

Mechanism of melatonin combined with calcium carbonate on improving osteoporosis in aged rats

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Abstract. The effects of melatonin and calcium carbonate on aged rats with osteoporosis (OP) were assessed. Forty female Sprague-Dawley (SD) rats aged 15 months were randomly divided into a model group (group OP), melatonin group (group M), calcium carbonate group (group Ca) and melatonin combined with calcium carbonate group (group M+Ca), while 10 rats aged 3 months were set as the control group (group NC). The changes of bone density and bone mineral level of lumbar vertebra and bilateral femur in rats of each group were observed. The levels of serum calcium, phosphorus, superoxide dismutase (SOD), malondialdehyde (MDA) and glutathione peroxidase (GSH-Px) in rats of each group were determined. Compared with those in group NC, bone density of lumbar vertebra and bilateral femur and bone mineral level were distinctly reduced, serum calcium and activities of SOD and GSH-Px were obviously decreased, and MDA content was remarkably increased in rats of groups OP, M and Ca; the differences were statistically significant ($P<0.05$ or $P<0.01$); compared with that in group OP, bone density of lumbar vertebra and bilateral femur and bone mineral level were remarkably increased, serum calcium and activities of SOD and GSH-Px were obviously increased, and MDA content was remarkably decreased in rats of groups M, Ca and M+Ca; the differences were statistically significant ($P<0.05$ or $P<0.01$); compared with those in groups M and Ca, bone density of lumbar vertebra and bilateral femur and bone mineral level were obviously elevated, serum calcium and activities of SOD and GSH-Px were evidently elevated, and MDA content was remarkably decreased in rats of group M+Ca; the differences were statistically significant ($P<0.05$). Melatonin and calcium carbonate can significantly improve antioxidative ability in

rats with osteoporosis, increase bone density, elevate serum calcium level and reduce bone mineral loss, thus preventing and treating osteoporosis, and the combination displays more remarkable effects.

Introduction

Osteoporosis (OP) is a systemic disease characterized by the reduction of bone matrix and mineral, resulting in the destruction and rupture of trabecular bone and the increase of fragility of bone, thus reducing biomechanical properties (1). Along with increase in age, the absorption of calcium by the body decreases with the reduction of osteoblast production, which leads to the gradual increase of bone loss, finally resulting in the occurrence of OP (2). The prevention and treatment of OP include the adequate intake of vitamin D and calcium, proper exercise and other lifestyle interventions (3). Melatonin is a hormone mainly secreted by the pineal gland, which has antioxidant, scavenging free radicals, ameliorating sleep, antitumor, regulating immune response of the body and other biological functions (4,5). Studies have indicated that (6,7) melatonin can mediate the secretion of thyroxine and calcitonin in the body, thereby regulating calcium metabolism *in vivo*. In recent years, melatonin has been applied by some scholars in the prevention and treatment of OP (8). However, there is no report on melatonin combined with calcium carbonate to prevent and treat OP. Hence, by adopting melatonin combined with calcium carbonate in senile female rats, this study aimed to observe the effects of the combination on bone density, bone mineral and oxidative stress, so as to provide the basis for clinical prevention and treatment of OP.

Materials and methods

Materials

Experimental animals. Ten female specific pathogen-free (SPF) Sprague-Dawley (SD) rats aged 3 months, weighing 180-250 g, were selected. Forty female SPF SD rats aged 15 months were enrolled, which displayed osteoporosis via comparison with rats aged 3 months through determination by dual-energy X-ray absorptiometry. Fifty experimental animals were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (license no. SCXK 2012-0002; Shanghai, China). The rats were kept in cage with controlled temperature and light cycles

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(24°C and 12/12 light cycles) and free access to food and water. The humidity was 40%. The study was approved by the Ethics Committee of Dezhou People's Hospital (Dezhou, China).

Experimental reagents. Calcium carbonate, melatonin (Sigma-Aldrich, San Francisco, CA, USA), and kits of superoxide dismutase (SOD), malondialdehyde (MDA) and glutathione peroxidase (GSH-Px) (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) were prepared.

Experimental instruments. Dual-energy X-ray absorptiometry (Hologic, Marlborough, MA, USA); fully automatic biochemistry analyzer (Mindray Biomedical Electronics Co., Ltd., Shenzhen, China); continuous-wavelength multifunctional microplate reader (Tecan, Salzburg, Austria).

