Melatonin Administration Aids Bone Healing in Diabetic Rats

Kaya, G.S.,[1] Kaya, M.,[2*] Gursan, N.,[3] Kiziltunc, A.,[4] Alp, H.H.,[4] Balta, H.,[3] Hayirli, A.[5]

[1] Department of Oral and Maxillofacial Surgery, Faculty of Dentistry, Atatürk University, Erzurum, Turkey.
[2] Department of Surgery, Faculty of Veterinary Medicine, Atatürk University, Erzurum, Turkey.
[3] Department of Pathology, Faculty of Medicine, Atatürk University, Erzurum, Turkey.
[4] Department of Biochemistry, Faculty of Medicine, Atatürk University, Erzurum, Turkey.
[5] Department of Animal Nutrition and Nutritional Disorders, Faculty of Veterinary Medicine, Atatürk University, Erzurum, Turkey.

* **Corresponding author:** Dr. Mahir Kaya, Department of Surgery, Faculty of Veterinary Medicine, Atatürk University, Erzurum, Turkey. Tel: +904422315527, Fax: +904422360881. E-mail: kayamahir@gmail.com

ABSTRACT

This randomized experimental study was conducted to determine the effect of melatonin on bone healing processes in diabetic rats with bone defects. Adult male Sprague Dawley rats (n = 64) were divided into diabetic and control groups. The diabetic group was administered with streptozotocin (60 mg/kg BW, (intraperitoneally) I.P.), whereas the control group was administered with saline containing 6% ethanol intraperitoneally. Defects were created in the left tibias, and rats were then administered with either saline or melatonin (10 mg/kg BW, I.P.) for 21 or 42 days. Overall, the diabetic rats had significantly higher serum glucose concentrations than the control rats (462 vs. 118 mg/dl; $P<0.0001$). Melatonin administration increased serum glucose by 5% in the control groups and decreased it by 43% in the diabetic groups ($P<0.0001$). Serum melatonin concentrations in the control rats were lower than in the diabetic rats (666 vs. 745 pmol/L; $P<0.006$). Melatonin administration increased serum melatonin concentrations to a similar extent in both groups (by 15%). Diabetes was associated with pathologies (degranulation, vacuolization, degeneration, and necrosis) in pancreatic β cells and aggravated inflammation indicators ($P<0.0001$ for all). Melatonin administration considerably decreased the number of rats with pancreatic β cell pathologies (63%) and depressed inflammation indicators (from $P<0.004$ to $P<0.0008$). Melatonin administration increased osteogenesis indicators (collagen, cartilage, and osteocyte intensities) (2.0 vs. 1.0; $P<0.04$) and numerically decreased inflammation indicators (macrophage, lymphocyte, and polymorph nuclear leukocyte) (1.0 vs. 2.0) at a greater extent in the diabetic rats than in the control rats. In conclusion, postoperative administration of melatonin supports bone healing in diabetic rats apparently through alleviating pancreatic cytopathology and hyperglycemia.

Keywords: Melatonin, bone healing, diabetes mellitus, rat.

INTRODUCTION

Diabetes mellitus is a common and serious metabolic disorder associated with many functional and structural complications. It is one of the most frequently diagnosed endocrinopathies in both humans (1) and household pets (cats and dogs) (2-5). Obesity and physical inactivity are major predisposing factors in both humans (1) and pet animals (6, 7). Diabetes increases the risk of fracture (8) and interferes with healing processes (9, 10) by slowing bone formation.

The pineal gland hormone, melatonin (N-acetyl-5-methoxytryptamine) has a variety of physiological, immunological and biochemical functions. It is a direct endogenous free-radical scavenger and indirectly exerts chemoprotective, immunostimluatory and myelostimulatory effects (11), which are important in the pathogenesis and treatment of diabetes complications (12). Moreover, melatonin interacts with insulin (13), and consequently alleviates the adverse effects of diabetes by lowering blood glucose levels (14) and

enhancing antioxidant defense capacities (15) to detoxify harmful reactive oxygen species (16). As a strong scavenger, melatonin may diminish the negative effects of oxidative stress, which is an important cause of diabetes-related complications. Melatonin injection has been shown to decrease the amount of superoxide radicals in rat plasma and to increase total antioxidative capacity as well as the activity of antioxidative enzymes, including superoxide dismutase and glutathione peroxidase (17).

