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ORIGINAL ARTICLE

Basic Study

Gene expression analysis of cytokines and MMPs in melatonin and rhBMP-2 enhanced bone remodeling

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Abstract

BACKGROUND

In the medical and dental fields, there is a need for studies of new therapeutic approaches for the treatment of bone defects that cause extensive bone loss. Melatonin may be an important endogenous biological factor for bone remodeling, and growth factors may enhance the repair process.

AIM

To evaluate the gene expression of cytokines (IL-1β, IL-6, IL-10 and TNF-α), markers of osteoclastogenesis (RANK, RANKL and OPG) and MMPs (MMP-1, MMP-2, MMP-8 and MMP-13) from the treatment of melatonin associated with an osteogenic membrane and rhBMP-2 on the recovery of a bone injury.

METHODS

Sixty-four rats were used and divided into 9 experimental groups and were formed according to the treatment carried out in the region of the bone lesion, which varied between the combination of 1, 10 and 100 μmol/L of melatonin. Gene Expression analysis was performed using real time-PCR by reading the concentration of total RNA and reverse transcription.

RESULTS

There were differences between groups when compared with clot or scaffold control, and improvement with a higher concentration of melatonin or rhBMP-2. The combination melatonin (1 μg) with 5 μg of rhBMP-2, using the guided bone regeneration technique, demonstrated some effects, albeit mild, on bone repair of critical bone defects.

CONCLUSION

This indicates that the approach for administering these substances needs to be reassessed, with the goal of ensuring their direct application to the affected area. Therefore, future research must be carried out, seeking to produce materials with these ideal characteristics.

Key Words: Bone repair; Melatonin; Gene expression; Rhbmp-2; Scaffold; Tissue engineering; Guided bone regeneration

Core Tip: Melatonin is believed to be an important endogenous biological factor for bone remodeling. Furthermore, growth factors such as bone morphogenetic proteins (rhBMPs) have been used to repair bone defects. The aim of this study was to assess the expression of cytokine genes (IL-1β, IL-6, IL-10 and TNF-α), markers of osteoclastogenesis (RANK, RANKL and OPG) and MMPs (MMP-1, MMP-2, MMP-8 and MMP-13) from the treatment of melatonin associated with an osteogenic membrane and rhBMP-2 on the recovery of a bone injury.

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INTRODUCTION

Melatonin (N-acetyl-5-methoxy-tryptamine) is a substance easily found in nature, present in animals and vegetables[1]. It is secreted by the pineal gland during the nocturnal phase of the day[2]. Melatonin is capable of performing the function of a circadian synchronizer of the function of all peripheral tissues, including bone tissue[3] maintaining metabolism in synchrony with the day-night cycle. Therefore, osteogenesis should take place during the night when melatonin levels are at their peak. Melatonin has the potential to regulate bone tissue cells that are active throughout the daytime[4], in this way, it intensifies the importance of melatonin in the osseintegration process.

Studies on melatonin show it has a positive effect on osteogenesis, such as the anabolic action in which it acts on osteoblast cells and also the anti-abortion action in which melatonin acts on osteoclast cells[5]. Experimental studies have verified that melatonin has an action on osteoblast cells, acting on cell proliferation[6] and differentiation[7]. The action of melatonin on osteoblastic cells is decisive for mesenchymal stem cells to be directed to transition into osteogenic pathways[8]. Research conducted in living organisms has demonstrated that the local administration of melatonin can promote bone formation around titanium implants, thus improving osseointegration[9]. In addition to melatonin stimulating osteoblastic action, there is also an increase in bone morphogenetic proteins (BMP-2)[10].

Various methods for restoring bone defects have been explored in the literature. BMP-2 is found in minute amounts in connective tissue cells, collagenous fibers, and non-collagenous components of the bone structure[11]. BMP-2, produced through advanced genetic engineering, plays an osteoinductive role by acting on mesenchymal cells, converting them into osteoprogenitor cells that promote bone formation[12]. To facilitate the repair of large bone defects, guided bone regeneration (GBR) has shown promising outcomes. GBR utilizes membranes to protect the repair area from endothelial cell migration that inhibits bone formation, prevents epithelial cell infiltration, and helps reduce bone resorption. Recent discussions suggest that biomembranes serve not only as passive barriers but also as bioactive components. Additionally, GBR can aid in maintaining the mechanical stability of the site[13]. One of the main pathways of osteoclast differentiation and activation involves the recently discovered system, RANK-RANKL (RANK ligand)-OPG (osteoprotegerin). RANKL is a crucial molecule for the differentiation of hematopoietic progenitor cells into mature osteoclasts, exerting its effects by binding to the RANK receptor. Conversely, osteoclastogenesis is blocked in the presence of OPG[14], since when OPG is linked to RANKL, it cannot bind to RANK, thus making it impossible for osteoprogenitor cells to differentiate into osteoclasts and there is no bone resorption. The imbalance of this RANK/RANKL/OPG system is observed in osteoporosis, rheumatoid arthritis, periodontal disease. MMPs constitute a family of endopeptidases, with broad-spectrum

hydrolytic activity towards extracellular proteins. MMPs belong to a family of at least 20 members, the product of genes, homologous or pseudohomologous, related to each other[15]. These enzymes can be classified according to structural and functional criteria, into four large families of different substrate specificity[16]: Collagenases (MMP1, MMP8 and MMP13), gelatinases (MMP2 and MMP9), stromalysins (MMP3, MMP7, MMP10, MMP11 and MMP26) and membrane metalloproteins (MMP14, MMP15, MMP16, MMP17, MMP24 and MMP25, also known as MT1-MMP to MT6-MMP, respectively).

Real-time PCR (qPCR) is widely regarded as the ideal method for quantifying gene expression and is often used to validate techniques with higher throughput but overall lower sensitivity, such as microarray analysis. qPCR operates by using fluorescent dyes to measure the transcription of the target gene, with the cycle number at which these dyes/transcripts are detected indicating the relative or absolute quantity of the target molecules. The sensitivity of qPCR makes it a powerful tool for assessing gene expression, particularly when sample quantities are limited or when the transcript is present at low levels. However, this sensitivity also necessitates meticulous attention to experimental design and procedural execution[17]. Cytokine secretion is a rapid response, a self-limiting event. In general, cytokines are not stored as pre-formed molecules and their synthesis begins with transcription, therefore it is extremely important to evaluate gene expression, considering that its analysis will give a more accurate perspective of the body reaction[18].

In a preliminary study[19], our group of researchers carried out qualitative and quantitative evaluations with histopathological and immunohistochemical analyzes under a light microscope. Given the need for a complementary study, with new analyses, we aim to evaluate the effects of administration of topical melatonin in different concentrations on bone repair in surgical bone defects using the GBR technique, associated or not with rhBMP-2, by assessing the expression of genes involved in signaling molecules (IL-1β, IL-6, IL-10 and TNF-α), markers of osteoclastogenesis (RANK, RANKL and OPG) and MMPs (MMP-1, MMP-2, MMP-8 and MMP-13).

