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Evaluation of the effectiveness of local Melatonin applied at different doses on healing of bone defect in rat tibias

Evaluación de la efectividad de la melatonina local aplicada a diferentes dosis en la curación de defectos óseos en tibias de rata

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ABSTRACT

This study aimed to investigate the effect of local melatonin application on bone healing in critical size defects created in rat tibias. 30 Spraque Dawley rats selected for the experimental setup were divided into three groups, each with 10 rats. In the control–defect group (n=10), only defects were created in the tibia bones of the rats, while 1.2 mg and 3 mg melatonin were applied locally to the defect–melatonin dose 1 (n=10) and defect melatonin dose 2 (n=10) groups, respectively. At the end of the experiment the subjects in all the groups were euthanized after an 8-week healing period and defect healing was calculated with histological bone healing percentage. The Shapiro–Wilk test and the Kolmogorov–Smirnov test were used to evaluate the conformity of the data to a normal distribution. One–way ANOVA was used to determine whether there was a difference between the groups due to the normal distribution of the data, and Tukey's honestly significant difference (HSD) test was used in pairwise comparisons to determine from which group the difference originated. The healing rate in the defect melatonin dose 2 group was calculated as 53.2 ± 7.38% and the healing rate in the control group was calculated as 44 ± 6.38%, which was a significant increase (P=0.008); however, when the difference between the defect melatonin dose 1 group (53 ± 7.1%) and the defect melatonin dose 2 group (53. 2± 7.38%) was evaluated statistically, no significant difference was found.The bone healing percentage in the groups where local melatonin was applied was found to be statistically significantly higher compared to the control group (P<0.05). No statistically significant difference was found between the groups where local melatonin was applied in terms of bone healing percentage (P>0.05). Within the limits of this study, it can be said that local melatonin application applied at two different doses had a positive effect on bone healing, however, no difference was observed between the applied local melatonin doses in terms of bone healing.

Key words: Bone; defect; local melatonin; rat tibia

RESUMEN

El objetivo de este estudio fue investigar el efecto de la aplicación local de melatonina en la cicatrización en defectos óseos de tamaño crítico creados en tibias de ratas. Se distribuyeron aleatoriamente 30 ratas Sprague–Dawley en tres grupos. En el grupo control con defecto (n=10), no recibió tratamiento en la zona afectada. En el grupo con dosis 1 de melatonina (n=10), se aplicaron tópicamente 1,2 mg de melatonina liofilizada en la zona afectada. En el grupo con dosis 2 de melatonina (n=10), se aplicaron tópicamente 3 mg de melatonina liofilizada en la zona afectada. Al final del experimento, los sujetos de todos los grupos fueron sacrificados después de un período de cicatrización de 8 semanas y se calculó la cicatrización del defecto con el porcentaje de cicatrización ósea histológica. Se utilizaron las pruebas de Shapiro–Wilk y de Kolmogorov–Smirnov para evaluar la conformidad de los datos con una distribución normal. Se empleó un ANOVA de una vía para determinar si existía una diferencia entre los grupos debido a la distribución normal de los datos, y la prueba de diferencia honestamente significativa (HSD) de Tukey se empleó en las comparaciones por pares para determinar de qué grupo provenía la diferencia. La tasa de cicatrización en el grupo de dosis defectuosa de melatonina 2 se calculó como 53,2 ± 7,38 % y la tasa de cicatrización en el grupo de control se calculó como 44 ± 6,38 %, lo que fue un aumento significativo (P=0,008); sin embargo, cuando se evaluó estadísticamente la diferencia entre el grupo de dosis defectuosa de melatonina 1 (53 ± 7,1 %) y el grupo de dosis defectuosa de melatonina 2 (53,2 ± 7,38 %), no se encontró ninguna diferencia significativa. Se encontró que el porcentaje de cicatrización ósea en los grupos a los que se les aplicó melatonina local fue significativamente mayor en comparación con el grupo de control (P<0,05). Sin embargo, cuando se compararon los grupos a los que se aplicó melatonina local, no se detectó diferencia estadísticamente significativa en términos de cicatrización ósea (P<0,05). Como resultado, se demostró que la melatonina tuvo un efecto positivo en la consolidación ósea, independiente de la dosis empleada en este estudio.

