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Melatonin promotes fracture healing in the rat model

Melatonin sıçan modelinde kırık iyileşmesini hızlandırır

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Objectives: In this study, we investigated the effect of melatonin on fracture healing in the rat tibia model by using biochemical and histopathologic methods.

Materials and methods: In this study 80 male Sprague-Dawley rats were randomized into two groups, a control group (Group 1) and melatonin group (Group 2) with eight rats per group according to the day of sacrifice (Days 1, 3, 7, 14 and 28). The fractures were produced by the manual breakage using plate-bending devices, placed at the distal 3rd of the right tibia. Group 2 received 30 mg/kg/day melatonin and group 1 1% alcohol in saline 5 ml/kg/day intraperitoneally during the experiment. Plasma Malondialdehyde (MDA) levels, activity of superoxide dismutase (SOD) and myeloperoxidase (MPO) were measured biochemically. The fracture healing was evaluated using a five-point scale defined by Allen et al.

Results: Malondialdehyde levels in group 2 decreased at days 3, 7, 14, and 28 compared to control values ($p<0.05$). Superoxide dismutase activity in group 2 decreased at days 3, 7 and 14, and returned to the 1st day value after 28 days. Myeloperoxidase values in group 2 decreased at days 1, 3, and 7 ($p<0.001$). Histopathological specimens of healed tibias showed two animals with complete cartilaginous union, five with incomplete bony union and one with complete bony union in the group 2. In contrast, in the group 1, six rats showed complete cartilaginous union and two showed incomplete cartilaginous union ($p<0.05$).

Conclusion: The administration of melatonin maybe beneficial in suppressing the effects of free oxygen radicals and regulating antioxidant enzyme activity in the fracture healing process.

Key words: Antioxidant; fracture healing; malondialdehyde; melatonin; myeloperoxidase; superoxide dismutase.

Amaç: Bu çalışmada sıçan tibia modelinde melatoninin kırık iyileşmesi üzerine olan etkileri biyokimysal ve histolojik yöntemle araştırıldı.

Gereç ve yöntemler: Bu çalışmada 80 adet erkek Sprague-Dawley sıçan kontrol grubu (Grup 1) ve melatonin grubu (Grup 2) olarak iki gruba ayrıldı, her gruptan sekizer sıçan 1, 3, 7, 14 ve 28. günlerde sakrifiye edilecek şekilde randomize edildi. Kırıklar, plak eğme cihazı kullanılarak elle kırma yöntemiyle sağ tibianın distal 1/3'ünde oluşturuldu. Grup 2'ye deney süresince 30 mg/kg/gün melatonin, grup 1'e ise salin solüsyonu içerisinde %1'lik alkol 5 ml/kg/gün intraperitoneal yolla verildi. Plazma malondialdehit (MDA) seviyeleri ile süperoksit dismutaz (SOD) ve miyeloperoksidaz (MPO) aktiviteleri biyokimyasal yolla ölçüldü. Kırık iyileşmesi Allen ve ark.nın beş dereceli skalası kullanılarak değerlendirildi.

Bulgular: Malondialdehit seviyesi, grup 2'de 3, 7, 14 ve 28. günlerde kontrol değerlerine göre düşük bulundu. ($p<0.05$) Süperoksit dismutaz aktivitesi grup 2'de 3, 7 ve 14. günlerde düşük olmasına rağmen, 28. günde ilk günkü değerine ulaştı. Miyeloperoksidaz seviyesi grup 2'de 1, 3. ve 7. günlerde azalmış idi ($p<0.001$). Histopatolojik olarak iyileşen tibiaların örneklerinde: grup 2'de iki sıçanda tam kıkırdak bütünleşmesi, beşinde tam olmayan kemik bütünleşmesi ile birinde tam kemik bütünleşmesi görüldü. Buna karşın, grup 1'de altı sıçanda kıkırdak yapıda tam bütünleşmeyle birlikte yalnızca ikisinde tam olmayan kıkırdak bütünleşme gözlendi ($p<0.05$).

Sonuç: Sonuç olarak melatonin uygulaması serbest oksijen radikallerinin zararlı etkilerini baskılayıp, antioksidan enzim aktivitelerini düzenleyerek kırık iyileşme sürecini olumlu yönde etkilemektedir.

Anahtar sözcükler: Antioksidan; kırık iyileşmesi; malondialdehit; melatonin; miyeloperoksidaz; superoksit dismutaz.

