BALNEO RESEARCH Balneo and PRM Research Journal English Edition
e ISSN 2734 - 8458 p ISSN 2734 - 844X ROMANIAN ASSOCIATION OF BALNEOLOGY

Research article

Influence of melatonin on systemic inflammatory status and bone histopathological modifications in female rats with surgically induced menopause

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Citation: Ciortea et al., Influence of melatonin on systemic inflammatory status and bone histopathological modifications in female rats with surgically induced menopause , *Balneo and PRM Research Journal* **2022**, *13*(1): 493.

Academic Editor(s):
Constantin Munteanu

Reviewers:
Carmen Nistor Cseppento
Mariana Rotariu

Received: 15.02.2022
Accepted: 18.03.2022
Published: 21.03.2022

Publisher's Note: Balneo and PRM Research Journal stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

ABSTRACT: Background. Melatonin, N-acetyl-5-methoxy-tryptamine is the major secretion product of the pineal gland with important anti-inflammatory and antioxidant properties, also being an important marker of bone remodelling associated with menopause. **Objectives.** The aim of our study was to evaluate the effect of the co-administration of melatonin and estrogen on systemic inflammatory status and bone histopathological modifications in surgically induced menopausal female rats. **Materials and methods.** The study was performed on a number of 40 female rats, Wistar breed, which underwent bilateral surgical ovariectomy. Within 14 days postoperative, hormone replacement therapy with estrogen or estrogen with melatonin was initiated, in different doses. The treatment was administered for 12 consecutive weeks. At the end of the treatment we measured the serum levels of IL-6 and TNF-α. The femoral bones were harvested after sacrificing the animals and the thickness of the cortical bones was measured and histologically analysed.
Results. Serum values of inflammatory markers were negatively correlated with melatonin administration, the differences being more important at higher doses of melatonin (for both IL-6 and TNF-α the difference between group E_2M with estrogen substitution and melatonin in double dose and control group W, without hormone replacement, was highly statistically significant with $p < 0.0001$). Bone diameters improved in the case of female rats that received hormone replacement with estrogen and higher dose of melatonin ($p = 0.0004$ between group E_2M, with hormone replacement and group W, control group). **Conclusions.** Melatonin improved inflammatory status and bone histopathological changes in ovariectomized female rats.

Keywords: *melatonin, estrogen replacement therapy, inflammation, low bone density*

1. INTRODUCTION

Bone tissue undergoes a continuous process of remodelling, involving environmental and growth factors, cytokines and systemic hormones such as estradiol, parathyroid hormone and growth hormone (1), as well as vitamins D and K (2).
Recently, the genetic factors involved in bone metabolism have been studied, being admitted the polygenic nature of the phospho-calcium imbalance. Although the results remain inconclusive and controversial both for the general population and for the Romanian population (3,4).

Melatonin is a marker of the bone remodelling process, being inversely correlated with bone histopathological changes associated with menopause (5). Melatonin has a dual action in the bone, on the one hand promoting the differentiation and action of osteoblasts, by increasing the expression of osteoprotegerin in these cells, on the other hand preventing the differentiation of osteoclasts and neutralizing free radicals responsible for bone resorption. Thus, melatonin contributes to the hormonal modulation of bone cells, being more and more often highlighted in the etiopathogenesis of postmenopausal osteoporosis especially since its secretion gradually decreases with the aging of female patients (6). Studies demonstrate the influence of melatonin on bone turnover, leading to increased markers of bone formation and decreased bone resorption in postmenopausal female rats (7), with similar effects among human subjects (8). To validate the effects of melatonin on bone remodelling and bone turnover, adequate concentrations of serum estradiol are required, studies showing that melatonin and estrogen have cumulative effects on bone changes in postmenopausal osteoporosis (9). It is also known that low serum estrogen levels are the main cause of bone histopathological changes in the first 5-10 years after menopause, being responsible for the high turnover of bone remodelling during this period, with the predominance of bone resorption (10,11). Also, the decrease of melatonin secretion is directly correlated with the increase of the risk of falling and implicitly with the appearance of fractures with various locations, accompanied by the decrease of the life quality among the elderly population (12).

