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Impact of Local Melatonin Application on Expression Level of Osteogenic Marker (Runx2) and Post-orthodontic Relapse in Ovine Model: A Split Mouth Study

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ABSTRACT

Background: Retaining teeth in their proper alignment after orthodontic treatment can be highly challenging. This study aimed to estimate the effectiveness of locally injected melatonin in improving bone remodelling and reducing post-orthodontic relapse.

Methods: The third incisors on the right and left quadrants of six sheep were removed and the second and fourth incisors were brought close together by using a sectional orthodontic appliance. Melatonin solution was injected near and parallel to the mesial surface of the second incisor and the distal surface of the fourth incisor on one quadrant while the other quadrant received 1% dimethyl sulphoxide as a control. Four weeks later, the orthodontic appliance was debonded and the teeth were allowed to move back. By utilizing digital analysis, the relapse distance of the approximated incisors was measured at 21 and 42 days respectively after appliance removal. Histological and mRNA expression analysis of osteogenic marker (Runx2) were conducted to evaluate the periodontal space width and the surrounding alveolar bone of the melatonin-treated and control side incisors.

Result: clinical and histological measurements showed that the approximated incisors in the melatonin-treated quadrant had a significantly shorter relapse distance ($p \leq 0.05$), significantly smaller periodontal ligament width ($p \leq 0.05$) and significantly larger new bone area formation ($p \leq 0.05$) than in the control quadrant. The mRNA expression level of the osteogenic marker (Runx2) was significantly larger ($p \leq 0.05$) in the melatonin-treated quadrant than in the control quadrant. The outcomes of this research suggest that melatonin can enhance bone remodelling and reduce post-orthodontic relapse in sheep.

Key words: Melatonin, Orthodontic Relapse, qRT-PCR, Runx2, Sheep.

INTRODUCTION

Orthodontic therapy is an affordable and effective approach for the correction of maligned teeth and gaining a beautiful smile. Retaining teeth in their proper positions following treatment tends to be the most difficult aspect of an orthodontic treatment plan. Relapse might be defined as any undesirable movement of a tooth away from its corrected position after orthodontic treatment of malocclusion (Littlewood *et al.,* 2017). The precise etiology of the relapse remains incompletely understood and is thought to encompass a wide range of elements. Proposed causes include pressure from muscles and soft tissues, elongated periodontal and gingival fibres, changes in the location of the teeth and size of the arch, facial growth, inability to treat the exact issue, and continuous bone remodelling (Maleeh *et al.,* 2016).

Developing a biological approach for retaining teeth after orthodontic treatment remains a significant need in the field of orthodontics. It has been reported that various substances, including prostaglandin, rBMP-2, osteoprotegerin, injectable platelet-rich fibrin, magnesium oxide, olive oil and bisphosphonates, can be applied topically to regulate bone remodelling to reduce the likelihood of post-orthodontic relapse (Al-Fakhry and Al-Sayagh, 2022; Al-Hamdany *et al.,* 2017; Han *et al.,* 2010; Hassan *et al.,* 2010; Mohammed *et al.,* 2023; Okamoto *et al.,* 2009).

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Melatonin is an endogenous hormone that is generated rhythmically within the pineal gland, by the light/dark cycle (Zawilska *et al.,* 2009). Melatonin is crucial in several physiological processes such as circadian entrainment, blood pressure control and immunological function (Dominguez-Rodriguez *et al.,* 2010). In the last few years, there has been a significant amount of focus placed on the studies and uses of melatonin in the hard tissues, including teeth and bony tissues. Cesur *et al.,* (2018) concluded that Melatonin administration systemically during rapid maxillary expansion treatments accelerates new bone development and shortens the retention phase

To the best of our knowledge, no previous research has investigated the efficacy of the local application of melatonin

melatonin in reducing post-orthodontic relapse. Thus, the main objectives of this study were to evaluate clinically, histologically and on the level of molecular biology the effectiveness of melatonin in improving bone remodelling and hence, reducing the relapse of orthodontically moved teeth.