MATERIALS AND METHODS

Animals

Sixty-four adult male *Wistar* albino rats (250 g) were used, from the Central Animal Bioterium PUSP RP-University of São Paulo. These animals were kept in plastic cages with a maximum of 3 animals each, separated according to group and kept in an environment with a stable temperature (22 ± 2 ℃), with a controlled light cycle (12 hours light and 12 hours dark), fed with food for small rodents and water ad libitum. The methodology presented here was evaluated and approved by the Animal Experimentation Ethics Committee of FORP-USP, with process number 2017.1.294.58.4. This experimental protocol followed the ARRIVE (Animal Research: Report of *in vivo* Experiments) checklist, and the animals were monitored throughout the experiment.

Surgical technique for creating bone lesions

The animals were weighed to correctly provide the anesthetic dose. For all experimental procedures, the animals received general anesthesia, with the association of 80 mg/kg of Ketamine Hydrochloride (Cetamim-Rhobifarma Indústria Farmacêutica Ltda-Cotia-SP-Brazil) and 6 mg/kg of Xylazine Hydrochloride (Zilazin-Rhobifarma Indústria Farmacêutica Ltda-Cotia-SP-Brazil), intramuscularly into the biceps femoris of the right paw. In cases where there was no necessary effect of the anesthetic, anesthesia was supplemented with half the initial dose applied.

The anesthetized animals underwent trichotomy of the skin covering the central region of the calvaria and then disinfection with polyvinylpyrrolidone iodide (Indústria Química e Farmacêutica Rioquímica Ltd, Brazil). After the asepsis technique, a sagittal incision was made through the skin of the skull and periosteum, creating a full-thickness flap. After the incision, the subcutaneous connective tissue was dissected to expose the calvaria. In the exposed calvaria, bone defects were created bilaterally, both equidistant from the median sagittal suture, using a trephine-type surgical drill with 6 mm external diameter (Kavo, São Paulo, Brazil). With the aid of an electric motor for implants, set at 3000 RPM, the calvaria was perforated, under constant and abundant irrigation with 0.9% saline solution (Figure 1).

Treatments and experimental groups

To investigate the isolated and synergistic osteogenic potential of melatonin on the recovery of calvarial bone injury, the animals were distributed into 8 experimental groups, with 8 animals each. Melatonin (1, 10 and 100 μM), bone morphogenetic protein (5 μg BMP-2) and osteogenic membrane in the form of a sponge or scaffold (Lumina-Coat Criteria®) were applied bilaterally to each animal, and the experimental groups were formed according to according to the treatment carried out in the region of the bone lesion, as shown in the table below.

Post-operative care

After treatment, the edges of the incised skin tissue were repositioned in the midline and united using sutures with 4.0 silk thread (Ethicon, Johnson & Johnson, São José dos Campos, SP, Brazil), in order to restore the integrity of skin tissue. Then, each animal received treatment with the analgesic dipyrone 100 mg/kg animal weight, subcutaneous injection and the antibiotic Small Veterinary Pentabiotic (Fort Dodge®, Campinas, SP, Brazil), intramuscular injection, in a volume of 0.1 mL/100 g of weight.

Euthanasia of animals and obtaining samples

After the pre-determined period of two weeks for the creation of the bone defect, the animals were anesthetized with the

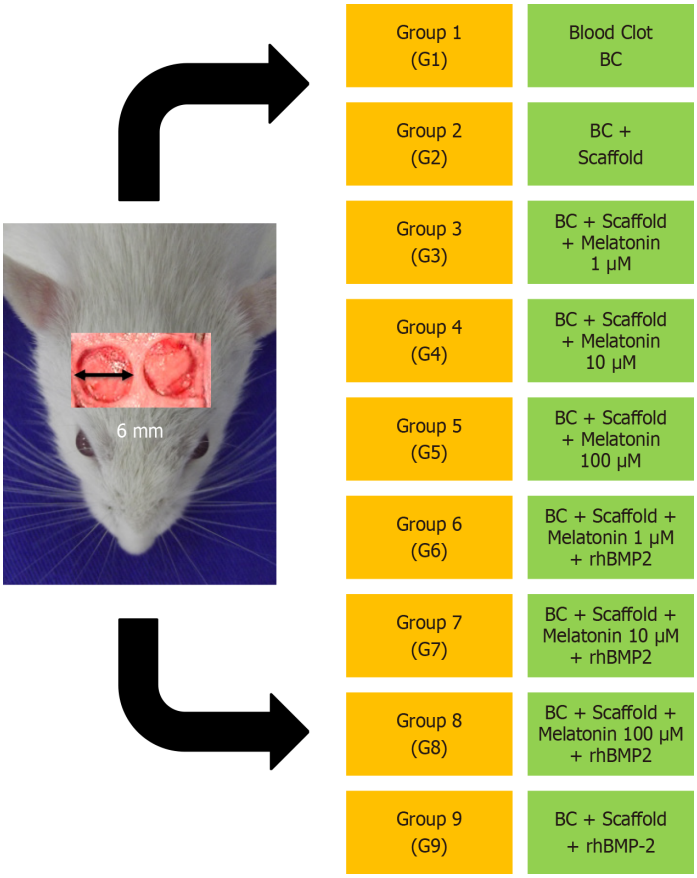

Figure 1 Schematic representation of the procedure for creating the defect in the calvarial bone. The bone defects were created bilaterally, both equidistant from the median sagittal suture, using a 6 mm diameter trephine surgical drill.

combination Xylazine + Ketamine (75-100 mg/kg and 5-10 mg/kg, respectively), injected intramuscularly. After confirming the loss of postural reflex and pain sensitivity, the animals were decapitated and the head dissected to obtain the calvaria. As the two calvarial lesions received the same treatment in each animal, samples from the left side of the calvaria were used for histological and immunohistochemical analyzes[19] while samples obtained from the right side were used for molecular analysis.

Assessment of gene expression by real time-PCR

Extraction of total RNA: The samples were crushed in liquid nitrogen (N2) in the crucible, and the material in the form of frozen powder was transferred to a 2.0 mL polypropylene tube, where RNA extraction began by adding Trizol®. This was followed by incubation for 15 minutes on ice and centrifugation at 10000 g, for 20 minutes, at 4 ºC. At the end of centrifugation, 3 distinct phases were visualized. The aqueous phase, containing RNA, was carefully aspirated and transferred to another tube, where 1 volume of isopropanol was added at -20 ºC. After incubation for approximately one day at -20 ºC (a phase in which precipitation of the RNA) was followed by centrifugation at 10000 g for 20 minutes, at 4 ºC. The supernatant obtained was discarded, the pellet (RNA) resuspended with 75% ethanol (-20 ºC), to remove impurities. After incubation for 15 minutes at room temperature, a new centrifugation at 10000 g at 4 ºC for 10 minutes followed and the supernatant was discarded again. The pellet, which represents the RNA obtained, was left to dry for 15 minutes at room temperature, on the bench, and was then dissolved in 40 µL of DNase and RNase-free ultrapure H_2O. The RNA concentration was estimated by OD of the solution, using spectrophotometry (NanoDrop 2000c-Thermo Scientific, United States). To analyze the purity of the RNA, the device calculates the ratio between the sample reading in absorbance at 260 nm and 280 nm. The samples showed absorbance 260/280 between 1.7 and 2.0. Total RNA quantifications ranged from 0.3 to 0.7 µg/µL.

