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ORIGINAL ARTICLE

Melatonin can Ameliorate Radiation-Induced Oxidative Stress and Inflammation-Related Deterioration of Bone Quality in Rat Femur

Zelal Ünlü Çakir,[1] Can Demirel,[1,6] Sevil Cagiran Kilciksiz,[2] Serkan Gürgül,[3] S. Burhanedtin Zincircioğlu,[4] and Nurten Erdal[5]

ABSTRACT—The aim of the present study was to evaluate the radioprotective effects of melatonin on the biomechanical properties of bone in comparison to amifostine (WR-2721). Forty Sprague Dawley rats were divided equally into 5 groups namely; control (C), irradiation (R; single dose of 50 Gy), irradiation + WR-2721 (R + WR-2721; irradiation + 200 mg/kg WR-2721) radiation + melatonin 25 mg/kg (R + M25; irradiation + 25 mg/kg melatonin), and radiation + melatonin 50 mg/kg (R + M50; irradiation + 50 mg/kg melatonin). In order to measure extrinsic (organ-level mechanical properties of bone; the ultimate strength, deformation, stiffness, energy absorption capacity) and intrinsic (tissue-level mechanical properties of bone; ultimate stress, ultimate strain, elastic modulus, toughness) features of the bone, a three-point bending (TPB) test was performed for biomechanical evaluation. In addition, a bone mineral density (BMD) test was carried out. The BMD and extrinsic properties of the diaphyseal femur were found to be significantly higher in the R + M25 group than in group R ($p < 0.05$). A significant increase was observed in R + M50 ($p < 0.05$) in comparison to group R in the cross-sectional area of the femoral shaft and elastic modulus parameter. The protective effect of melatonin was similar to that of WR-2721. Thus, biomechanical quality of irradiated bone can be ameliorated by free radical scavenger melatonin.

KEY WORDS: bone quality; biomechanics; radiotherapy; radioprotection; antioxidants; inflammation; melatonin; WR-2721.

INTRODUCTION

High-dose irradiation has detrimental effects on the bone tissue such as osteoradionecrosis, sclerosis, loss of bone mass, and bone fracture [1, 2]. A recent study found that a cumulative incidence of pelvic fracture was 13 % in females after radiotherapy over 5 years [3]. Studies have shown that irradiation can deteriorate bone formation as it may hinder osteoblast proliferation and differentiation, induce cell-cycle arrest and direct cell death, damage microvascular structures, decrease collagen production, and may cause a reactive inflammatory reaction [1, 2, 4–8]. Radiotherapy-related bone necrosis usually results in sclerotic bone change of the trabeculae and cortex accompanied by decreased mechanical strength and repair capacity.

[1] Department of Biophysics, Faculty of Medicine, Gaziantep University, Gaziantep, TR-27310, Turkey
[2] Department of Radiation Oncology, Kolan International Hospital, Istanbul, TR-34000, Turkey
[3] Department of Biophysics, Faculty of Medicine, Gaziosmanpaşa University, Tokat, TR-60000, Turkey
[4] Department of Radiation Oncology, Faculty of Medicine, Dicle University, Diyarbakır, TR-33169, Turkey
[5] Department of Biophysics, Faculty of Medicine, Mersin University, Mersin, TR-33169, Turkey
[6] To whom correspondence should be addressed at Department of Biophysics, Faculty of Medicine, Gaziantep University, Gaziantep, TR-27310, Turkey. E-mail: cdemirel27@gmail.com

As a result, the bone becomes more vulnerable to stress fractures [9].

Studies have shown protection from functional and histopathological damage by various agents administered both before and after irradiation [10–12]. Free radical scavenging is one of the mechanisms to protect from radiation damage on normal tissue and it relies on the assumption that radiation damage to cells is primarily caused by free radicals formed by the radiolysis of water [13]. It is suggested that the first approved radioprotective drug, amifostine, provides major radioprotection for bone cells while it does not protect tumor cells [14].

