Melatonin dietary supplement as an anti-aging therapy for age-related bone loss

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Abstract

Introduction: Previous studies have shown that melatonin, an antioxidant molecule secreted from the pineal gland, is a positive regulator of bone mass. However, melatonin potential effects on bone mass have never been investigated in old population yet. The aim of this study was to assess the effects of dietary melatonin supplementation on mass accrual and biomechanical properties of old rat femora.

Methods: Twenty 22-months-old male Wistar rats were divided into 2 randomly assigned groups. The first group was treated for 10 weeks with melatonin, whereas the second group left untreated (control). Rat femurs were collected, and their phenotypes and biomechanical properties were investigated by micro-computed tomography, histomorphometry and 3-point-bending test. Statistical analyses were performed by Student's two-tailed unpaired t-test. In all experiments, a value of $p < 0.05$ was considered significant.

Results: Rats treated with melatonin had higher bone volume, bone trabecular number, trabecular thickness and cortical thickness in comparison to control group. Histomorphometric analyses confirmed the increase of bone volume in melatonin-treated rats. In agreement with these findings, melatonin-treated rats demonstrated with higher bone stiffness, flexural modulus and ultimate load compared to controls.

Conclusion: These compelling results are the first evidence indicating that dietary melatonin supplementation is able to exert beneficial effects against age-related bone loss in old rats; improving the microstructure and biomechanical properties of aged bones.

Introduction:

Bone tissue consists of an inorganic phase (~65%) and an organic phase (~35%)[1]. Bone hosts distinct cell types such as osteoblasts, osteocytes, osteoclasts and mesenchymal stem cells[1,2]. Bone undergoes a remodeling process where mature bone is removed by osteoclasts (a process called bone resorption) and replaced by new bone by osteoblasts (a process called bone formation)[1]. In normal physiological conditions, bone remodeling is coupled tightly to maintain a steady state between bone resorption and bone formation[3]. With increasing in age, bone capacity to respond to different stimuli that promotes bone mass (such as vitamin D, growth hormone and IGF-I) decreases[4,5]. Indeed, aging bone has been shown to suffer from a reduction in stem cells differentiating into osteoblasts, an increase in bone marrow adiposity as well as a longer life span for mature osteoclasts[6]. These age-related changes shift the balance of bone remodeling towards bone resorption, leading to a substantial decrease in both bone quality and mechanical properties[6].

Interestingly, age-related bone changes occur alongside the observed decline in function of the neuroendocrine system[7]. One of the neuroendocrine hormones that declines with age is melatonin[8]. Melatonin, a very powerful broad spectrum antioxidant molecule[9], is synthesized from serotonin at the pineal gland[10]. Melatonin binds to its membrane-bound G protein-coupled receptors: MT1 and MT2, which are widely distributed in body tissues[11], including both osteoblasts and osteoclasts[12].

Melatonin is known to control body circadian rhythm[13], body core temperature[14], blood pressure[15], immune system[16] as well as both bone remodeling arms: bone formation and bone resorption[17]. Moreover, previous research suggested a possible role of melatonin in the process

of aging[18], and thus melatonin supplements might act as an anti-aging therapy for age-related body changes including the immune, cardiovascular and nervous systems[19-21]. However, the potential role of melatonin supplement as an anti-aging therapy for age-related bone changes has never been investigated yet. One interesting finding that links melatonin to age-related bone changes is that as melatonin secretions and MT1 receptors expression by osteoblasts tend to decline with age, similarly to bone mass accrual[22,23].

All these linking findings indicate that melatonin supplement might be act as an anti-aging therapy for bone deteriorations in old population. Accordingly, here we have conducted this *in vivo* study in which we investigated the effects of dietary supplementation of melatonin on architecture and biomechanical properties of old rats bones.

