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BONE BIOLOGY

Systemic administration with melatonin in the daytime has a better effect on promoting osseointegration of titanium rods in ovariectomized rats

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Aims

This study examined whether systemic administration of melatonin would have different effects on osseointegration in ovariectomized (OVX) rats, depending on whether this was administered during the day or night.

Methods

In this study, a titanium rod was implanted in the medullary cavity of one femoral metaphysis in OVX rats, and then the rats were randomly divided into four groups: Sham group (Sham, n = 10), OVX rat group (OVX, n = 10), melatonin day treatment group (OVX + MD, n = 10), and melatonin night treatment group (OVX + MN, n = 10). The OVX + MD and OVX + MN rats were treated with 30 mg/kg/day melatonin at 9 am and 9 pm, respectively, for 12 weeks. At the end of the research, the rats were killed to obtain bilateral femora and blood samples for evaluation.

Results

Micro-CT and histological evaluation showed that the bone microscopic parameters of femoral metaphysis trabecular bone and bone tissue around the titanium rod in the OVX + MD group demonstrated higher bone mineral density, bone volume fraction, trabecular number, connective density, trabecular thickness, and lower trabecular speculation (p = 0.004) than the OVX + MN group. Moreover, the biomechanical parameters of the OVX + MD group showed higher pull-out test and three-point bending test values, including fixation strength, interface stiffness, energy to failure, energy at break, ultimate load, and elastic modulus (p = 0.012) than the OVX + MN group. In addition, the bone metabolism index and oxidative stress indicators of the OVX + MD group show lower values of Type I collagen cross-linked C-telopeptide, procollagen type 1 N propeptide, and malondialdehyde (p = 0.013), and higher values of TAC and SOD (p = 0.002) compared with the OVX + MN group.

Conclusion

The results of our study suggest that systemic administration with melatonin at 9 am may improve the initial osseointegration of titanium rods under osteoporotic conditions more effectively than administration at 9 pm.

Cite this article: *Bone Joint Res* 2022;11(11):751–762.

Keywords: Osteoporosis, Titanium rods, Melatonin, Bone mass, Osseointegration

Article focus

- The purpose was to observe the effect of systemic administration of melatonin by day and night on osseointegration in ovariectomized rats.

Key messages

- This study found that melatonin treatment at 9 am may be useful for improving implant osseointegration.

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doi: 10.1302/2046-3758.1111.BJR-2022-0017.R2

Bone Joint Res 2022;11(11):751–762.

Strengths and limitations

- This is a novel exploration of the effect of melatonin treatment at different timepoints on the osseointegration of titanium rods in the state of osteoporosis, which provides a theoretical basis for improving the clinical efficacy of melatonin.
- This study did not further examine the specific mechanism and blood melatonin changes, so further research is needed.

Introduction

Total joint replacements are cost-effective and highly successful surgical procedures that have successfully reduced chronic joint pain and improved quality of life, especially for older adults with advanced joint disease and end-stage osteoarthritis.[1,2] However, poor bone/implant interface contact (due to a variety of reasons including loosening, persistent pain, and infection) remains an issue, leading to the necessity for implant revision surgery.[1,3] Over 70,000 annual revision procedures are performed for failed implants in the USA alone, with aseptic loosening being the major indication for revision.[4,5] Moreover, the number of annual total joint replacement revisions is expected to increase to over 350,000 by the year 2030.[4,5] Therefore, one approach to reducing the risk of implant loosening is to improve bone formation around the implant, and attempt to improve early stability of the implant sufficiently to subsequently acquire long-term stability.[6,7] At present, scholars have attempted to promote implant fixation through local or systemically delivered drugs, such as bone morphogenetic protein-2 (BMP-2) and parathyroid hormone, to promote bone regeneration and osseointegration.[8-12]

Melatonin (N-acetyl-5-methoxytryptamine) is an indole hormone mainly secreted by the pineal gland, which has been demonstrated to be involved in many biological processes including tumour growth inhibition, sleep, immune response, and reproductive control.[13,14] In recent years, a number of studies have confirmed that the absence of melatonin or its inefficient production have an important impact on bone quality and remodelling.[15,16] Moreover, melatonin can promote osteoblast differentiation and inhibit the formation of osteoclasts by regulating BMP-responsive signal transduction pathways (SMAD), extracellular signal-regulated kinase (ERK), and nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) signalling pathways.[17,18] Although multiple studies have confirmed that melatonin can promote the osseointegration of titanium implants,[19-21] there have been no reports on whether the effect on osseointegration is influenced by daytime or night-time administration. This is particularly important given that the release capacity of this hormone by the pineal gland is influenced by circadian rhythms.

Postmenopausal osteoporosis is one of the most common bone diseases, characterized by loss of trabecular bone, destruction of bone microarchitecture, and decreased bone strength.[22,23] Low levels of oestrogen in postmenopausal osteoporosis cannot counteract the inflammatory response in the body, thereby aggravating the ageing and apoptosis of osteoblasts and promoting the proliferation and differentiation of osteoclasts. This in turn accelerates the bone remodelling cycle, severely affecting the normal bone metabolic balance.[24] Female Sprague Dawley (SD) rats have been used widely to establish an osteoporosis model after bilateral ovariectomy to simulate the physiological state of oestrogen deficiency in women after menopause, resulting in bone loss.[10,25–27] Therefore, this study aims to establish whether daytime or night-time administration of melatonin can influence the osseointegration of titanium rods in the femoral metaphysis of ovariectomized SD rat.

Methods

Animals and reagents. A total of 40 12-week-old healthy female SD rats (weighing between 220 and 250 g) were used in this study. The animals were raised under controlled conditions (temperature 20°C ± 3°C, humidity 45% ± 5%). The animal experiment was approved by the Experimental Animal Ethics Committee of our institution. Melatonin used in this study was purchased from Sigma-Aldrich (USA).

Animal experiments. All osteoporosis models of oestrogen deficiency induced by bilateral ovariectomy were established as described previously.[26,28,29] The animals were anaesthetized with 2% pentobarbital sodium (50 mg/kg) and placed in the prone position, the back hair was removed, and the skin was disinfected. The skin and subcutaneous tissue were incised layer by layer until the ovaries were exposed in the abdominal cavity. In the Sham group, only the adipose tissue around the ovary was removed, while in the OVX group both fallopian tubes were ligated, and both ovaries were removed. After the operation, the abdominal cavity and wounds on both sides were sutured; 12 weeks after sham operation (Sham) and bilateral ovariectomy (OVX), all rats were randomly divided into four groups by a random number table: Sham group (Sham, n = 10), OVX rat group (OVX, n = 10), OVX rats + melatonin day treatment group (OVX + MD, n = 10), and OVX rats + melatonin night treatment group (OVX + MN, n = 10). Next, the titanium implants (external diameter (1.2 mm) and length (20 mm); Zhejiang Guangci Medical Appliance, China) were introduced across the left femoral condyle of the rats into the medullary canal (Supplementary Figure a) as described previously.[30,31] The animals were anaesthetized with 2% pentobarbital sodium (50 mg/kg) and placed in the prone position, the hair of left lateral condyle was removed, and the skin was disinfected. The skin and subcutaneous tissue were incised layer by layer until the femoral condyle was exposed. A hole from the lateral femoral condyle to the medial femoral condyle was prepared using a medical slow drill, and a titanium rod was then implanted into the medullary canal through this channel. After the operation, the subcutaneous tissue and wounds were sutured. The rats classified to OVX + MD and OVX + MN were treated with melatonin intraperitoneally with a dose of 30 mg/

