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Local application of melatonin associated or not to xenogeneic material, in critical defects of rat calvaria

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ABSTRACT

Melatonin (MLT) is a hormone that can stimulate bone formation and inhibit bone resorption, among other functions. ***Aim:*** *To evaluate the effect on new bone formation of MLT applied locally to critical defects created in the calvaria of rats, compared to the effect of Bio-Oss® xenogeneic bone substitute (BO), by analyzing histomorphometry, microtomography and gene expression.* ***Materials and Method:*** *Two critical defects (5.0 mm in diameter) were created in the calvaria of 36 adults male Wistar rats. The rats were divided randomly into two groups: a test group, in which one of the defects was filled with MLT, and the other with MLT with Bio-Oss® (MLTBO), and a control group, in which one of the defects was filled only with the clot (C), and the other with BO. The rats were euthanized 30 days after surgery. Samples of the calvaria containing the critical defects were collected for analysis by histomorphometry, microtomography, and the expression of the genes for type I collagen (COL-I), osteopontin (OPN) and bone morphogenetic protein 2 (BMP-2).* ***Results:*** *A qualitative improvement was observed in bone healing when MLT was used, though there was no statistical difference in the quantification of newly formed bone (p>0.05). Micro-CT showed that bone volume was significantly smaller in absence of BO (p=0.006). Bone trabeculae thickness (p=0.590) and number (p=0.150) were not significantly affected by MLT. Regarding the expression of the genes COL-I, OPN and BMP-2, no significant differences were observed between the MLT, BO and MLTBO groups.* ***Conclusion:*** *Topical application of MLT resulted in a qualitative improvement in bone healing, although it did not affect bone formation quantitatively. In the absence of BO, less bone volume and less bone trabecular thickness were observed.*

Keywords: *calvaria - critical bone defects - bone mineral density - melatonin - bone regeneration.*

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Aplicação local de melatonina em defeitos em calvárias de ratos associada a material xenógeno

RESUMO

A melatonina (MLT) é um hormônio sintetizado e secretado pela glândula pineal, e que, dentre outras atividades e funções, tem capacidade de estimular a formação e inibir a reabsorção óssea. ***Objetivo:*** *avaliar o efeito da aplicação local do hormônio MLT na neoformação óssea, em defeitos críticos produzidos na calvária de ratos, por meio de análise histomorfométrica, microtomográfica e de biologia molecular, comparando-a com um substituto ósseo de origem xenogênica (Bio-Oss®).* ***Materiais e Método:*** *foram utilizados 36 ratos Wistar machos adultos, nos quais foram produzidos dois defeitos críticos de 5,0 mm de diâmetro cada, que receberam diferentes tratamentos alocados de forma randomizada: no grupo controle, os animais tiveram um dos defeitos preenchidos apenas com coágulo sanguíneo (C) e o outro com substituto ósseo xenógeno (Bio-Oss®, BO); no grupo teste, um dos defeitos foi preenchido apenas com MLT e, o outro, recebeu a associação da MLT ao material sintético xenógeno (MLTBO). Todos os animais foram eutanasiados após 30 dias do pós-operatório. As amostras das calotas contendo os defeitos críticos foram coletadas para análises histomorfométricas, microtomográficas e da expressão gênica de colágeno do tipo I (COL-I), osteopontina (OPN) e proteína óssea morfogenética 2 (BMP-2), por meio de PCR em tempo real.* ***Resultados:*** *Após análise dos dados pode-se observar que não houve diferença estatística na quantificação de neoformação óssea (p>0.05), porém, melhora qualitativa foi observada na cicatrização, quando a MLT foi utilizada. Quanto aos par®metros microtomográficos, foi observado que com ou sem MLT, o volume ósseo foi significativamente menor na ausência de BO. A espessura (p = 0,590) e número (p = 0,150) de trabéculas não foram significativamente afetados pelo uso da MLT. Quanto à expressão gênica de COL-I, OPMN e BMP-2, não foram observadas diferenças significativas entre os grupos MLT, BO e MLTBO.* ***Conclusão:*** *conclui-se que a aplicação tópica de MLT, associada ou não ao BO não afetou quantitativamente a neoformação óssea, porém resultou em uma melhora qualitativa na cicatrização. Adicionalmente, na ausência de BO foi observada menor volume ósseo e menor espessura das trabéculas.*

Keywords: *calvaria - critical bone defects - bone mineral density - melatonin - bone regeneration.*

INTRODUCTION

Melatonin (MLT) or N-acetyl-5-methoxytryptamine is an indoleamine centrally produced by pinealocytes in the pineal gland and released directly into the blood, which acts as a hormone[1]. It also acts on bone formation, promoting osteoblastic differentiation by stimulating the formation of new bone matrix[2-7]. Considering bone metabolism, MLT seems to act directly on osteoclasts through several mechanisms, potentially interfering with their function, thereby inhibiting bone resorption[1,8]. Some studies have shown that topical application of melatonin promotes new bone formation and regeneration in bone defects [9-12].

MLT plays a role in reducing cell degeneration by stimulating the production of type I collagen (COL-I), thereby modulating osteoblastic activities[13,14]. MLT can also participate in regulating the inhibition of bone resorption, which occurs through the negative regulation of osteoclasts mediated by inhibition of the RANKL molecule action[8,15].