Melatonin also has strong anti-inflammatory and antioxidant effects by inhibiting the secretion of important cytokines such as TNF-α and IL-6 (13-15) both molecules being mediators of the acute phase response negatively correlated with bone mineral density values. Both TNF-α and IL-6 destabilize the balance of phospho-calcium homeostasis at the bone level, tilting it in favour of bone resorption (16,17). The mechanisms by which these cytokines influence the degree of bone mineralization are still debated in the literature but the serum level of inflammation markers is higher among patients with low bone mineral densities compared to those with bone mineral densities within normal range (18-20).

Thus, the evaluation of specific markers for inflammation in the case of patients with low bone mineral density becomes important for a correct therapeutic option. IL-6 inhibitors may be an alternative treatment for postmenopausal patients with bone loss in order to improve inflammatory status (21), as well as the administration of exogenous melatonin with a very good safety profile (22) on the one hand for the bone-forming beneficial effects, as well as for the reduction of the serum values of the inflammation markers (7,13).

Objectives

All the above evidence supports the idea that exogenous administration of melatonin under appropriate serum estradiol levels may improve inflammatory status and bone histopathological changes associated with menopause. The aim of our study was to evaluate the effect of melatonin co-administered with estrogen on systemic inflammatory status and bone histopathological modifications in surgically induced menopausal female rats.

Materials and methods

The study was conducted on 40 female Wistar rats according to the norms of ethics, with the approval of the University's Ethical Committee. The animals were housed in a controlled environment as showed in the Figure 1. Human bilateral ovariectomy surgical technique has been adapted for female rats (7).

After the completion of the 14 days post-ovariectomy period, which is required to confirm the status of ovarian failure, hormone replacement therapy (estrogen mono therapy) combined with melatonin treatments was started. The treatment was administrated for 12 weeks, following the veterinary posology. The levels of serum estradiol were measured before and after the bilateral ovariectomy in all study participants.

The ovariectomized rats were randomly separated into 4 groups (W, E, E_M and E_2M) as explained in Figure 1.

All 40 animals maintained clinically healthy, without any post-surgical complications until the end of the experiment. None of them was excluded during the study.

Upon completion of the treatment, the orbital sinuses were punctured for blood samples and the serum levels of IL-6 and TNF-α were anlysed.