kg (Sigma-Aldrich) at 9 am and 9 pm, respectively.[32] After 12 weeks of treatment, the rats undergoing bone defect surgery were killed using an overdose of 2% pentobarbital sodium (100 mg/kg). Serum and femur samples were then harvested. Femora were fixed at 4°C with 4% paraformaldehyde for 24 hours and later evaluated by histology, biomechanics, and micro-CT assessment. Whole blood was frozen at -80°C for later use. ARRIVE guidelines were followed for the in vivo experiment, and an ARRIVE checklist is included in the Supplementary Material to show that these guidelines were adhered to.

Micro-CT scanning. The distal femur (n = 5/group) with the titanium rod was analyzed with an anisotropic voxel size 10 μm using micro-CT (Bruker Skyscan 1272 system; Bruker, Belgium). The voltage was set to 55 kV, 114 mA, with a thickness of 0.048 mm per slice in medium-resolution mode, 1,024 × 1,024 reconstruction matrix, and 200 ms integration time. These images and parameters of trabecular bone with a distance of 1 mm proximal from the end of the growth plate in the femoral metaphysis were compared among the four groups to confirm the effect of melatonin treatment in the rat implant osteoporosis model. For evaluation of bone formation around the titanium rod, the central 250 μm-diameter region around the surface of the titanium rod was defined by drawing a circular contour as a consistent volume of interest (VOI) (Supplementary Figure b). After 3D reconstruction, bone mineral density (BMD), bone mineral content (BMC), bone volume fraction (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), and trabecular separation (Tb.Sp) were automatically determined for identification of osteoporosis, while BMD, BV/TV, Tb. N, Tb. Th, Tb.Sp, and the mean connective density (Conn.D) in VOI regions were used to evaluate new bone formation, using a protocol provided by the manufacturer of the micro-CT scanner as previously described.[10,33]

Mechanical testing. Pull-out tests and three-point bending tests were performed using a materials testing system (Electron E1000; Instron, UK). The femora (n = 5) containing the titanium rods were first subjected to epiphyseal separation to expose the titanium rod for pull-out tests. The mechanical test was performed at a cross-head speed of 1.00 mm/minute. Then, these fixed specimens were subjected to pull-out tests and load-displacement curves used to acquire mechanical parameters including the strength of fixation, energy failure, and interface stiffness, according to previous reports.[27,34] The right femora (n = 5) without the titanium rod underwent a three-point bending test as previously reported.[35] The results of bone biomechanical tests were shown by energy at break, ultimate load, and elastic modulus.

Histological evaluation. Following micro-CT scanning, these femur tissue specimens containing titanium rods (n = 5) and those without titanium rods (n = 5) were all transferred to 10% neutral-buffered formalin. The undecalcified femur specimens were cut and ground to a thickness of 40 to 50 μm using Saw Microtome Leica SP1600 (Leica, Germany). The sections were fixed with 70% ethanol, subjected to von-Gieson staining, and embedded in methyl methacrylate. Based on the observation results of bone tissue slices, the percentage of bone implant contact (BIC) and the bone area ratio (BA) were calculated to assess the effects of different treatments on osseointegration in ovariectomized rats according to established methods.[10,33] The sections of the femoral metaphysis were used to observe the changes of trabecular bone after treatment with different interventions.

The remaining femora (n = 5) after pull-out tests were placed into a decalcification solution of 10% ethylenediaminetetraacetic acid (EDTA) and 4% phosphate-buffered formalin solution for 28 days at 4°C. Subsequently, bone tissue was fixed in 10% (v/v) formalin, embedded in paraffin, and sliced into 5 mm-thick sections. Briefly, 3% hydrogen peroxide was used to block endogenous peroxidase for ten minutes, and trypsin was used to expose the antigen for 20 minutes. The sections were irrigated and incubated overnight with the commercially available specific osteocalcin antibodies (1:100, Abcam, UK) and tartrate-resistant acid phosphatase (TRAP) (1:300, Abcam) at 4°C. Finally, the slides were incubated with the corresponding goat anti-rabbit secondary antibody (1:2,000, Abcam) for 30 minutes and counter-stained with diaminobenzidine and haematoxylin. All sections were examined and photographed under a light microscope and analyzed using Image Pro Plus software (Media Cybernetics, USA).

Analysis of bone formation and resorption markers. Blood samples were obtained in a serum separator tube from the direct cardiac puncture when the rats were killed. Sera were stored at -80°C until analysis. Serum type 1 collagen N-terminal propeptides (P1NP, bone formation marker) were evaluated using a mouse enzyme immunoassay (EIA) kit (Immunodiagnostic Systems, UK). Type 1 collagen crosslinked C-telopeptide (CTX-1, bone resorption marker) levels in the serum were measured using the Serum CrossLaps (CTX-1) enzyme-linked immunosorbent assay (ELISA) kit (Immunodiagnostic Systems). The serum levels of oxidative stress-related indicators such as superoxide dissemination (SOD), malondialdehyde (MDA), and total antioxidant capacity (TAC) were measured using commercial biochemical kits from Nanjing Jiancheng Bioengineering Institute (China) according to the manufacturer's instructions.

Statistical analysis. The data were collected and reported as the mean and standard deviation (SD) for the final result of each experiment. Normality of the variables was checked using the Kolmogorov-Smirnov test. The independent-samples *t*-test and one-way analysis of variance (ANOVA) were used to test differences among different groups. SPSS Statistics 21.0 software (IBM, USA) was used for analyses. A p-value < 0.05 was considered statistically significant.