MATERIALS AND METHODS

Study setting

The study was conducted on 2023 in the animal household of the College of Veterinary Medicine/University of Mosul. The University Mosul Dental School's research ethics committee reviewed and endorsed the research protocol (Approval No. UoM. Dent. 23/18 on 02.05.2023). A comprehensive examination was carried out by veterinarians on Awassi sheep to assess their general, periodontal, and dental health. The sheep were quarantined for weeks before the beginning of the experiment (Henry *et al.,* 2022). The following formula was performed to calculate the sample size:

$$n = DF/k + 1$$

Where,

n = Number of subjects per group,

DF= The between-subject error (that is, the within-subject DF) based on the acceptable range was set to 10.

k = Number of groups.

Following the necessary adjustments to the obtained value, the final sample size was calculated as the sample size per group.

Study design

The study comprised six adult male sheep (weight 55 kg; age 3 years). Each sheep possesses a total of eight permanent incisors, with four located on each side. To determine the effects of melatonin, a prospective randomized split-mouth experimental trial was conducted. The right and left sides of the sheep were randomly assigned to either the melatonin group (MG) or the control group (CG) based on a digitally produced sequence of random numbers. The study involved the second and fourth incisors on each side and the third incisor on each side was removed. Throughout the intended experiment, the sheep were anaesthetized numerous times with ketamine (22 mg/kg IM) and xylazine (0.2 mg/kg IM). Each sheep had its third incisors removed on both the right and left sides and the areas were permitted to heal for a week. The sheep were anaesthetized and the labial surfaces of the second and fourth incisors on each quadrant were polished using pumice powder (Produits Dentaires SA., Switzerland) and a dental polishing brush using low-speed handpiece (Coxo Medical Instrument CO., LTD., China). Then, teeth surfaces were etched with phosphoric acid 38% (Pulpdent Co., USA) for thirty seconds, washed with water for fifteen seconds and air dried. Standard 0.022 slot edgewise metal brackets of equilibrium® 2 series (Dentaurum GmbH and Co., Germany) were bonded using TrueBond LC (IOS Corp., USA) and cured by Eighteeth Curing Pen E (Changzhou Sifary Medical Technology Co., China) for twenty seconds from each direction. Each bracket was attached at the midpoint of the tooth's long axis, in a mesiodistal direction. The brackets were all of equal height, to ensure there were no differences in vertical alignment among the bonded incisors. The two incisors on either side were then joined together using sectional 0.017×0.025 stainless steel orthodontic archwire and their ends were bent to make a non-traumatic end. An elastomeric chain (Orthometric, Marília, Brazil) was applied to the second and fourth incisors to achieve a starting force of 150 grams. Three times per week, the elastomeric chain was replaced until the second and fourth incisors had completely approximated. Then the brackets on the approximated teeth were passively ligated using stainless steel ligature wire 0.01 inch. (Dentaurum GmbH and Co., Germany) for four weeks (Fig 1).

Melatonin (N-Acetyl-5-methoxytryptamine) was prepared for injection by dissolving 10 mg melatonin powder (Chem-Impex INTL INC., USA) in 1 millilitre of 1% dimethyl sulfoxide (Chem-Impex INTL INC., USA) solution.

A melatonin injection was administered to a randomly selected side of each animal utilizing a disposable one-unit insulin syringe equipped with a 25-gauge microneedle. (Forlong Medical Corp., China). The injection was given adjacent and parallel to the mesial surface of the second incisor and the distal surface of the fourth incisor at the mucogingival junction, penetrating through the attached gingiva into the oral mucosa. The dose was divided, with 0.5 ml injected into the labial side and another 0.5 ml injected into the lingual side of the vestibular mucosa.

A volume of 1 millilitre of 1% dimethyl sulfoxide solution was injected on the opposite side using the same injection technique, serving as a control. The injection was performed two times weekly for four weeks.

Clinical assessment

After completing the injection procedure, the fixed orthodontic device was debonded utilizing bracket removal pliers (Dentaurum., Germany) and an intraoral impression Zhermack Zetaplus A Silicone impression Material (Badia Polesine (RO), Italy) loaded into a customized resin tray will be obtained immediately after the orthodontic appliances removal and 21, 42 days later of post-orthodontic relapse to create dental models, All impressions were poured with improved die stone (Elite Rock Dental Stone; Zhermack, Badia Polesine, Italy). All stone models were scanned with an E1 lab scanner (3Shape Co., USA) to generate three-dimensional models in stl. file format. Utilizing the Viewbox software (V 4.0.1.7; dHal Software, Greece), The models were aligned according to the mandibular occlusal plane, which was determined by the position of the incisal edge of the lower incisors. Two planes were constructed at right angles to the occlusal plane of the mandible. The first plane was initially sketched on the distal surface of the second incisor, precisely at the location of the furthest contact region. A second plane was sketched to make contact with the most

Table 1: Primer Sequences for housekeeping gene (GAPDH) and target gene (Runx2).