Melatonin is known for its supportive effect on the bone structure [15–18]. The free radical scavenging cascade reaction is a unique feature of melatonin, and it makes melatonin different from other conventional antioxidants [19–21]. Its interaction with free radicals appears to be a long process involving many of its derivatives. Melatonin's receptor-dependent functions can be listed as circadian rhythm regulation, sleep, and cancer inhibition. Receptor-independent actions, on the other hand, are related to its ability to act in the detoxification of free radicals, which protects critical molecules from the destructive effects of oxidative stress under ischemia/reperfusion injury, ionizing radiation, and drug toxicity, and some others [19–21].

In the study on bone grafts exposed to high doses of radiation, it has been reported that the use of different free radical protectors contribute to the formation of biomechanically stable bone grafts [22]. The related literature has suggested that scavenging free radicals can reduce gamma radiation-induced biochemical damage to the bone's collagen, which will occur together with a decrease in the extent of biomechanical impairment secondary to gamma radiation sterilization [22].

We therefore hypothesized in our study that, due to its strong free radical scavenging ability, melatonin may have a radioprotective function on the biomechanistic properties of a bone exposed to radiation. In the present study, by using geometric parameters and biomechanical parameters of radiation-damaged bones, the radioprotective effect of melatonin application was examined in comparison to that of amifostine.

MATERIAL AND METHODS

Animal Preparation and Experimental Protocol

All experiments and protocols described in the present study were performed in accordance with the guidelines of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes and also approved by the Medical Faculty Experimentation Ethics Committee of Gaziantep University. A total of 32 male Sprague Dawley rats (12-week-old; 181 ± 11 g) were used. They were housed under standardized condition (12 h light/dark cycle, 21 ± 2 °C, relative humidity 50–70 %) and provided free access to standard rat nutrients and purified drinking water *ad libitum*. Following 1 week of acclimatization prior to the experimental procedures, the rats were assigned randomly into 4 equal-sized groups (8 per group), namely control (C), irradiation (R), irradiation plus 25 mg/kg melatonin (R + M25), irradiation plus 50 mg/kg melatonin (R + M50), and irradiation plus 200 mg/kg amifostine (WR2721; R + WR). C rats received no medication or irradiation, but 2.2 ml of saline was injected intraperitoneally (ip.). The rats in groups R, R + M25, R + M50, and R + WR received γ-irradiation, as a single dose of 50 Gy, and the left leg of the each rat was subjected to irradiation at a distance of 80 cm. The ^{60}Co radiation was delivered from two opposite fields. A cobalt-60 teletherapy unit (ALCYON-II, CGR, MeV, France) was used for all irradiations.

Besides irradiation, rats in R received 2.2 ml of saline (ip.), while the rats in R + WR, R + M25, and R + M50 received 200 mg/kg WR-2721 (ip., Ethyol flacon, Er-Kim Ilac, Istanbul) and 25 and 50 mg/kg of melatonin (ip., Sigma-Aldrich, Interlab Inc., Istanbul, Turkey), respectively. Saline, melatonin, and WR-2721 injections in the irradiation groups were done 15 min before the irradiation. The study was terminated after 4 weeks. At termination, the left femur of each animal was harvested under Ketalar (Eczacıbası, Turkey) anesthesia (70 mg/kg, intramuscularly) and the femur length (L_0; mm) was measured with a digital clipper for the biomechanical evaluation. Later, femurs were stored at −20 °C until densitometric and mechanical testing.

Densitometric Measurements

After thawing at room temperature, femurs of rats in each group underwent densitometric evaluation. Whole femur bone mineral density (BMD) was measured by dual-energy X-ray absorptiometry (DEXA; Norland 45 XR, Norland Scientific Instruments, Fort Atkinson, WI, USA), and the analysis was performed using Illuminatus-based DEXA software (Version 4.2.0) in a "small subject" protocol. Results were represented as grams per square centimeter.