Materials and Methods:

Animals

All experimental procedures of this study were performed following the guidelines for Ethical Care of Experimental Animals of the European Union, approved by the Ethical Committee for Animal Studies of the Complutense University (Madrid, Spain). Twenty 22-months-old male Wistar rats were used in this study. All animals were housed in polycarbonate cages, subjected to a 12-h light–dark cycle at the constant temperature of 23 ℃ and fed a standard laboratory rat diet (A.O4 Panlab, Barcelona, Spain) and water ad libitum. Rats were randomly divided into two assigned groups (n = 10). The first group of rats was treated with melatonin diluted in drinking water at dosages of 10 mg/Kg/day. Melatonin was dissolved in absolute ethanol and added to the drinking water in a final concentration of 0.066%. Water bottles were covered with aluminum foil to be protected from light. The second group of rats were left

untreated as a control (only water and vehicle-treated). After 10 weeks of treatment, the animals were sacrificed by decapitation. Rat femurs were collected and fixed in buffered formaldehyde for further analyses.

Micro-CT scan analysis

Micro CT analyses were performed as previously described[24]. Briefly, proximal right femur of each rat was analyzed by 3D-micro CT at X40 magnification with a SkyScan 1072 (Bruker-Microct, Kontich, Belgium) and using bone-analysis software (Version 2.2f, Skyscan, Kontich, Belgium). Parameters were acquired with a rotation of 0.7 degree between each picture with the X-ray source set at 100 kV and 98 µA. The segmentation of the image was made by a global threshold and a voxel size of 21.90 × 21.90 × 21.90 µm; the same threshold setting was used for all the samples. The following 3D morphological parameters were evaluated for the 20 femurs: bone volume fraction (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N) and cortical thickness (Ct.Th)[25].

Bone histology

Twenty rats left femurs were fixed for 24 hr in n 4% PFA/PBS, dehydrated in graded ethanol series before being embedded in methyl methacrylate resin according to standard[26]. Undecalcified sections of the embedded rats femurs were stained by basic fuchsin methylene blue. Bone volume-to-tissue volume ratio (BV/TV) was measured on seven-micrometer sections.

Mechanical testing

A three-point breaking test was performed on the midshaft of the 20 right femurs obtained from all mice as previously described[24,27]. Briefly, a commercial bench-mounted vertical tensile/ compression tester (Instron 5569, Instron Corp., Canton, MA) was used. The span of two support points was 20 mm, and the deformation rate was 0.1 mm/min. The extrinsic

parameters: young modulus and stiffness were calculated from the resulting load-displacement curves. Stiffness is a measure of resistance of bone to displacement; Young's modulus is a measure of stiffness related to the shape of the object; ultimate force is the maximum force that bone can resist; work to failure is the energy required to break bone.

Statistical analyses

All results are shown as descriptive outcomes (mean ± standard deviation). Normal distribution of data was checked by the Shapiro-Wilks statistical test. Statistical analyses were performed by Student's two-tailed unpaired t-test. In all experiments, a value of $p < 0.05$ was considered significant as indicated by a single asterisk.

Results

Melatonin treatment favors bone mass, bone microarchitecture and bone biomechanical properties

We examined bone structural parameters by 3D Micro CT of rats treated with melatonin and those left untreated (control). In figure 1 and 2, rats treated with melatonin had higher bone volume (BV/TV=30.9±4.6%), bone trabecular number (Tb.N=9.6±0.6/mm), trabecular thickness (Tb.Th=0.8±0.2mm) and cortical thickness (Ct.Th=0.5±0.1mm) in comparison to control group (BV/TV=25.0±3.7%; Tb.N=8.3±0.5/mm; Tb.Th=0.6±0.1mm; Ct.Th=0.4±0.1mm). Histomorphometric analyses confirmed the increase of bone volume in melatonin-treated rats (BV/TV=32.5±1.1%) compared to control (BV/TV=27.3±1.4%, Figure 3). Analysis of bone mechanical properties showed higher bone stiffness (453±155N/mm), flexural modulus (2182±1084N/mm^2) and ultimate load (181±50N) in melatonin-treated rats than in controls (Stiffness=307±75N/mm; Flexural modulus=1332±976N/mm^2; ultimate force= 148±36N, Figure

4). However, femurs of melatonin-treated rats did not exhibit a significant improvement in work to failure (300 ±147N*mm) compared to control (244 ± 126N*mm).