Palabras clave: Hueso; defecto; melatonina local; tibia de rata

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INTRODUCTION

Bone tissue, one of the hardest types of tissue in the body, is dynamic and vascularized, with the ability to renew itself [1]. Protecting internal organs, providing support, and enabling movement are the main functions of bone tissue [2]. It also serves as a depot for minerals such as phosphate and calcium, playing a role in mineralization. Additionally, it has a function in hematopoiesis [3, 4].

Trauma, tumors, and inflammation can cause bone defects. Osteoporosis is considered a primary cause of fractures in elderly individuals [4, 5]. Various growth factors and hormones affect osteoblasts and osteoclasts during the bone defect healing process [6]. Grafts are generally used to treat defects. Since autogenous bone grafts, considered the gold standard, have limited sources, require a secondary surgical site, and carry a morbidity risk, alternative treatment approaches are being investigated [7].

Recent research has shown positive effects of melatonin on bone metabolism. Besides being a hormone that regulates circadian rhythm, melatonin is noteworthy for its antioxidant, anti–inflammatory, and osteoblastic activity–enhancing properties [8]. By regulating osteoblastogenesis and osteoclastogenesis through circadian effects, it supports bone regeneration and bone development [9].

For melatonin production and secretion from the pineal gland, sympathetic innervation and stimulation of the pineal gland with norepinephrine are required [10]. Melatonin hormone is synthesized in many tissues and organs of the body such as the retina, bone marrow and intestinal mucosa. Melatonin also serves as an important hormone for bone metabolism. It has been stated that the decrease in melatonin synthesis seen with aging is associated with osteoporosis and bone loss [11].

Melatonin can accelerate the osteogenic process in bone tissue and support bone healing by reducing oxidative stress. When the literature is examined, it has been shown that melatonin increases the production, proliferation and differentiation of osteoblastic cells involved in bone formation and also supports the mineralization of the bone matrix, which is important for bone formation [12]. Another study revealed that melatonin reduces bone resorption by inhibiting osteoclastic activity, which accelerates bone destruction [13].

In an experimental study conducted by Jung *et al.*, it was reported that melatonin application increased the healing of bone defects [14]. However, it can be stated that the information regarding the dose–dependent effects of melatonin application is not sufficient. When evaluated from this perspective, the effect of melatonin application on bone healing when applied at different doses seems worth examining.

This study aimed to assess the effects of locally applied melatonin at varying doses on the healing of rat bone defects through histological analysis.

MATERIALS AND METHODS

Experimental animals and study groups

This study was conducted with the approval of the Firat University Animal Experiments Local Ethics Committee (Approval Number: 2023/18-03, Date: 13 November 2023) at the Firat University Experimental Research Center. Thirty Sprague–Dawley rats were included in this study and divided into three groups:

In the control–defect group (n=10), 4 mm diameter and 4 mm depth cavities were opened in the cortico–cancellous bone of the metaphyseal parts of the right tibial bones of the subjects, and no additional treatment was applied during the eight–week experimental setup. The defect–containing healed bone tissue was collected, decalcified, and subjected to histological analysis, and new bone formation in the defect was calculated as a percentage.

Defect melatonin dose 1 group (n=10): 4 mm diameter and 4 mm depth cavities were opened in the cortico–cancellous bone of the metaphyseal parts of the right tibial bones of the subjects. After the bone defects were created, 1.2 mg of lyophilized melatonin powder (Sigma–Aldrich, St. Louis, MO, USA) was applied topically to the defects [15]. After the surgical procedure, no treatment was applied to the rats for 8 weeks. The defect–containing healed bone tissue was removed, decalcified, and subjected to histological analysis. New bone formation in the defect was calculated as a percentage.

