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Effects of ferric ammonium citrate on iron accumulation, bone turnover and bone density in ovariectomized rat models with osteoporosis

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ABSTRACT

To analyze the effect of ferric ammonium citrate on iron accumulation, bone turnover and bone density in ovariectomized rat models with osteoporosis, 40 female SD rats were randomized into four groups, that were, sham-operated, model, low and high-dose ferric ammonium citrate groups (i.e. low and high-dose groups) respectively, each of them had ten rats. Except for the sham-operated group, bilateral ovariectomy was performed in the other groups to establish models with osteoporosis; one week after the operation, those in the low and high-dose groups were given 90 mg/kg and 180 mg/kg ferric ammonium citrate, respectively. Those in the other two groups received isodose saline for nine weeks, with the frequency of twice per week. The changes in bone tissue morphology, serum ferritin concentration, tibial iron content, serum osteocalcin, carboxyl terminal peptide (β - CTX), bone density, bone volume fraction and trabecular thickness were compared. Results showed that the rats in the low and high-dose groups contained a higher concentration of serum ferritin and tibial iron content compared to the other groups ($P < 0.05$). In contrast to the model group, the bone trabeculae in the low and high-dose groups were sparse in morphology and increased in spacing. It was obvious that the rats contained more osteocalcin and β - CTX in the model group, the low and high-dose groups versus the sham-operated group ($P < 0.05$), and those had more β - CTX in the high-dose group versus the model group and the low-dose group ($P < 0.05$). The bone density, bone volume fraction and trabecular thickness of the rats in the model group, the low and high-dose groups showed lower versus the sham-operated group ($P < 0.05$); those in the low and high-dose groups significantly presented lower bone density and bone volume fraction versus the model group ($P < 0.05$). Iron accumulation can aggravate osteoporosis in ovariectomized rats, and its mechanism may be associated with accelerating bone turnover, promoting bone absorption, reducing bone density and sparsely trabecular structure. Therefore, it is particularly important to understand iron accumulation in postmenopausal osteoporosis patients.

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Introduction

Osteoporosis is a metabolic bone disease with abnormal bone remodeling, manifested by the destruction of bone microstructure caused by the decrease of bone density and bone quality. It usually occurs in middle-aged and elderly people, and can be clinically manifested as pain, shortening of body length, hunchback and decreased respiratory function. Osteoporotic fractures are prone to occur due to increased bone fragility and decreased bone strength, with limited post-fracture mobility and complications, which bring a heavy burden to patients and their families (1). At present, there are many clinical theories about the etiology of osteoporosis, among which the relationship between abnormal iron metabolism and osteoporosis has become the focus of attention at home and abroad (2). Iron, as an important trace element in living organisms, is vital for maintaining normal physiological activities and can participate in various enzymatic reactions as a coenzyme. Type I collagen is the primary organic component that resides in bone, and the iron ion is involved in collagen formation, so iron plays a key role in the formation of bone and bone mass maintenance (3). Osteoporosis mainly includes primary, secondary and idiopathic osteoporosis. Postmenopausal osteoporosis is the main type of primary osteoporosis, which is caused by insufficient estrogen secretion due to postmenopausal ovarian dysfunction (4). Some scholars have found that postmenopausal osteoporosis in women is related to estrogen reduction, especially to iron accumulation (5). Therefore, this study aims to observe the impact of ferric ammonium citrate on iron accumulation, bone turnover and bone density of these rat models by simulating the phenomenon of iron accumulation in postmenopausal women, establishing ovariectomized rat models with osteoporosis and inducing iron accumulation through the intervention of ferric ammonium citrate (FAC).

Materials and Methods

Experimental animals

All animal experiments are in full compliance with the ethics regulations of laboratory animals. Forty female SD rats aged 3 months, weighing 230-270g, were selected as

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subjects, which were bought from Beijing Vital River Laboratory Animal Technology Co., Ltd. The rats were raised in the animal laboratory of our hospital at room temperature 23-27 ℃, with humidity ranging from 50% to 60% and cycle light for 24 hours. They were freely given water and food and adaptively fed with a normal diet for 1 week for the experiment.

Laboratory reagents and instruments

Ferric ammonium citrate was bought from Sigma Corporation in the USA; Chloral hydrate was bought from Sinopharm Chemical Reagent Co., Ltd.; Serum ferritin ELISA kit was purchased from AmyJet Scientific; The ELISA kit of mid-osteocalcin and β-type I collagen carboxyl-terminal peptide was purchased from IDS in the UK; Estrogen ELISA Kit was purchased from R&D in the USA; Hematoxylin and eosin were bought from ZSGB-BIO; Potassium ferricyanide was bought from Sinopharm Chemical Reagent Co., Ltd.;

The centrifuge was bought from Shanghai Anting Scientific Instrument Factory; Micropipettor was obtained from Eppendorf China Ltd.; The pressure steam sterilizer was obtained from Shanghai Huaxian Medical Nuclear Instrument Co., Ltd.; The thermostatic water bath was obtained from Shanghai Yuejin Medical Equipment Factory; ELX-800 microplate reader was purchased from BIO-TEK, USA; TX223L analytical balance was obtained from Shimadzu (Shanghai) Global Laboratory Consumables Co., Ltd.; Different types of surgical knife handles, medical needles and threads, ophthalmic scissors and other surgical instruments were obtained from Shanghai Medical Instrument Co., Ltd.

