LIPUS treatment for 1 day. Extensive research has been performed to show the anabolic effect of LIPUS on different types of cells, including gingival cells,[34] however, research performed on CGMCs is limited. El-Bialy *et al*.[33] showed that it may be possible that CGMCs require alternative parameters of LIPUS treatment, such as intensity and length of exposure, compared to parameters used currently in the treatment of HGMCs. This may explain the overall decreased effect of OIGF and OIGF + LIPUS treatment.

Future studies should not allocate different treatments to neighboring teeth in order to avoid the possibility of cross-contamination of treatments, especially those that can dissipate through tissues, such as ultrasound. This study also did not biologically track the injected OIGFs. Biologically labelling these cells would allow tracking of these cells to be determined and to know whether or not if these cells became incorporated into the target tissue and used in tissue repair. Finally, the present study used identical ultrasound application parameters that are used in studies involving human gingival cells. Future studies may also be directed to understanding the possible mechanisms, of which OIGF, LIPUS and BMP2 might be involved in repairing OITRR and possible interrelationship between these mechanisms for possible optimum combination of these treatment modalities in treating/prevention of OITRR.

CONCLUSION

This study showed that OIGF, LIPUS, and BMP-2 may have a possible effect on the repair of OIRR, however, more optimal parameters of ultrasound use and improved experimental design are required in order to further investigate this effect.

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Conflicts of Interest

There are no conflicts of interest.

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