Discussion:

Aging is associated with a reduction in the function of most physiological systems of the organism, including bone. Age-related bone changes are characterized by deterioration of bone architecture, loss of bone mass and increase in susceptibility to fractures[6,28], and it is a serious public health problem in the aging population[29]. Accordingly, several studies were conducted to find new therapeutic approaches to prevent or to slow down age-related bone deterioration. Nowadays, the only approved drugs that are commonly prescribed to improve bone quality are Bisphosphonates, Denosumab, Parathyroid Hormone, Selective Estrogen Receptor Modulators and Estrogen/Progesterone Hormone therapy[30]. However, even though these drugs are successful therapies for age-related bone changes and diseases, their use is limited due to the high cost and side effects[31-36]. In our study and for the first time, we provided the first evidence for the effects of melatonin as an anti-aging therapy for age-related bone changes.

The chronic treatment with 10 mg/kg/day of melatonin for 10 weeks involves the oral intake of high amounts of melatonin. Our group has been studying effects of melatonin on aging-related parameters since nearly twenty years, such as oxidative stress, inflammation and apoptosis on several organs like heart[37,38], pancreas[39], liver[40,41], and brain[42]. The influence of different doses of melatonin (1 mg/kg/day to 50 mg/kg/day) on different organs has also been studied. Since 10 mg/kg/day had showed more beneficial effects on pancreas[39], and heart[37,38] than 1 mg/kg/day, we thought that 10 mg/kg/day of melatonin should be more effective on bone than 1 mg/kg/day.

Melatonin was diluted in the drinking water, according to previous papers[37-42]. Rats are night-time animals. During the day they normally sleep, so more than 90% of the total daily water is drunk during the night.

Male animals have been used in this study and in the majority of our experiments[37-40,42]. Male rats are not dependent of cycle variations and represent a well established model of aging.

Melatonin supplement favors bone mass accrual in old rats

This study was conducted on 22-months-old rats which is equivalent to 60 years of age in humans[43]. Following 10 weeks of treatment with melatonin (treated group), which is equivalent to 6 years in human age[43], *versus* those left untreated (control), femur bone phenotype was analyzed by micro CT-scan, histology and biomechanically.

Micro CT-scan provided additional advantages over classical densitometers such as DXA, the most popular tool to measure bone mineral density. Indeed, micro CT-scan is able to measure separately cortical and trabecular tissues, while DXA measure the addition of them, trabecular plus cortical, without discriminating both tissues[44]. In our study, melatonin-treated old rats expressed higher bone volume, trabecular number and larger trabecular and cortical thickness compared to control. Findings from micro-CT analyses were also confirmed by histomorphometric analyses. The results of this study are in agreement with previous studies conducted on young mice[23,45]. In which, daily intraperitoneal injections of melatonin were able to significantly enhance BMD in mice tibiae[45] and enhance cortical thickness[23].

Biomechanical properties of rats femurs collected from our experiments were analyzed by three-point bending test, the most popular method for the characterization of long bone biomechanical properties[46]. Melatonin-treated rats demonstrated with higher biomechanical

properties than those rats left untreated (control group). Our results are in agreement with previous studies, in which it has been shown that melatonin treatment is able to improve bone mechanical properties in young animals[47,48]. However, to the best of our knowledge our study is the first evidence demonstrating that melatonin treatment improves bone biomechanical properties in old animals.

Mechanism by which melatonin favors bone mass accrual

Melatonin has been proposed to affect the bone formation arm of the bone remodel model[17,23,49-52]. *In vitro* studies have shown that melatonin regulates the mesenchymal stem cells (MSCs) differentiation towards osteogenic lineage, rather than adipogenic lineage[49], by enhancing the expression of runt-related transcription factor 2 (Runx2) and bone morphogenetic protein (BMP)-2 and BMP-4, thereby affecting the Wnt signaling pathway[50]. Also, melatonin has been shown to be able to reduce the osteoblasts differentiation time[51] and to increase synthesis of alkaline phosphatase[52], osteopontin, osteocalcin or procollagen type I C-peptide (PICP)[23,50,53,54], markers for bone formation.

On the other hand, it has been shown that melatonin can also regulate the bone resorption arm of bone remodeling[17,55]. Indeed, *in vitro*, *in vivo* and clinical studies demonstrated that melatonin is capable to reduce RANKL[45,56,57] and increase OPG[45,56] expression by osteoblasts, leading to a net anabolic effect on bone. Also, some of our *in vivo* studies have shown that melatonin reduces the mRNA expression of TNF-α and IL-1[58], markers for osteoclastogenesis.