Results

Animal experiment. Only one animal death occurred in the ovariectomy surgery. Three rats died during or after

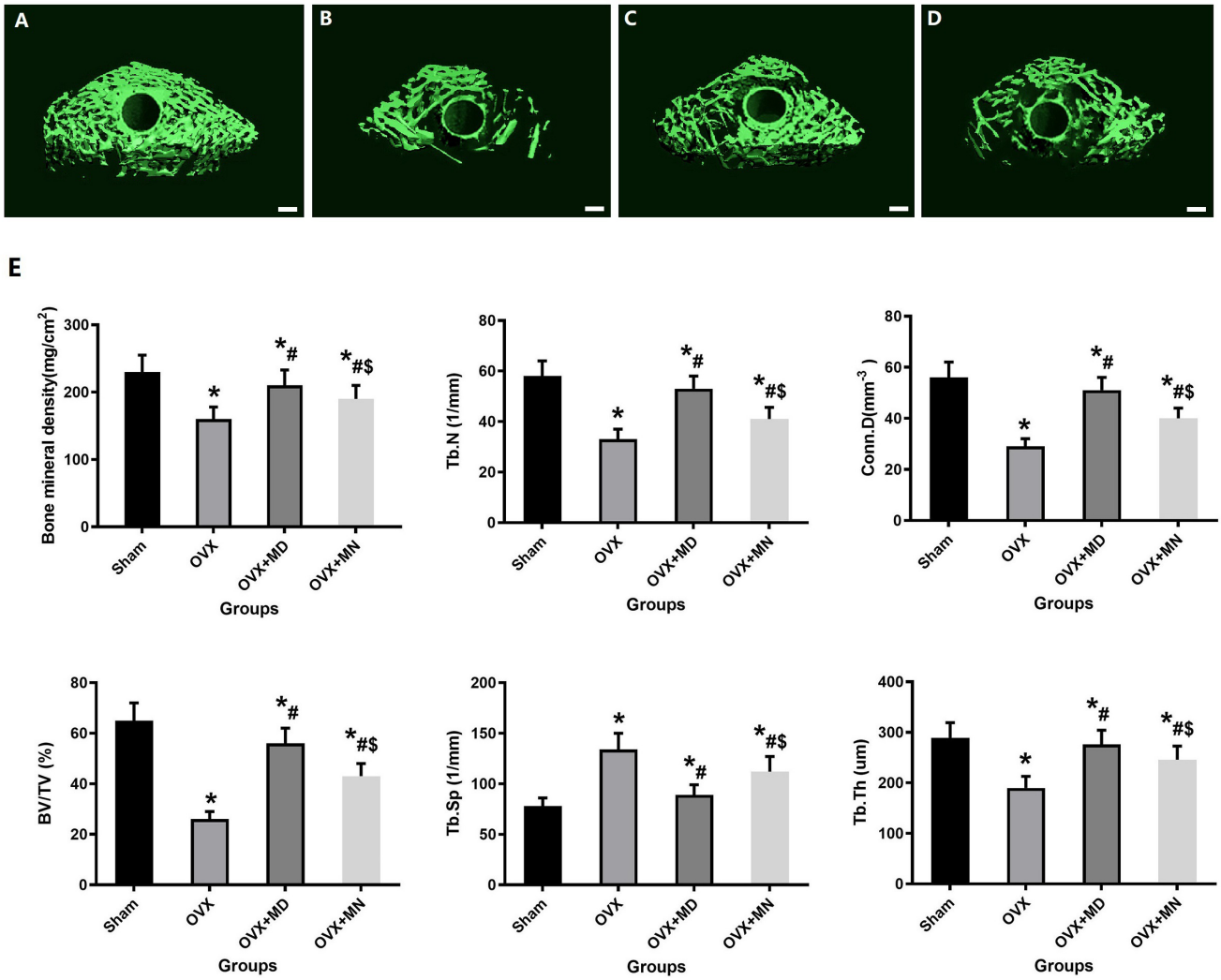

Fig. 1

a) to d) Micro-CT 3D reconstruction clearly shows that melatonin treatment can significantly improve the poor osseointegration of titanium rods in ovariectomized (OVX) rats: a) Sham group; b) OVX group; c) OVX + melatonin day treatment (MD) group; and d) OVX + melatonin night treatment (MN) group. e) The quantitative results of bone microscopic parameters around the titanium rod include bone mineral density, bone volume fraction (BV/TV), trabecular thickness (Tb. Th), trabecular number (Tb. N), connective density (Conn. D), and trabecular separation (Tb.Sp). There were five specimens per group; error bars in the figure indicate standard deviation. $ = vs Sham group; p = 0.015; # = vs OVX, p = 0.002; *vs OVX + MD group, p = 0.003, all independent-samples *t*-test and one-way analysis of variance.

titanium rod implantation, including one rat in the Sham group, one in the OVX + MD group (one day after surgery), and one in the OVX + MN group (two days after surgery).

The influence of different intervention treatments on the osseointegration of titanium rods. The results of osseointegration for the titanium rod in the femoral medullary cavity from the four groups of rats with different intervention via micro-CT evaluation are shown in Figures 1a to 1d. Bone formation around the titanium in the OVX group was inferior compared with the Sham group. After melatonin treatment, the bone formed around the implant was increased in the OVX + MD and the OVX + MN group compared with the OVX group. In the melatonin treatment groups, a substantial increase in bone tissue was observed around the surface of the implant. However, the bone that formed around the titanium rod in the OVX + MN group was lower than that in the OVX + MD group. Figure 1e illustrates the quantification of BMD, BV/TV, Tb. Th, Tb. N, Conn. D, and Tb.Sp among four groups. Compared to the Sham group, oestrogen deficiency can significantly reduce bone microscopic parameters including lower BMD, BV/TV, Tb. N, Conn.D, Tb. Th, while it can also increase Tb.Sp ($p = 0.002$). After melatonin treatment for 12 weeks, bone microscopic parameters of the OVX + MD group show higher BMD, BV/TV, Tb. N, Conn. D, Tb. Th, and lower Tb.Sp ($p < 0.05$) compared with the OVX + MN group. These findings indicate that melatonin