Geometric Measurements

To examine the geometric features of the femurs, cross-sectional area (cortical area; CSA, mm^2) at the femoral mid-shaft in both medio-lateral (ML) and anterior-posterior (AP) planes were measured by computerized tomography (Toshiba Aquilion 64 Slice CT, Toshiba American Medical Systems, Tustin, CA, USA) with a slice thickness of 0.5 mm. The endosteal (END) and periosteal (PER) diameters at the femoral mid-shaft in ML and AP planes were measured for calculating cross-sectional moment of inertia (CSMI; mm^4). The data were analyzed using Vitrea software (Version 2.0, Minnetonka, MN, USA) and also evaluated by point counting method.

Biomechanical Test

For the biomechanical evaluation, three-point bending (TPB) test was performed to measure extrinsic (organ-level mechanical properties of bone: the ultimate strength, deformation, stiffness, energy absorption capacity) and intrinsic (tissue-level mechanical properties of bone: ultimate stress, ultimate strain, elastic modulus, toughness) features of the bone. After performing the densitometric and geometric measurements, samples were tested using a biomaterial testing machine (MAY TPBM 2113, Commat Ltd., Ankara, Turkey). For the test, the femur bone was mounted horizontally on two supporting bars located at a distance of 14 mm, centered on the supports, and the load was applied vertically in all tests to the mid-shaft of the femoral diaphysis in the ML plane with a constant speed of 1 mm/s until failure [23, 24]. Load–deformation data were recorded and analyzed using Logger Pro software (Version 3.8.3, Vernier Software & Technology, Orlando, FL, USA). Ultimate strength (the force causing bone fracture; N), ultimate displacement (deformation at the fracture point; mm), stiffness (the slope of the linear (elastic) part of the load–deformation curve, N/mm), and energy absorption capacity (the area under the load–deformation curve within the elastic range; mJ) were determined from this curve. The load–deformation curve obtained from each specimen was converted into stress–strain curve to evaluate the intrinsic features of bone. The ultimate stress (MPa), ultimate strain (mm/mm), elastic modulus (GPa), and toughness (MPa) were determined from this curve. Stress–strain curves were generated using Logger Pro software (Vernier Software & Technology) according to the following formulas [25]:

$$\sigma_U = F_U \cdot \left(\frac{L \cdot C}{4 \cdot CSMI_{AP}}\right); \varepsilon_U = d_U \cdot \left(\frac{12 \cdot c}{L^2}\right); E = S \cdot \left(\frac{L^3}{48 \cdot CSMI_{AP}}\right); u = U \cdot \left(\frac{3 \cdot c^2}{CSMI_{AP} \cdot L}\right)$$

For the calculations, c (mm) is the half-diameter of the mid-shaft in the load direction (ML direction; $c = PER_{ML}/2$). The moment of inertia about the bending axis, $CSMI_{AP}$ (mm^4), was calculated according to the following formula [26]:

$$CSMI_{AP} = \frac{\pi}{64} \cdot \left[\left(PER_{AP}^3 \cdot PER_{ML}\right) - \left(END_{AP}^3 \cdot END_{ML}\right)\right]$$

Statistical Analysis

The results were expressed as a mean ± standard deviation of mean (SD). Data analyses were performed on SPSS version 10.0.6 for Windows. Values were checked for normal distribution using the Shapiro–Wilk test, and the results were evaluated with one-way analysis of variance (ANOVA). Post hoc Kruskal–Wallis test was utilized to identify differences between the experimental groups. $p < 0.05$ was the value accepted as statistically significant.

RESULTS

Geometric Properties and Bone Marrow Density of the Diaphyseal Femur

Table 1 shows the BMD and geometric properties of the diaphyseal femur. Experimental groups were homogenous considering the femur length. Femur BMD in group R was significantly lower when compared to group C ($p < 0.05$). BMD was significantly higher in the R + M25 group than in the R group ($p < 0.05$) and in the C group ($p < 0.05$). BMD was significantly higher in the R + WR group than in the R group ($p < 0.05$). There was a significant reduction in the cross-sectional area of the femoral shaft in group R ($p < 0.05$), while a significant increase was determined in group R + M50 ($p < 0.05$) when compared to group R. There was a significant reduction in the cross-sectional area of the femoral shaft in the R + WR when compared group C ($p < 0.05$).