Establishment and grouping of osteoporotic rat models

The rats were anesthetized intraperitoneally by being injected with chloral hydrate at the dose of 0.01ml/g, the hair in the surgical area of the back was cut off and disinfected with iodophor, the skin was cut along the soft ribs on the left and right sides of the back, the incision was about 2.0cm, the subcutaneous tissue was separated and the muscle was dissected to go into the abdominal cavity, white fat was seen in the operation area, then the fat layer was pushed aside to find out the uterus and pull it out in order to show green bean-sized and dark red or brownish yellow ovaries and bright red fallopian tubes, the fallopian tubes under ovaries were clipped with tissue forceps and ligated with Mersilk. After ovaries (including part of the fallopian tube) were excised, the abdominal cavity and muscle layer were sutured intermittently, and then the muscle and skin were sutured. The other ovary was removed by the same method. Only the periovary and abdominal fat were removed in the sham-operated group.

40 rats were randomly split into four groups: sham-operated, model, low and high-dose groups. Each of them had 10 rats. Following the operation, intraperitoneal injection with ferric ammonium citrate was performed for the rats in the low and high-dose groups, at the dose of 90mg/kg and 180mg/kg twice a week for 9 weeks, and those in the other two groups were given equivalent normal saline respectively.

Test methods

1) HE staining was used to detect bone morphology in rats: The femur tissue of rats for each group was collected and fixed at room temperature with 10% formaldehyde solution, then decalcified with a decalcifier 20 times the volume of the specimen, and the specimen was then dehydrated with a totally-enclosed automatic dehydrator. Following the dehydration, HE staining (hematoxylin-eosin staining) and neutral gum sealing were performed for tissue sections. The histopathological features were observed and photographed with an intelligent biological image navigator.

2) Iron accumulation in rats: The changes in rats for each group were tested by enzyme-linked immunosorbent assay (ELISA) and the tibial iron content was determined with the atomic absorption method.

3) Serum bone turnover of rats: The levels of serum osteocalcin and β-CTX of rats in each group were detected by ELISA.

4) Bone density of rats: The femur of rats was scanned by micro-CT, and the parameters like bone density, bone volume fraction and bone trabecular thickness were recorded.

Statistical methods

In this study, all measurement data were expressed by ($\bar{x}$ ±s), an independent-sample t-test was adopted to compare the mean between two groups, and for the comparison of multiple groups, we used the variance analysis. P<0.05 was considered to be a significant difference, and the data were analyzed by the SPSS20.0 software package.

Results

Comparison of iron accumulation in rats

It was evident that the rats in the low and high-dose groups contained higher serum ferritin and iron in the tibia versus the other two groups (P < 0.05), but the concentration of serum ferritin showed no obvious difference between the model group and sham-operated group (P > 0.05), and the iron in tibia showed lower in the model group versus the sham-operated group (P < 0.05). The differences in both the low and high-dose groups showed statistical significance (P<0.05). See Table 1.

Table 1. Comparison of iron accumulation in rats ($\bar{x}$ ±s).

Grouping	Number of cases	Serum ferritin (μg/L)	Iron content in the tibia (μg/g)
sham-operated group	10	1000.35±105.15	85.26±10.33
Model group	10	1100.44±110.85	47.68±9.78*
low-dose group	10	4000.85±216.48*#	165.26±17.36*#
High-dose group	10	4503.78±254.39*#△	260.78±16.74*#△

Note: Versus the sham-operated group, *P<0.05; Versus the model group, #P<0.05; Versus the low-dose group△(P < 0.05).

Comparison of bone histomorphology of rats

The results of HE staining indicated that bone trabeculae in the sham-operated group were plump, complete, arranged neatly, regular and connected into a network. In the model group, the bone trabeculae became thinner obviously and the spacing increased. The bone trabeculae in the low-dose group were sparsely arranged, broken and distorted, and the morphology and structure were poor. The spacing between bone trabeculae in the high-dose group increased significantly, showing very sparse, fractured and distorted.

Comparison of serum bone turnover of rats

In contrast to the sham-operated group, the rats contained more osteocalcin and β-CTX in the other three groups ($P<0.05$), and those had an higher level of β-CTX in the high-dose group versus the model group and low-dose group, and the difference had statistical significance ($P<0.05$). See Table 2.

Comparison of bone density of rats

It was obvious that the rats in the model group, low and high-dose groups had a higher bone density, bone volume fraction and trabecular thickness versus the sham-operated group ($P<0.05$), and the first two indexes in the low and high-dose ammonium ferric citrate groups were obviously lower versus the model group ($P<0.05$). The difference in bone density between the low and high-dose groups showed statistical significance ($P < 0.05$). See Table 3.