In dentistry, MLT is a potential adjuvant in dental implant osseointegration[16,12], and used in the treatment of oral cancer[17], as an analgesic, and to induce new bone formation after extraction of third molars[18], as well as to repair damage caused by periodontitis[19-21]. It has also been shown to influence *in vitro* osteoblastic function, improve bone regeneration when administered by mouth daily to ovariectomized mice[22], and increase bone strength in naturally aged mice[23].

Studies using MLT have been conducted with the aim of improving dental implant surfaces, bone substitutes and materials that can accelerate osseointegration[11,14,24,25]. However, there is still little information about the MLT mechanism of action in bone matrix formation, especially when it is administered locally.

The aim of this study was therefore to evaluate the effect of local application of MLT on new bone formation when placed in critical defects created in the calvaria of rats, compared to MLT with a xenogeneic bone substitute (Bio-Oss®), through histomorphometry, microtomography and analysis of COL- I, osteopontin (OPN) and bone morphogenetic protein 2 (BMP-2) gene expression.

MATERIALS AND METHOD

Sample characteristics

Thirty-six healthy male rats (*Rattus norvegicus* – Wistar) were selected, 3 months old, and weighing approximately 300g. The animals were acclimatized for a period of 10 days before the surgical procedures, with water and food *ad libitum*, housed in cages with wood shavings, at controlled temperature (21ºC) and lighting (12/12-hour light-dark cycles), at the vivarium at *Faculdade São Leopoldo Mandic*, after approval by the Ethics Committee (2019/012, SLM. CEUA. F8-00).

Sample size was calculated using the equation for finite population recommended by Zar (2010): n = (z2. dp2) / d2 + z2 (dp2 / N), in which n = finite population; z = confidence interval (α = 0.05 or z = 1.96); d = error (20%); dp = variance (25%). The calculation was: n = (1.962. 252)/202+1.962. (252/16) = 4.37. Adjusted for expected death of animals, 6 animals were allocated for each analysis, totaling 36 rats.

Experimental design

Two circular defects were created in the calvaria of each rat (see Surgical Procedure, below). The animals were randomized into two groups and treated as follows: a) 18 rats were treated with MLT, and had one defect filled with 0.015g of MLT (Active Pharmaceutica, China) - MLT group; and the other defect filled with a combination of 0.025g of xenogeneic bone substitute (Bio-Oss®, Geistlich Biomaterials, Switzerland) and 0.0075g of MLT (Active Pharmaceutica, China) - MLT/BO group; and b) 18 rats had one defect filled with 0.050g of xenogeneic bone substitute (Bio-Oss® Geistlich Biomaterials, Switzerland) - BO group; and the other defect filled only with the blood clot - C group. After the applications, the defects were covered with a single collagen membrane (Bio-Gide®, Geistlich Biomaterials, Switzerland). Six animals from each group were used for histomorphometric analysis, 6 for microtomographic analysis, and 6 for analysis of COL-I, OPN and BMP-2 gene expression (Fig. 1).

Surgical procedures

The animals were weighed and anesthetized according to body weight by intramuscular injection in the outer thigh with a ketamine solution (10 mg/kg/IM) (Francotar®, Virbac do Brasil Industria e Comércio LTDA, Roseira, SP, Brazil) and xylazine hydrochloride (75mg/kg/IM) (Virbaxil® 2%, Virbac do Brasil Industria e Comércio LTDA, Roseira, SP, Brazil), which act as a anesthetic, and as a sedative