Future research

Findings of our study are the first evidence indicating that melatonin supplement is able to affect bone remodeling and biomechanical properties in old animals. Accordingly, future large-scale

prospective randomized clinical trials should be conducted in order to investigate melatonin as a potential therapy for age-related bone changes. Moreover, *in vivo* and clinical studies assessing biochemical bone markers and bone density measurements will have to be performed to explore the effect of melatonin supplement on bone structure in old population.

Conclusion:

Our findings demonstrate that dietary melatonin supplementation increases bone mass accrual and bone biomechanical properties in old male rats. These findings suggest that melatonin supplement could improve the health of bones in old population, although additional clinical studies are needed to confirm these potentially important findings.

Authors disclosure statement: authors disclose any commercial associations that might create a conflict of interest in connection with submitted manuscripts.

All of authors inform that no competing financial interest exist regarding this manuscript.

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Figure legends

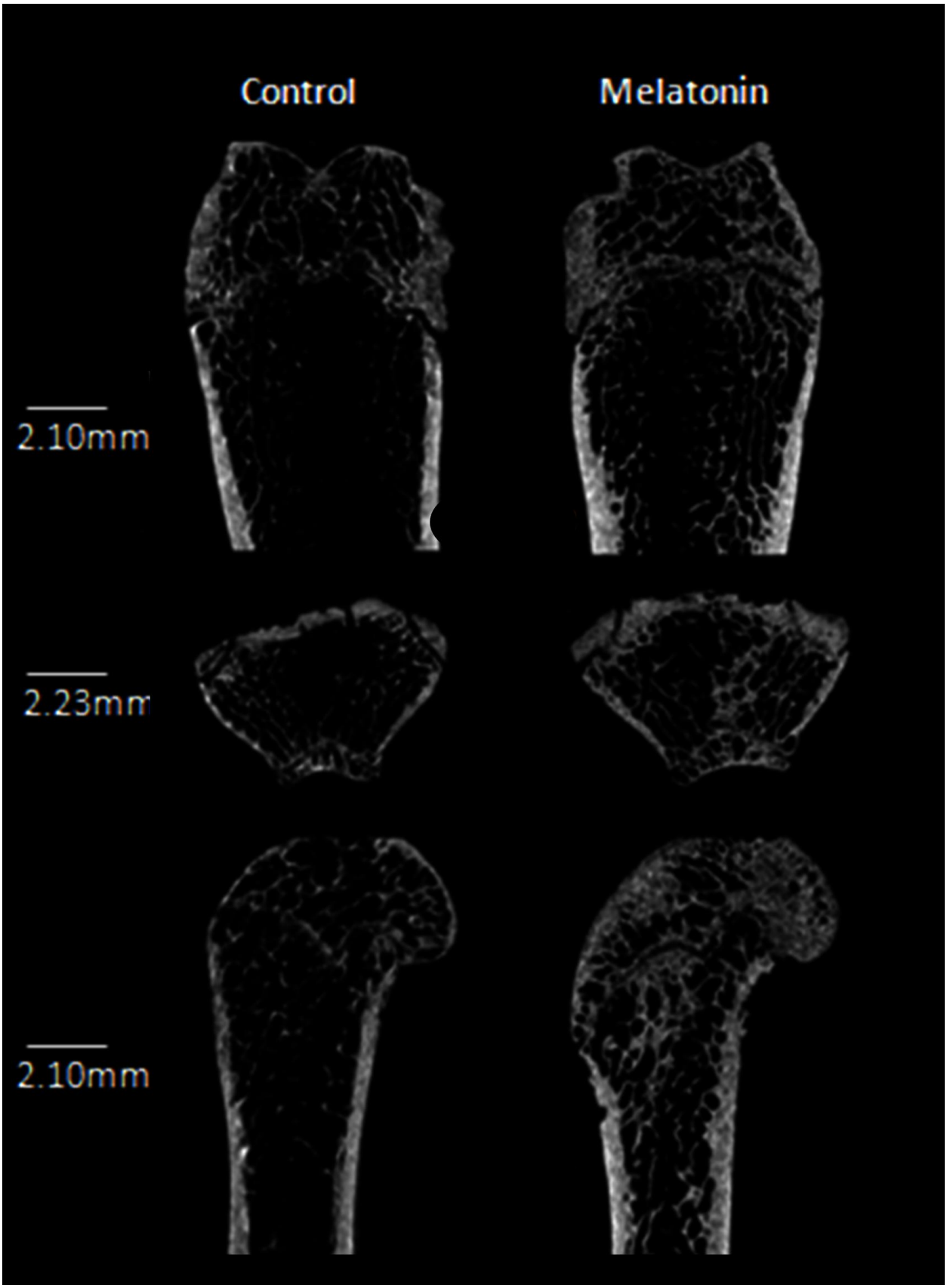

Figure 1. Micro-CT sections (top row=Coronal; middle row=Sagittal; bottom row= transversal) of the femurs retrieved from aged rats treated with melatonin or those left with no treatment (control). It is visually obvious from the images that melatonin-treatments increases bone mass accrual compared to the control group.