Methods

Grouping and medication of experimental animals. Forty senile female SPF rats were adaptively fed for one week, followed by being randomly divided into four groups with 10 animals in each group, namely model group (group OP), melatonin group (group M), calcium carbonate group (group Ca) and melatonin combined with calcium carbonate group (group M+Ca), while 10 rats aged 3 months were set as the control group (group NC). All rats were reared in an SPF system, fed with ordinary feedstuff, and ate freely during the experiment. Rats in group M were treated with 40 mg/kg melatonin via intragastric administration once a day; rats in group Ca received 20 mg/kg calcium carbonate via intragastric administration once a day; rats in group M+Ca were treated with 40 mg/kg melatonin combined with 20 mg/kg calcium carbonate via intragastric administration once a day; rats in groups NC and OP were given intragastric administration of normal saline every day for consecutive 12 weeks.

Measurement of bone density. After the end of the last intragastric administration, rats were treated with fasting for solids not liquids for 8 h. The rats were weighed, followed by intraperitoneal injection of 50 mg/kg 2% pentobarbital sodium for anesthesia. The four limbs of rats were fixed, which were placed at supine position under the probe of dual energy X-ray absorptiometry; it should keep a vertical state of vertebra and horizontality of bilateral femur. The bone density of lumbar vertebrae, L4-L6, and left and right femurs was measured by dual-energy X-ray absorptiometry.

Detection of serum indexes. After the measurement of bone density, the blood was extracted from abdominal aorta and centrifuged at $2,030 \times g$ at 4°C for 10 min. The serum was separated and cryopreserved at -80°C. Serum calcium and phosphorus were detected by fully automatic biochemical analyzer. Serum SOD, MDA and GSH-Px were treated by sequential sample loading according to the instructions for the detection. The content of SOD in each group was measured at 550 nm by continuous-wavelength multifunctional enzyme analyzer. The content of MDA in each group was determined at 532 nm, and the content of GSH-Px in each group was detected at 412 nm.

Determination of bone mineral level. The left femur of rats was isolated, and the attached soft tissue was removed. Femur was roasted in the oven at 110°C for 48 h to the constant weight. The bone dry weight was weighed by an analytical balance, and the ratio of bone dry weight to body mass was calculated. Then, the femur was incinerated in a chamber-type

electric resistance furnace at 700°C for 6 h. The ash weight was weighted by an analytical balance, and the ratios of bone ash weight to body mass and bone ash weight to bone dry weight (%) were calculated.

Statistical analysis. Experimental results were expressed as mean \pm SD. The data were statistically analyzed by SPSS 20.0 software (IBM Corp., Armonk, NY, USA). The independent-samples t-test was used for comparisons between two groups, and one-way analysis of variance (ANOVA) was utilized for comparisons among multiple groups and Least Significant Difference was used for post hoc. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Changes of bone density of rats in each group. Compared with that in group NC, bone density of lumbar vertebra and bilateral femur was distinctly reduced in rats of groups OP, M and Ca ($P < 0.05$ or $P < 0.01$); compared with that in group OP, bone density of lumbar vertebra and bilateral femur was remarkably increased in rats of groups M, Ca and M+Ca ($P < 0.05$); compared with that in groups M and Ca, bone density of lumbar vertebra and bilateral femur was obviously elevated in rats of group M+Ca ($P < 0.05$); there was no significant difference in comparison of bone density between groups M+Ca and NC ($P > 0.05$). The results indicated that melatonin and calcium carbonate could significantly ameliorate bone density in rats with osteoporosis, and the combination displayed more remarkable effects (Table I).

Bone mineral metabolism in rats of each group. Compared with those in group NC, bone dry weight, the ratio of bone dry weight to body mass, bone ash weight and the ratios of bone ash weight to body mass and bone ash weight to bone dry weight of rats in groups OP, M and Ca were significantly reduced ($P < 0.05$ or $P < 0.01$); compared with those in group OP, these indexes of rats in groups M, Ca and M+Ca were obviously increased ($P < 0.05$ or $P < 0.01$); compared with those in groups M and Ca, these indexes of rats in group M+Ca were remarkably elevated ($P < 0.05$); the comparisons in these indexes between groups M+Ca and NC had no distinct differences ($P > 0.05$). The results revealed that melatonin and calcium carbonate could significantly reduce bone mineral loss in rats with osteoporosis, and the combination displayed more remarkable effects (Tables II and III).