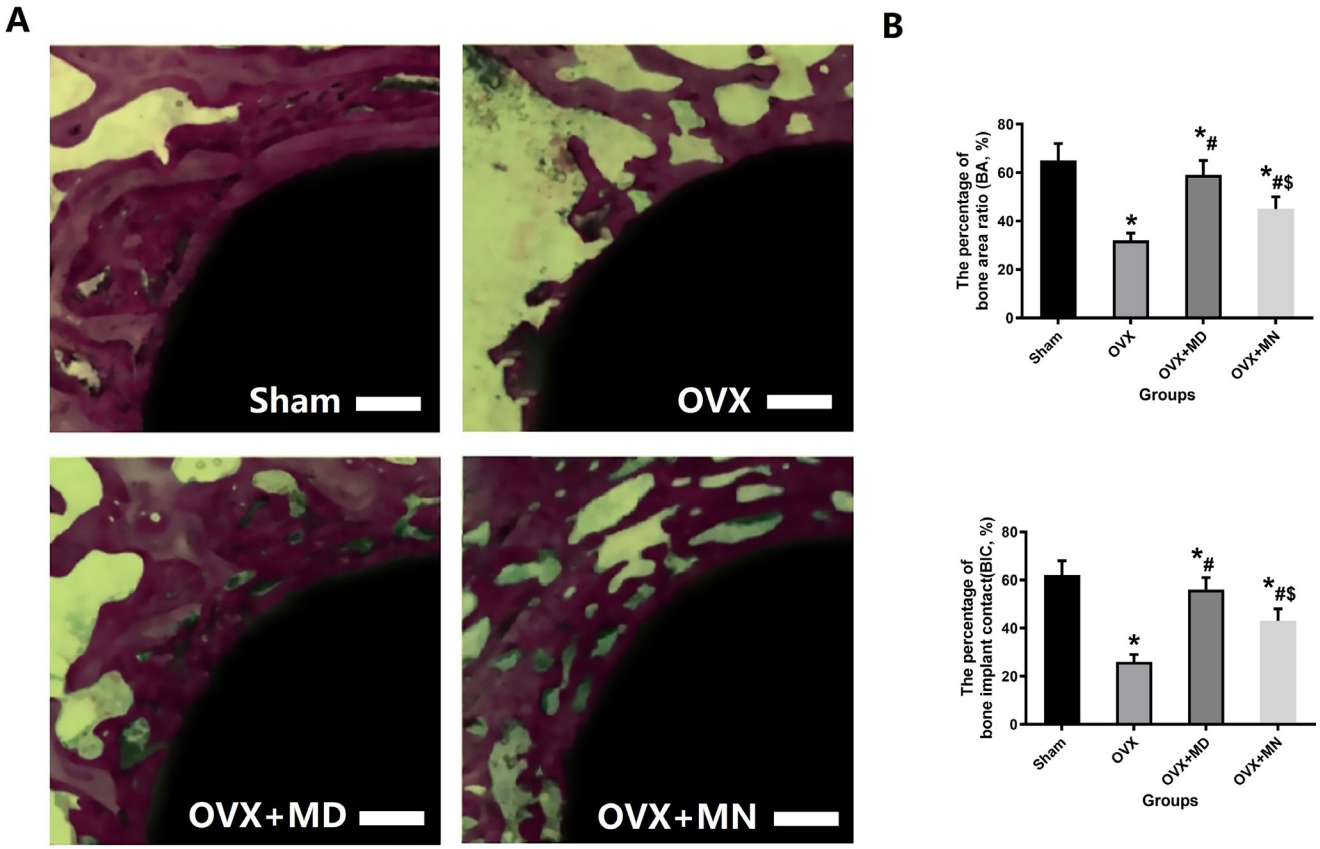

Fig. 2

Melatonin treatment can significantly improve bone formation around the titanium rod and increase the connection of bone tissue and internal implants in an ovariectomized (OVX) rat model. a) Bone formation around titanium rods under different intervention conditions, as shown by Von-Gieson staining (×10). b) The percentage of bone implant contact (BIC) and bone area ratio (BA). There were five specimens per group; error bars in the figure indicate the standard deviation. * = vs Sham group, p = 0.018; # = vs OVX, p = 0.005; $ = vs OVX + melatonin day treatment (MD) group, p = 0.005, all independent-samples *t*-test and one-way analysis of variance. MN, melatonin night treatment.

treatment could enhance osseointegration when administered during the day rather than at night.

Histological analysis. As shown in Figure 2, representative histological images show bone regeneration around titanium rods in OVX rats with different intervention treatments for 12 weeks: a large amount of new bone tissue was observed to surround the implant in the Sham group. However, due to osteoporosis we can only observe a small amount of bone tissue formation surrounding the titanium rod, while more bone tissue was found around the titanium rod in the OVX + MD group and the OVX + MN group. The quantitative results are represented by BIC and BA, as shown in Figure 2b. Compared with the Sham group, significantly reduced levels of BA and BIC were observed in the OVX groups (p = 0.023), while BA and BIC increased significantly after melatonin treatment. After melatonin treatment for 12 weeks, histological parameters of the OVX + MD group showed higher BIC and BA (p = 0.011), compared with the OVX + MN group. Histological results show that systemic treatment with melatonin could increase bone tissue formation around titanium rods and promote the connection between bone tissue and titanium rods. Furthermore, the effect of melatonin administration on the osseointegration of titanium rods at 9 am was significantly higher than that of melatonin administered at 9 pm, as demonstrated through histological analysis (p = 0.003).

Biomechanical analysis. The results of pull-out tests for titanium rods, including fixation strength, interface stiffness, and energy to failure, in each group of rats are shown in Figure 3. Osteoporosis can significantly reduce fixation strength, interface stiffness, and energy to failure of the titanium rod compared with the Sham group (p = 0.001, independent-samples *t*-test and one-way ANOVA). Moreover, melatonin can improve the push-out force for the titanium rods compared with the OVX group (p = 0.003). In addition, the biomechanical parameters of the OVX + MD group show higher values of fixation strength, interface stiffness, and energy to failure (p = 0.007) compared with the OVX + MN group. These results indicate that melatonin treatment can enhance the stability of the titanium implant in the marrow cavity of OVX rats. Furthermore, the effect of daytime melatonin administration on the stability of the titanium implant significantly

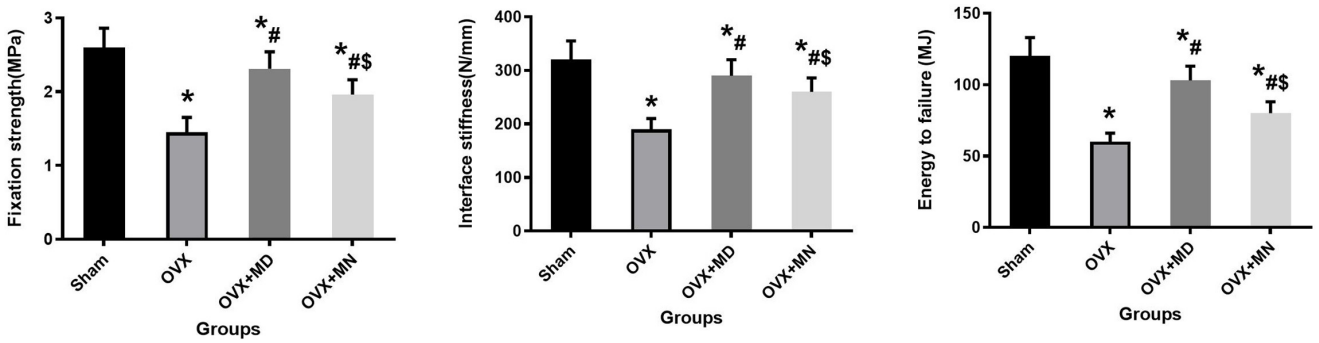

Fig. 3

These graphs show that melatonin treatment can significantly improve mechanical pull-out data, including fixation strength, interface stiffness, and energy to failure of the titanium rod, in an ovariectomized (OVX) rat model. There were five specimens per group; error bars in the figure indicate standard deviation. * = vs Sham group, p = 0.009, independent-samples *t*-test and one-way analysis of variance; # = vs OVX, p < 0.05; $ = vs OVX + melatonin day treatment (MD) group, p < 0.05. MN, melatonin night treatment.