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In the early stages of fracture healing, there is bleeding from the damaged bone ends and associated soft tissues, and clot formations around the fragments. Reduced blood flow to a fracture results in regional ischemic injury in this period. Consequently, fracture callus and the medullary cavity during external callus formation show low oxygen tension.[1,2] In this period, myeloperoxidase (MPO) plays a fundamental role in oxidant production of neutrophils, which are potential source of ROM and are considered to be major effective cells in cellular injury long after tissue ischemia has been reversed. The enzymes, neutrophyl myeloperoxidase and others, released from the polymorphonuclear leukocytes (PMNL) increase the reactive oxygen metabolites (ROM) and cause tissue injury/damage. These ROM which attack polyunsaturated fatty acids in the cell membranes are degraded by this process with consequent disruptions of membrane integrity, known as lipid peroxidation.[3-5] Superoxide dismutase (SOD) is an antioxidative metalloenzyme which catalyses the dismutation of O^{-2} into O_2 and H_2O_2 which affords protection against free radical damage, it can be associated with increased oxidative stress.[6,7]

Melatonin (MEL) (Sigma, St. Louis, MO, USA), produced by the pineal gland, has been shown to be an effective antioxidant and free radical scavenger, and has in vivo and in vitro activity because of its lipophylic property which can easily enter the nucleus and other organelles. In addition to this, it protects the cell and deoxyribonucleic acid (DNA) from the oxidative damage.[8-11] Also, in recently published studies, the in vitro osteoblastic differentiation and bone formation effect has been demonstrated.[12] However, the effect of MEL on fracture healing in rat tibia model has not been studied to date.

In this study, we aimed to investigate the effect of MEL on fracture healing in rat tibia model, and to show the role of MEL in antioxidant defense system and fracture healing by biochemical and histopathologic methods.

MATERIALS AND METHODS

Animals

Eighty male Sprague-Dawley rats weighing 287 to 332 g (average 302 g) were used in this study. The rats were kept in a room at a constant temperature 23±2 °C, with 14/10 hours light and dark cycles, in individual cages and fed standard rat chow. All procedures were performed in the Experimental Animals Breeding and Research Center of Medical Faculty of Erciyes University Animal care was carried out with the prior approval of the University Animal Experimental Ethics Committee, and was in full compliance with Turkish Law 6343/2, Veterinary Medicine Deontology Regulation 6.7.26, and with the Helsinki Declaration of Animal Rights.

Experimental groups

The animals were randomly divided into two groups: control (Group 1; n=40) and MEL (Group 2; n=40), with eight rats per group according to the day of sacrifice (Days 1, 3, 7, 14 and 28). The total duration of the experiments in each group was the same. Rats in group 1 were determined as control, and group 2 as the melatonin group. The fractures were produced by the manuel breakage using plate-bending devices, placed at the distal third of the right tibia under Ketamine (20 mg/kg) and Xylazine (5 mg/kg) anesthesia. The rat was performed anterolateral incision, exposing the tibial fracture line where a 0.8 mm to 1 mm Kirschner wire (K-wire) was employed to make an opening of the medullar canal placed in to the foot and procedure was completed retrograde approach. After each procedure, all animals were X-rayed anteroposteriorly (AP) and laterally.

Group 2 received 30 mg/kg/day MEL intraperitoneally (i.p.) during the experiment. Melatonin was dissolved in absolute ethanol and further dilutions were made in saline. The final concentration of ethanol was 1%. The group 1 was administrated 1% alcohol in saline (5 ml/kg/day) i.p. during the experiment.

Biochemical assay

Blood samples for biochemical testing were taken from the right femoral vein of the rats. After separating the plasma, samples were stored at –20 °C till analyses. Plasma malondialdehyde (MDA) levels were measured by the double heating method of Jain[13] and expressed in nmol/mL. Enzymatic activity of SOD was measured according to Sun et al.[14] and was expressed as U/mL. Myeloperoxidase activity was determined as described by Klebanoff[15] and specific activity was expressed as U/L.

Histopathological analysis

Both the right and left legs were amputated for histological examinations. Tissues were embedded in paraffin wax, and 5 μm-thick sections after decalcification (12% Ethylene diamine tetraacetic acid) were stained with Hematoxylen-Eosine (H-E) in routine fashion and examined by light microscopy. The fracture healing was evaluated using a five-point scale defined by Allen et al.[16] According to those authors, at grade IV the bone union is complete, at grade III the bone union is not complete yet due to the presence of a small amount of cartilage in the callus, at grade II there is a well-formed hyaline cartilage bridge connecting both ends (complete cartilage union), at grade I the cartilage union is incomplete with fibrous element retention in the chondral plate, and at grade 0 there is an absence or delay in fracture repair, characterized by the presence of cartilage among the fragments and remnants of hematoma or another fluid (pseudarthrosis).