Changes of serum calcium and phosphorus levels in rats of each group. Compared with that in group NC, serum calcium level was significantly reduced in rats of groups OP, M and Ca ($P < 0.05$ or $P < 0.01$); compared with that in group OP, serum calcium level was obviously increased in rats of groups M, Ca and M+Ca ($P < 0.05$); compared with that in groups M and Ca, serum calcium level was remarkably elevated in rats of group M+Ca ($P < 0.05$); the comparison in this level between groups M+Ca and NC had no distinct difference ($P > 0.05$). There was no evident difference in comparison of serum phosphorus level in rats among groups ($P > 0.05$). The results revealed that melatonin and calcium carbonate could significantly increase serum calcium level in rats with

Table I. Comparisons of bone density of lumbar vertebra and bilateral femur in rats among groups.

Groups	No.	Lumbar vertebra	Left femur	Right femur
NC	10	0.252±0.014	0.248±0.017	0.249±0.017
OP	10	0.177±0.021 ^b	0.172±0.016 ^b	0.174±0.018 ^b
M	10	0.210±0.018 ^{a,c}	0.212±0.021 ^{a,c}	0.215±0.015 ^{a,c}
Ca	10	0.221±0.018 ^{a,c}	0.219±0.016 ^{a,c}	0.220±0.022 ^{a,c}
M+Ca	10	0.248±0.020 ^{d-f}	0.243±0.018 ^{d-f}	0.245±0.019 ^{d-f}

Compared with group NC, ^aP<0.05 and ^bP<0.01; compared with group OP, ^cP<0.05 and ^dP<0.01; compared with group M, ^eP<0.05; compared with group Ca, ^fP<0.05.

Table II. Comparisons of bone dry weight in rats among groups.

Groups	No.	Body mass (g)	Bone dry weight (mg)	Bone dry weight/body mass (g/kg)
NC	10	332.35±18.71	580.16±23.68	1.75±0.09
OP	10	328.91±17.22	497.47±25.10 ^b	1.51±0.06 ^b
M	10	330.64±12.16	532.04±27.82 ^{a,c}	1.61±0.09 ^{a,c}
Ca	10	334.11±14.40	538.48±20.98 ^{a,c}	1.62±0.10 ^{a,c}
M+Ca	10	331.52±17.84	571.60±22.73 ^{d-f}	1.72±0.13 ^{c-f}

Compared with group NC, ^aP<0.05 and ^bP<0.01; compared with group OP, ^cP<0.05 and ^dP<0.01; compared with group M, ^eP<0.05; compared with group Ca, ^fP<0.05.

Table III. Comparisons of bone ash weight in rats among groups.

Groups	No.	Body mass (g)	Bone ash weight (mg)	Bone ash weight/body mass (g/kg)	Bone ash weight/bone dry weight (%)
NC	10	332.35±18.71	382.87±20.09	1.15±0.06	65.99±1.13
OP	10	328.91±17.22	290.04±18.53 ^b	0.88±0.08 ^b	58.30±0.94 ^b
M	10	330.64±12.16	331.61±16.78 ^{b,c}	1.00±0.05 ^{a,c}	62.33±0.81 ^{a,c}
Ca	10	334.11±14.40	335.29±19.24 ^{a,c}	1.01±0.05 ^{a,c}	62.27±0.90 ^{a,c}
M+Ca	10	331.52±17.84	374.83±18.33 ^{d-f}	1.13±0.04 ^{d-f}	65.58±0.77 ^{d-f}

Compared with group NC, ^aP<0.05 and ^bP<0.01; compared with group OP, ^cP<0.05 and ^dP<0.01; compared with group M, ^eP<0.05; compared with group Ca, ^fP<0.05.

osteoporosis, and the combination displayed more remarkable effects (Table IV).

Changes of serum SOD, GSH-Px activity and MDA content in rats of each group. Compared with those in group NC, serum SOD and GSH-Px activities in rats of groups OP, M and Ca were significantly reduced (P<0.05 or P<0.01), and MDA content was obviously increased (P<0.05 or P<0.01); compared with those in group OP, serum SOD and GSH-Px activities in rats of groups M, Ca and M+Ca were obviously increased (P<0.05 or P<0.01), and MDA content was obviously decreased (P<0.05 or P<0.01); compared with those in groups M and Ca, serum SOD and GSH-Px activities in rats of group M+Ca were remarkably elevated (P<0.05), and MDA content was distinctly reduced (P<0.05); the comparison in these levels between

Table IV. Comparisons of calcium and phosphorus levels in rats among groups.