Melatonin is considered an important mediator in osteogenesis (18, 19) through shortening the osteoblast differentiation period (20). Studies of osteoporosis and menopause confirm the influence of melatonin on bone metabolism; indeed, the low melatonin level in menopausal women maybe one of the reasons for their susceptibility to orthopedic problems (21). Despite the well-known effects of melatonin on bone tissue in non-diabetic subjects (22), the effects of melatonin on bone healing in diabetic subjects has not been studied. Therefore, this study was conducted to examine the effects of melatonin administration on the healing process in diabetic rats with tibial defects.

MATERIALS AND METHODS

Animals and Management

Sixty-four male Sprague Dawley rats weighing an average of 260 g (220-340 g) at 8 weeks of age were obtained from The Atatürk University Experimental Medical Application and Research Center and used in full compliance with the 1975 Helsinki Declaration of Animal Rights (revised in 2000) after the approval of the Atatürk University Faculty of Veterinary Sciences Ethical Committee on Animal Care and Use. Rats were kept in pairs in cages in a room with a 12-hour daylight/darkness cycle and an ambient temperature of 23±2°C and humidity of 55±10% throughout the study (23). Standard rat chow (Bayramoglu Feed Co., Erzurum, Turkey) and water were provided *ad libitum*.

Experimental Groups

Rats were distributed into 4 groups in a 2×2 factorial arrangement with 2 health statuses (control-healthy and diabetic) and 2 agent administrations (saline and melatonin). Each group was further divided according to time of agent administered (21 and 42 days), yielding 8 replications per group.

Pineal indole melatonin (Sigma, St. Louis, MO, USA) was freshly dissolved in saline containing 6% ethanol (total volume of 1 ml/kg). Daily i.p. injections of 10 mg/kg BW were administered postoperatively using a 1-mL syringe with a 25-gauge needle, with 6% ethanol saline as a vehicle; experimental rats received the melatonin injection, whereas control rats received 6% ethanol saline only. To avoid interfering with the daily circadian rhythm, melatonin and saline were administered at 23:00-24:00 hrs under dim light by illumination from an adjacent room.

Diabetes Induction

Prior to experimentation, rats were grouped by their live weights to avoid variability in body weight. After overnight fasting, 32 rats were injected intraperitoneally (i.p.) with streptozotocin (STZ; Sigma, St. Louis, MO, USA), at a dose of 60 mg/kg BW in 0.1 M cold citrate buffer (pH 4.5) to induce Type I diabetes mellitus (24). In order to prevent the possibility of fatality due to STZ-induced hypoglycemia caused by massive insulin release, rats were administered a 5% dextrose solution for 24 hrs beginning 6 hrs after STZ administration. Diabetes induction was verified 72 hrs after STZ administration using a glucometer (Optium Xceed Glucometer; Abbott, Chicago, IL, USA). The rats were monitored for 14 d to ensure that blood glucose levels had stabilized. Rats with fasting blood glucose levels above 220 mg/dl were considered diabetic.