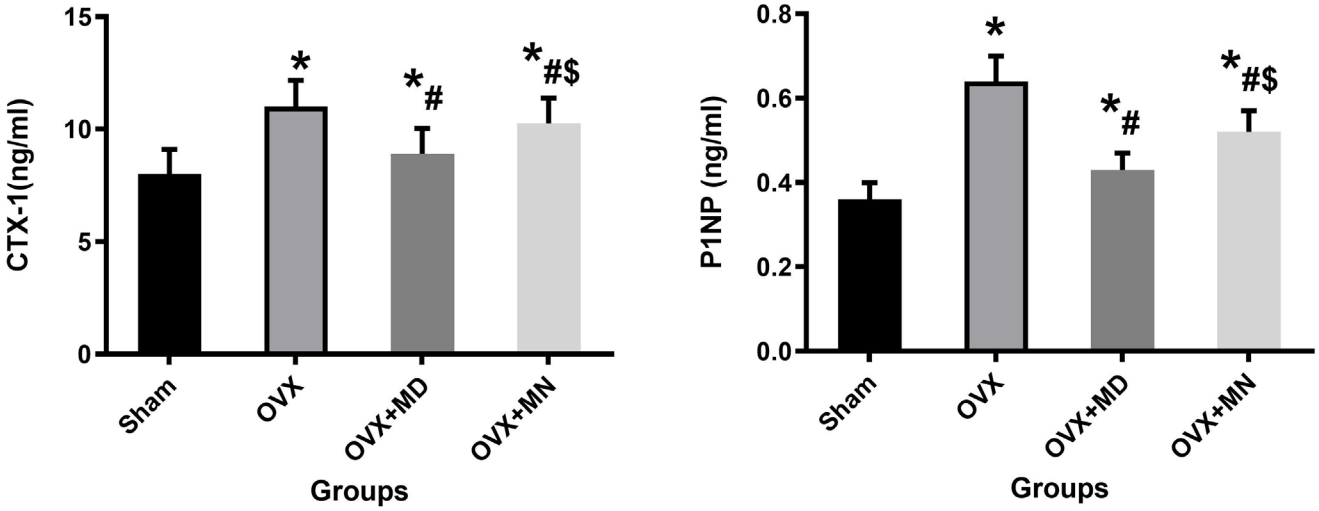

Fig. 4

These graphs show that melatonin treatment can significantly reverse the imbalance of bone metabolism in an ovariectomized (OVX) rat model. There were five specimens per group; error bars in the figure indicate standard deviation. * = vs Sham group, p = 0.011; # = vs OVX, p = 0.005; $ = vs OVX + melatonin day treatment (MD) group, p = 0.002, all independent-samples *t*-test and one-way analysis of variance. MN, melatonin night treatment.

improved compared to night-time melatonin administration, as demonstrated through biomechanical analysis.

Bone metabolism index analysis. The results of bone metabolism indicators were detected at the end of the experiment, as shown in Figure 4. Compared with the Sham group, the level of CTX-1 and P1NP increased in OVX rats. Compared with the OVX group, serum CTX-1 and P1NP decreased after melatonin treatment. In addition, the bone metabolism index of the OVX + MD group showed lower values of CTX-1 and P1NP (p = 0.014) compared with the OVX + MN group. These results indicate that melatonin can reverse the imbalance of bone metabolism in OVX rats. Furthermore, the effect of melatonin administration on decreasing the values of CTX-1 and P1NP at 9 am is more obvious than that at 9 pm, as shown through bone metabolism indicators.

Analysis of oxidative stress indicators. The results of oxidative stress indicators were detected 12 weeks after implantation, as shown in Figure 5. Compared with the Sham group, serum TAC and SOD decreased significantly in OVX rats (p = 0.012, independent-samples *t*-test and one-way ANOVA), while the level of MDA increased. Compared with the OVX group, serum TAC and SOD increased after melatonin treatment, while MDA decreased significantly. In addition, the oxidative stress indicators of the OVX + MD group showed lower values of MDA (p = 0.001 independent-samples *t*-test and one-way ANOVA), while TAC and SOD increased significantly compared

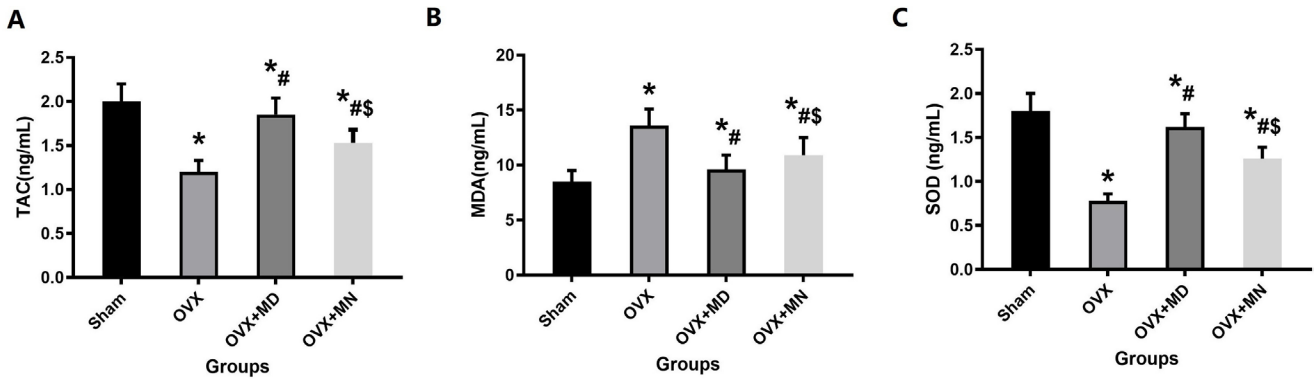

Fig. 5

These graphs show that melatonin treatment can significantly reduce the levels of oxidative stress in an ovariectomized (OVX) rat model. a) Serum total antioxidant capacity (TAC) level. b) Serum malondialdehyde (MDA) level. c) Serum superoxide dissemination (SOD) level. There were five specimens per group; error bars in the figure indicate standard deviation. * = vs Sham group, p = 0.001; # = vs OVX, p = 0.012; $ = vs OVX + melatonin day treatment (MD) group, p = 0.016, all independent-samples *t*-test and one-way analysis of variance. MN, melatonin night treatment.

with the OVX + MN group. These results indicate that melatonin can reverse the imbalance of oxidative stress in OVX rats. Furthermore, the effect of melatonin administration on decreasing the values of oxidative stress at 9 am is greater than that at 9 pm, as shown via bone metabolism indicator analysis.