Gene	Forward	Reverse
GAPDH	5′-ACAGTCAAGGCAGAGAACGG-3′	5′-CCAGCATCACCCCACTTGAT-3′
RUNX2	5′-TTCGCCTCACAAACAACCAC-3′	5′-GTGCTCGGATCCCAAAAGAA-3′

mesial contact area of the mesial surface of the fourth incisor. The post-orthodontic relapse is quantified by measuring the linear distance, which runs parallel to the mandibular occlusal reference plane, between the two constructed planes. Measurements were obtained utilizing the ruler tool in the Viewbox software, Fig (2,3).

On 21 and 42 days of orthodontic appliance removal 3 sheep were slaughtered, and a dense diamond saw (Cadence Inc., USA) was utilized to precisely cut the anterior portion of each mandible 5 mm distal to the fourth incisor. A sterile 4-mm diameter trephine drill (NTI-Kahla GmbH, Germany) using a low-speed handpiece (COXO Medical Instrument Co., Ltd., China) and profuse Sodium Chloride 0.9% Irrigation Solution (Jedux Parenteral Private Limited, India) was employed to remove bone and root tissue from the distal aspect of the fourth incisor of all samples parallel to the visible inclination of the fourth incisor root through full thickness of the alveolar bone. Then, the removed tissues were placed in sterile micro-centrifuge tubes containing 1.5 ml of DNA/RNA shield lysis solution (Zymoresearch, Irvine, USA) and stored at -20°C for further RNA extraction and qRT-PCR analysis

Histological assessment

The specimens were fixed for three days in a 10% neutral buffered formalin solution, followed by decalcification for five to six weeks in an 8% hydrochloric and 8% formic acid solution. Histological section were treated based on standard protocol.

RNA extraction and cDNA synthesis

Samples in micro-centrifuge tubes were crushed using a pestle and mortar and subjected to ultrasonic cell disruption using Vibra-Cell 500 processor (Sonics and Materials, Inc., Newtown, USA). RNA was extracted from the homogenate according to the manufacturer's protocol using AddPrep Total RNA Extraction Kit (Add Bio Inc., Yuseong-gu, South Korea). Then, the extracted RNA concentration was quantified spectrometrically using a Nanodrop 2000 (Thermofisher Scientific Inc., USA). 2 µl of extracted RNA was used for the synthesis of complementary DNA (cDNA) using AddScript cDNA Synthesis Kit (Add Bio Inc., Yuseong-gu, South Korea). Thermal cycler steps of cDNA Reverse Transcription were 25°C for five minutes, then 42°C for thirty minutes and lastly 5°C for five minutes. The resultant cDNA was stored at -20°C until further qRT-PCR was carried out.

Gene selection and primer sequence

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was chosen as the housekeeping gene and Runt-related transcription factor 2 (RUNX2) as the target gene (Table 1) (Dash and Singh, 2024).

Quantitative Real-Time Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR)

The mRNA expression levels of the reference and target genes were determined using Sybr green quantitative reverse transcriptase polymerase chain reaction (qRT PCR) assays as per manufacturer instruction (Add Bio Inc., Yuseong-gu, South Korea) (Table 2) (Panigrahy *et al.,* 2023; Vyas *et al.,* 2022).

Statistical analysis

Statistical analysis was conducted using SPSS V26 (Statistical Package for Social Science, IBM Inc., USA). The data were tested for their normal distribution by using the Shapiro-Walk test. Comparison between the Melatonin group (MG) and Control group (CG) regarding relapse distance, periodontal ligament width and new bone formation area was done on 21 and 42 days respectively after removal of the orthodontic appliance using independent sample t-test or Mann Whitney U test depending on a normal distribution of data. A significance level of 0.05 (two-tailed) was used to determine statistical significance for all analyses.

RESULTS AND DISCUSSION

Relapse distance and relapse percentage

Based on millimetric measurements obtained from digital analysis of 3-dimensional models, significantly more

Table 2: Thermal profile of GAPDH and RUNX2 mRNA expression.