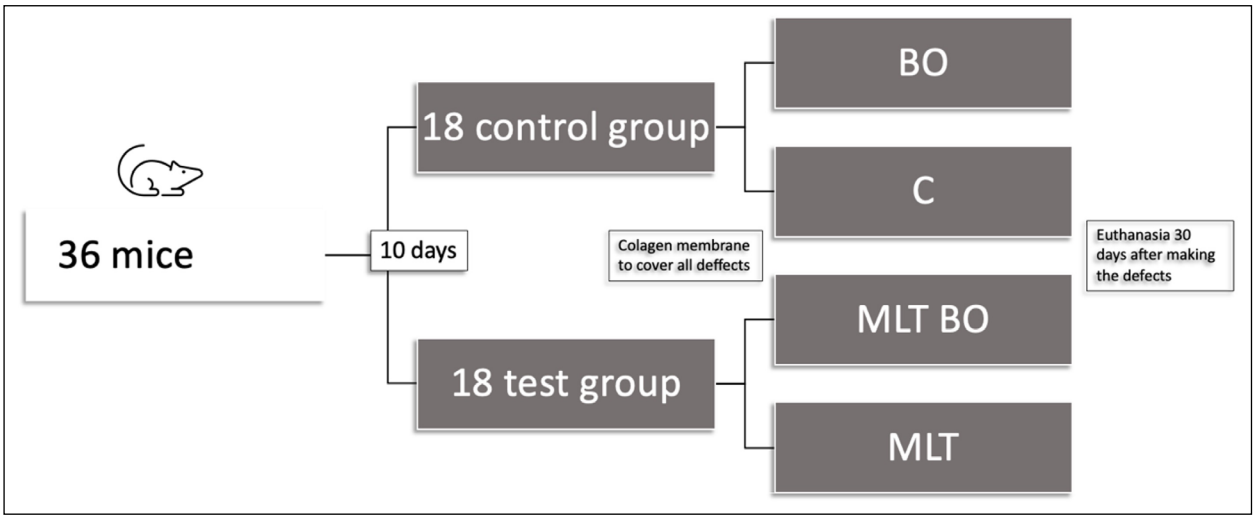

Fig. 1: Flowchart of the study experimental design

and muscle relaxant respectively. After confirming analgesic effectiveness, the animals were marked with their number on the tail, shaved in the calvarial region and disinfected with iodinated alcohol. A straight sagittal incision measuring approximately 1.5 cm was made in the median region of the cranium, followed by wide lateral divulsion and exposure of the calvaria. Two circular defects were created in the calvaria, laterally to the sagittal suture, under constant irrigation with saline, using a 5mm trephine drill (Neodent®, Curitiba, Brasil) with a Neodent® Neosurg Pro implant motor (Neodent®, Curitiba, Brasil) and a SG20 Handpiece (Neodent®, Curitiba, Brasil), at 1200 rpm, taking the necessary care to avoid causing additional injuries (Fig. 2).

The filled critical defects were covered with collagen membrane (Bio-Gide®, Geistlich Biomaterials, Switzerland) and sutured with simple stitches using a 4-0 silk thread (Ethicon® Johnson & Johnson Johson & Johnson do Brasil Comercio de Produtos para Saude Ltda,Brasil). A sterile gauze soaked in 0.9% saline solution was kept over the animals' eyes throughout surgery to prevent the corneas from drying out.

In the immediate postoperative period, the animals were medicated with a single 0.3 mL dose of 5% levofloxacin antibiotic (Isofarma Indústria Farmacêutica Ltda, Precabura, Ceara, Brasil), and 0.3 mL of dipyrone analgesic (Algivet®, Vetnil, Louveira, São Paulo, Brasil) every 12 hours for 3 days.

Thirty days after surgery, all the animals were euthanized with deepening anesthesia using barbiturate [sodium thiopental (71-73-8) 150mg/kg and lidocaine (137-58-6) 10mg/mL]

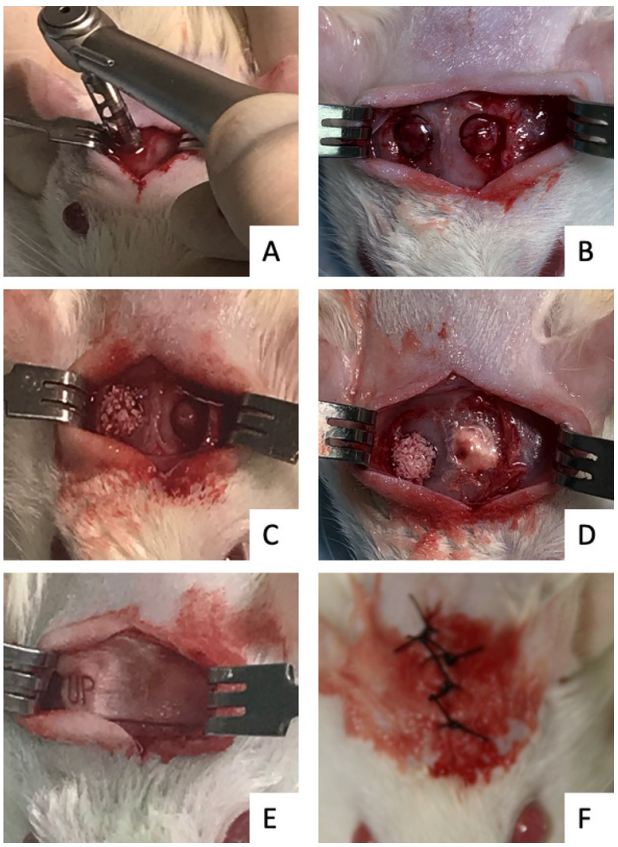

Fig. 2: Surgical sequence of the experiment
A) Creation of defects with a 5mm trephine; B) Cranial perforations; C) Control group, with defects filled with BO (left) and Clot (right); D) Test group, with defects filled with MLT/BO (left) and MLT (right); E) Installation of the membrane covering the defects after insertion of the biomaterials without suture; F) Simple suture with silk thread.

intraperitoneally[26]. After euthanasia, the calvaria was removed using a 2 cm diameter diamond disc with a handpiece, washed with 0.9% saline solution, identified, and stored for subsequent bone analysis.

Bone sample analysis

Bone histomorphometry

For the histological procedures, the calvaria specimens were sectioned into blocks, fixed in a 10% buffered formalin solution (pH 7.4) for 24 hours at room temperature, and then decalcified in a 20% formic acid solution (Merck, Darmstadt, Germany) for 5 days. Once the mineral portion had been removed, the specimens were washed in running tap water, dehydrated with ethyl alcohol, and cleared with alcohol/xylene using a Leica TP 1020 tissue processor (Leica Biosystems, Nussloch, Germany). Then, they were impregnated with liquid paraffin and cut into 4 µm histological sections using a Leica RM2245 microtome (Leica Biosystems, Nussloch, Germany) to prepare slides corresponding to each bone defect. The sections were stained with hematoxylin-eosin (HE), and coverslips were applied using Permount® resin mounting medium (Fisher Scientific, USA).

The histological slides were analyzed by a blinded calibrated researcher (JLD), using a Nikon Eclipse Ci-S microscope (Japan) at 40x objective. The Image J software developed by the US National Institutes of Health (NIH) was used to quantify the area of new bone formation on images in JPEG format, at software default parameters (Fig. 3).