Statistical analysis

Mann-Whitney U-test was used to compare the plasma oxidant-antioxidant activities (MDA, MPO, and SOD) between the groups. Daily variation was evaluated by Friedman analysis in each group. The analysis of parameters in each group was carried out using Wilcoxon rank test. Chi-square was used to compare the histological parameters between the groups. Statistical significance was set at a level of $p<0.05$.

RESULTS

Biochemical results

Malondialdehyde levels decreased gradually in group 1 and regained the first three days values (0.96±0.03 nmol/ml) after 28 days (0.95±0.25 nmol/ml; $p<0.05$). Malondialdehyde levels in group 2 decreased at the days 3, 7, 14, and 28 (0.32±0.04 nmol/ml, 0.41±0.22 nmol/ml, 0.6±0.24 nmol/ml, 0.21±0.03 nmol/ml, respectively) as compared to control values ($p<0.05$). The lowest MDA values were obtained on day 28 in the group 2 (0.21±0.03 nmol/ml; $p<0.001$; Table I).

Superoxide dismutase activity in group 2 decreased at the days 3, 7, and 14 (0.82±0.06 U/mL, 0.97±0.11 U/mL, 0.64±0.25 U/mL) as compared to control values, and regained the first day value (1.19±0.16 U/mL) after 28 days (1.23±0.1 U/mL), and stayed level as control ($p<0.001$). However, the SOD level did not change, and similar values were obtained during the study in the group 1 ($p>0.05$; Table I).

Myeloperoxidase activity decreased gradually in the group 1 and regained the first day values (126.72±24.72 U/L) after 28 days (101.51±17.18 U/L; $p<0.001$). Myeloperoxidase values in group 2 decreased at the days 1, 3, and 7 (86.45±6.42 U/L, 51.08±6.06 U/L, 69.3±19.49 U/L, respectively) as compared to control values, and regained similar values after 14 days (72.82±22.38 U/L) and stayed level as control ($p<0.001$; Table I).

Histopathological evaluation

According to the criteria of Allen et al.[16] used to assess histopathological specimens of healed tibias, there were two animals with complete cartilaginous union, five with incomplete bony union and one with complete bony union in the group 2 (Figure 1). In contrast, in the group 1, six animals showed complete cartilaginous union and two

TABLE I

Plasma MDA, SOD and MPO levels on days 1, 3, 7, 14, and 28 in control and melatonin groups (Mean±SD)

Days	MDA (nmol/ml)		SOD (U/mL)		MPO (U/L)	
	Control (n=8)	MEL (n=8)	Control (n=8)	MEL (n=8)	Control (n=8)	MEL (n=8)
1	0.54±0.27[a]	0.59±0.28[f]	1.31±0.07[a]	1.19±0.16[f]	126.72±24.72[a]	86.45±6.42[f]
3	0.96±0.03[b]	0.32±0.04[g]	1.26±0.02[b]	0.82±0.06[g]	74.02±17.07[b]	51.08±6.06[g]
7	1.1±0.11[c]	0.41±0.22[h]	1.34±0.03[c]	0.97±0.11[h]	119.43±5.49[c]	69.3±19.49[h]
14	1.14±0.39[d]	0.6±0.24[k]	1.31±0.1[d]	0.64±0.25[k]	64.57±21.29[d]	72.82±22.38[k]
28	0.95±0.25[e]	0.21±0.03[m]	1.41±0.41[e]	1.23±0.1[m]	101.51±17.18[e]	101.41±6.62[m]
	p<0.05	p<0.001	p>0.05	p<0.001	p<0.001	p<0.001

p<0.05: Control group MDA: Between a-b, a-c*, a-d*, a-e* and b-c*; MEL group MDA: Between f-g*, f-h*, f-m*, g-k*, g-m*, h-k* and h-m*; MEL group SOD: Between f-g*, f-h*, f-k*, g-h*, g-k*, h-k*,h-m* and k-m*; Control group MPO: Between a-b*, a-d*, b-c*, b-e*, c-d*, c-e* and d-e*; MEL group MPO: Between f-g*, f-h*, f-m*,g-k*, g-m* and h-m*.