Table 1. Geometric Properties and Bone Mineral Density (BMD) of the Rat Diaphyseal Femur

Parameters	C	R	R + M25	R + M50	R + WR
Length (mm)	28.63 ± 0.69	27.85 ± 0.48	27.77 ± 0.61	27.86 ± 0.59	28.62 ± 1.12
Bone mineral density (g/cm^2)	0.104 ± 0.003	0.092 ± 0.009^{a†}	0.111 ± 0.002$^{a\dagger,b''}$	0.093 ± 0.003	0.104 ± 0.002^{b†}
Cross-sectional area (mm^2)	10.75 ± 0.63	7.00 ± 0.53^{a†}	8.40 ± 1.02	8.67 ± 0.31^{b†}	7.67 ± 0.31^{a†}

Each group consisted of 8 rats.
WR amifostine, *M* melatonin, *C* control rats that were treated with 2.2 ml of saline, *R* rats which hind legs were exposed to 50 Gy and were treated with 2.2 mL of saline, *R + M25* rats that were exposed to the same irradiation procedure as R rats plus treatment with 25 mg kg^{-1} melatonin, *R + M50* rats that were exposed to the same irradiation procedure as R rats plus treatment with 50 mg kg^{-1} melatonin, *R + WR* rats that were exposed to the same irradiation procedure as R rats plus treatment with 200 mg kg^{-1} amifostine
$^{\dagger}p < 0.05$, $^{*}p < 0.01$, $^{''}p < 0.001$, $^{\ddagger}p < 0.0001$
^{a} Compared to C rats
^{b} Compared to R rats

Mechanical Parameters of the Diaphyseal Femur

Table 2 shows the mechanical parameters of the diaphyseal femur. After irradiation, all "mechanical endpoints except for elastic modulus and ultimate stress were significantly lower in group R than in group" C ($p < 0.05$). In R + M25, while there was a statistically significant ($p < 0.05$) decrease in all mechanical parameters except for stiffness, energy absorption capacity and AP direction $CSMI_{AP}$ in comparison to group C, a statistically significant ($p < 0.05$) increase was observed in ultimate displacement, ultimate strength, stiffness, and energy absorption capacity parameters with 25 mg/kg melatonin application in comparison to group R. Only the elastic modulus value increased after 50 mg melatonin application compared with group R ($p < 0.05$). Likewise, a decrease ($p < 0.05$) was found in all values except for energy absorption capacity and AP direction $CSMI_{AP}$ in the mechanical parameters of R + M50 in comparison with group C. With the amifostine application, a significant increase ($p < 0.05$) was absorbed in the ultimate displacement, ultimate strength, energy absorption capacity, and toughness parameters of R + WR compared to group R. There was also an increase in the ultimate stress and elastic modulus parameters of this group in comparison with group C ($p < 0.05$). A decrease ($p < 0.05$) was observed in the same group in ultimate strength, stiffness, and AP direction $CSMI_{AP}$ in comparison to group C.