After all areas of new bone formation had been measured on each slide, they were added, to provide a single value in micrometers, which was considered for statistical analysis. Qualitative analysis was performed to identify the following aspects of new bone formation: presence/absence of inflammatory infiltrate, location of new bone formation (close to or distant from residual bone), and presence of mature lamellar bone.

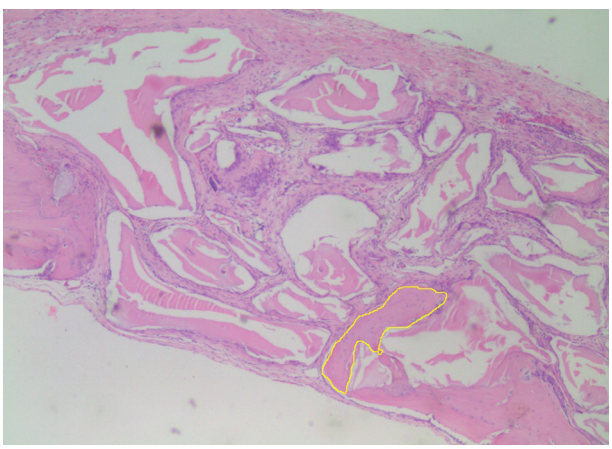

Fig. 3: Photograph of a histological slide analyzed by Image J Software. Measurement of an area of new bone formation highlighted by the yellow line

Microtomography

The calvaria samples were fixed in 10% buffered formaldehyde solution (pH 7.4) and sent for micro-CT analysis to the Brazilian National Nanotechnology Laboratory (LNNano) of the Brazilian Center for Research in Energy and Materials (CNPEM), Campinas, SP. The micro-CT images were acquired using a SkyScan 1272 *micro-CT* scanner (Bruker, Kontich, Belgium) at the following *acquisition* parameters: 11µm voxel size, 360º gantry rotation, 0.4º rotation step, 2 frames and 0.5mm aluminum filter.

Images were reconstructed with NRecon software (Bruker, Kontich, Belgium) at the following standardization parameters: ring artifact correction = 5, smoothing = 0 and beam hardening artifact correction = 0. The reconstructed images were reoriented using the Dataviewer software (Bruker, Kontich, Belgium), according to each defect, considering the convexity of the calvaria, so that each side was analyzed separately by rotating a standard vertical axis (Fig. 4).

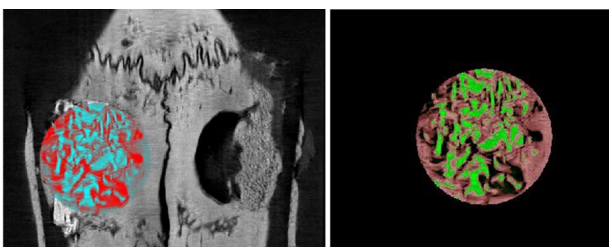

Fig. 4: Microtomographic images – segmentation of the bone defect (defect filled with BO)

The groups of reoriented images were then analyzed in the CTAn software (Bruker, Kontich, Belgium). The region of interest delimited consisted of a disc-shaped volume, 5 mm in diameter, with a height according to the height of each calvaria. Image segmentation was performed automatically, with the exclusion of any shades of gray that stood out (which is characteristic of the BO material). After image segmentation, the parameters of bone quantity (bone volume) and bone quality (thickness and