Reverse transcription: Subsequent to reading the total RNA concentration, reverse transcription (RT) was performed. In this process, samples containing RNA were treated with DNAse I (RNAse free DNAse I-Invitrogen NZ Ltd, Auckland, New Zealand) prior to real time PCR in order to remove any residue of genomic DNA in a final volume of 22 µL. Then, 20 µL of the previous solution containing total RNA treated with DNase I, oligo dT (100 µg/mL), 10 mmol/L of each dNTP (5'-triphosphate deoribonucleotide-dATP, dCTP, dGTP and dTTP) were added. Invitrogen Life Technologies® (Carlsbad, CA, United States), 5X First-Strand buffer and 2 µL of enzyme (200 U/µL) M-MLV Reverse Transcriptase. The reaction occurred in a cycle of 65 ºC for 10 minutes, followed by 37 ºC for 70 minutes and 5 minutes at 95 ºC.

Real-time PCR (Q-PCR): The cDNA obtained in the reverse transcriptase reaction from the mRNA was amplified in Real-time PCR, in which fluorescence quantification was evaluated by the ABI Prism 7700 sequence detector (Applied

Table 1 Primers in the 5´-3´ sequence for assessment of gene expression by real time-PCR

Name of the animal's mRNA	Sense	Antisense
IL-1β	CCTATGTCTTGCCCGTGGAG	CACACACTAGCAGGTCGTCA
IL-6	CACTTCACAAGTCGGAGGCT	TCTGACAGTGCATCATCGCT
IL-10	GAAGGACCAGCTGGACAACA	GGGGCATCACTTCTACCAGG
TNF-α	ATGGGCTCCCTCTCATCAGT	GCTTGGTGGTTTGCTACGAC
RANK	CCCTTGCAGCTCAACGTGGATA	GTGCTTCCATCTTCCCAAGGA
RANKL	CCTGTACTTTCGAGCGCAGA	AGTCGAGTCCTGCAAACCTG
MMP-13	ACCCAGCCCTATCCCTTGAT	TCTCGGGATGGATGCTCGTA
OPG	CAACCGAGTGTGCGAATGTG	TTGCAAACCGTGTTTCGCTC
MMP-1	CCCAAATCCCATCCAGCCAA	ATTGAGCTCAGCTTCTGGCAT
MMP-8	ACCCAATGGAATCCTTGCCC	GGGTACATCAAGGCACCAGG
MMP-2	AAGGATGGAGGCACGATTGG	CCTTGGGGCAGCCATAGAAA

Biosystems, Foster City, CA, United States). Thus, 2 μL of the RT reaction product was used in a reaction buffer with 5 μL SYBER Green PCR master mix, 900 nM of rat primers (Table 1). The emitted fluorescence was captured by the device and analyzed by the program (ABI Prism 5700 detector-Applied Biosystems). The reaction had two phases: The first at 50 °C for 2 minutes to activate the enzyme; the second, at 95 °C for 10 minutes for denaturation. Then, 50 cycles of 3 phases: First at 95 °C, for 20 seconds (denaturation), the second at 58 °C, for 30 seconds (annealing) and the third at 72 °C, for 30 seconds (extension).

The real-time RT-PCR cycle starting point (CT) was evaluated in duplicate for each sample. Each CT is related to the PCR cycle number and represents the intensity of the fluorescence emitted by the RTPCR product amplified from the target gene, being inversely proportional to the mRNA content of the sample. The calculation of CT variation (ΔCT) was done by subtracting the CT value of the gene of interest (neuroglobin and cytoglobin) from the CT value of the gene used as reference (cyclophilin). Variations between samples were normalized by the mean variation in the CT value (ΔCT) of control animals. This obtained value (ΔΔCT) was used to calculate the relative expression of the genes evaluated through 2-DDCT expression, represented in arbitrary units[20]. The gene sequences needed for designing primers for expression studies were retrieved from the NCBI database (https://www.ncbi.nlm.nih.gov/pubmed), primer design and potential hairpin structures evaluation was carried out using Primer3 software (http://primer3.ut.ee/).

Statistical analysis

Gene expression experiments were performed in triplicate, and all data were expressed as mean ± SD. Statistical analysis was performed using GraphPad Prism 8.0.1 software (GraphPad, United States). One-way ANOVA with Tukey post-test was used to determine significant differences between groups, and $P \geq 0.05$ does not represent statistically significant values.

RESULTS

No complications were observed throughout the healing period in any of the animals, and no negative responses were observed at any of the bone injury locations. The inclusion of the scaffold in the bone defect complicated the repair process; in group G2 (blood clot + scaffold), the outcomes for bone formation and immunohistochemistry were notably lower compared to all other groups.

In the expression of IL-1β, there was a significant difference ($P \leq 0.05$) only between groups G1 and G7 (0.9127 ± 0.098 and 0.7695 ± 0.1309, respectively) (Figure 2A and B). However, in IL-6 expression there was a significant difference between groups G3 and G8 (0.7482 ± 0.1547 and 1.227 ± 0.1598), G4 and G7 (0.6055 ± 0.2494 and 1.082 ± 0.2098), G4 and G9 (0.6055 ± 0.2494 and 1.177 ± 0.1522), G5 and G7 (0.7185 ± 0.2213 and 1.082 ± 0.2098), and finally between G5 and G8 (0.7185 ± 0.2213 and 1.227 ± 0.1598), respectively (Figure 2C and D).

The results of IL-10 expression showed a significant difference ($P \leq 0.05$) between groups G1 and G9 (1.007 ± 0.1298 and 0.3501 ± 0.048), group G2 with G5, G6 and G9 (0.8582 ± 0.066 with 0.7273 ± 0.007, 1.094 ± 0.1297 and 0.3501 ± 0.048), group G3 with G6 and G9 (0.7437 ± 0. 052 with 1.094 ± 0.1297 and 0.3501 ± 0.048), group G4 and G6 (0.5555 ± 0.1601 and 1.094 ± 0.1297), G5 with G6 and G9 (0.7273 ± 0.007 with 1. 094 ± 0.1297 and 0.3501 ± 0.048), between G6 and G9 (1.094 ± 0.1297 and 0.3501 ± 0.048) and, finally, between G7 and G9 (0.9050 ± 0.3501 ± 0.048), respectively (Figure 3A and B).

With regard to TNF-α expression, there was a statistically significant difference ($P \leq 0.05$) between the G2 group with G3 and G5 (0.8582 ± 0.066 with 0.7437 ± 0.052 and 0.7273 ± 0.07), G3 with G5, G6, G7 and G8 (0. 7437 ± 0.052 with 0.7273 ± 0.07, 1.094 ± 0.1297, 0.9050 ± 0.087 and 0.6588 ± 0.3287), of G5 with G6, G7 and G8 (0.7273 ± 0.07 1.094 ± 0.1297, 0.9050 ± 0.087 and 0.6588 ± 0.3287), respectively (Figure 3C and D).