Tibial Defect Creation

Under general anesthesia with xylazine (5 mg/kg, Bayer, Istanbul, Turkey) and ketamine (75 mg/kg, Eczacıbaşı, Lüleburgaz, Turkey), the left hind leg of each rat was shaved, the skin was disinfected using povidone iodine, and an incision (1 cm) was made in the anteriomedial aspect of the tibia. A full-thickness flap was reflected in order to expose the bone surface of the left tibia. Under profuse saline solution irrigation, a unicortical circumferential critical-size defect (3.0×3.0 mm) was created on the methaphyseal area of the tibia with a 3.0 mm wide trephine burr at a rotary speed not exceeding 1,500 rpm (25). The soft tissue was repositioned and fascia and skin layers were surgically closed. Animals were closely monitored after surgery, but their functional activity was not restricted. Antibiotic (amoxicillin, 150 mg/kg intramuscularly for 3 d, Bremer Pharma, Warburg, Germany) and analgesic (buprenorphine 0.05 mg/kg subcutaneously every 12 hrs

for 3 d, Reckitt and Colman, Richmond, VA, USA) were administrated following surgery to prevent infection and minimize pain. No complications were noted during the postsurgical period.

Measurements

Half of the rats in each group were sacrificed with thiopental (200 mg/kg, i.p., Abbott, Campoverde di Aprilia, Italy) administered 21 days after the tibial defect creation and the other half at 42 days. Blood samples were collected from the heart under anesthesia prior to sacrifice, and pancreases and left tibias were collected post-sacrifice for biochemical and histopathological examination.

Biochemical Analysis

Blood glucose levels were measured using a glucometer (Optium Xceed Glucometer; Abbott, Chicago, IL, USA). Blood samples were then centrifuged at 3,000 g for 15 min, and serum melatonin concentrations were measured using ELISA (USCN Life Science Inc., Wuhan, China) in a microplate reader (BioTek, PowerWave XS, California, CA, USA) at 450 nm optical density and a standard range of 0-2000 ρmol/L.

Histopathological Analysis

Pancreatic tissue samples were fixed in 10% buffered neutral formaldehyde and Bouin's fixatives for light microscopic examination, passed through a standard alcohol dehydration-xylene sequence and embedded in paraffin. General pancreatic structure was evaluated by applying Mallory's triple stain to 5-6 μm-thick sections cut from tissue blocks and by examining them under a light microscope (Olympus BX51; Olympus Corp., Tokyo, Japan) at x40 magnification. Samples were evaluated by two pathologists blinded to the sample groups and scored on a graded scale, as follows: 0 = no necrosis; 1 = slight degenerative changes (≤ 25% of islets affected); 2 = mild necrosis (≤ 50% of islets affected); 3 = severe necrosis (≤ 75% of islets affected).

Bone tissue samples were fixed in 10% buffered formalin and decalcified in hydrochloric acid/formic acid solution 20 times their own volume, with fresh solution provided daily until decalcification was complete (about 96 hrs). Specimens were routinely embedded in paraffin and then thoroughly washed under running tap water. The sections (5 μm) were placed on polylysine-coated slides, stained with hematoxyline and eosin (H&E), randomized and examined under a light microscope (Olympus BX51; Olympus Corp., Tokyo, Japan) at x40 magnification by two pathologists blinded to the specimen groups. Polymorph nuclear leukocyte, lymphocyte and macrophage infiltrations as well as osteocyte density, cartilage formation and collagen accumulation were evaluated semi-quantitatively on a graded scale based on degree of healing, as follows: 0 = absent; 1 = mild; 2 = moderate; 3 = remarkable.

Statistical Analysis

To achieve 10% increase in serum melatonin concentration and to consider this elevation significant at α error of 0.05 and power (1 – β error) of 0.90, power analysis yielded 7 rats per group. Blood and bone metabolites (continuous variables) were analyzed using two-way ANOVA as repeated measures. The linear model included the main effect of health status (healthy vs. diabetic) and agent administration (saline vs. melatonin) and their interaction as whole-plot terms and time, and time by whole-plot term interactions as sub-plot terms (26). Moreover, correlation between blood glucose level and inflammation and osteogenesis markers was attained. In the analyses of bone histopathological parameters (discrete variables), Kruskal-Walis test was employed. Statistical significance was set at $P<0.05$.