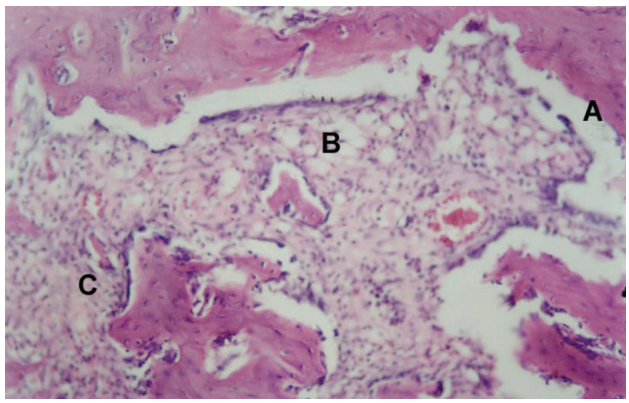

Figure 1. Photomicropgraphs showing complete bony union in the melatonin group (H-E x 200). A: Mature osteocytes; B: Bone marrow; C: Osteoblastic activity.

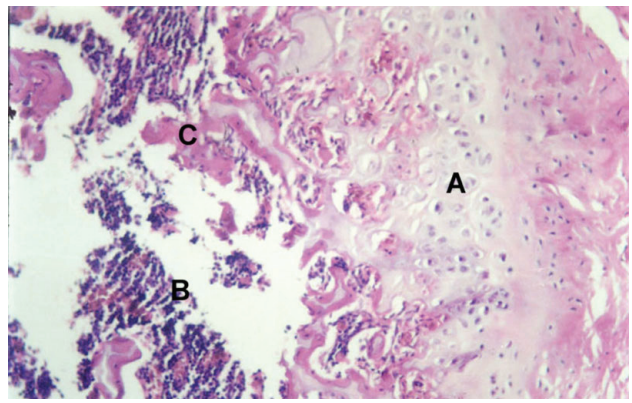

Figure 2. Photomicropgraphs showing incomplete bony union in the control group (H-E x 200). A: Cartilage island; B: Bone marrow; C: Endochondral ossification.

showed incomplete cartilaginous union (Figure 2). Fracture healing was significantly more advanced in the group 2 ($p<0.05$). Radiological results showed complete bony union in the group 2 (Figure 3).

DISCUSSION

McKibbin[1] suggested that fracture callus and medullary cavity during external callus formation show low oxygen tension and that arteriolar vasoconstriction develops in the fracture site because of interruption of vascular supply in the beginning of this period. Following this period, with the arteriolar vasodilatation and increase of reperfusion in the fracture zone, inflammatory cells (leukocytes, macrophage and mast cells) migrate to the hematoma fluids.[17-18] In contrast, blood flow increased not only at the fracture site, but also throughout the cortex on day nine,[19] which results in the production of free oxygen radicals as in ischemia and reperfusion injury.[3,4] Göktürk et al.[20] reported that free oxygen radicals have a negative effect on fracture healing. Durak et al.[4] reported that the cause of negative effect is the activation of polymorphonuclear leukocytes (PMNL) affected fracture healing negatively in experimental animal model.

The pineal hormone MEL was recently shown to have a free radical scavenging ability which reduces lipid peroxidation and has also been