Defect melatonin dose 2 group (n=10): 4 mm diameter and 4 mm depth cavities were opened in the cortico–cancellous bone of the metaphyseal parts of the right tibial bones of the subjects. After the bone defects were created, 3 mg of lyophilized powder melatonin (Sigma–Aldrich, St. Louis, MO, USA) was applied topically to the defects [15]. The defect–containing healed bone tissue was removed, decalcified, and subjected to histological analysis. New bone formation in the defect was calculated as a percentage.

Surgical procedures

To provide general anesthesia, 10 $mg \cdot kg^{-1}$ Xylazine (Rompun; Bayer, Germany) and 40 $mg \cdot kg^{-1}$ Ketamine (Ketasol; Richter Pharma, Wels, Austria) were injected intramuscularly [15]. The tibias of the rats were shaved, and the area was washed with betadine. To reach the cortico–cancellous bone of the metaphyseal part of the tibia, a 5 mm incision was made from the top of the tibia bone with a number 15 scalpel following aseptic protocols. With the help of special burs, bone defects of 4 mm in diameter and 4 mm in depth were created under sterile physiological serum perfusion. To ensure standardization of bone defects, all defects were made by the same person. Furthermore, the marking on the drill bit was taken into account to ensure a standard depth. After local melatonin application, the soft tissues were sutured to their original positions. After the surgical procedures, antibiotics (Cefazolin sodium 40 $mg \cdot kg^{-1}$, iespor 250, I.E. Ulagay, Türkiye) and analgesics (Tramadol hydrochloride 0.1 $mg \cdot kg^{-1}$, Contramal, Abdi Ibrahim, Türkiye) were administered intramuscularly. Eight weeks after surgery, the healed bone tissue containing the defect was removed, decalcified, and subjected to histological analysis to calculate new bone formation in the defect as a percentage.

Histopathological analysis

The subjects in all the groups were euthanized after an 8–week healing period. After euthanasia, the decalcified tibia bones were placed in separate tissue tracking cassettes (Isolab GmbH, Wertheim, Germany) and fixed in 10% neutral formalin solution. After being washed under running tap water for approximately 2 hours (h), they were passed through alcohol, Xylene and paraffin series in an automatic tissue tracking device (Leica TP 1020, Wetzlar, Germany) and blocked vertically with paraffin in a tissue blocking device (Leica EG 1150 H, Wetzlar, Germany). Serial 3–5 micron thick sections were taken from the paraffin blocks via a rotary microtome (Leica RM2125, Wetzlar, Germany) on positively charged slides (Thermo Fischer Scientific, Superfrost, Massachusetts, U.S.A.). The prepared sections were subjected to hematoxylin–eosin (HE) staining via an automatic tissue staining machine (Leica Autostainer XL, Wetzlar, Germany).

In the evaluations, the amount of new bone formation (NBF) was determined by measuring the callus tissue in the area where the defect was created. While performing this measurement, the area where no new bone formed was subtracted from the entire callus area, and the area filled with bone was calculated. The percentage of new bone formation was obtained by dividing the area filled with bone by the total callus area.

Statistical analysis

SPSS version 22 was used for the statistical evaluation of the data. The Shapiro–Wilk test and the Kolmogorov–Smirnov test were used to evaluate the conformity of the data to a normal distribution. One–way ANOVA was used to determine whether there was a difference between the groups due to the normal distribution of the data, and Tukey's honestly significant difference (HSD) test was used in pairwise comparisons to determine from which group the difference originated. Mean and standard deviation values were used when the data it was noted that were presented. Notably, $P<0.05$ was considered significant.

RESULTS AND DISCUSSION

In this study, examining the effect of melatonin on bone healing, each group was monitored for eight weeks, and the bone healing rates were evaluated via histological analysis after healing. In the control–defect group, the healing rate was 44 ± 6.38%, indicating the healing capacity of the bone (FIG. 1).