RESULTS

Serum Metabolites

Blood glucose concentrations of diabetic rats on post-induction day 21 and 42 were, respectively, 4.4 and 3.5 times greater than those of controls ($P<0.0001$; Table 1). Melatonin administration resulted in 27% and 44% reductions in blood glucose concentrations on day 21 and 42, respectively as compared to saline administration ($P<0.0001$; Table 1). Following melatonin administration, there were slight increases in blood glucose concentrations in the control rats (4.2 and 6.1%), whereas there were considerable decreases in blood glucose concentrations in the diabetic rats (-33 and -54%) on days 21 and 42 ($P<0.0001$; Table 1).

Serum melatonin concentrations of the control rats were lower than those of the diabetic rats (664 vs. 713 ρmol/L on d 21 and 667 vs. 778 ρmol/L on day 42, $P<0.006$; Table 1). As compared to saline administration,

Table 1: Effects of melatonin administration on blood glucose (mg/dl), and serum melatonin (ρmol/L) concentrations in diabetic rats with tibial defect*

Parameter	Control rats (n=32) Saline (n=16)	Control rats (n=32) Melatonin (n=16)	Diabetic rats (n=32) Saline (n=16)	Diabetic rats (n=32) Melatonin (n=16)	SEM	Significance of Effects, P< Diabetes	Significance of Effects, P< Melatonin	Significance of Effects, P< Interaction
Glucose								
day 21	111.7	116.4	598.1	402.3	31.4	0.0001	0.0001	0.0001
day 42	118.4	125.6	578.3	268.0	29.4			
Melatonin								
day 21	582	746	677	749	40	0.006	0.002	0.330
day 42	628	706	750	805	38			

* Type I diabetes was induced in half of the rats by administering streptozotocin (60 mg/kg body weight, i.p). Tibial defects were then created in all rats, which were then administered either saline or melatonin (10 mg/kg body weight, i.p.) for 21 or 42 d. n = 8 for control and diabetic rats on each day measurement.
[1] Time effect, P<0.0001.

melatonin administration resulted in 19% and 10% increases in serum melatonin concentrations on days 21 and 42, respectively (*P*<0.002; Table 1). However, the extent of increase in serum melatonin concentration upon melatonin administration to the control (28 and 14%) and diabetic (11 and 7%) rats did not differ both on days 21 and 42 (P<0.33; Table 1).

Pancreas Histopathology

Islets of Langerhans in the control groups injected with saline and melatonin appeared histologically normal (Figures 1a-b). In the diabetic groups, islets of Langerhans showed degranulation, vacuolization, degeneration and necrosis of β cells (Figure 1c); however, notably fewer histological changes were observed in the diabetic rats administered melatonin when compared to the diabetic rats administered saline (Figure 1d). Severe necrotic and vacuolized pancreatic β cells were observed in more diabetic rats than controls (Table 2), and the number of diabetic rats administered melatonin with severe necrotic and vacuolized pancreatic β cells was considerably lower when compared to the diabetic rats administered saline.

Bone Histopathology

Inflammation indicators, i.e. polymorph nuclear leukocyte (*P*<0.0001), lymphocyte (*P*<0.0001) and macrophage (*P*<0.004) infiltrations, were more severe in the diabetic rats than the control rats (Table 3). The degree of infiltration decreased with melatonin administration (Table 3) at a similar extent in both groups. Cellular infiltration decreased considerably over time independently of health status.

Table 2: Semi-quantitative analysis of the pancreatic β cells in diabetic rats with tibial defect (n=8)*

Groups	Islet necrosis and vacuolization[1] 0	1	2	3
Control + saline	8	0	0	0
Control + melatonin	7	1	0	0
Diabetes + saline	0	1	3	4
Diabetes + melatonin	5	2	1	0

* Type I diabetes was induced in half of the rats by administering streptozotocin (60 mg/kg body weight, i.p.). Tibial defects were then created in all rats, which were then administered either saline or melatonin (10 mg/kg body weight, i.p.) for 42 d.
[1] Values represent numbers of rats. β cell necrosis and vacuolization were graded using light microscopy, as follows: 0 = no necrosis; 1 = slight degenerative changes (≤ 25% islets affected); 2 = moderate necrosis and vacuolization (≤ 50% islets affected); 3 = extensive and marked necrosis and vacuolization in central zone of the islets (≤ 75% islets affected).