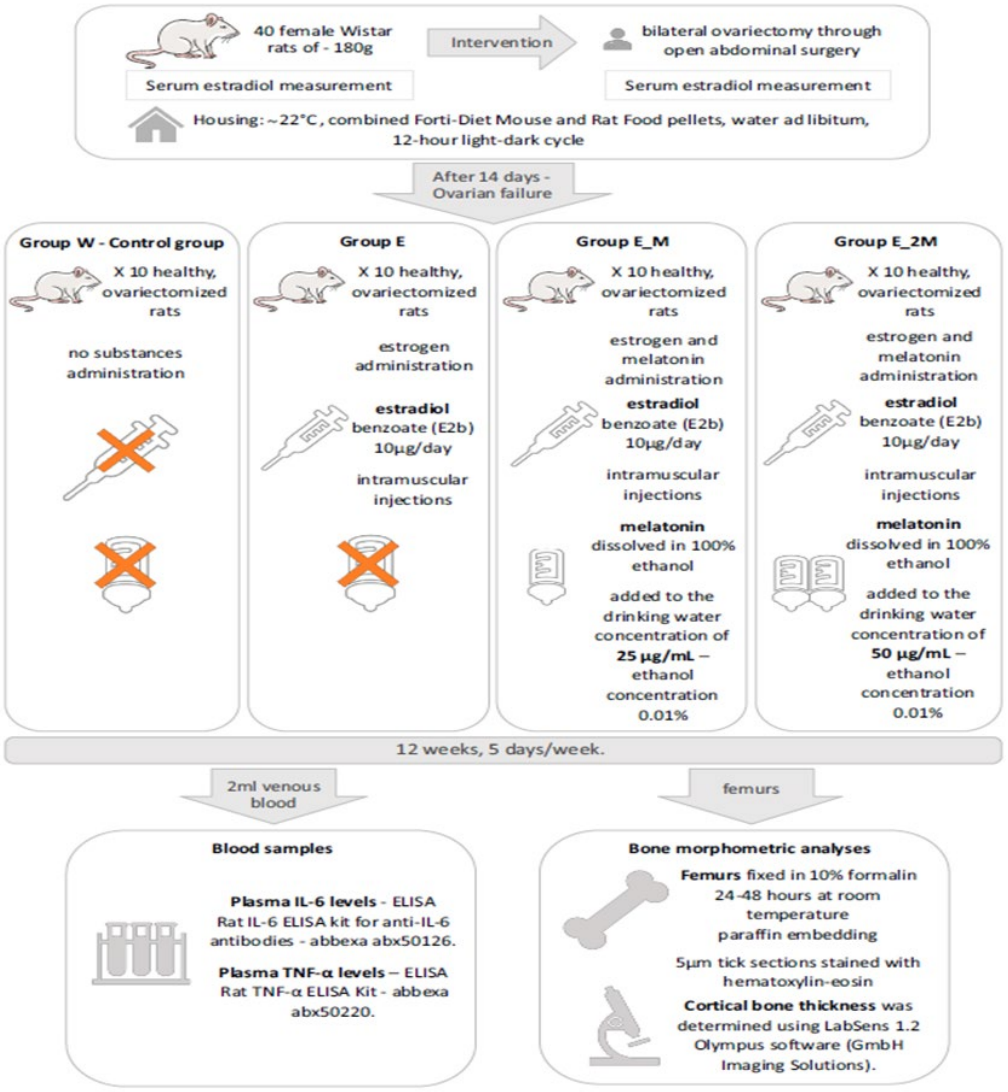

Figure 1. Materials and methods

After rats slaughtering, femurs were harvested, fixed in 10% formalin for 24-48 hours at room temperature and processed for paraffin embedding. 5µm tick sections obtained from formalin fixed paraffin embedded *(FFPE)* blocks were stained with hematoxylin-eosin in order to perform morphometric analyses. Cortical bone thickness was determined using LabSens 1.2 Olympus software (GmbH Imaging Solutions). Two different persons were responsible with the cages labeling and correct treatment administration and the ones responsible with conducting the experiments were not involved in the sampling procedures.

Statistical analyses were performed using MedCalc version 19 software and Microsoft Excel Analysis Tool Pack.

Normal distribution was tested by Kolmogorov-Smirnov test. Data were expressed as means ± standard deviation (SD). Comparison between groups was performed using Student t-test for paired or for independent samples or ANOVA, followed by Bonferroni correction for analysis of more than 2 groups. Analysis of correlations was based on Pearson correlation coefficient. Statistical significance threshold was set at α=0.05.

Results

The mean and standard deviation (SD) for all the variables studied are presented in Table 1 for each study group.

Table 1. *Variables Mean and Standard Deviation (SD)*

Study group	Variables Mean±SD				
	E_pre	E_post	Il-6	TNF-alfa	Diameter
W	37.4±9.8	17.9±2.4	0.0768±0.0049	0.1074±0.0046	57.66244±5.619852
E	36.9±6.3	35.6±1.9	0.0664±0.0042	0.0872±0.0045	65.4165±14.90199
E_M	35.0±5.4	35.8±1.3	0.0577±0.0035	0.0785±0.0047	66.0815±11.57504
E_2M	39.1±4.7	36.0±1.8	0.0469±0.0025	0.0709±0.0035	78.29467±9.552679

W -control group, E -group with estradiol replacement therapy, E_M -group with estradiol replacement therapy and melatonin in simple dose, E_2M -group with estradiol replacement therapy and melatonin in double dose, E_pre -estrogen value before therapy, E_post -estrogen value after therapy.