The influence of different interventions treatment on the femoral metaphysis trabecular structure and BMD. The results of melatonin treatment on the femoral metaphysis trabecular structure and BMD from the four groups of rats via micro-CT evaluation and histological analysis are shown in Figures 6a to 6d. The BMD and trabecular bone structure in the OVX group were inferior compared with the Sham group. After melatonin treatment, the bone mass and BMD were increased in the OVX + MD and OVX + MN groups compared with the OVX group. In the melatonin treatment groups, substantial increased bone mass and BMD were observed at femoral metaphysis. However, the bone mass and the BMD in the OVX + MN group were lower than those in the OVX + MD group. Figure 6e illustrates the quantification of BMD, BV/TV, Tb.Th, Tb. N, Conn. D, and Tb.Sp among the four groups. Compared to the Sham group, oestrogen deficiency can significantly reduce bone microscopic parameters including lower BMD, BV/TV, Tb. N, Conn.D, and Tb. Th, while it can also increase Tb.Sp (p = 0.001, independent-samples *t*-test and one-way ANOVA). After melatonin treatment for 12 weeks, bone microscopic parameters of the OVX + MD group showed higher BMD, BV/TV, Tb. N, Conn.D, Tb. Th, and lower Tb.Sp (p = 0.004) compared with the OVX + MN group. These findings indicate that melatonin treatment could improve BMD and bone mass when administered during the day.

Three-point bending test. The results of a three-point bending test for femoral shaft, including energy at break, ultimate load, and elastic modulus, in each group of rats are shown in Figure 7. Osteoporosis can significantly reduce energy at break, ultimate load, and elastic modulus of the femoral shaft compared with the Sham group (p = 0.003). Moreover, melatonin can improve the energy at break, ultimate load, and elastic modulus for the femoral shaft compared with the OVX group (p = 0.021, independent-samples *t*-test and one-way ANOVA). In addition, the biomechanical parameters of the OVX + MD group showed higher values of energy at break, ultimate load, and elastic modulus (p = 0.004, independent-samples *t*-test and one-way ANOVA) compared with the OVX + MN group. These results indicate that melatonin treatment can enhance the mechanical characteristics of the femoral shaft in OVX rats. Furthermore, the effect of melatonin administration on the mechanical parameters of the femoral shaft at 9 am is significantly better than that at 9 pm, as shown via biomechanical analysis (p = 0.013, independent-samples *t*-test and one-way ANOVA).

Immunohistochemical analysis. To determine the underlying mechanisms of melatonin's positive effects, we next performed immunohistochemical analysis of bone tissue, as shown in Figure 8. The expression of OC was upregulated in the OVX + MD and OVX + MN groups. In addition, TRAP staining showed an increase in the number of osteoclasts in OVX rats, which decreased after the administration of melatonin; TRAP-positive signals in the OVX + MD and OVX + MN groups were significantly lower than in the OVX group (p = 0.001). Additionally, OC staining and TRAP staining were performed on distal femur, which more comprehensively confirmed that daytime melatonin treatment can reduce bone turnover markers and increase bone formation markers.

Discussion

As an endocrine hormone influenced by the circadian rhythm, melatonin is produced with low levels expressed during the day and higher levels at night.[36,37] This raises the interesting question of whether the administration of melatonin at different timepoints influences the osseointegration of titanium rods during osteoporosis. In

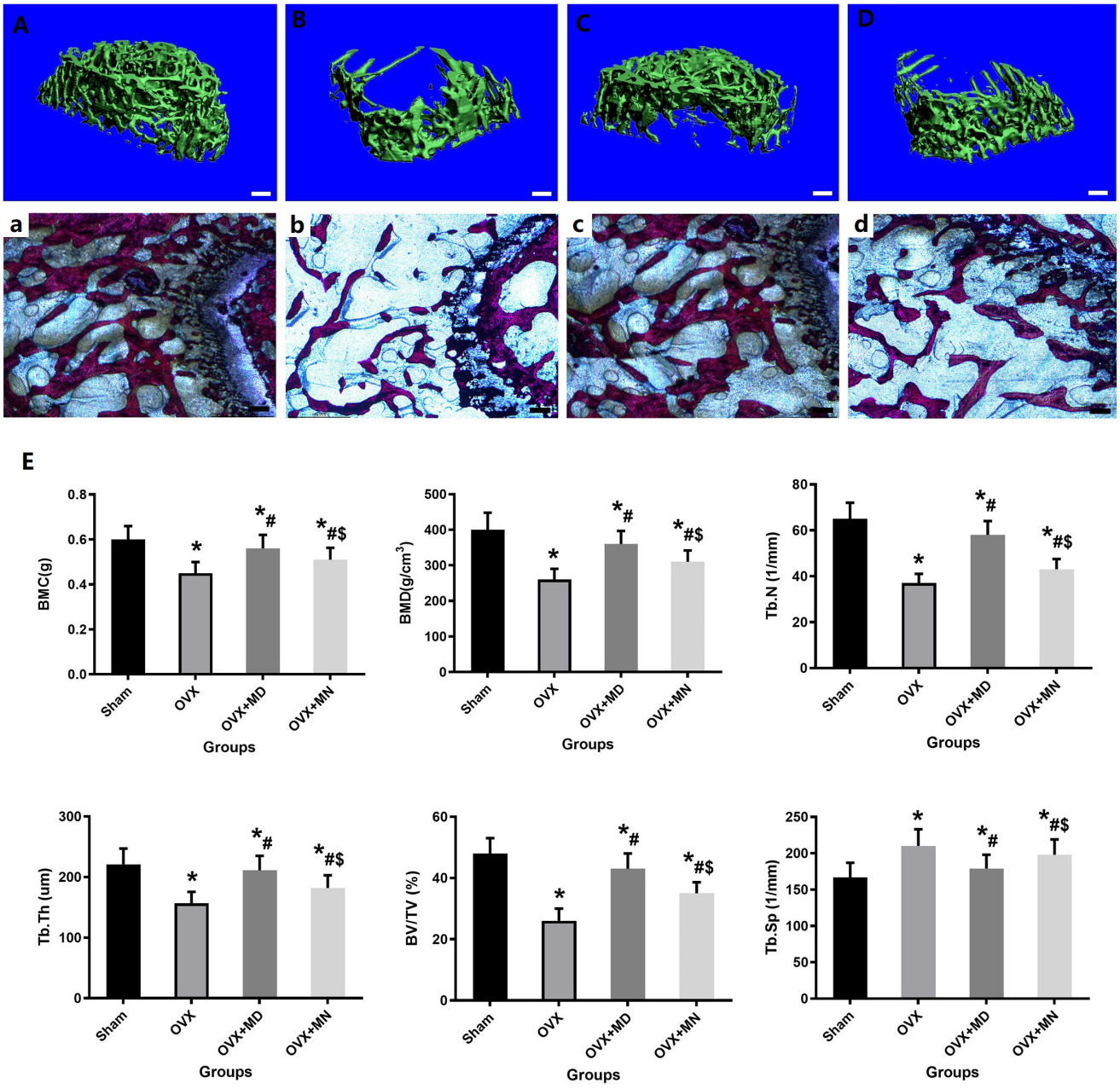

Fig. 6

a) to d) Micro-CT 3D reconstruction and histological results clearly show that melatonin treatment can significantly improve the poor osseointegration of titanium rods in ovariectomized (OVX) rats, as also shown by Von-Gieson staining (×10): a) Sham group; b) OVX group; c) OVX + melatonin day treatment (MD) group; and d) OVX + melatonin night treatment (MN) group. e) The quantitative results of bone microscopic parameters of the femoral metaphysis trabecular structure include bone mineral density (BMD), bone volume fraction (BV/TV), trabecular thickness (Tb. Th), trabecular number (Tb. N), connective density (Conn. D), and trabecular separation (Tb.Sp). There were five specimens per group; error bars in the figure indicate the standard deviation. * = vs Sham group, p = 0.001; # = vs OVX, p = 0.013; $ = vs OVX + MD group, p = 0.027, all independent-samples t-test and one-way analysis of variance.