Discussion

Osteoporosis is the most common disorder of bone metabolism. With the coming of an aging society, the incidence of osteoporosis is increasing gradually, and it is common among postmenopausal women (6). Since the discovery of iron accumulation in postmenopausal osteoporotic women, the relationship between iron metabolism and osteoporosis has become a hot topic in this field (7). Iron ion is an essential trace element in the human body. It is needed in red blood cell function and tissue oxidative stress and other activities. In recent years, it has been proved that iron accumulation is not only related to blood system diseases and brain iron metabolism but also inhibits bone formation and promotes bone absorption through oxidative stress and cell differentiation, proliferation and apoptosis. Iron accumulation has become an independent risk factor for osteoporosis, and its potential mechanism is that iron may be involved in DNA synthesis in cellular physiological activities, abnormal iron metabolism may lead to metabolic disorders in every system, and too much iron in the body may promote oxidation of lipids in the cell membrane and cause damage to cells and tissues to a certain extent (8,9). In addition, iron can involve in the Fenton reaction and produce active oxygen promoting c-jun kinase binding transcription factor FoxO, and then mediate apoptosis, reduce cell reproductive capacity and bone mass (10). Some foreign researchers found that iron accumulation can produce too much reactive oxygen, resulting in reduced bone mass and destruction of bone tissue by establishing rat models (11,12). By observing the proliferation and differentiation of bone marrow mesenchymal stem cells (BMSCs), it was found that bone formation or lipid differentiation was not balanced. Antioxidant therapy could partly alleviate the damage to bone marrow cells (13).

In this study, the osteoporosis model was established by bilateral ovariectomy and intervened with ferric ammonium citrate to investigate the impact of iron on bone turnover and bone density among ovariectomized rats. The results evidently revealed that the rats in the low and high-dose groups contained more serum ferritin and iron in the tibia versus the other two groups ($P<0.05$), suggesting that the intervention can obviously promote iron accumulation in rats. Additionally, the HE staining showed that the bone trabeculae of rats in the low-dose group were sparsely arranged, fractured and distorted, and the morphological structure was poor, in contrast with the sham-operated group and model group. The spacing between bone trabeculae in the High-dose group increased significantly, showing very sparse, fractured and distorted, which indicates that iron accumulation may aggravate osteoporosis in ovariectomized rats. The levels of osteocalcin and β-CTX were detected to evaluate bone turnover in ovariectomized rats after 9 weeks of iron intervention. Osteocalcin is a commonly used clinical indicator of bone formation, and

Table 2. Comparison of serum bone turnover ($\bar{x}$ ±s).

Grouping	Number of cases	Osteocalcin (ng/ml)	β-CTX (ng/ml)
Sham-operated group	10	251.36±20.74	40.26±9.33
Model group	10	349.78±18.60*	48.16±10.20*
Low-dose group	10	347.22±17.63*	50.79±11.54*
High-dose group	10	360.27±20.11*	75.26±15.95*#△

Note: Versus the sham-operated group, *P<0.05; Versus the model group, #P<0.05; Versus the low-dose group△ (P < 0.05).

Table 3. Comparison of bone density ($\bar{x}$ ±s).

Grouping	Number of cases	Bone density (mg/mm^3)	Bone volume fraction (%)	Trabecular thickness (mm)
Sham-operated group	10	419.26±47.11	56.24±4.68	0.15±0.07
Model group	10	295.55±51.20*	32.28±5.16*	0.13±0.02*
Low-dose group	10	256.34±63.74*#	26.68±6.02*#	0.12±0.03*
High-dose group	10	203.75±50.52*#△	24.36±5.41*#	0.10±0.06*#

Note: Versus the sham-operated group, *P<0.05; Versus the model group, #P<0.05; Versus the low-dose group△ (P < 0.05).

it is the main non-collagen protein in bone matrix (14). β-CTX is the most valuable clinical method for evaluating osteoclast activity and bone resorption (15). The results indicated that the rats in the model group, low and high-dose groups had higher levels of osteocalcin and β-CTX versus the sham-operated group ($P<0.05$), and those in the high-dose group showed a higher level of β-CTX versus the model group and low-dose group ($P<0.05$), suggesting that bone turnover of ovariectomized rats is accelerated and osteoporosis is aggravated by promoting bone resorption. Micro-CT scan showed that the rats in the model group, low and high-dose groups presented lower bone density, bone volume fraction and trabecular thickness versus the sham-operated group ($P<0.05$), those in the low and high-dose groups had lower bone density and bone volume fraction versus the model group ($P<0.05$), it further proves that iron accumulation can aggravate osteoporosis of ovariectomized rats.

In summary, iron accumulation can aggravate osteoporosis in ovariectomized rats, which may be associated with accelerating bone turnover, promoting bone resorption, decreasing bone density and thinning trabecular structure. Therefore, it is vital to learn about the iron accumulation of patients with postmenopausal osteoporosis.

Acknowledgments
Not applicable.

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

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