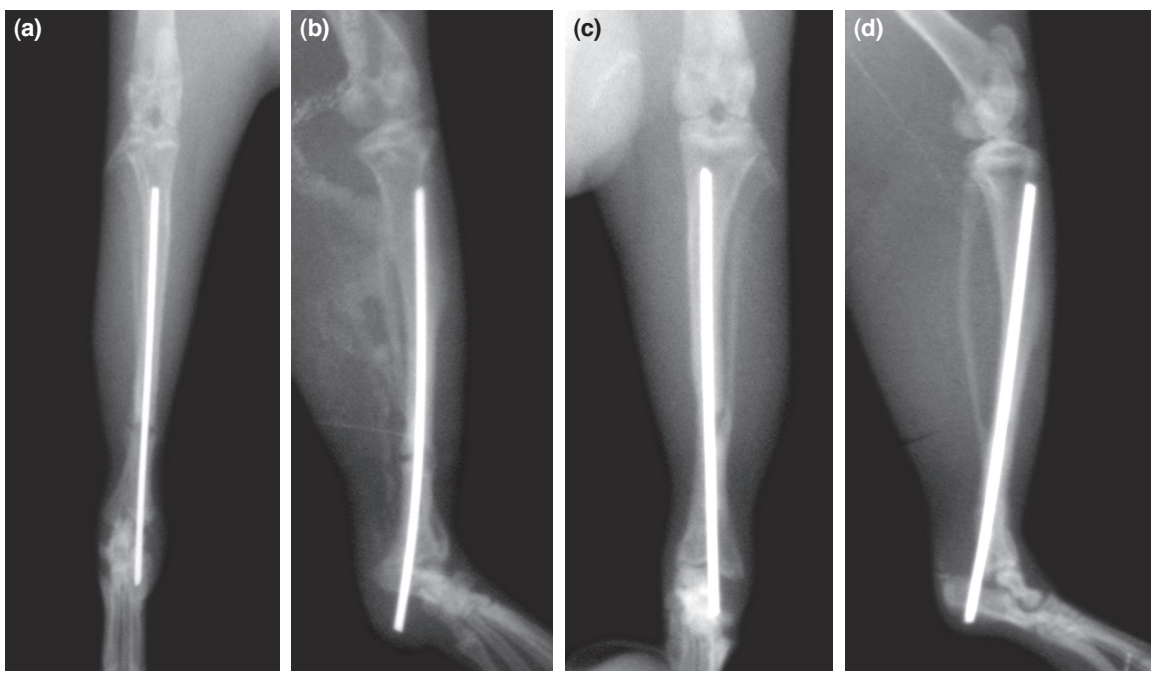

Figure 3. Radiological evaluation of fracture healing at 28 days. a-b: Control group; c-d: Melatonin group.

shown to scavenge the hydroxyl radical. The antioxidant activity of MEL probably derives from its stimulatory effect on SOD, glutathione reductase and glucose-6-phosphate dehydrogenase, and its inhibitory action on nitric oxide synthase. Finally, MEL acts to stabilize cell membranes, thereby making them more resistant to oxidative attacks.[8-11,19,21]

Our results demonstrate that antioxidant MEL inhibits MDA elevation significantly and that it reverses back to control levels ($p<0.05$). The cause of low levels of MDA in the first few days is the presence of hyaluronic acid.[22] Furthermore, based on current findings, the protective effect of MEL, in part, may be attributed to its stimulatory effect on SOD release, similar to MPO activity ($p<0.001$).

It is also evident from previously reported studies that the osteoclasts generate high levels of superoxide anions during bone resorption, which contributes to the degradative process. One mechanism for their removal is via the protective superoxide-scavenging enzyme SOD.[23] Oxygen-derived free radicals, and particularly the superoxide anion, are intermediaries in the formation and activation of osteoclast. Parathyroid hormone, interleukin-1, and tumor necrosis factor stimulated bone resorption was inhibited by natural and recombinant SOD, an enzyme which depletes tissues of superoxide anions.[24] The studies suggest that MEL dose-dependently increases the proliferation normal human bone cells and human osteoblastic cell lines. Melatonin also increases procollagen type Ic-peptide production in these cells.[25] In another study, MEL was shown to modulate in vitro expression of rat bone sialoproteins, including alkaline phosphatase, osteopontin, secreted protein and osteocalcin.[12] Similar study, demonstrated that MEL possessed an enhancing action on proliferation and differentiation of human osteoblast, but its mechanism unclear. The mechanism of this stimulatory effect of bone formation may be direct actions of MEL on osteoblasts as demonstrated by Roth et al.[12] or on the one hand, by indirect actions via secretion of growth hormone and cortisol or downregulation of the receptor activator of nuclear factor kappa-light-chain-enhancer of activated B cells ligand-mediated osteoclast formation activation stimulated by MEL.[26] Therefore, MEL appears to be capable of promoting osteoblast differentiation and mineralization of matrix in vitro.

Melatonin is a significant free radical scavenger and antioxidant at both physiological and pharmacological concentrations[8] but the antioxidant effect of MEL in humans probably occurs at pharmacological concentrations. No serious side effects or risks have been associated with the use of MEL. The physiological dose-dependent effects of the hormone, however, have not yet been fully evaluated in those receiving large doses for prolonged time periods.[27] Lieberman et al.[28] suggested that MEL, administered in pharmacological quantities (240 mg/day), has significant but short acting sedative-like properties. In recent studies, MEL at pharmacological doses (30 mg/kg) showed significant protective effects against skeletal muscle ischemia-reperfusion injury.[29] In addition to its ability to directly neutralize a number of free radicals and reactive oxygen and nitrogen species, MEL stimulates several antioxidative enzymes, which increases its efficiency as an antioxidant. Also, MEL stimulates osteoblast differentiation and mineralization of matrix and it may regulate osteoclastic activity via SOD in vitro. Therefore, the effect of MEL in fracture healing depends in part on the free radical scavenging and osteoblastic-osteoclastic regulatory properties of MEL.

In conclusions, MEL has a positive effect on fracture healing at pharmacological doses and may be beneficial as a supportive agent in fracture cases.

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Declaration of conflicting interests

The authors declared no conflicts of interest with respect to the authorship and/or publication of this article.

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