recent years, several studies have shown that systemic or topical administration of melatonin could be used to accelerate the process of osseointegration.[32,38,39] However, research in this area is still sparse and limited. Therefore, based on the widely used OVX rat model, the aim of this study was to explore the effect of melatonin treatment at 9 o'clock in the night and 9 o'clock in the day on the osseointegration of titanium rods in osteoporotic rats. The experimental results reveal to us an important and expected phenomenon: that melatonin administered at different times can promote the osseointegration of the titanium rod in the medullary cavity of the femoral metaphysis in OVX rats. Interestingly, daytime melatonin injections are more effective at promoting trabecular

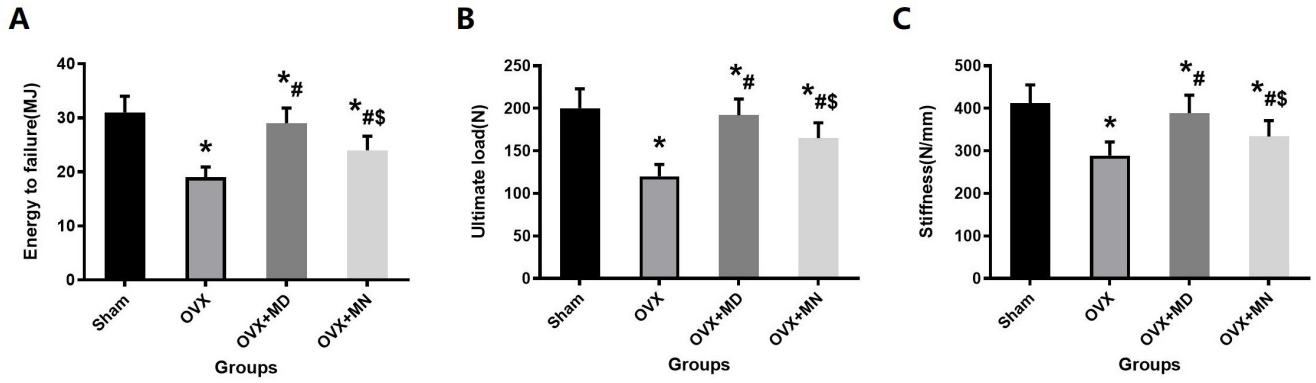

Fig. 7

Melatonin treatment can significantly improve the data of three-point bending test for femoral shaft including a) energy to failure, b) maximum load, and c) stiffness in each group of rats. There were five specimens per group; error bars in the figure indicate the standard deviation. * = vs Sham group, p = 0.002; # = vs ovariectomized rats (OVX), p = 0.015; $ = vs OVX + melatonin day treatment (MD) group, p = 0.001, all independent-samples *t*-test and one-way analysis of variance. MN, melatonin night treatment.

bone formation and improving osseointegration than night-time melatonin injections.

In this study, we built a reliable bilateral OVX rat model confirmed by extensive research,[26,28] which can reproduce the pathological condition of postmenopausal osteoporosis. At 12 weeks after surgery, the bone mineral density and bone mass of the rats in the OVX group were found to have rapidly decreased. This bone state is consistent with the physiological state of postmenopausal osteoporosis, which suggested that the postmenopausal osteoporosis model constructed by the first operation in this study is satisfactory and successful. Subsequently, based on the OVX rat model, we performed a second operation to build a standard implantation model in the femoral medullary cavity. In the experiment, only a small amount of bone tissue formed around titanium rods in the OVX group through histological evidence and micro-CT analysis. The results of the biomechanical analysis further assessed the harmful effects on the stability of mechanical fixation by bilateral ovariectomy through pull-out tests. As observed in the OVX group, the osseointegration of the titanium rods cannot be obtained satisfactorily without intervention by medication or other means under osteoporotic conditions.

In the study, we have demonstrated the beneficial effect of systemic treatment with melatonin on new bone around a titanium implant, with significantly increased microscopic parameters of bone tissue in the VOI region including BMD, BV/TV, Tb.Th, Tb.N, and Conn. D in OVX rats. The biomechanical testing results further demonstrate the potentially beneficial role of melatonin in improving implant stability. Our results show that systemic medication with melatonin could increase titanium implant osseointegration, improve bone formation surrounding the implant, and enhance implant fixation for 12 weeks in OVX rats, regardless of whether the melatonin was administered at 9 am or 9 pm. Interestingly, a large amount of bone tissue formed around the titanium rod was observed in the OVX + MD group, when compared with the OVX + MN group. Better osseointegration of the titanium rod after melatonin treatment at 9 am may indicate greater bone formation around the implant, which may be associated with the enhanced activity of osteoblasts or lower osteoblast activity.

In order to confirm the possible mechanism of this result, we further examined the levels of the serum bone metabolism indicators, oxidative stress indicator, and evaluated the changes in the trabecular structure and mechanical parameters of the femoral metaphysis on the other side of the rat. Owing to the increased reactive oxygen species production in postmenopausal osteoporosis, studies have indicated that the increased levels of oxidative stress induced by oestrogen deficiency are a major pathogenic factor of restricted bone formation and related cell injury.[40,41] Simultaneously, oxidative stress is an important mediating factor leading to osteoporosis in the elderly.[42] The level of MDA, TAC, and SOD2 can directly reflect the level of oxidative stress in cells and in vivo.[43,44] Due to oxidative stress being reported to play an important role in bone repair in osteoporosis, the serum indicators of oxidative stress including SOD2, MDA, and TAC were measured and evaluated. The serum TAC and SOD2 level decreased significantly (p = 0.001), while the level of MDA increased in OVX rats. Serum TAC and SOD2 increased after melatonin treatment, while MDA decreased significantly (p = 0.001), which were similar to reports of other studies.[43,44] The results of the bone metabolism index showed that the bone formation index (P1NP) was significantly reduced (p = 0.001), while the bone resorption index (CTX-1) decreased, and melatonin treatment can reverse this imbalance of bone metabolism. Histology, micro-CT imaging, and biomechanical testing assessed the potentially beneficial role of melatonin treatment in the changes in trabecular microstructural properties and parameters in the distal femur metaphysis. Combined with the nocturnal peak secretion of melatonin[13,14] and the dose-dependent nature of its therapeutic effect,[45] we deduced that daytime melatonin