Step	Temperature	Duration	Cycles
Enzyme activation	50°C	2 min	Hold
Denaturation	95°C	10 min	40
Annealing	94°C	15 sec	
extension	60°C	1 min	

Table 3: Comparison of orthodontic relapse between studied groups.

Groups	Time (days)	Relapse distance		
		mm	%	p value
MG (n=6)	21	2.28±0.37	37.53	0.004
CG (n=6)		3.36±0.80*	55.37	
MG (n=3)	42	2.40±0.18	39.47	0.012
CG (n=3)		3.41±0.18*	56.09	

Data expressed as Mean±SD, *indicate significantly higher at p<0.05 using independent sample t test, MG= Melatonin group, GC= Control group, mm= Millimeter

relapse was observed in CG than MG on 21 and 42 days after appliance removal (Table 3).

Periodontal ligament width and New bone formation

Histological measurement revealed a substantial difference in the periodontal ligament width mean between MG and CG at 21 and 42 days after appliance removal, respectively (Table 4).

Microscopical findings in the alveolar bone adjacent to the mesial surface of the second incisor root of MG at 21 and 42 days after appliance removal revealed outstanding and variable observations. Newly produced bone, both mature and immature, with several groups of osteoblasts, was seen along the boundary of the alveolar socket and within the periodontal space, delineating the remodelled region of the socket wall. Several bony spicules with newly produced blood vessels were also seen adjacent to the bone surface (Fig 4).

Based on the histological measurement of the new bone formation area a significant difference in the mean was observed between MG and CG at 21 and 42 days respectively after appliance removal (Table 5).

RUNX-2 mRNA expression analysis

The RUNX-2 mRNA expression level was inclined to up-regulate continuously with a maximum 12.20-fold change at 42 days and 11.56-fold change at 21 days after appliance removal in MG which was significantly higher (p d" 0.05) than CG (Table 6).

The sheep model is appropriate for studies focusing on the dentoalveolar structure and bone remodelling due to the morphological and periodontal similarities between an adult sheep's lower incisors and human teeth (Tokhtah and Alhadlaq, 2022a). The assessment of sheep for a healthy periodontium and teeth posed difficulties, leading to a restricted number of sheep being included in the study. This research utilized a split-mouth design to minimize the influence of possible confounding variables, such as body weight, dental hygiene and the rate of tooth movement. This study is the first to assess the effectiveness of injectable melatonin in increasing post-orthodontic stability, based on our present understanding. In the present study, The melatonin solution was administered via submucosal injection, a mode of drug delivery that is widely regarded as safe and suitable for clinical use (Zeitounlouian *et al.,* 2021). In this study, the injected melatonin was efficacious in reducing post-orthodontic relapse, as demonstrated by a three-dimensional model, histological and genetic analysis. As compared to a study by Hassan *et al.* (2010), who have created trans gingival channels that penetrate the periodontium using mini-screws, and then injected Dynagraft material., the aforementioned procedure in this research can be more clinically acceptable by orthodontic patients is less invasive (Cheng *et al.,* 2023; Li, *et al.,* 2023).

Histological findings of this study of newly formed bone extending from the alveolus toward root cementum decreasing the width of the periodontal ligament in MG could be the main contributor to reducing post orthodontic relapse noticed in this group. Similar results were observed by Hassan *et al.,* (2010), who used Dynagraft material to reduce post orthodontic relapse in ovine models. Equivalent results were noticed by Tokhtah and Alhadlaq (2022b), after the injection of bisphosphonate gel into the periodontal space in goat models. Furthermore, active bone remodelling, as observed by the presence of osteoblasts and osteoclasts

Table 4: Comparison of periodontal ligament width between studied groups.

Groups	Time (days)	Width (µm)	p value
MG (n=3)	21	445.2±49.3	0.004
CG (n=3)		535.4±69.3*	
MG (n=3)	42	356.8±50.5	0.0001
CG (n=3)		462.1±56.6*	

Data expressed as Mean±SD, *indicate significantly higher at p<0.05 using independent sample t test, MG= Melatonin group, GC= Control group, µm= Micrometer.

Table 5: Comparison of new bone formation area between studied groups.