The serum levels of estradiol in premenopausal female rats did not differ significantly between the 4 groups ($p=0.8$), while the serum levels of estradiol in the postmenopausal period differed significantly in each of the three groups with estradiol substitution (E, E_M, E_2M) compared with the control group (W) ($p<0.0001$), but without differences between groups E, E_M and E_2M ($p>0.05$) (Fig. 2).

Serum levels of IL-6 and TNF-α significantly differed ($p<0.0001$) from the control group in all three groups with estrogen replacement (group E) or estrogen replacement co-administered with melatonin (groups E_M and E_2M), as well as between each of the 3 groups (E, E_M and E_2M) (Fig.3, Fig.4). Thus, melatonin administration beside estrogen replacement brought supplementary dose-dependent benefits in reducing inflammation markers such as IL-6 and TNF-α.

Regarding the values of the femoral bone diameters, they significantly differed between the control group (W) and the group that received estrogen replacement and the simple dose of melatonin (E_M) ($p=0.046$), respectively between the control group (W) and the group that received estrogen replacement and the double dose of melatonin (E_2M) ($p=0.0004$). No statistically significant difference was found between the control group (W) and the group that received only estrogen replacement (E) ($p=0.083$).

When comparison was made between the groups receiving estrogen replacement, there was no statistically significant difference in femoral bone diameters between group E (with only estrogen replacement) and group E_M (with estrogen replacement and simple dose of melatonin) ($p=0.922$). On the other hand, femoral bone diameters were significantly larger in group E_2M (estrogen replacement and the double dose of melatonin) than in group E_M ($p=0.034$) or in group E ($p=0.029$). (Fig. 5). Bone diameter values improved as estrogen and melatonin were administered to the three study groups, the improvement being dependent on the presence of estrogen and the dose of melatonin administered.

The results have shown an increase in bone diameters as the serum values of IL-6 and TNF-α decreased, with an inverse relationship between markers of inflammation and changes in bone structure, but without statistical significance of the correlations.

The histological images of the femoral bones differed between the four groups in the study, the repair processes being more important for group E_2M.

Discussion

In a review published in 2019 which included several in vivo and in vitro studies in experimental animal models the anti-inflammatory effects of melatonin (N-acetyl-5-methoxy-tryptamine) were highlighted in an impressive number of pathologies with damage to various organs, in different circumstances.(13). The involvement of melatonin in angiogenesis, apoptosis, free radicals scavenging and increased imunity has been shown.

Previous studies demonstrated that melatonin has important oncostatic properties. Blood levels of melatonin is inversely correlated with the rate of tumor proliferation in patients with endometrial cancer (23). Melatonin has been shown to have anti-inflammatory properties in several autoimmune diseases such as multiple sclerosis, systemic lupus erythematosus, rheumatoid arthritis, type I diabetes (24) and also cardiovascular and renal protective effects associated with high blood pressure and myocardial ischemia (25). Due to its anti-inflammatory and antioxidant properties, several clinical trials have aimed to monitor the beneficial effects of melatonin administration in patients infected with SARS-CoV2 during the COVID-19 pandemic. In critically ill patients diagnosed with COVID-19, melatonin is an adjunct in reducing the effects of cytokine storm triggers and therefore excessive inflammation; in addition it would decrease anxiety levels and improve sleep in these patients by regulating the sleep-wake mechanism and the circadian rhythm. These studies also recall the high safety profile of melatonin (15,26).

Melatonin proved its anti-inflammatory role in the present study by reducing the serum values of IL-6 and TNF-α inflammation markers in groups of rats with estrogen plus/minus melatonin substitution (E, E_M, E_2M) compared to the control group, without hormone replacement. The differences were also statistically significant between the groups with estrogen substitution (group E) and estrogens with melatonin in different doses (groups E_M and E_2M). The serum values of the two inflammation markers studied were inversely correlated with the doses of melatonin administered being significantly lower in group E_2M as compared to group E_M.