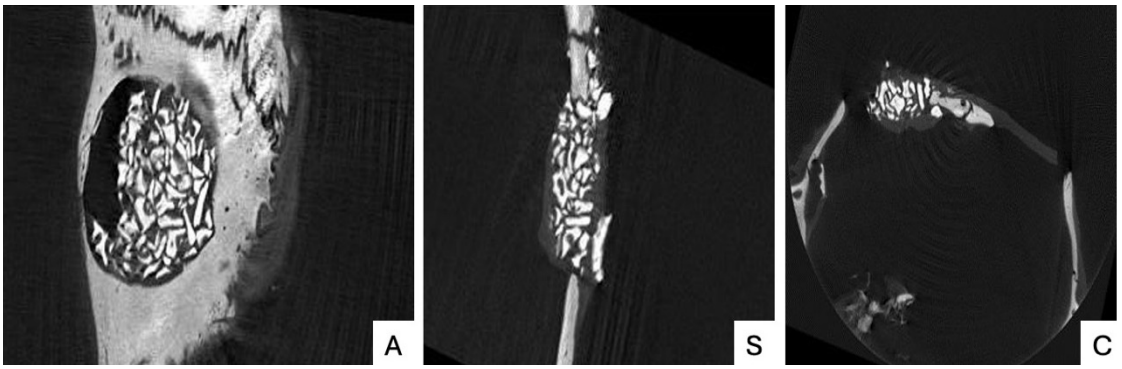

Fig. 5A: Axial (A), sagittal (S) and coronal (C) section - defect filled with BO

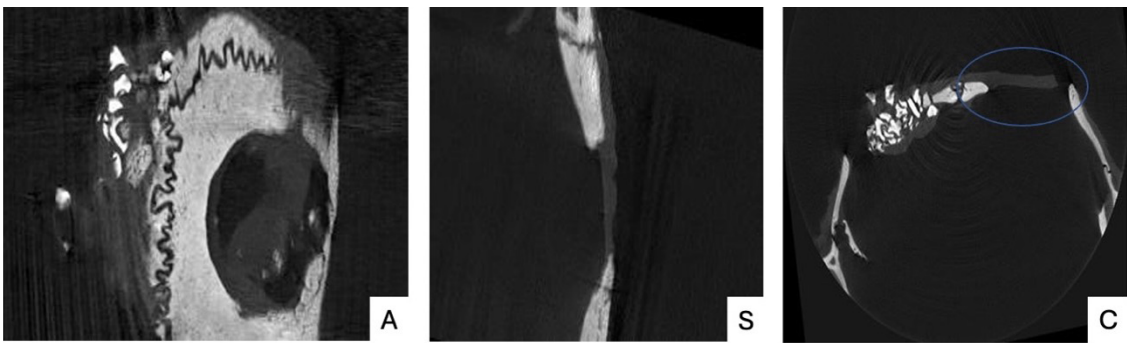

Fig. 5B: Axial (A), sagittal (S) and coronal (C) section - defect filled with MLT

number of trabeculae) were calculated and tabulated for subsequent statistical analysis (Fig. 5A and B).

Analysis of gene expression

Total RNA was extracted using Trizol reagent (Thermo Fisher Scientific, São Paulo, Brazil) according to the manufacturer's instructions. Briefly, the newly formed tissue was collected from each defect, crushed, and homogenized with 1 mL of Trizol. The aqueous and organic phases were separated by adding 0.2 mL of chloroform, followed by centrifugation (12000 g, 15 minutes, 4ºC). RNA was precipitated from the aqueous phase with 0.5 mL of isopropanol (12000 g, 15 minutes, 4 ºC), washed with 75% ethanol and resuspended in water. For reverse transcription PCR (RT-PCR), 1µg RNA samples were treated with 1U DNAse I enzyme. The cDNA synthesis was performed using a RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, São Paulo, Brazil) according to the manufacturer's instructions. Briefly, the following reagents were mixed: 1µg of RNA, 0.5 µg of oligo(dT)18, 1 mM of the dNTP mix, 200U of RevertAid H Minus M-MuLV Transcriptase and 20U of RiboLockRNAse Inhibitor. The reaction ran at 42 °C for 60 minutes, after which it was halted by heating at 70 °C for 5 minutes.

For quantitative PCR (qPCR, also called real-time PCR), primer pairs (oligonucleotides) for OPN, COL-I, BMP2 genes (Table 1) were designed for amplification of their regions of interest, using the Primer Express software (Life Technologies, São Paulo, Brazil). Amplification reactions occurred using 40 ng of cDNA and 0.3 µM of primer pairs, added to Maxima SYBR Green qPCR Master Mix (Thermo Fisher Scientific, São Paulo, Brazil). The reaction conditions were 10 minutes at 95 °C, followed by 40 cycles at 95 °C, 15 seconds, and at 60 °C, 1 minute. The 7500 Fast Real Time PCR System (Life Technologies, São Paulo, Brazil) was used. Expression levels were quantified using

Table 1. Primer sequence.

Oligonucleotide	Sequence
O–N - rat F	5'-AAGCCTGACCCATCTCAGAA-3'
O–N - rat R	3'-ATGGCTTTCATTGGAGTTGC-5'
COL–1 - rat F	AAGCCTGACCCATCTCAGAA
COL–1 - rat R	ATGGCTTTCATTGGAGTTGC
BMP2 – rat F	5'-ACTACCAGAAACGAGTGGGAA-3'
BMP2 – rat '	5'-GCATCTGTTCTCGGAAAAACCT-3'

COL1 = type 1 collagen, OPN = osteopontin, BMP2 = Human Bone Morphogenetic Protein-2, F = forward, R = reverse.

an SDS software (Life Technologies, São Paulo, Brazil), and the relative expression between samples was calculated according to the threshold cycle (Ct) comparison method, based on the formula 2-ΔΔCt. To normalize expression levels, the GAPDH gene was used as a reference gene (internal reaction control).

Statistical analysis

Data referring to histomorphometry, microtomography and biomolecular analysis were tested for normal distribution and homoscedasticity. Then, treatments were compared using t*wo*-way *ANOVA* for *randomized blocks*. Tukey's test was used for histomorphometry and microtomography, Kruskal-Wallis test for biomolecular analysis, and Dunn's test for multiple comparisons. All statistical analyses were conducted at a 5% significance level, using a SPSS 23 software (SPSS INC., Chicago, IL, USA).

RESULTS

Bone histomorphometry

For the sample size of this study and for the measures of dispersion observed within each of the four treatments, no statistically significant differences ($p = 0.869$) were identified in the mean values of new bone formation when the defects in the calvaria were treated with BO and/or MLT (Fig. 6).