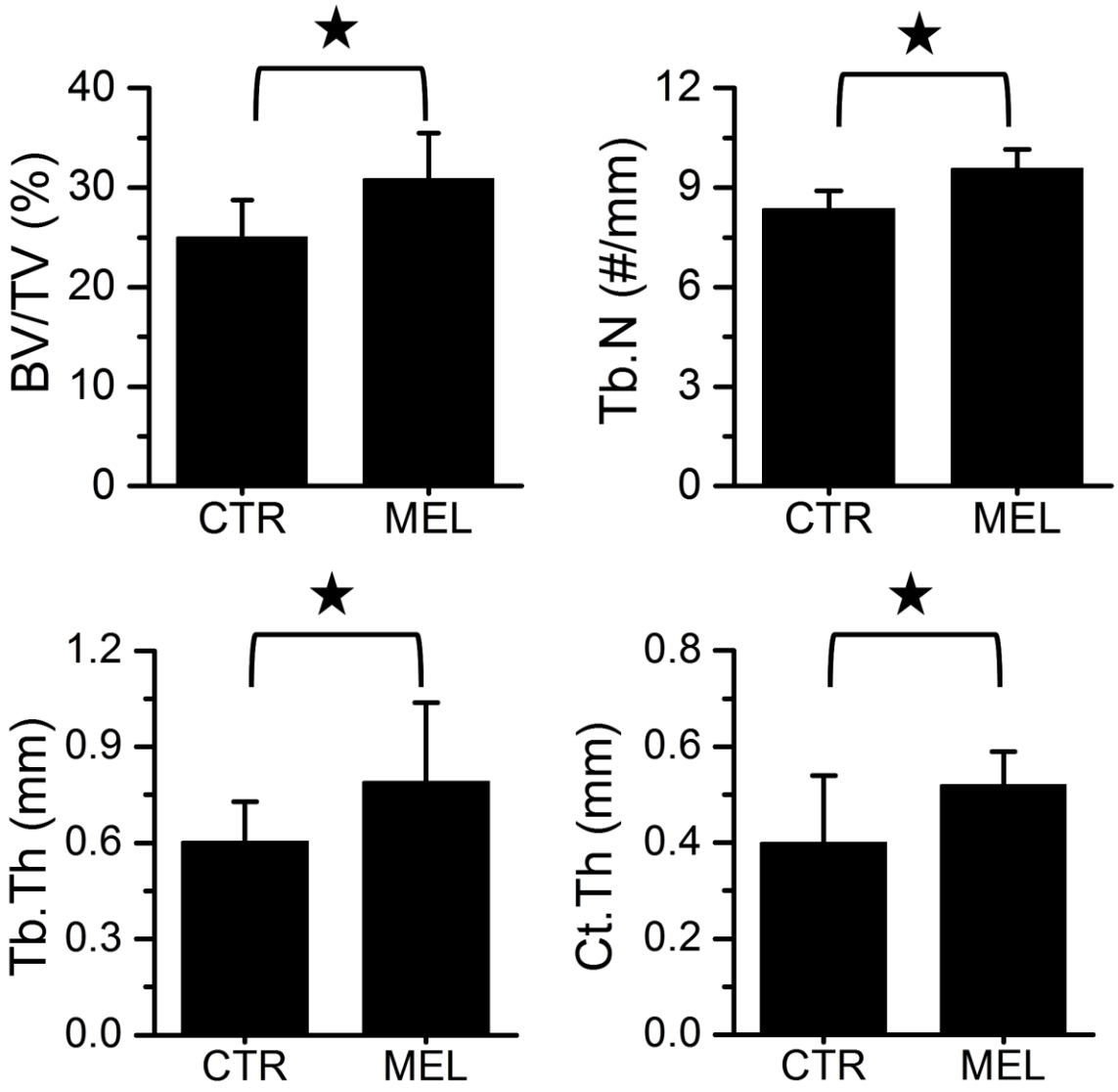

Figure 2. Morphometrical micro-CT analysis of femurs retrieved from aged rats treated with melatonin or those left with no treatment (control). Rats treated with melatonin demonstrated higher bone volume percentage (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th) and cortical thickness (Ct.Th) compared control group rats. Values are mean±SD. *p<0.05.

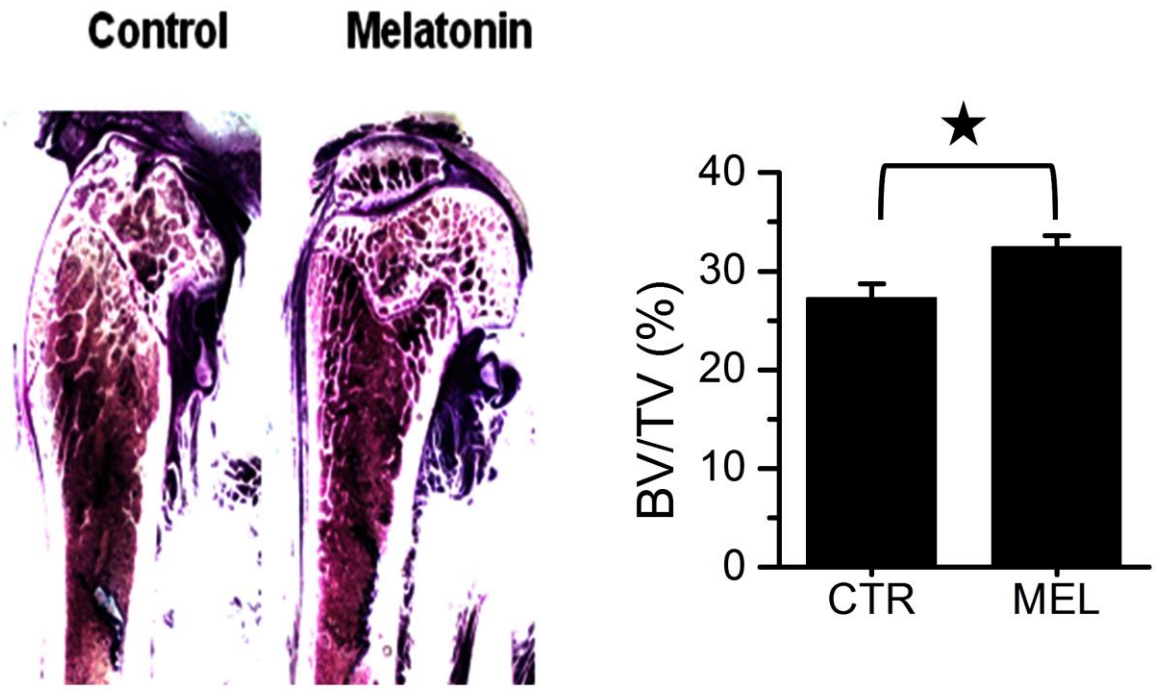

Figure 3. Histological sagittal sections of the femurs retrieved from aged rats treated with melatonin or those left with no treatment (control). It is visually obvious from the images melatonin-treatment increases the bone mass accrual compared to control group. Histomorphometric analyses demonstrated that rats treated with melatonin had higher bone volume percentage (BV/TV) than control group. Values are mean ±SD. *$p<0.05$.