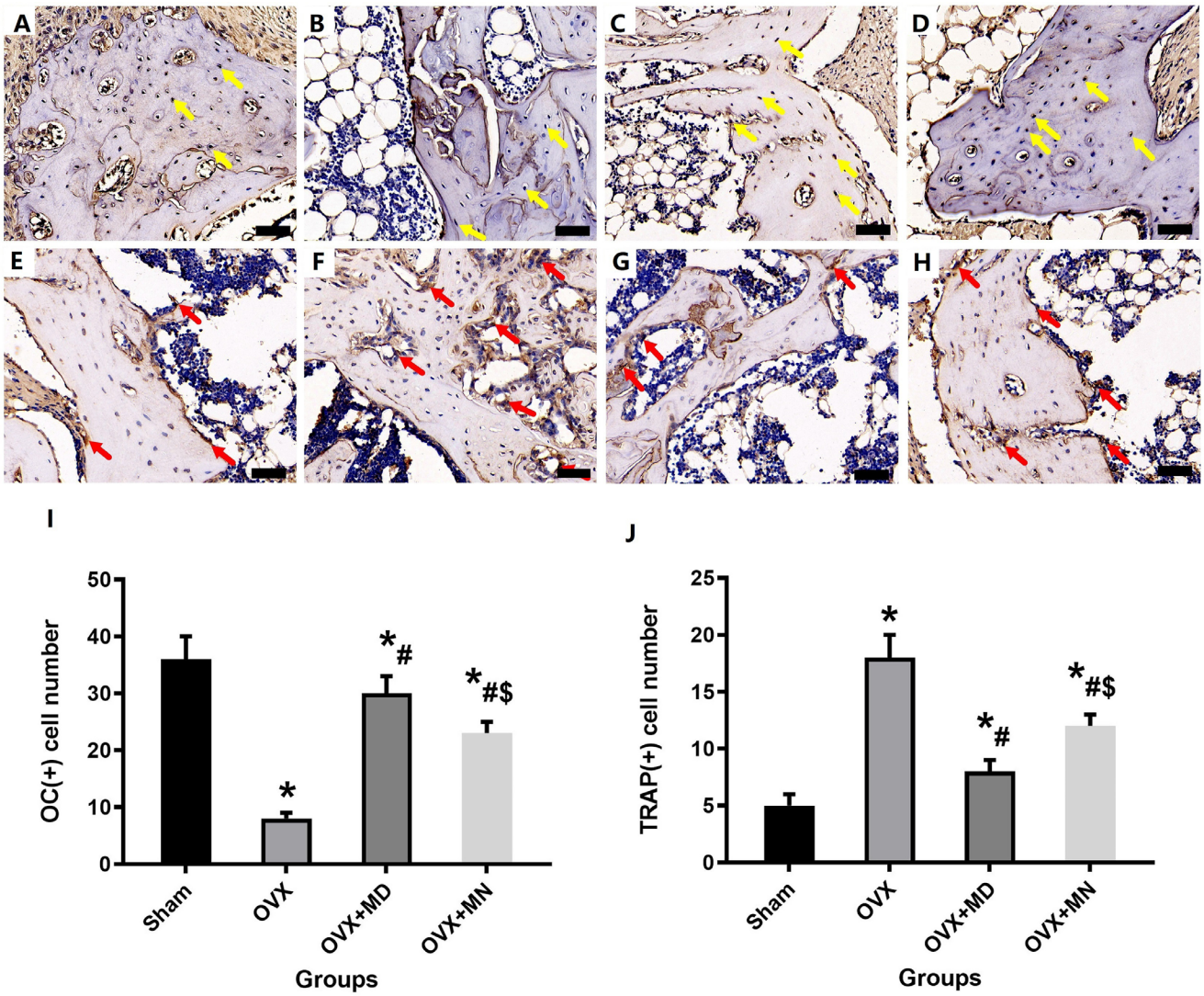

Fig. 8

Melatonin treatment improves the number of osteoblasts and reduces the number of osteoclasts in osteoporosis model rats. a) to h) After the administration of melatonin, bone tissue around the titanium rod in the distal femur was detected by osteocalcin (OC) staining (yellow arrows represent osteoblasts; magnification ×400) and tartrate-resistant acid phosphatase (TRAP) staining (red arrows represent osteoclasts; magnification ×400). i) The numbers of OC-stained osteoblasts and j) TRAP-stained osteoclasts were quantified. a) and e) Sham group; b) and f) ovariectomized rats (OVX) group; c) and g) OVX + melatonin day treatment (MD) group; and d) and h) OVX + melatonin night treatment (MN) group. There were five specimens per group. * = vs Sham group, $p < 0.05$; # = vs OVX, $p < 0.05$; $ = vs OVX + MD group.

therapy is able to compensate for the lack of melatonin in the body and maintain a high level of melatonin in the body when administered during the daytime (9 am) while administration at night (9 pm) may cause a negative hormonal feedback effect, resulting in a blocked release of melatonin from the pineal gland and therefore a low level in the body overall.

As far as we know, this is the first study of the effect of systemic administration of melatonin at 9 am (daytime) and 9 pm (night-time) on the osseointegration of titanium rod under osteoporotic conditions. Nevertheless, this study had several deficiencies. First, the mechanisms underlying the effects of systemic administration of melatonin at different times should be elucidated. Second, the changes in serum melatonin and oestrogen were not further evaluated. Finally, the lack of a pineal gland destruction group could affect the applicable value of the research.

In summary, our study suggests that systemic administration of melatonin could improve the initial osseointegration of titanium implants under osteoporotic conditions regardless of daytime or night-time timepoint. With that said, the effect of melatonin administration on the osseointegration of titanium implants at 9 am is significantly better than that at 9 pm.

Supplementary material

ARRIVE checklist, representative radiograph of femoral rod implants in all four groups, and micro-CT region of interest used for bone parameter analysis.

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Funding statement:
- The authors disclose receipt of the following financial or material support for the research, authorship, and/or publication of this article: this study was supported by a grant from National Natural Science Foundation of China (82002322), Funding of "Peak" Training Program and "Panfeng" Innovation Team Project for Scientific Research of Yijishan Hospital, Wannan Medical College (grant no. GF2019G04, PF2019005, GF2019T02, and PF2019007) and Young and Middle-aged Key Project of Wannan Medical College (WK2020ZF16).

Acknowledgements:
- Many thanks to Min Yang and Tianlin Li for their guidance on the experiments.

Open access funding
- The open access fee for this study was self-funded.