Osteogenesis indicators improved significantly in the diabetic rats following melatonin administration. Osteocyte density decreased following diabetes induction (*P*<0.0001) and increased following melatonin administration (*P*<0.0007; Table 3), with more notable improvements in diabetic rats when compared to the control rats (*P*<0.05; Table 3). Diabetes induction was also accompanied by compromised collagen accumulation and cartilage

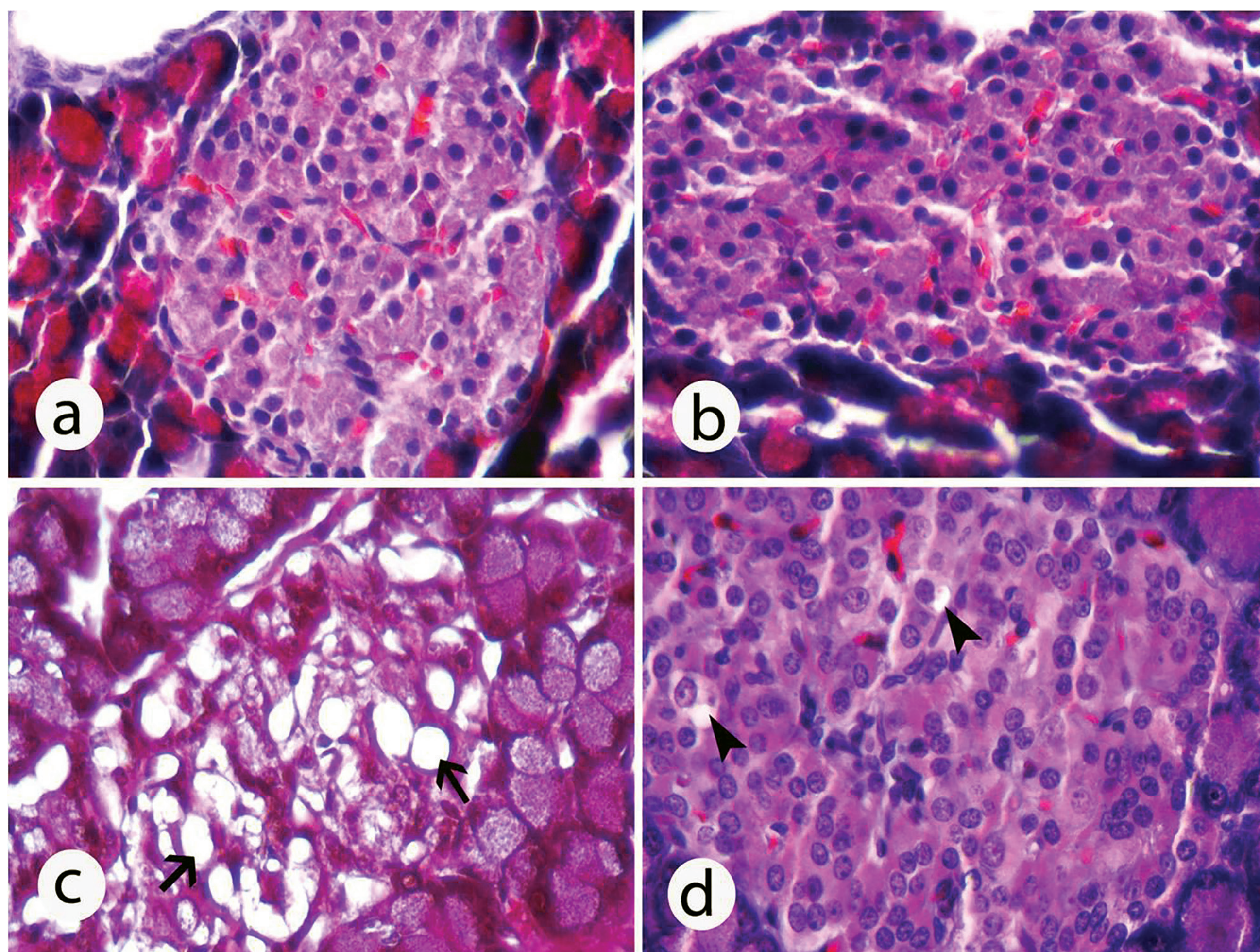

Figure 1: Histological structures of Langerhans islets in traumatized control and diabetic rats on day 42 post-tibial defect creation (H&E×100). Control + saline (a), control + melatonin (b), diabetes + saline (c), diabetes + melatonin (d). Arrows indicate numerous degenerative and vacuolized β cells in diabetic rats administered with saline and arrow heads indicate a few degenerative cell in diabetic rats treated with melatonin.

formation (*P*<0.0001 for both; Table 2). Melatonin administration enhanced both collagen accumulation (*P*<0.0004) and cartilage formation (*P*<0.0002). The increase in collagen accumulation was significantly greater in the diabetic rats when compared to the control rats, whereas the degree of cartilage formation was unaffected by health status (*P*<0.04; Figure 2, Table 2).

Overall, blood glucose levels showed a positive correlation with polymorph nuclear leukocyte (*r* = 0.56; *P*<0.0001), lymphocyte (*r* = 0.48; *P*<0.0002), and macrophage (*r* = 0.39; *P*<0.003) infiltrations and a negative correlation with osteocyte density (*r* = -0.47; *P*<0.0002), collagen accumulation (*r* = -0.56; *P*<0.0001), and cartilage formation (*r* = -0.52; *P*<0.0001).

DISCUSSION