Groups	Time (days)	Area (μm^2)(Mean±SD)	p value
MG (n=3)	21	80129.8±4946.1*	0.0001
CG (n=3)		31976.8±4392.1	
MG (n=3)	42	369447.1±4118.1*	0.0001
CG (n=3)		132708±3818.3	

Data expressed as Mean±SD, *indicate significantly higher at p<0.05 using independent sample t-test, MG= Melatonin group, GC= Control group, μm^2= Square micrometer.

Table 6: RUNX-2 mRNA expression level and Fold change of studied groups.

Groups	Time	Mean Ct Runx2	Mean Ct GAPDH	Mean ΔCt study sample sample	Mean ΔCt Calibrator expression	Fold change of Runx2 gene ($2^{-\Delta Ct}$)	Runx2 mRNA expression	p value
MG	21	24.42	23.93	0.48	4.01	11.56	0.72	0.0001*
CG	21	26.25	24.20	2.05	4.01	3.90	0.24	
MG	42	24.68	24.27	0.40	4.01	12.20	0.76	0.0001*
CG	42	27.13	25.15	1.98	4.01	4.10	0.25	

*indicate significantly higher at p<0.05 using independent sample t test, MG= Melatonin group, GC= Control group, Ct= Cycle threshold.

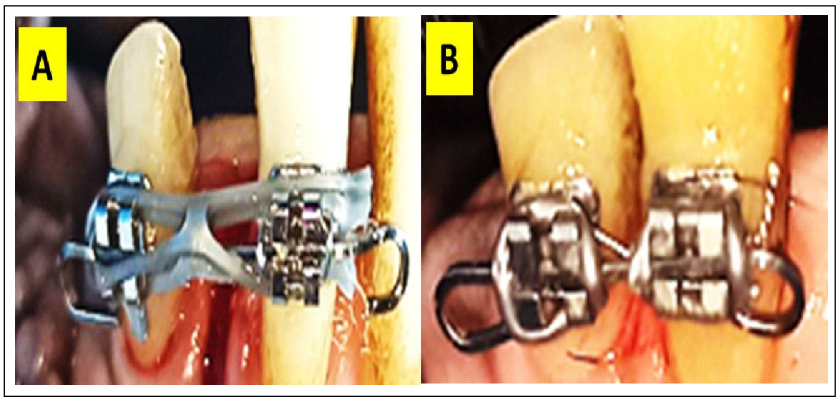


Fig 1: (A) Post-extraction space of third incisor and orthodontic appliance *in situ*. (B) post-extraction space closure and passive ligation of brackets on the second and fourth incisors with stainless steel ligature wire.

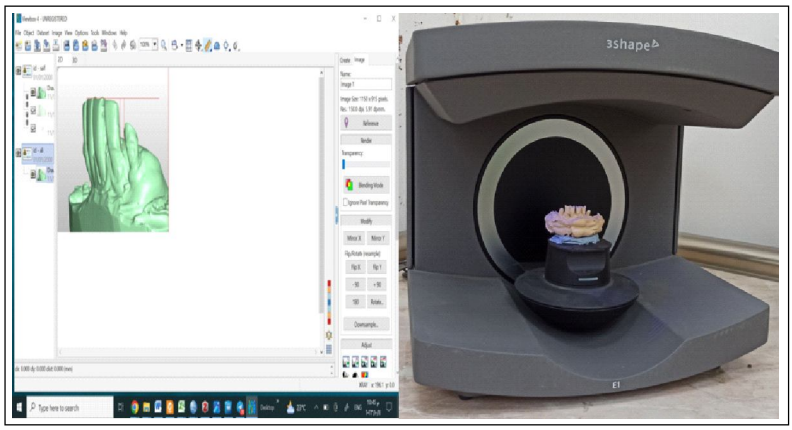


Fig 2: Three-dimensional scanning of study models using E1 3shape lab scanner (3Shape co., USA) and measuring the magnitudes of post-orthodontic relapse using Viewbox software (dHal Software, Greece).

around the newly produced bone islands in the periodontal ligament in the MG is in accordance with a study by Cesur *et al.* (2018), in which the systemic administration of melatonin in Wistar albino rats models resulted in enhanced new bone formation and might shorten the retention phase in rapid maxillary expansion treatment.