For qualitative analysis, the slides were digitized and evaluated using a 40X microscope objective, regarding the following aspects: presence/absence of inflammatory infiltrate, location of new bone formation (close to or distant from the residual bone)

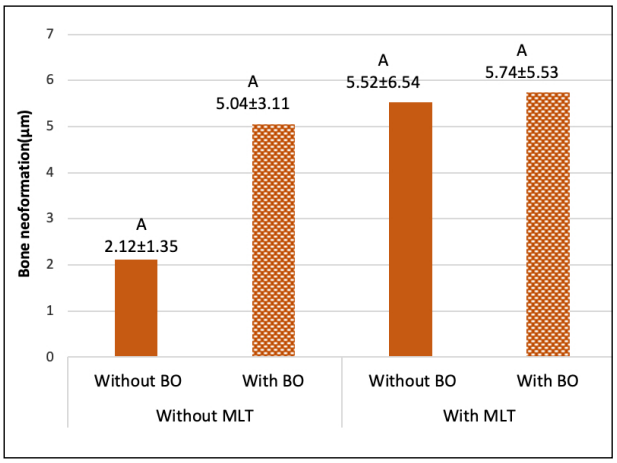

Fig. 6: Bar chart of mean values of new bone formation in defects created in the calvaria of rats, with or without BO and MLT. Legend: Same capital letters indicate that treatments do not differ significantly (ANOVA and Tukey's test, alpha=5%).

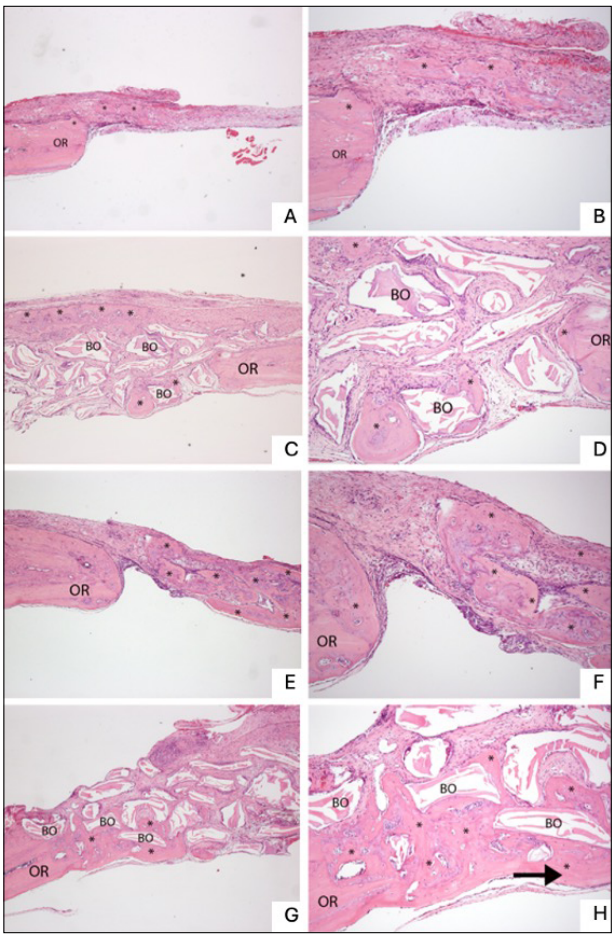

Fig. 7: Photomicrograph of HE-stained histological slides of bone defects

Left column (H&E) original magnification X40; Right column (H&E) original magnification X100. Histological characteristics: A) Sample of bone defect filled with clot (control) showing small areas of newly formed bone (*) mainly in the area adjacent to the residual bone (OR) at the edge of the defect. B) Higher magnification of the previous image showing areas of newly formed bone and absence of significant inflammatory infiltrate. C) Sample of bone defect filled with Bio-Oss® showing areas of newly formed bone (*) in an area adjacent to the residual bone (OR) and permeated with biomaterial particles (BO). D) At higher magnification, biomaterial particles can be seen near the newly formed bone and the absence of significant inflammatory infiltrate. E) Sample of a bone defect filled with MLT, showing a relevant amount of newly formed bone (*) even in areas distant from the residual bone (OR). F) The newly formed bone (*) can be observed in greater detail in absence of significant inflammatory infiltrate. G) Sample of bone defect filled with MLT and Bio-Oss® showing a large amount of newly formed bone (*). H) At higher magnification, extensive new bone formation can be seen amidst the biomaterial particles (BO) and the absence of significant inflammatory infiltrate (H&E original magnification X40). In some areas, the newly formed bone exhibits characteristics of mature lamellar bone (arrow).

and presence of mature lamellar bone, as identified in the histological analysis (Fig. 7).
Histological findings showed that MLT promoted an improvement in bone healing, with greater bone formation in the group with the addition of MLT. In addition, the presence of bone formation distant from the remaining bone and the presence of mature lamellar bone were observed in the MLT group.

Microtomography

For volume data, two-way analysis of variance for randomized blocks demonstrated a statistically significant interaction between the use of BO and MLT ($p = 0.006$). Bone volume was significantly smaller when MLT was used in the absence of BO. It was also verified that when MLT was used, the bone volume was significantly lower in the absence of BO (Fig. 8).

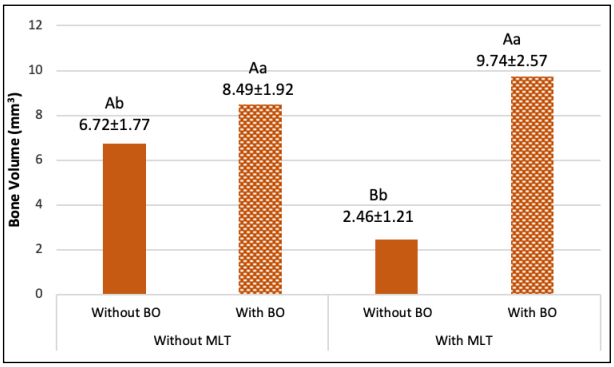

Fig. 8: Bar chart of bone volume in defects created in the calvaria of rats, with or without BO and MLT. Legend: for data on thickness (p = 0.860) and number (p = 0.167) of trabeculae, two-way analysis of variance for randomized blocks indicated that there was no statistically significant interaction between the bone substitute and melatonin.