The low antioxidative capacity of pancreatic β-cells makes them highly susceptible to oxidative changes due to hyperglycemia (27). Cell damage by free radicals has been shown to lead to β-cell degranulation or necrosis in STZ-induced diabetic rats, resulting in a decrease in insulin secretion and an increase in blood glucose (28). In previous studies, melatonin (50 mg/kg) treatment has been shown to cause a sharp decrease in serum glucose, a slight increase in serum insulin concentrations and partial regeneration/proliferation of islet β-cells (29, 30). Similar findings were observed in the current study using 10mg/kg melatonin (Figure 1, Table 2). Other studies showed that melatonin exerted similar protective effects on the liver (31) and kidneys (32) of diabetic rats. This

Table 3: Effects of melatonin administration on the healing process indicators in diabetic rats with tibial defect*

Parameter**	Control rats (n=32) Saline (n=16)	Control rats (n=32) Melatonin (n=16)	Diabetic rats (n=32) Saline (n=16)	Diabetic rats (n=32) Melatonin (n=16)	Significance of Effects, *P*< Diabetes	Significance of Effects, *P*< Melatonin	Significance of Effects, *P*< Interaction
PNL[1]							
d 21	2.00	1.00	3.00	2.00	0.0001	0.0008	0.10
d 42	0.00	0.00	2.00	1.00			
LYM[1]							
d 21	2.00	1.00	3.00	2.00	0.0001	0.0007	0.35
d 42	0.50	0.50	1.50	1.00			
MRFJ[2]							
d 21	2.00	1.00	2.50	2.00	0.004	0.004	0.60
d 42	1.00	1.00	2.00	1.00			
OSTD[1]							
d 21	1.00	1.50	0.00	1.00	0.0001	0.0007	0.05
d 42	2.50	2.50	1.00	2.00			
COLL[1]							
d 21	1.00	2.00	0.00	1.00	0.0001	0.0004	0.04
d 42	3.00	3.00	1.00	2.00			
CRTL[1]							
d 21	1.00	2.00	0.00	1.00	0.0001	0.0002	0.19
d 42	3.00	3.00	1.00	2.00			

* Type I diabetes was induced in half of the rats by administering streptozotocin (60 mg/kg body weight, i.p.). Tibial defects were then created in all rats, which were then administered either saline or melatonin (10 mg/kg body weight, i.p.) for 21 or 42 d. n = 8 for control and diabetic rats on each day measurement.
** PNL: polymorph nuclear leukocyte infiltration; LYM: lymphocyte infiltration; MRFJ: macrophage infiltration; OSTD: osteocyte density; COLL: collagen accumulation; CRTL: cartilage formation; [1] Time effect, P<0.0001; [2] Time effect, P<0.001.