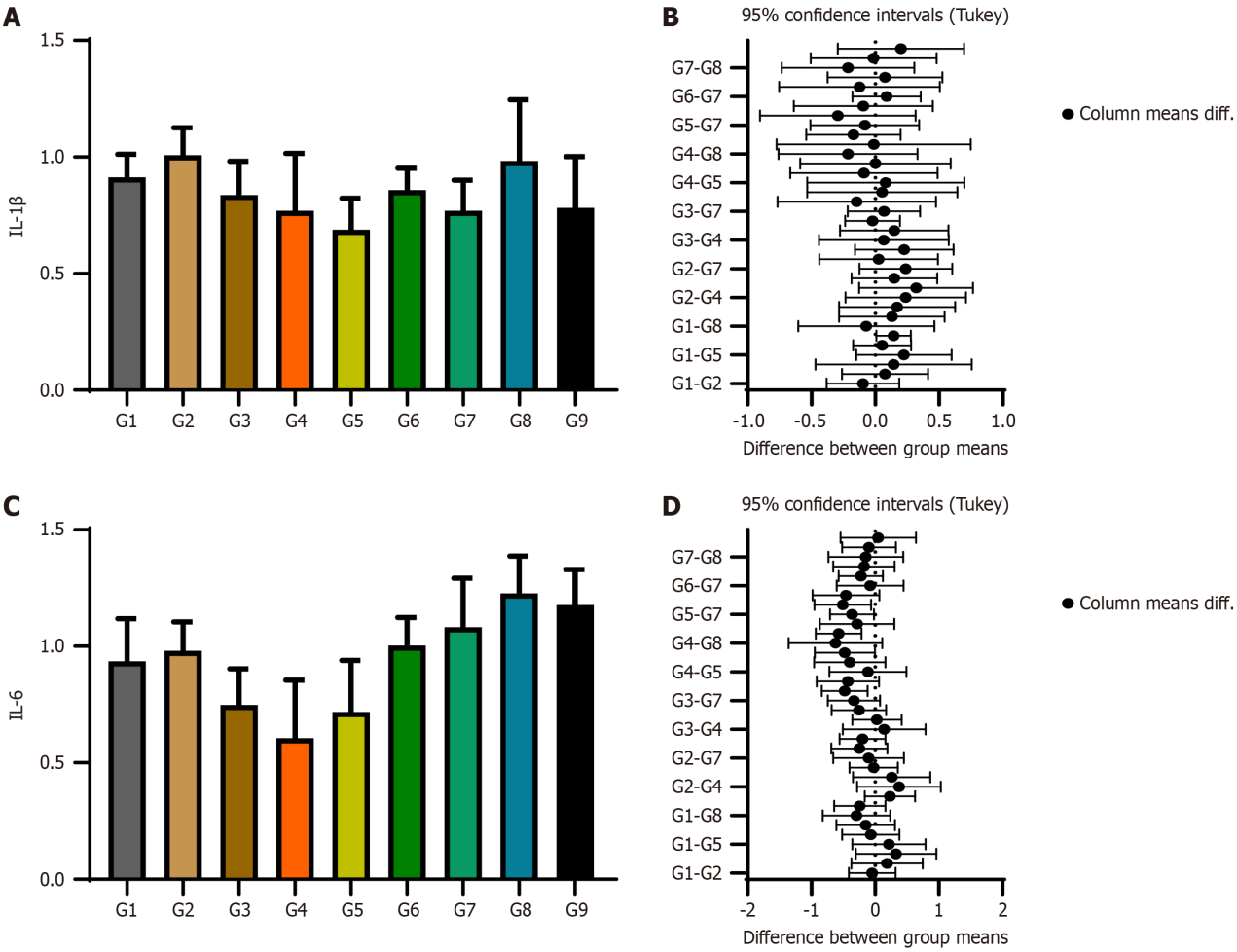

Figure 2 Expression of IL-1β and IL-6 genes. A and B: Expression of IL-1β: Mean and standard deviation column graph with the confidence intervals in their respective groups (G1-G9); C and D: Expression of IL-6: Mean and standard deviation column graph with the confidence intervals in their respective groups (G1-G9). G1: Control; G2: Blood clot and Scaffold; G3: Blood clot + Scaffold + Melatonin 1 μg; G4: Blood clot + Scaffold + Melatonin 10 μg; G5: Blood clot + Scaffold + Melatonin 100 μg; G6: Blood clot + Scaffold + Melatonin 1 μg + rhBMP2; G7: Blood clot + Scaffold + Melatonin 10 μg + rhBMP2; G8: Blood clot + Scaffold + Melatonin 100 μg + rhBMP2; G9: Blood clot + Scaffold + rhBMP2.

In the osteoclastogenesis markers, the results showed the following condition: In RANK, group G6 showed a significant difference ($P \leq 0.05$) with groups G1, G3, G4 and G5 (1.243 ± 0. 1176 with 0.8645 ± 0.082, 0.8661 ± 0.088, 0.8740 ± 0.1377 and 0.7466 ± 0.08, respectively) (Figure 4A and B); in RANKL there was only a significant difference between G4 and G7 (1.293 ± 0.1115 and 0.9829 ± 0. 1114, respectively) (Figure 4C and D); in OPG there was a significant difference between group G9 and groups G1, G2, G3, G5, G6 and G7 (1.371 ± 0.3127 with 0.9685 ± 0.1097, 0.8790 ± 0.092, 1.034 ± 0.1645, 1.034 ± 0.1445, 0.7525 ± 0. 1217 and 0.8266 ± 0.092); between G4 and groups G2, G6 and G7 (1.234 ± 0.1460 and 0.8790 ± 0.092, 0.7525 ± 0.1217 and 0.8266 ± 0.092) and finally between G6 and G8 (0.7525 ± 0.1217 and 1.136 ± 0.2337), respectively (Figure 4E and F).

In MMPs markings, specifically MMP-1, there was a significant difference ($P \leq 0.05$) only between G6 and G8 (1.093 ± 0.089 and 1.379 ± 0.1484, respectively) (Figure 5A and B). There was a significant difference in MMP-2 between G6 with G3 and G4 (0.7691 ± 0.1178 with 1.392 ± 0.1531 and 1.470 ± 0.2421, respectively) (Figure 5C and D).

Finally, in MMP-8 marking, there was a significant difference ($P \leq 0.05$) between G4 (0.9006 ± 0.1) with G6 (0.6840 ± 0.068) and G8 (1.689 ± 0.2808), as well as a difference between G6 (0.6840 ± 0.068) and G8 (1.689 ± 0.2808) (Figure 6A and B). In MMP-13, there was a significant difference between G5 (1.861 ± 0.094) and G1 (0.982 ± 0.1706), G2 (0.9492 ± 0.1171) and G7 (1.41 ± 0.5028) (Figure 6C and D).