For thickness ($p = 0.860$) and number ($p = 0.167$) of trabeculae, there was no statistically significant interaction between BO and MLT. The thickness ($p = 0.590$) and number ($p = 0.150$) of trabeculae were also not significantly affected by MLT, either in the presence or the absence of BO. However, the use of BO had a statistically significant influence on the thickness ($p = 0.001$) and number ($p < 0.001$) of trabeculae. Trabecular thickness was significantly smaller in the presence of BO than when BO was absent, whether associated with MLT (Fig. 9). A significantly greater number of trabeculae ($p<0.05$) was observed in the presence of BO, with or without the use of MLT (Fig. 10).

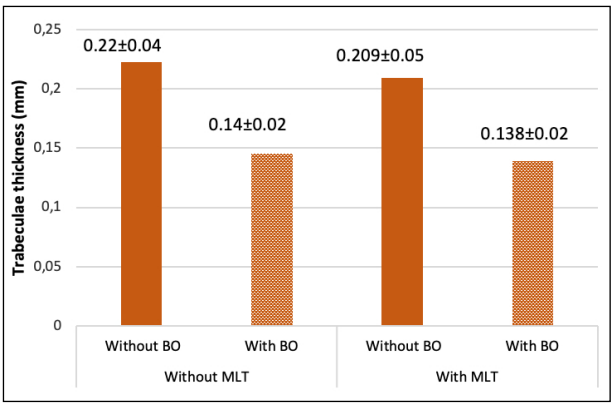

Fig. 9: Bar chart of trabecular thickness in defects created in the calvaria of rats, with or without BO and MLT.

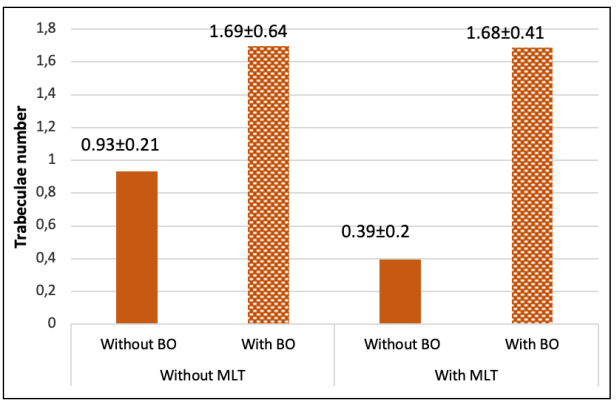

Fig. 10: Bar chart of the number of trabeculae in defects created in the calvaria of rats, with or without BO and MLT.

Quantification of COL-I, OPN and BMP-2 gene expression

The control group showed significantly higher COL-I gene expression than the MLT group ($p=0.0291$). No statistical differences were observed between BO and MLTBO ($p=0.9183$). The control group also showed significantly higher COL-I gene expression than the BO group ($p=0.0374$). There was no significant difference in the comparisons between MLT and BO ($p>0.9999$), MLT and MLTBO ($p=0.7855$), or BO and MLTBO ($p=0.9183$).
Regarding OPN gene expression, there was a tendency for better results in the groups in which MLT was used, though the difference was not statistically significant.
Regarding BMP-2 gene expression, the defects in the control group showed significantly higher gene expression than the defects in the MLTBO group ($p=0.0216$). For the other comparisons, no statistical differences were observed ($p>0.05$).

DISCUSSION

MLT acts on bone tissue by inducing osteoblastic differentiation from mesenchymal cells, and by inhibiting the action of osteoclasts[27-30]. Several studies have suggested positive effects of MLT on periodontal treatment and dental implant osseointegration[12,16,23,24]. However, there is no complete understanding of the MLT action on bone formation and of MLT behavior when associated with biomaterials. The present study demonstrated that the local administration of MLT, alone or associated with BO, induced more qualitative effect than quantitative effect on bone formation, according to the parameters evaluated.

The histomorphometric findings showed that the different treatments did not influence new bone formation differently. Even so, in the histological analysis, the MLT group showed presence of bone formation distant from the remaining bone, and presence of mature lamellar bone, as observed in previous studies[9,11,14,24,31]. Furthermore, in the present study, MLT promoted greater bone formation than the control, but there was no statistical significance, possibly because a single application of MLT may not suffice to trigger a significant quantifiable effect. It could be hypothesized that the combined administration of systemic MLT during the post-surgical period could provide longer MLT action and greater impact on the histological results.

Based on the microtomographic analysis, MLT did not significantly influence parameters of quantity (bone volume) and of quality (bone trabecular thickness and bone trabeculae number). The bone volume inside the defects was significantly smaller when MLT was used in the absence of BO.

Other authors have used Cone Beam Computed Tomography (CBCT) to evaluate the effects of topical MLT application on bone regeneration of isolated vertical defects created with plastic capsules in rat calvaria[11]. The capsules used in the test group were filled with 10 mg of MLT powder[11]. Micro-CT images showed a significant increase in bone volume in the test group, suggesting that MLT induced vertical bone regeneration in isolated defects[11].