In the present study, the mRNA level of RUNX-2 a typical marker of osteoblast differentiation was highly expressed in MG as compared to CG and this was in accordance with Zhu *et al.* (2020), as they analyzed the effect of melatonin on osteoblastic differentiation in Osteoblast precursor MC3T3 E1 cells. They observed that the expression levels of RUNX-2 showed a remarkable increase in response to additional melatonin. Moreover, the findings of our research were in agreement with the findings of a research conducted by Zhang *et al*. (2010), who noticed that melatonin directly inhibits human mesenchymal stem cells adipogenic differentiation and significantly improves osteogenic differentiation by inhibiting peroxisome proliferator-activated receptor gamma expression while increasing RUNX-2 expression.

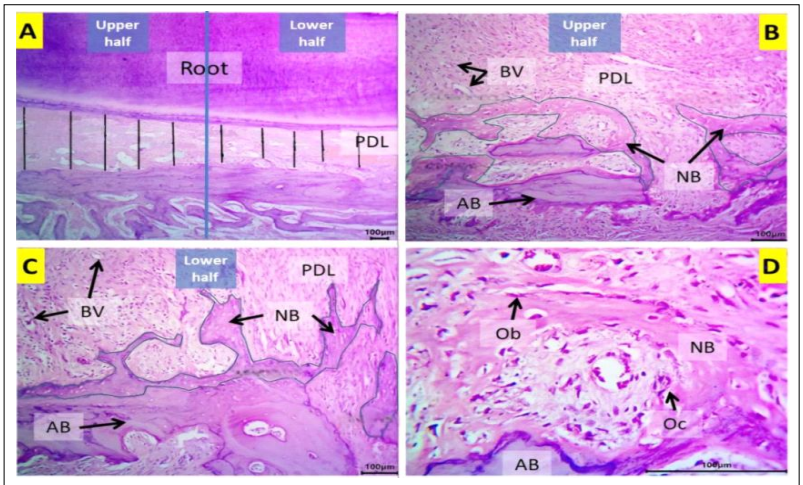
Previous research has shown that melatonin is an efficient osteoblastic substance when applied locally. Tresguerres *et al.,* 2012 indicated that a 3 mg local melatonin administration during surgical implant integration might boost the trabecular bone-implant connection as well as the trabecular bone area density.

Another more recent study by Golpasandhagh *et al.* (2023) revealed that the topical application of melatonin gel can serve as a bone formation stimulant, as evidenced by a higher rate of ossification in the melatonin groups compared to the control group, with the effect being dose-dependent. Moreover, the role of melatonin in enhancement of osteoblast differentiation is not limited to expression of Runx2 marker, it also enhances osteoblast differentiation through the BMP-2/Smad signaling pathway (Niu, *et al.,* 2023).

In contrast, the digital analysis of three-dimensional models revealed that teeth of the CG experienced a more pronounced relapse at 21 and 42 days, respectively, after the removal of the orthodontic appliance than the teeth of MG. Histological examinations revealed increased periodontal ligament width and areas of osteoclastic activity, indicative of a substantial relapse that occurred.

Nevertheless, there were some limitations in this study such as several animals, study duration and one concentration of melatonin used, It is recommended to administer greater dosages of the medication in future research and compare them with the current concentration

Fig 3: (A) Occlusal view of incisors teeth 42 days after appliance removal. (B) Lateral view of incisors of melatonin injection side. (C) Lateral view of incisors of dimethyl sulphoxide injection side.



(A) periodontal ligament width measurement, (B) upper half (apical area), (C) lower half (cervical area) and (D) New bone formation (NB) with clusters of osteoblast (Ob) and osteoclast (Oc). Periodontal ligament (PDL), Blood vessels (BV) and Alveolar bone (AB). H and E stain, (A: 100X; B: 100X; C: 400X), Scale bar= 100µm.

Fig 4: Histological section of the second incisor root and the surrounding bony socket of the MG at 21 days after orthodontic appliance removal.

to establish the most effective dose of melatonin for reducing post-orthodontic relapse.

CONCLUSION

Within the limitation of this study, our preliminary results indicate that melatonin could improve bone remodelling and potentially reduce post-orthodontic relapse in sheep and it has the potential to be part of the biomodulation protocol of orthodontic relapse control.

Conflicts of interest

The authors declare that they have no conflicts of interest. The authors received no specific funding for this work.

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