DISCUSSION

The current research examined the effects of applying melatonin topically in various concentrations, either alone or combined with rhBMP-2, to enhance bone repair in surgical defects using the GBR technique. This approach shows promise as a novel, safe alternative for treating bone injuries, breaks and transplants in clinical settings. The findings validated the secure application of the suggested combination and determined a safe and efficient dosage for the trial. To date, no studies have evaluated bone repair using the methods outlined in this investigation. The detailed histological

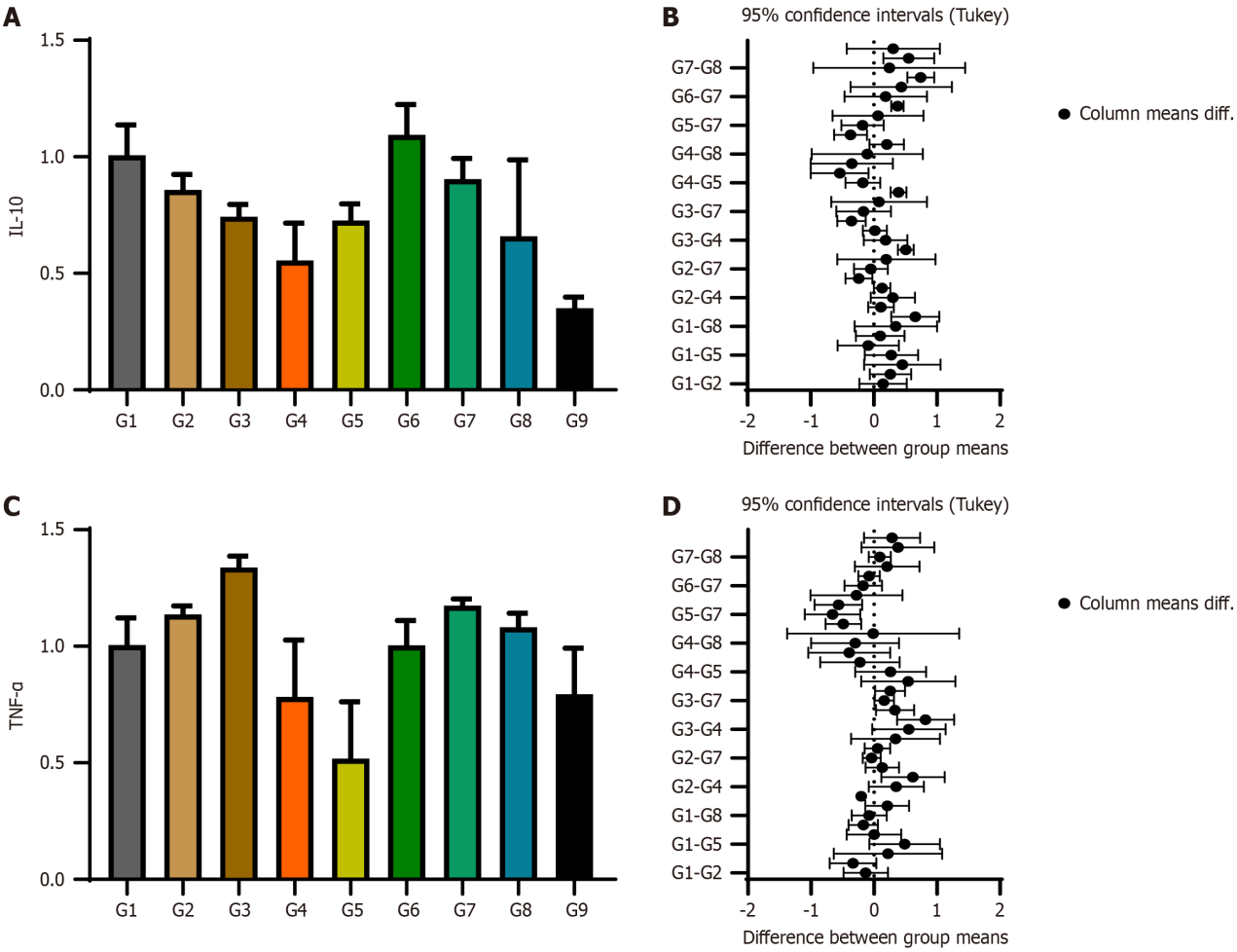

Figure 3 Expression of IL-10 and TNF-α genes. A and B: Expression of IL-10: Mean and standard deviation column graph with the confidence intervals in their respective groups (G1-G9); C and D: Expression of TNF-α: Mean and standard deviation column graph with the confidence intervals in their respective groups (G1-G9). G1: Control; G2: Blood clot and Scaffold; G3: Blood clot + Scaffold + Melatonin 1 μg; G4: Blood clot + Scaffold + Melatonin 10 μg; G5: Blood clot + Scaffold + Melatonin 100 μg; G6: Blood clot + Scaffold + Melatonin 1 μg + rhBMP2; G7: Blood clot + Scaffold + Melatonin 10 μg + rhBMP2; G8: Blood clot + Scaffold + Melatonin 100 μg + rhBMP2; G9: Blood clot + Scaffold + rhBMP2.

analyses utilized in the current research provided deeper insights into the bone tissue's response to different treatments and assessed the biological processes involved in bone repair. This contrasts with most studies that rely on single or multiple tissue sections, which limit the sensitivity of histological data. The histological techniques used here, including advanced microscopy and sophisticated software combined with the researchers' expertise in histomorphometry, expand the range of variables examined and increase the precision of tissue reaction measurement, thereby refining quantitative analysis techniques[21].

Bone injuries in rat calvaria are commonly utilized in scientific research for histological studies and analytical imaging. This is due to the bones' stability in terms of movement and their uniform structure, along with their unique embryological origin through intramembranous ossification. The model employed in this study was specifically created to replicate a severe bone injury, as these injuries are especially challenging to heal due to their resistance to complete spontaneous recovery. Severe bone injuries in the calvaria are used to assess various materials as potential and safe options for restoring bone structure, particularly in the craniofacial area[22,23]. To choose a material that promotes bone regeneration, ensuring its safety for the body is essential. Key factors to assess include the material's biocompatibility, its ability to facilitate osteogenesis, and its characteristics related to osteoinduction and osteoconduction[24].