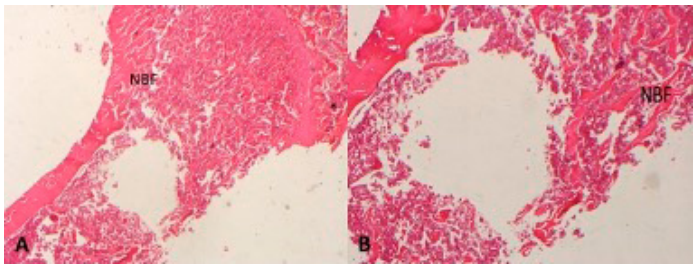

FIGURE 1. Histological images of the Control–Defect group; (A: 20×; B: 40× magnification; Hematoxylin and eosin). NBF: new bone formation, *: fibrosis

In the defect melatonin dose 1 group, the healing rate was 53 ± 7.21%, indicating a significant increase in bone healing (FIG. 2) (P=0.008)

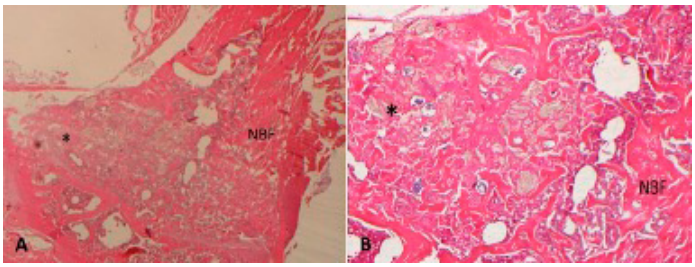

FIGURE 2. Histological images of the Defect Melatonin Dose 1 group; (A: 20×; B: 40× magnification, Hematoxylin and eosin). NBF: new bone formation, *: fibrosis

The healing rate in the defect melatonin dose 2 group was calculated as 53.2 ± 7.38%, which was a significant increase (FIGURE 3) (P=0.008); however, when the difference between the defect melatonin dose 1 group and the defect melatonin dose 2 group was statistically evaluated, no significant difference was found. (Tukey HSD, ^{a1}: 0.016, ^{a2}: 0.018) (TABLE I).

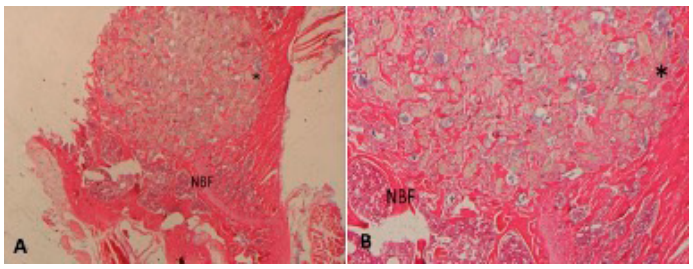

FIGURE 3. Histological images of the Defect Melatonin Dose 2 Group; (A: 20×, B: 40× magnification, Hematoxylin and eosin). NBF: new bone formation, *: fibrosis

TABLE I
New bone formation (NBF) ratios of the groups

Groups	N	Mean (NBF) (%)	Std. Dev.	*P**
Control–defect	10	44.0	6.38	
DMLT Dose 1^{a1}	10	53.0	7.21	0.008
DMLT Dose 2^{a2}	10	53.2	7.38	

These findings indicate that melatonin has a positive effect on bone healing, with no significant difference observed between different doses, suggesting that melatonin may reach a plateau effect at a certain dose.

Melatonin is a hormone with positive effects on bone healing and exerts these effects through endocrine, paracrine, and autocrine pathways [16, 17]. Studies have shown that melatonin supports new bone formation by increasing osteoblastic activity

in bone tissue, thus potentially accelerating the bone healing process [16, 18]. It is thought that these effects of melatonin occur through its capacity to suppress reactive oxygen species and inflammation [19]. Melatonin's regulatory role in oxidative stress plays a significant role in bone healing because oxidative stress can adversely affect osteoblast function [20]. In one study, following tooth extraction, the delay in wound healing was due to increased reactive oxygen species (ROS) at the tooth extraction site, whereas locally applied melatonin neutralized the increased oxidative and nitrosative stress in the blood due to its antioxidant effect. This reduces inflammation and damage in the oral cavity and accelerates wound healing [21].