Groups	No.	Calcium (mmol/l)	Phosphorus (mmol/l)
NC	10	2.68±0.15	1.55±0.13
OP	10	1.93±0.20 ^b	1.52±0.17
M	10	2.27±0.18 ^{a,c}	1.56±0.21
Ca	10	2.36±0.21 ^{a,d}	1.53±0.19
M+Ca	10	2.59±0.17 ^{d-f}	1.55±0.17

Compared with group NC, ^aP<0.05 and ^bP<0.01; compared with group OP, ^cP<0.05 and ^dP<0.01; compared with group M, ^eP<0.05; compared with group Ca, ^fP<0.05.

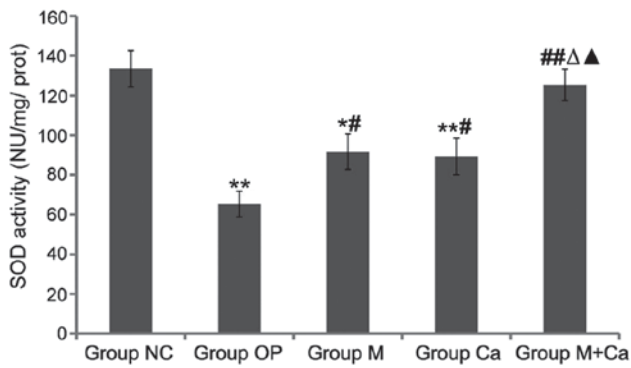


Figure 1. Comparison of superoxide dismutase (SOD) activity among groups. Compared with group NC, * $P < 0.05$ and ** $P < 0.01$; compared with group OP, # $P < 0.05$ and ## $P < 0.01$; compared with group M, $^{\Delta}P < 0.05$; compared with group Ca, $^{\Delta}P < 0.05$.

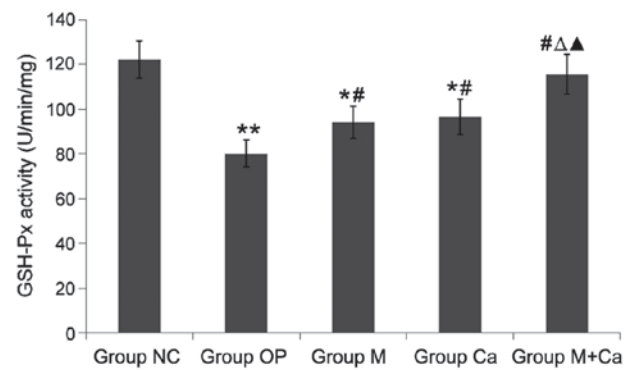


Figure 3. Comparison of glutathione peroxidase (GSH-Px) activity among groups. Compared with group NC, * $P < 0.05$; ** $P < 0.01$; compared with group OP, # $P < 0.05$; compared with group M, $^{\Delta}P < 0.05$; compared with group Ca, $^{\Delta}P < 0.05$.

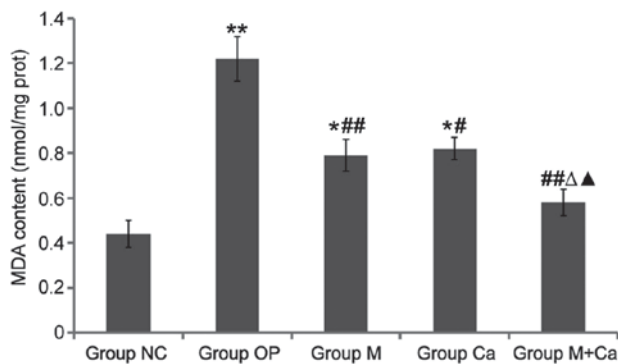


Figure 2. Comparison of malondialdehyde (MDA) content among groups. Compared with group NC, * $P < 0.05$ and ** $P < 0.01$; compared with group OP, # $P < 0.05$ and ## $P < 0.01$; compared with group M, $^{\Delta}P < 0.05$; compared with group Ca, $^{\Delta}P < 0.05$.

groups M+Ca and NC had no distinct differences ($P > 0.05$). The results revealed that melatonin and calcium carbonate could significantly increase serum SOD and GSH-Px activities and reduce MDA content, and the combination displayed more remarkable effects (Figs. 1-3).