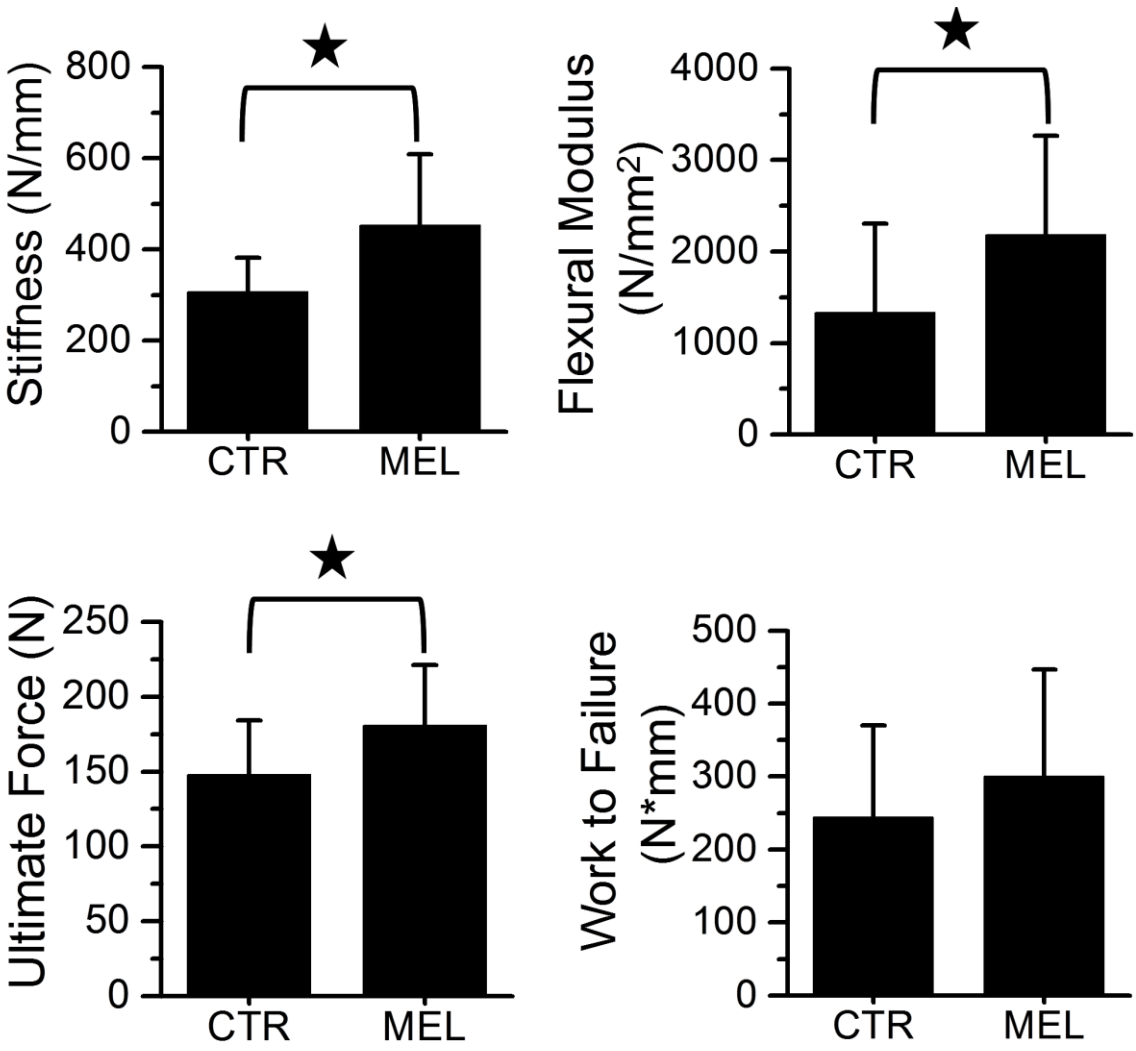

Figure 4. Mechanical analyses of femurs retrieved from aged rats treated with melatonin or those left with no treatment (control). Rats treated with melatonin demonstrated higher bone stiffness, flexural modulus and ultimate force compared to control. Values are mean ±SD.

* $p<0.05$.