Table 2. Mechanical Parameters of the Rat Diaphyseal Femur

Parameters	C	R	R + M25	R + M50	R + WR
Ultimate displacement (mm)	0.61 ± 0.22	0.13 ± 0.09$^{a''}$	0.22 ± 0.06$^{a'',b\dagger}$	0.16 ± 0.01$^{a''}$	0.58 ± 0.18^{b†}
Ultimate strength (N)	60.78 ± 0.79	40.31 ± 4.31$^{a''}$	51.06 ± 2.12$^{a'',b*}$	46.22 ± 3.50$^{a''}$	49.34 ± 1.37$^{a'',b*}$
Stiffness (N/mm)	586.4 ± 34.1	424.7 ± 29.7$^{a''}$	524.7 ± 62.8^{b†}	415.7 ± 39.0$^{a''}$	455.5 ± 57.1^{a†}
Energy absorption capacity (mJ)	19.87 ± 8.52	10.85 ± 0.52^{a*}	14.41 ± 3.16^{b†}	13.01 ± 2.59	15.82 ± 2.19^{b*}
Moment of inertia ($CSMI_{AP}$ (mm^4))	7.18 ± 0.46	4.70 ± 0.93^{a*}	6.13 ± 1.55	5.48 ± 0.39	3.55 ± 0.24^{a†}
Ultimate stress (MPa)	60.26 ± 5.52	67.78 ± 23.54^{a*}	58.03 ± 11.43^{a†}	61.36 ± 4.05^{a†}	94.87 ± 5.54^{a*}
Ultimate strain (mm/mm)	0.083 ± 0.016	0.031 ± 0.002^{a*}	0.032 ± 0.011^{a*}	0.020 ± 0.001$^{a''}$	0.061 ± 0.018
Elastic modulus (GPa)	0.76 ± 0.16	2.10 ± 0.64	2.19 ± 0.95^{a†}	3.17 ± 0.09$^{a'',b\dagger}$	2.12 ± 0.82^{a†}
Toughness (MPa)	3.25 ± 1.63	2.31 ± 0.48^{a†}	2.32 ± 0.52^{a†}	2.28 ± 0.48^{a†}	3.31 ± 0.40^{b†}

Each group consisted of 8 rats.
WR amifostine, *M* melatonin, *C* control rats that were treated with 2.2 ml of saline, *R* rats which hind legs were exposed to 50 Gy and were treated with 2.2 mL of saline, *R + M25* rats that were exposed to the same irradiation procedure as R rats plus treatment with 25 mg kg^{-1} melatonin, *R + M50* rats that were exposed to the same irradiation procedure as R rats plus treatment with 50 mg kg^{-1} melatonin, *R + WR* rats that were exposed to the same irradiation procedure as R rats plus treatment with 200 mg kg^{-1} amifostine
$^{\dagger}p < 0.05$, $^{*}p < 0.01$, $^{''}p < 0.001$, $^{\ddagger}p < 0.0001$
^{a} Compared to C rats
^{b} Compared to R rats

DISCUSSION

The effect of melatonin as a radioprotector has been the subject of many studies on tissues and organs due to its antioxidant property [27]. Melatonin's molecular weight is 232.278 g/mol. It has been found that compounds above 300 g/mol molecular weight cannot penetrate the bone structure [18]. Studies have shown that melatonin both affects bone metabolism and supports skeletal growth and bone formation, mainly by inhibiting bone resorption [15, 17]. Although melatonin is known for its supportive effect on the bone structure, there are very few studies on its protective effects on bone biomechanical properties concerning radiation-related bone damage [15, 16, 28, 29]. In the present study, the radioprotective effect of melatonin on the biomechanical parameters of bone was investigated on rats with bone damage in the lower extremity and findings were obtained regarding its protective effect from radiation-dependent bone damage. In addition, it is considered that the positive findings obtained from the application of amifostine may have additional contribution in expanding the future clinical use of amifostine and provide evidence to comparatively evaluate the radioprotective effect of melatonin on bone tissue.

Radiation may lead to various lesions due to direct interaction with DNA or indirectly by damage caused by free radicals. Damage due to radiation is a process which has direct relation to inflammation [13]. It has been referenced that melatonin decreases damage occurring due to inflammation in radiation and several inflammation models other than radiation (burn damage, sepsis, ischemia/reperfusion, *etc*.) [19, 28, 30–33]. Since it can directly scavenge toxic free radicals which may cause inflammatory response and associated tissue destruction, melatonin decreases macromolecular damage in all organs [32, 33].