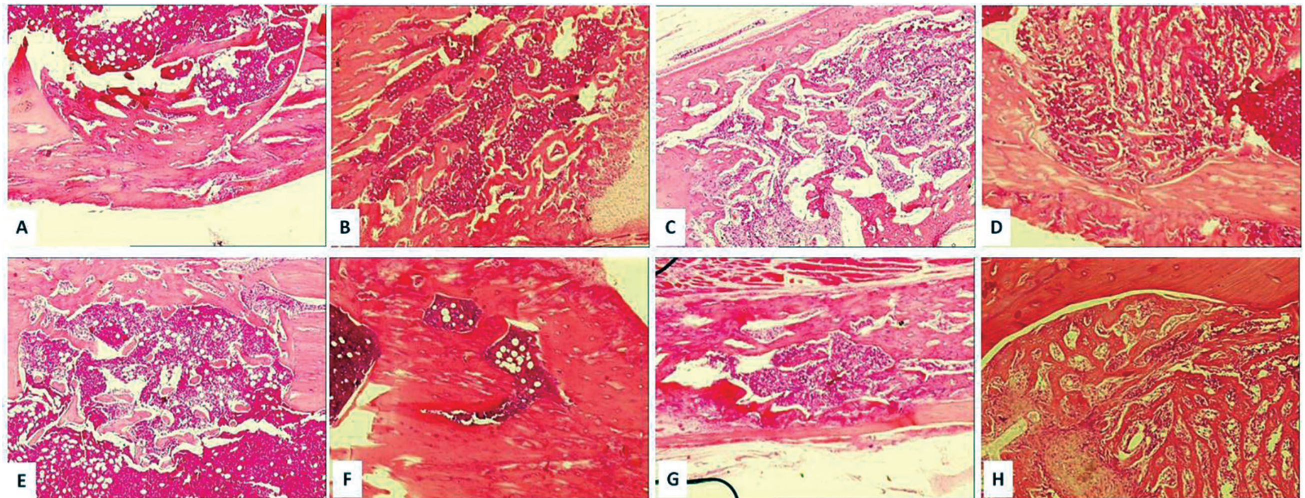

Figure 2: Histological structures of defective areas of the tibia in traumatized control and diabetic rats (H&E×100). Control + saline, minimal osteocyte density and cartilage formation on day 21 (A). Control + melatonin, minimal inflammation and notable collagen accumulation and cartilage formation on day 21 (B). Diabetes + saline, severe inflammation on day 21 (C). Diabetes + melatonin, moderate inflammation and notable collagen accumulation and cartilage formation on d 21 (D). Control + saline, minimal inflammation, increased osteocyte density and cartilage formation on day 42 (E). Control + melatonin, less inflammation 1 and more notable collagen accumulation and cartilage formation on day 42 (F). Diabetes + saline, less inflammation and more notable osteocyte density, collagen accumulation and cartilage formation on day 42 (G). Diabetes + melatonin, less severe inflammation and decreased5 osteocyte density, collagen accumulation and cartilage formation on day 42 (H).

is attributed to the immunostimulatory effect of melatonin, which stimulates the secretion of opioid peptides and cytokines from lymphocytes and prevents the translocation of nuclear factor-kB to the nucleus and its binding with DNA (33). Indeed, the reduction of hyperglycemia severity with melatonin treatment (34) also helps to reduce the severity of inflammation in diabetic subjects (14, 35).