One of the groups examined in this study involved using the animal's own autologous blood clot. This natural clot can serve as a biological factor for stimulate bone development, due to the presence of various cells that aid in this process. Grgurevic *et al*[25] demonstrated that the clot can act as a natural vehicle for rhBMP in bone formation. Reports indicate that melatonin, ROG, and rhBMP-2 have been utilized for effective bone repair. The authors noted that melatonin promotes osteogenesis, rhBMP-2 facilitates osteoinduction, and the collagen sponge supports osteoconduction. Specifically, melatonin encourages undifferentiated mesenchymal cells to become bone matrix-producing cells, while rhBMP-2 creates a conducive environment for the migration of osteogenic cells. Currently, it is recognized that melatonin influences bone metabolism, and its application promotes bone repair due to its osteogenic and anti-osteoclastic effects [26]. Wang *et al*[27] showed that the amount of rhBMP-2 is essential for bone formation. We chose a 5 μg dose of rhBMP-2 based on studies by Volpon *et al*[28] and Gonzaga *et al*[23], who found that this amount significantly aids bone formation in severe defects. rhBMP-2 is a well-researched protein crucial for several biological processes, especially those involving

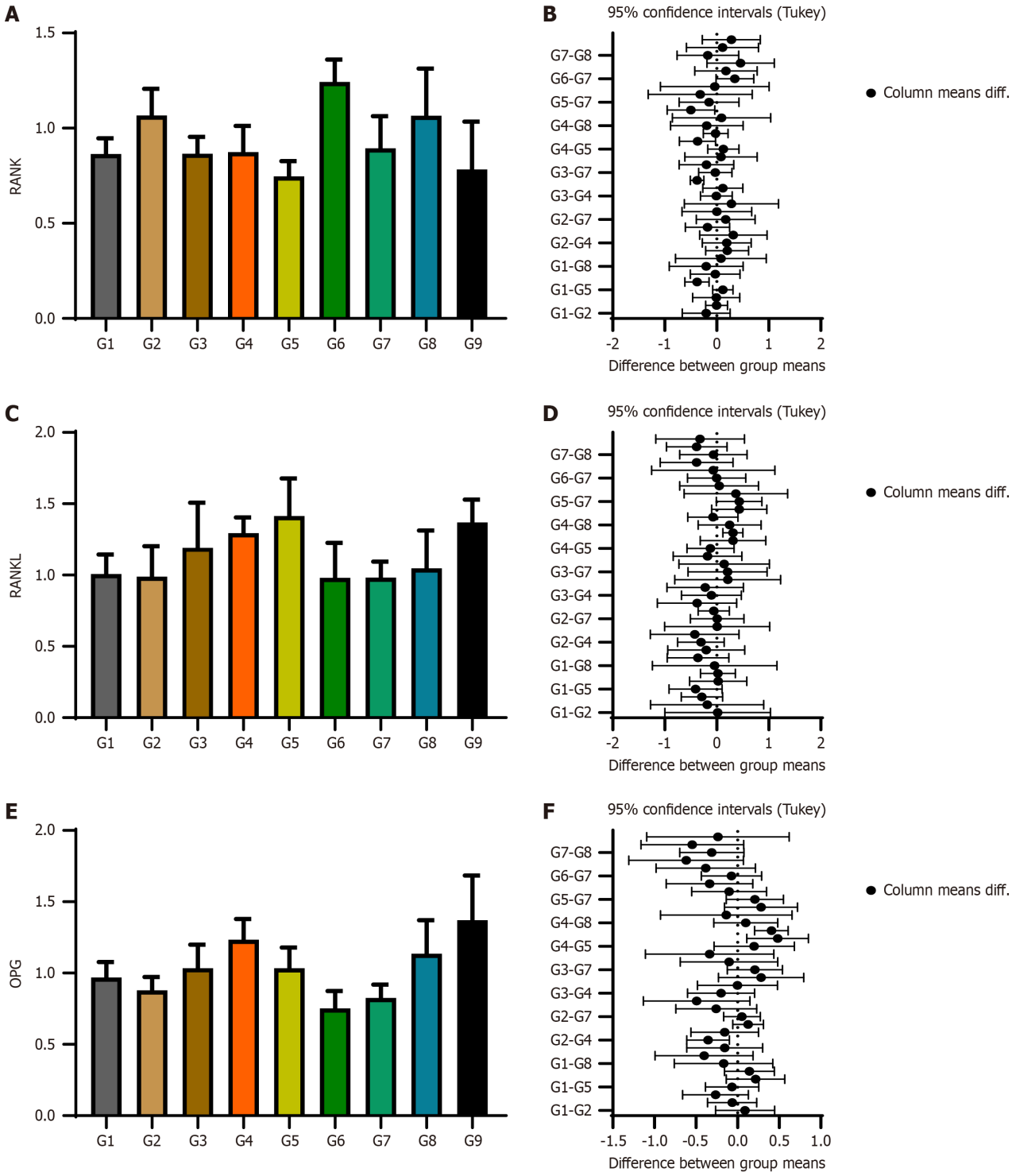

Figure 4 Expression of RANK, RANKL and OPG markers. A and B: Expression of RANK: Mean and standard deviation column graph with the confidence intervals in their respective groups (G1-G9); C and D: Expression of RANKL: Mean and standard deviation column graph with the confidence intervals in their respective groups (G1-G9); E and F: Expression of OPG: Mean and standard deviation column graph with the confidence intervals in their respective groups (G1-G9). G1: Control; G2: Blood clot and Scaffold; G3: Blood clot + Scaffold + Melatonin 1 μg; G4: Blood clot + Scaffold + Melatonin 10 μg; G5: Blood clot + Scaffold + Melatonin 100 μg; G6: Blood clot + Scaffold + Melatonin 1 μg + rhBMP2; G7: Blood clot + Scaffold + Melatonin 10 μg + rhBMP2; G8: Blood clot + Scaffold + Melatonin 100 μg + rhBMP2; G9: Blood clot + Scaffold + rhBMP2.

bone tissue. It plays a significant osteoinductive role in bone repair and regeneration by delivering morphogenic signals that direct the migration, growth, and transformation of mesenchymal cells into bone structures.

Nevertheless, using it by itself poses the drawback of rapid dissolution in the application environment, making it a costly material. Therefore, it necessitates a carrier material to ensure its slow, controlled, and secure release[29]. The GBR technique is a proven clinical method for reconstructing alveolar bone, primarily in dentistry. GBR works by blocking non-osteogenic tissues from the repair area and creating a bioactive space that promotes osteoconduction[30]. Therefore, GBR is well established in the literature for bone repair therapies[31,32]. Jung *et al*[33] found that combining a xenogeneic