Ren Haiwei et al. noticed in one study the same inverse relationship between serum melatonin levels and markers of inflammation (IL-6, IL-1, TNF-α) in female patients diagnosed with osteoporosis. On the other hand, the same study observed the positive correlation of the levels of inflammation markers with the markers of bone resorption. TNF-α induces stromal cell expression in osteoblasts and stimulates osteoclast activity while interleukins may promote cell proliferation of osteoclast precursors and increase the activity of these cells; all these mechanisms facilitating osteoresorption to the detriment of osteoformation (27).

At the same time, the effects of melatonin on bone turnover are well known, with a favourable influence on bone remodelling in postovariectomy female rats. For the validation of these effects it is necessary, as mentioned, adequate concentrations of serum estradiol (9,28).

With an adequate serum estradiol concentration provided by estrogen replacement in the three study groups (groups E, E_M and E_2M) and without significant differences in estradiol concentration between groups, in the present study melatonin was found to significantly influence bone remodelling. So, even if histological appearance was improved in all 3 study groups (groups E, E_M and E_2M), statistical significance was found only for the two groups with melatonin (E_M and E_2M) when compared to control group. On the other hand, in group E_2M (with estrogen substitution and double dose of melatonin) femoral bone diameters were found to be significantly larger than in group E_M (with estrogen substitution and simple dose of melatonin) and in group E (with only estrogen substitution), respectively.

Similarly to other studies, melatonin-induced bone changes were found to be dose-dependent (29-31).

The values of inflammation markers were inversely correlated with the values of bone diameter but they were not statistically significant. Even in the absence of correlations with statistical significance, it remains important to assess the inflammatory status in case of bone histopathological changes associated to menopause (20,21).

Melatonin remains one of the important hormones involved in bone remodelling. Numerous studies have shown the beneficial effects of melatonin on the proliferation of osteoblastic cells, stimulating the formation of type I collagen and bone proteins such as alkaline phosphatase, osteocalcin, osteopontin, and on the other hand its inhibitory

effects on osteoclast differentiation by decreasing RANK mRNA expression and increasing mRNA and osteoprotegerin levels (24,32,33).
Melatonin also indirectly influences bone metabolism by interacting with various systemic hormones such as estrogen and parathyroid hormone (9,34).
Ladizeski et al. emphasizes the role of estrogen in prolonging the effects of melatonin on bone remodeling in ovariectomized female rats (28).
In addition, melatonin eliminates superoxide anions resulting from the activity of osteoclasts during the process of bone resorption, its antioxidant effect being well known (24,34).
This information highlights the involvement of melatonin in the pathogenesis of menopausal-associated bone changes suggesting its potential value in the prevention and treatment of postmenopausal osteoporosis (35).
Limitations
One limitation of this study is the relative small number of animals and the limited extrapolation to possible effect of melatonin on human bone metabolism. The study also lacks a group of animals to which melatonin was administered exclusively.

Conclusions

Melatonin improved the inflammatory status associated with histopathological changes in menopausal bone and also improved bone remodelling in ovariectomized female rats in a dose-dependent manner.

Abbreviations
COVID-19 - "coronavirus disease 2019"
E2b - estradiol benzoat
ELISA – Enzyme-Linked- Immuno-Sorbent-Assay
IL-1- interleukin 1
IL-6 - interleukin 6
m ARN – messenger ribonucleic acid
SARS-COV-2 - severe acute respiratory syndrome coronavirus 2
TNF-α – tumor necrosis factor – alpha

Authors' contributions

V.M.C, I.M, A.L.P, L.I: Research concept and design; S.Ş, A.D.C, A.L.P: Collection and/or assembly of data; I.M.B, I.M, A.D.C, R.A.U: Data analysis and interpretation; V.M.C, I.M.B, I.M, R.A.U, L.I: Writing the article; V.M.C, I.M.B,L.I: Critical revision of the article; V.M.C, I.M.B, I.M, S.Ş, A.D.C, A.L.P, R.A.U and L.I: Final approval of the article
#All authors had equal contributions with the first author.

Conflicts of Interest: The authors declare no conflict of interest.

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