Radiation-related changes and fracture are rather associated with the repair and reshaping which plays a potential role in the pathogenesis of osteoradionecrosis. Among these changes are suppression of bone formation, an elevation of bone resorption, or even the destruction of the acellular components of the bone matrix with its organic and inorganic components or a combination them [34, 35]. Structural parameters are closely related to bone quality and toughness (bone strength) [36–38].

In our study, the presence of the statistically significant decreases observed in group R in the ultimate displacement, ultimate strength, stiffness, and energy absorption capacity, which are structural parameters of bones, is a strong proof that radiation deteriorates structural parameters. Stiffness of the femur is associated with the amount of ultimate strength and deformation and any decrease in these parameters is an indicator of crystallization in bone tissue [36, 37]. The decrease observed in the ultimate strength in group R in our study can be interpreted as the intensity of the radiation applied reduced femoral strength. Fracture energy is a significant indicator of fragility and flexibility of materials that are forced to be pulled [36, 37]. Increases observed in the structural parameters with the application of 25 mg/kg of melatonin can be considered as evidence for the positive contribution of the radioprotective effect of this dose on related parameters.

As also observed in our study, it is known that BMD, which is an indirect indicator of osteoporosis and bone damages in practice, decreases in the radiation-induced bone damage models [12, 36–38]. Damage to osteoblasts and osteocytes is considered to contribute significantly to the reduced BMD observed following irradiation [4, 5]. In the present study, the observation that melatonin 25 mg/kg application, like in the amifostine group, increased BMD in bones exposed to radiation at a statistically significant level may indicate the radioprotective effect of melatonin and amifostine applications prior to radiation on bone mineralization.

Biomechanical properties such as ultimate stress, ultimate strain, elastic modulus (Young module), and toughness are parameters that are used to evaluate bone fragility and strength [36–38]. Changes in these parameters, which indicate the biomechanical wholeness of the bone structure, are indicators of many conditions including crystallization, mineralization disorders, and collagen deformation, and therefore they show that the material properties of bone are damaged [36–38]. In our study, changes in the biomechanical parameters of group R indicate considerable deformation in bone quality due to radiation; in other words, they show degeneration, damaged bone wholeness, and that the bone's resistance to fragility has decreased. However, partial improvement was observed in these parameters with the 50 mg melatonin or amifostine application.

It has been reported that melatonin has a protective effect on bone growth in rats given fractional radiotherapy [28]. In a preclinical study, Suzuki *et al*. found that osteoclastic and osteoblastic activities were suppressed by melatonin [39]. The total bone mineral density of the femoral metaphysis significantly increased after the administration of 1-benzyl-2,4,6-tribromomelatonin orally in rats on a low-calcium diet [39]. With its abundance in the marrow, melatonin also increases matrix proteins in a dose-dependent manner [39]. Melatonin application has reduced bone loss in rats with ovariectomy [40].

The findings we obtained and some other studies as well suggest that the effect of melatonin on the bone is a

considerably complex effect determined by all the hormonal and structural factors and that this effect may be dependent on dose [37, 38, 41]. This protective effect of melatonin is considered to be the result of the antioxidant effect formed on the osteoblasts and osteoclasts which regulate bone formation and destruction. Considering all these findings obtained from biomechanical and densitometric parameters, it has been observed in the study that radiation has damaging effects on both structural and material properties and that the improvement in the structural parameters with the 25 mg/kg melatonin application is similar to amifostine.

In conclusion, it could be claimed that the present study has determined a radioprotective effect of melatonin on the biomechanical parameters and the mineral density of the bone against radiation-induced damage. Melatonin application against radiation damage can improve therapeutic index used in the clinic like amifostine and may be beneficial in clinical practices with the support of further research.

COMPLIANCE WITH ETHICAL STANDARDS

All experiments and protocols described in the present study were performed in accordance with the guidelines of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes and also approved by the Medical Faculty Experimentation Ethics Committee of Gaziantep University.

Conflict of Interest. The authors declare that they have no conflict of interest.

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