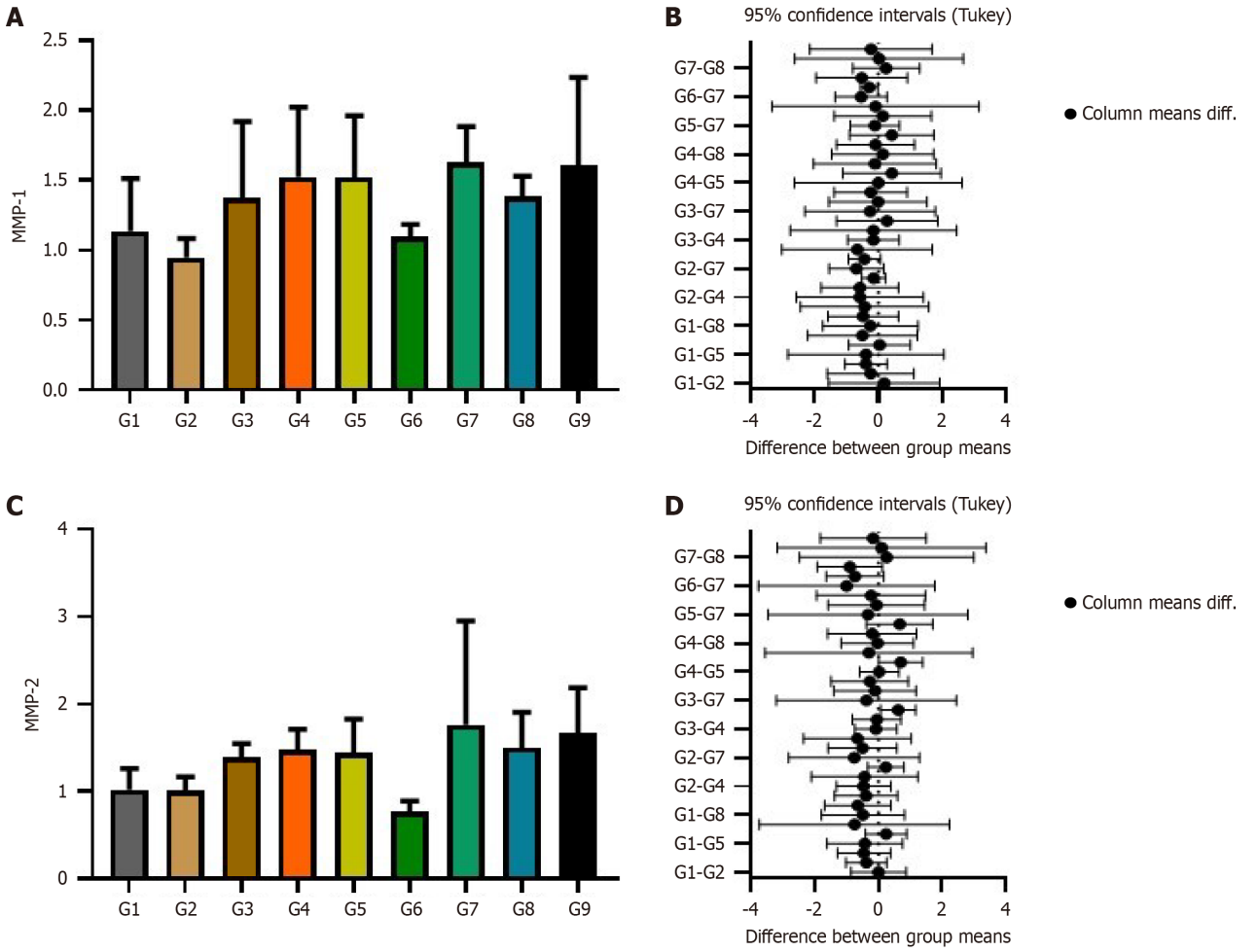

Figure 5 Expression of MMP-1 and MMP-2 markers. A and B: Expression of MMP-1: Mean and standard deviation column graph with the confidence intervals in their respective groups (G1-G9); C and D: Expression of MMP-2: Mean and standard deviation column graph with the confidence intervals in their respective groups (G1-G9). G1: Control; G2: Blood clot and Scaffold; G3: Blood clot + Scaffold + Melatonin 1 μg; G4: Blood clot + Scaffold + Melatonin 10 μg; G5: Blood clot + Scaffold + Melatonin 100 μg; G6: Blood clot + Scaffold + Melatonin 1 μg + rhBMP2; G7: Blood clot + Scaffold + Melatonin 10 μg + rhBMP2; G8: Blood clot + Scaffold + Melatonin 100 μg + rhBMP2; G9: Blood clot + Scaffold + rhBMP2.

bone mineral substitute with rhBMP-2 enhances bone regeneration maturation, highlighting rhBMP-2's potential to improve and accelerate GBR therapy. Similarly, Schorn *et al*[34] reported positive outcomes with vertical bone formation when using rhBMP-2 and VEGF in conjunction with a collagen carrier[35]. Wikesjö *et al*[36] also reported favorable outcomes with rhBMP-2 in bone regeneration[37-41]. As noted, osteoinductive proteins require a carrier because of their short biological duration, requiring gradual and sustained release. These carriers also serve as delivery systems and support cell growth. Combining an osteoinductive protein like rhBMP-2 with an osteoconductive material addresses challenges in current bone regeneration methods, which is why GBR was chosen for this study. Besides influencing mesenchymal cell differentiation, osteoblastic regulation, chemotaxis, and mitosis during bone repair[42], rhBMP-2 effectively speeds up bone regeneration. Consequently, significant bone repair was anticipated in groups 3, 4, 5, and 9, which had isolated combinations of melatonin with a scaffold and rhBMP-2 with a scaffold. However, group 9, which used a clot + scaffold + 5 μg rhBMP-2, did not show substantial bone formation compared to groups 3, 4, and 5 (clot + matrix + melatonin administered at concentrations of 1, 10, and 100 μg). High bone formation was anticipated in the aforementioned groups due to the osteogenic effects of melatonin or rhBMP-2 alone. This finding aligns with Sampath and Reddi[43], who showed that rhBMP-2 combined with collagen support can induce bone development at different locations, promoting bone regeneration. Similarly, Burkus *et al*[44] showed that rhBMP-2 in a degradable collagen sponge effectively promotes bone development. Our results suggest that the scaffold might have impeded bone regeneration by blocking the effects of melatonin and rhBMP-2. Groups 6 (blood clot + scaffold + 1 μg melatonin + 5 μg rhBMP-2) and 8 (blood clot + scaffold + 100 μg melatonin + 5 μg rhBMP-2), with varying melatonin concentrations, were the only ones to show a notable increase in bone formation compared to the others. This notable difference is likely due to the bone-inducing properties of rhBMP-2[45-47]. In this study, it was observed that melatonin had minimal impact on bone regeneration and gene expression related to improved regeneration and reduced adverse effects in groups 3, 4, and 5. This suggests that the scaffold significantly impaired melatonin's effectiveness in our model, unlike other studies where melatonin alone promoted bone formation[48,49]. Qualitative analysis showed that experimental groups 6, 7, 8, and 9 exhibited more bone trabeculae in the defect area, though substantial areas still needed repair compared to the remaining groups. Histological measurements revealed the most favorable outcomes for group 6 (blood clot + scaffold + 1 μg of