Zhang and colleagues reported that melatonin application increased the expression of bone–forming genes such as Runx2 and osteocalcin in osteoblast cells [22]. When the findings obtained by Zhang and colleagues are evaluated, it is suggested that melatonin may promote the production of bone–building cells and their transformation into bone tissue, thus supporting bone healing [22].

In studies investigating the relationship between melatonin application and bone tissue, it is seen that melatonin is applied with different methods. This study is a locally applied study, and there are also examples in the literature where melatonin was applied locally [15, 18, 20, 23]. Another method used in experiments is systemic administration via the intraperitoneal route [24, 25]. In a study where melatonin was administered systemically intraperitoneally, an increase in the volume of bone tissue in the femurs of mice was reported [25]. In another study examining bone formation in distraction osteogenesis with systemic melatonin use, significant results were reported [26]. Another study reported the use of melatonin administered by gavage in obesity [27].

The present study revealed that the effect of melatonin on bone defect healing was significant. In this study it was also supported by many studies in the literature. In an experimental study conducted by Dundar et al., they reported that by applying local melatonin to implant cavities created in rabbit tibias, melatonin provided the osteogenic property by stimulating osteoblastic cells [15]. A study by Zhou and colleagues also demonstrated that locally applied melatonin in the defect area promoted new bone formation and shortened the healing time, supporting in these findings [20]. Similarly, in a study by Munoz and colleagues, melatonin was applied to implant sites in the alveolar bone of dogs, and the results support our findings [18].

In another locally applied study, melatonin–containing and melatonin–free covers were placed on defects created in the skulls of male Fischer rats, and the bone volume was examined via CT between the first day after surgery and the 8th week. Researchers found a significant increase in bone volume in those treated with melatonin [28]. In a study where cranial bone defects were created using dental pulp stem cells with melatonin, it was stated that new bone formation increased compared to controls and an increase was provided in molecular pathways that are indicators of new bone formation [29]. In addition, it was reported that melatonin stimulated osteogenic and chondrogenic effects by triggering mesenchymal–derived stem cells [30]. In a study on bone healing in osteoporotic experimental animals, it was found that melatonin treatment increased bone mineralization and new bone formation, and its effects were further enhanced by the use of xenogeneic grafts [31].

When the bone healing data obtained in this study were evaluated, no statistically significant difference was found between the defect melatonin dose 1 group and the defect melatonin dose 2 group and a similar finding was reported in another study that reported no significant difference between doses [14]. This result, which is supported by another study, shows that determining the optimal dose rather than increasing the dose of melatonin in bone healing is important.

Along with other studies examining the relationship between melatonin and bone healing, the effect of melatonin in the treatment of bone tumors has also been investigated [32]. Some studies have reported that melatonin regulates bone metabolism, halting the growth of cancer cells and inhibiting the migration of osteosarcoma cells [33, 34]. Another study investigating the effect of melatonin in the treatment of osteosarcoma revealed that loading PLGA with melatonin resulted in the death of osteosarcoma cells, leading to positive results in treatment [35].

The study was conducted that by the studies reviewed, showing that melatonin could be used to treat bone defects, bone diseases, and bone tumors, either alone or in combination with other therapies, with further research.

CONCLUSION

As a result of this experimental study, it was concluded that local melatonin application significantly supports bone defect healing. In addition, it was observed that there was no significant difference in the healing of bone defects with different doses of local melatonin applied in this study. Although it can be stated that application of local melatonin has a positive effect on bone defect healing within the limits of this study, further studies are needed to contribute to a clearer understanding of the melatonin–bone healing relationship by investigating different application methods, dosages and biomaterials.

Conflicts of Interest

The authors declare that there are no known conflicts of interest.

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