Endocrine pancreas β-cells and pineal gland β-cells have interrelated functions (36). Both melatonin receptors 1A and 1B are expressed in the pancreatic islets, and these are upregulated in diabetes (30). Melatonin enhances insulin-receptor kinase and insulin-receptor substrate-1 phosphorylation, suggesting possible communication between melatonin and insulin pathways. Through its influence on insulin secretion, melatonin is capable of decreasing the severity of hyperglycemia. As in the present study (Table 1), previous rat studies showed that melatonin level was lower in Type 2 diabetic subjects (37) and higher in Type 1 diabetic subjects and STZ-injected animals (30) when compared to normal controls.

Bone remodeling is a constant and dynamic process in which osteoclasts resorb old bone and osteoblasts form new bone (22, 38). Melatonin influences the release of growth hormone and promotes bone formation (39, 40) by suppressing osteoclast activity; stimulating the formation and proliferation of a mineralized matrix and Type I collagen; increasing osteoblast alkaline phosphatase activity through the increased genetic expression of Type I collagen, osteopontin, bone sialoprotein and osteocalcin; and acting as an autacoid (a biological factor exerting its effects like a local hormone for a short period near the site of synthesis) to stimulate bone formation. In ovariectomized rats, decreased serum melatonin levels were accompanied by elevated bone resorption markers (41), suggesting that low melatonin during the post-menopause and post-andropausal periods may involve bone degradation in elderly subjects (42). In rats exposed to irradiation, melatonin exerted protective effects against cellular oxidation (43). The development of scoliosis and decreased vertebral stiffness in pinealectomized fish are indications of the role of melatonin in skeletal development (44); further evidence is apparent in the reductions in bone mineral density and osteocyte cell numbers reported in pinealectomized chickens (45).

As a result of hyperglycemia, diabetes mellitus compromises bone-tissue maintenance and osseous healing by increasing bone resorption, decreasing bone turnover, suppressing cell differentiation and delaying revascularization (10, 46, 47). Melatonin has been shown to speed the regeneration of cortical bone width and length around implants in rabbit (48) and rat tibias (49). When compared to the control rats, the diabetic rats exhibited poor osteointegration between bone marrow and implants, but this was improved with hyperglycemia treatment and the establishment of normoglycemia (50). In another study (9), a 50% reduction in bone formation on the contact surface between bone and tibial implants was found in diabetic rats when compared to the healthy rats, whereas insulin injections improved the ultrastructural characteristics of the bone-implant interface of the diabetic rats to a level similar to non-diabetic rats.

Based on these findings, the authors suggested metabolic control to be essential for osseointegration, as constant hyperglycemia delays the healing of bone surrounding implants. Moreover, osteoclasts generate free radicals, and melatonin administration enhances antioxidant status, thereby contributing to improved fracture healing (51). Collagen also plays an important role in every stage of wound healing, demonstrating a regulatory and stabilizing role in tissue formation (52). In human bone cells and osteoblastic cell lines, melatonin increases collagen accumulation (53, 54). In the present study, the control rats administered with melatonin for 21 and 42 days had reduced inflammatory markers and enhanced osteogenesis indicators (Figure 2, Table 3). These effects were more notable in the diabetic rats (Figure 2, Table 3).

This study demonstrated that hyperglycemia was associated with pathologies in pancreatic β cells and slowed down recovery process of tibial defect in diabetic rats. Melatonin administration caused a partial recovery in pancreas histopathology associated with lowered blood glucose levels, reduced the severity of inflammatory markers (polymorph-nuclear-leukocyte, lymphocyte and macrophage infiltration) and enhanced osteogenesis indicators (osteocyte density, collagen accumulation and cartilage formation).

In conclusion, bone healing can be enhanced by the administration of melatonin through alleviating pancreatic cytopathologies and indirectly controlling hyperglycemia in streptozotocin-induced diabetic rats with tibial defects.

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CONFLICT OF INTEREST

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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