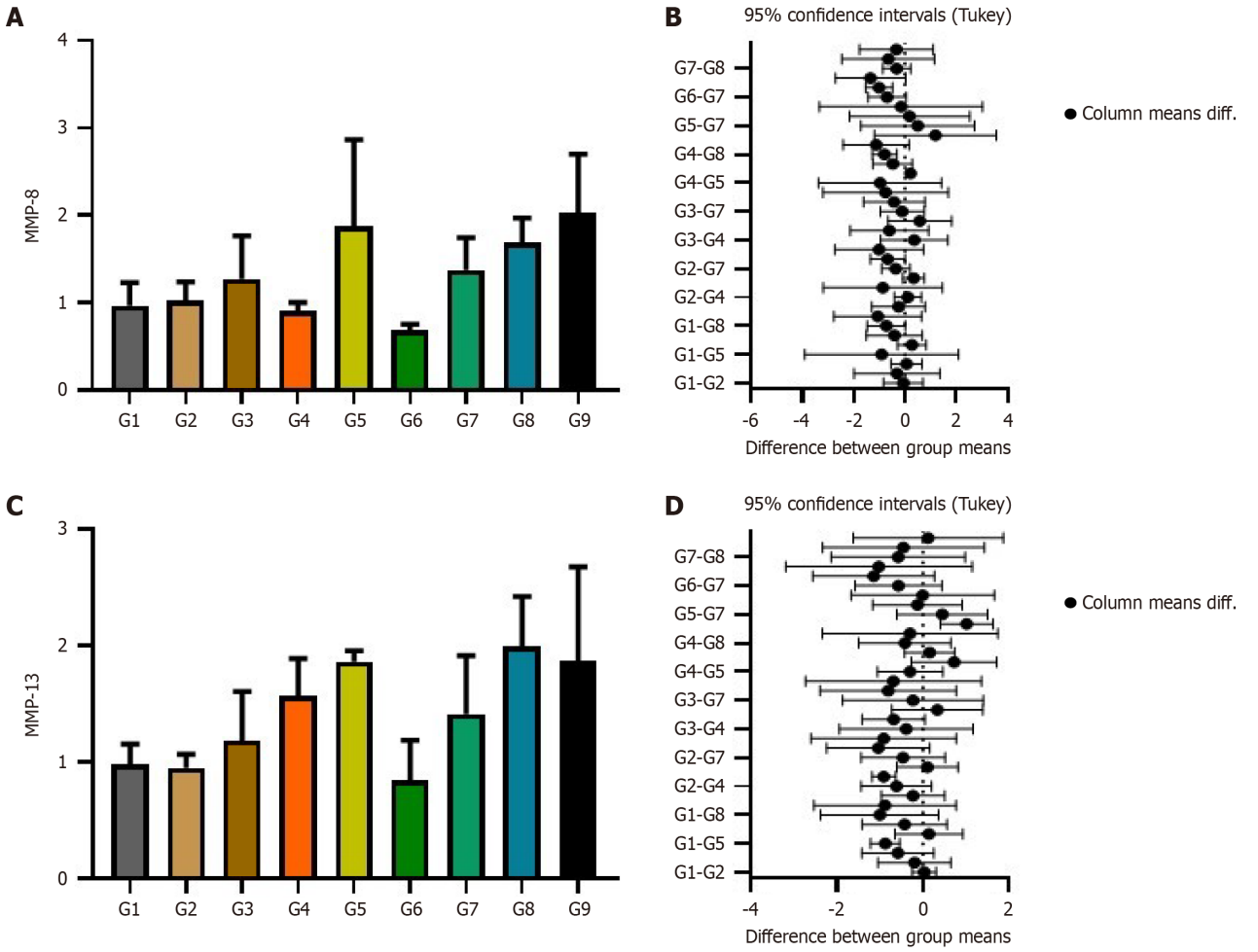

Figure 6 Expression of MMP-8 and MMP-13 markers. A and B: Expression of MMP-8: Mean and standard deviation column graph with the confidence intervals in their respective groups (G1-G9); C and D: Expression of MMP-13: Mean and standard deviation column graph with the confidence intervals in their respective groups (G1-G9). G1: Control; G2: Blood clot and Scaffold; G3: Blood clot + Scaffold + Melatonin 1 μg; G4: Blood clot + Scaffold + Melatonin 10 μg; G5: Blood clot + Scaffold + Melatonin 100 μg; G6: Blood clot + Scaffold + Melatonin 1 μg + rhBMP2; G7: Blood clot + Scaffold + Melatonin 10 μg + rhBMP2; G8: Blood clot + Scaffold + Melatonin 100 μg + rhBMP2; G9: Blood clot + Scaffold + rhBMP2.

melatonin + 5 μg of rhBMP-2), where the minimal amount of melatonin produced the strongest osteogenic response. Immunohistochemistry, using biomarkers for angiogenesis (CD31, VEGF), osteoclastogenesis (OPG), osteogenesis (osteocalcin, osteopontin, RUNX2), and pro-inflammatory factors (interleukin 1, 6, 10, TNF alpha), revealed weak staining for factors involved in bone repair, particularly in group 2. This supports the hypothesis that the scaffold inhibited bone repair. Nevertheless, the best outcomes were once again observed for sets 6 and 8, supporting the histological findings of improved regeneration in the cohorts treated with 1 and 100 μg of melatonin, combined with rhBMP-2. Real-time PCR (qPCR), a key molecular methodology, is highly effective for quantifying gene expression and is often used to validate higher-yield techniques with lower sensitivity, such as microarray analysis. qPCR utilizes fluorescent dyes to measure target gene transcription, with the cycle number at which these dyes/transcripts are detected indicating the relative or absolute abundance of the target molecules. Its sensitivity makes qPCR a valuable tool for assessing gene expression, particularly with limited samples or low transcript levels. However, this sensitivity necessitates meticulous experimental design and procedure execution[50]. Recent research has highlighted the regulation of BMP2-induced differentiation of C2C12 mesenchymal cells, showing that 22 mRNAs were significantly deregulated, which impacts bone formation and reduces inflammation[51].

BMP2 would certainly have effects on the gene expression of several parameters analyzed, however the use of the scaffold did not allow its correct action in injured tissues. Despite the different methodological situations found that resulted in the data presented here, this work proved to be innovative, as it showed the way for how these studies can be directed, considering that most studies related to gene expression in bones are directly correlated with implants bone[52-55]. It is worth highlighting that the experimental model used here, the rat, does not represent the ideal model for studies involving bone healing/repair, as it is an animal with an accelerated metabolism, therefore not enabling the precise study of the individual phenomena of this healing process, such as the which are addressed here. On the other hand, the rat allows for a good sample size and, therefore, a significant effect in terms of results based on the methodologies used here. Furthermore, some difficulties may be encountered in the methodology, such as the cost of the molecular material, the sensitivity of the technique and sample processing.

CONCLUSION

According to the methodologies used and within the limits they provide, the combination of melatonin (1 μg) with 5 μg rhBMP-2, through guided bone repair approach, managed to demonstrate some effects, albeit slight, on bone repair in severe defects in the rat calvaria, though the scaffold impeded this process in every groups. This indicates that the method for applying these substances needs to be reevaluated, focusing on their direct application to the defect without any substrate that affects clot formation, although with a possible compromise of the bioavailability of this material, an inherent characteristic of the materials. carriers. Therefore, future research must be carried out, seeking to produce materials with these ideal characteristics.

FOOTNOTES

Author contributions: Paulini MR participated in conceptualization, writing-original draft preparation, writing-review and editing, and visualization of the study; Montarele LF participated in conceptualization; Pitol DL participated in validation, investigation and data curation; Giannocco G participated in methodology and formal analysis; Pereira BF participated in methodology, validation and formal analysis; Buchaim DV participated in data curation and visualization; Reis CHB participated in validation and formal analysis; Buchaim RL participated in formal analysis, investigation and data curation, writing-original draft preparation, writing-review and editing, visualization and supervision; Issa JPM participated in writing-review and editing and supervision.

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