Such different results found in the current study in relation to cited study[11] could be due to a difference in the bone defects, since in the present study, the bone defects were truly critical because the bone was completely removed with a trephine drill, whereas in the aforementioned study[11], the defect was demarcated with a trephine, and perforations were made in the cortical bone, but it remained in place, which would increase the bone defect nutrition and accelerate bone formation in 4 weeks.

Regarding other microtomographic parameters analyzed in present study, the trabeculae thickness and number were not significantly affected by MLT. This agrees with another study on MLT, which found no significant differences in bone density measured using cone beam tomography[18]. Said study measured bone density between the dental alveoli before the extraction of third molars in humans, and again 60 days after surgery. The alveoli received either 3 mg of MLT or hydroxymethyl cellulose gel as placebo[18].

Another study evaluated the effect of topical application of MLT on accelerating bone formation in rabbit tibias, compared to xenogeneic grafts and with blood clots in control sites[14]. Radiographic evaluation after 60 days showed lower density in control defects compared to native bone, and greater bone formation in defects that received MLTBO compared to those that received only BO[14]. MLTBO resulted in an increase in formation of cortical bone in length and width in the initial stages (15 and 30 days), while the difference was not significant at the end of 12 weeks[14].

Those findings[14] differ from the outcomes of the present study, in which the application of MLT, alone or associated with BO, did not result in greater bone formation. For trabeculae thickness and number (bone quality parameters), there was no statistically significant interaction between BO and MLT. It is important to consider that the present analysis was performed at only one time point (30 days post-operatively) and used microtomography (a method that increases sensitivity and specificity of the analysis).

In addition to histomorphometry and microtomography, the present study analyzed COL-I, OPN and BMP- 2 gene expression, with the aim of understanding the influence of MLT, whether or not associated with BO, on the reconstruction of critical defects. Concerning bone metabolism, MLT stimulates the synthesis of type I collagen[3]. It also interacts with the cells and extracellular matrix of bone tissue, promoting the union of osteoblastic cells with the organic and mineral phases of the matrix, and controlling the functions of bone cells, such as the OPN gene[7]. MLT also promotes bone tissue repair,

acting concomitantly with the BMP-2 gene[32,33].

COL-I gene expression did not differ statistically between the group in which MLT was added to the xenogeneic bone material (MLT+BO) and the control group, though it was significantly higher in the control group than in the MLT and BO groups. This suggests that the addition of MLT to the biomaterial may increase COL-I protein production by osteoblasts. However, these results should be interpreted with caution, as they could vary depending on the MLT routes of administration (topical or systemic), as well as the MLT ideal dose, since MLT in small concentrations may not be able to change COL-I protein synthesis, while MLT in high doses could cause hyperoxidation and cell damage[29].

In the present study, OPN gene expression did not differ significantly between groups, even though it was higher in the groups in which MLT was applied. According to the literature, OPN protein is secreted by osteoblasts and osteoclasts at the end of the bone mineralization process, and directly or indirectly controls bone mass, bone mineral quantity, and bone orientation[7].

A previous study investigated the role of MLT in bone metabolism using an *in vitro* experimental model based on osteoblast cultures to observe COL-I and OPN gene expression[7]. It demonstrated that MLT upregulated gene expression and the secretion of COL-I and OPN proteins[7]. Those results differ from the present study, probably because the cited study was conducted *in vitro* with MLT in osteoblast cell culture, whereas the present study used MLT in bone tissue, which is a complex structure.

BMP-2 gene expression was significantly lower with MLT associated with BO than in the control group, while no differences were observed with the other groups. According to literature, BMP-2 protein is essential at the beginning of the healing process, and its greatest production occurs during the first days of the reparative process[32].

In the current study, BMP-2 gene expression was similar in the MLT, BO and MLTBO groups. Another study agglutinated MLT and BMP-2 protein in chitosan/hydroxyapatite (HAp) scaffolds, successfully attenuating osteoclastic differentiation induced by BMP-2[33]. The authors therefore recommended chitosan/HAp scaffolds loaded with MLT/BMP-2, which would have dual functions in bone regeneration: increasing bone formation and inhibiting osteoclastic activity[33].

It should be highlighted that it was difficult to compare the present findings with other studies in the literature, since there were major differences in methodology and experimental design. One of these differences is the time at which euthanasia was performed (in the current study, only once, at 30 days). Topical application of MLT might have more significant effects during the initial stages of the proliferative process of bone repair, increasing the production of osteoblasts and thereby inducing greater bone formation, as reported in some studies[9,14,21], though impossible to observe with the method used herein.

A recently published study[34] on calvarial defects in female rats with and without osteoporosis, in which MLT was applied with or without BO, found that in the osteoporosis group, the defects treated with MLT showed higher mineral filling than the other treatments. Such results were not observed in the group of systemically healthy rats. Thus, experimental designs using different species, with different health conditions, as well as different MLT concentrations and routes of administration make it difficult to compare results. Therefore, future research using a larger sample number, with euthanasia at earlier times, and with systemic administration of MLT could provide additional relevant insights regarding new bone formation in critical defects.

CONCLUSION

It can be concluded that the topical application of MLT, whether or not associated with a xenogeneic biomaterial, provided a qualitative improvement in healing, but did not have a quantitative effect on new bone formation. In the absence of the xenogeneic biomaterial used herein, lower bone volume and thinner bone trabeculae were observed.

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DECLARATION OF CONFLICTING INTERESTS

The authors declare no potential conflicts of interest regarding this research or the authorship and/or publication of this article.

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