Discussion

OP is mainly characterized by degenerative changes of bone tissue microstructure, osteopenia and increased bone fragility thus remarkably increasing fracture risk (9). OP is prevalent in elderly people, and age is a major risk factor of OP (10,11). In recent years, as China entered the time of the aged, the incidence of OP has increased year by year, and its fracture and other complications seriously affect people's lives. The study on OP has attracted wide attention of scholars in China and elsewhere. Calcium is one of the important trace elements in minerals of the body, and the main pathological change of OP is bone mineral loss (12). Therein, calcium deficiency is an important cause of OP (2). Thus, calcium supplementation is the most effective and safe method in the prevention and treatment of OP (13). Appropriate supplementation of calcium can enhance bone calcium levels and increase bone density, thereby significantly ameliorating bone formation (14). Adequate intake of calcium and vitamin D can prevent bone loss in

postmenopausal women, and is able to maintain bone density of femoral neck and lumbar vertebrae (15), thus reducing the incidence of fractures (16). The measurement of serum calcium and phosphorus can indirectly reflect bone metabolism in the body (17). This study revealed severely decreased bone density, bone mineral loss and reduced serum calcium level in elderly OP rats. The supplement of exogenous calcium carbonate can significantly increase bone density, elevate serum calcium level and reduce bone mineral loss in OP rats.

Melatonin is an indole hormone mainly secreted by pineal gland; the secretion is rhythmic, which is regulated by photoperiod and suprachiasmatic nucleus (18). The synthesis and secretion of melatonin is increased in the dark, and the amount of secretion at night is 5-10 times than that of in the daytime (19). When an organism enters old age, the circadian rhythm of melatonin gradually becomes gentle and even disappears (20). Melatonin has a wide range of biological functions, and its mechanisms are different (21). In terms of bone metabolism, melatonin can inhibit the proliferation and differentiation of osteoclasts, advance the proliferation and differentiation of osteoblasts and promote the mineralization of bone matrix through a variety of signaling pathways (22,23). Cell experiments have shown that melatonin can stimulate the proliferation of bone cells and osteoblasts in a dose-dependent manner (24). Moreover, it can upregulate the expression of runt-related transcription factor 2 (RUNX2) and inhibit the expression of peroxisome proliferator-activated receptor (PPAR- γ), thus promoting the differentiation of bone marrow mesenchymal stem cells into osteoblasts (25). Animal experiments revealed that melatonin can significantly increase bone density and trabecular bone volume in male mice, but distinctly reduce the size and number of osteoclasts (26). This study indicated that supplement of exogenous melatonin can significantly increase the bone density in OP rats, elevate serum calcium level and reduce bone mineral loss, which has a synergistic effect with calcium carbonate, and the combination displayed more remarkable effects.

The bone toxicity caused by reactive oxygen species plays an important role in the occurrence of OP (27). Excessive bone absorption in the organism increases the production of reactive oxygen species, which leads to the destruction of osteoclasts and osteoblasts, resulting in the reduction of bone

mass. With the increase of age, the level of free radicals in the body is gradually increased, while smoking, drinking and other unhealthy lifestyles can induce oxidative stress and elevate the level of free radicals, thus increasing bone absorption (28). Animal experiments revealed that the level of antioxidant stress in the body decreases with the reduction of melatonin levels. It can be seen that maintaining normal melatonin levels can reduce the damage of free radicals (29). This study indicated that melatonin and calcium carbonate can increase the activities of SOD and GSH-Px and reduce the production of MDA in OP rats, thereby exerting the effect of antioxidative stress; they have a synergistic effect, and the combination displayed more remarkable effects.

In conclusion, the study reveals severely decreased bone density, bone mineral loss, reduced serum calcium level and oxidative stress in OP rats, but the supplement of exogenous melatonin and calcium carbonate can significantly improve antioxidative stress ability, increase bone density, elevate serum calcium level and reduce bone mineral loss, thus preventing and treating osteoporosis. These two substances have a synergistic effect, and the combination displays more remarkable effects, which provides an experimental basis for the clinical prevention and treatment of osteoporosis. The related molecular biological mechanisms need to be further investigated.

Competing interests

The authors declare that they have no competing interests.

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