Chemico-Biological Interactions 279 (2018) 21–26

Contents lists available at ScienceDirect

Chemico-Biological Interactions

journal homepage: www.elsevier.com/locate/chembioint

Bone abnormalities in young male rats with iron intervention and possible mechanisms

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ARTICLE INFO

Keywords:
Iron
Osteoporosis
Reactive oxygen species
OPG
RANKL

ABSTRACT

Studies suggest iron overload may cause bone lesion. The mechanisms are not well understood at present. Therefore, this study was designed to observe the effect of iron overload on bone metabolism in young male rats and explore its possible mechanism. Eighteen SD rats were randomly assigned to iron-loading and control groups. Fe-dextran (250 mg/kg of body weight) was injected intraperitoneally into the rats from iron-loading group, every other day for 5 weeks. The bone mineral density (BMD) of femur, length and diameter of tibia, and histological microstructure of femur and vertebra was determined. The concentrations of serum superoxide dismutase (SOD) and malondialdehyde (MDA) were assayed by ELISA. The mRNA expression of cytokines was detected by real-time PCR. The results showed an obvious bone abnormality after iron intervention, such as significantly decreased content of Ca in bone tissue, shorter length of tibia, lower BMD of femur, and obvious lesion of bone microarchitecture. At the same time, with iron intervention, the concentrations of serum SOD decreased but MDA increased; the mRNA expression of osteocalcin and osteoprotegerin (OPG) decreased, whereas that of receptor activator of nuclear factor kappa B ligand (RANKL) and IL-6 increased significantly. In summary, iron overload indeed give rise to the abnormal changes of bone metabolism independently. Increased bone resorption, and probably decreased bone formation are involved in the process of bone lesion caused by iron overload. Oxidative stress and RANKL participate in the pathological process, and IL-6 may play a supporting role.

1. Introduction

Iron (Fe) is an essential nutrient with crucial biological functions. However, high concentration of Fe is harmful to both cells and tissues, including the bone tissue [1], which has been neglected in studies on iron metabolism for a long time, because more attention was being paid to calcium (Ca) and phosphorus (P) in studies on bone metabolism.

Abnormal bone metabolism in patients with iron overload, such as hemochromatosis and β-thalassemia major, has been described before [2,3]. However, whether the bone lesion was caused by iron excess could not be determined at that time, because patients with iron-loading often show a series of abnormalities such as vitamin D deficiency and hypogonadism, which can also interfere with bone metabolism. Thereafter, more clinical studies reported bone abnormalities that appeared in patients with iron-loading conditions, such as thalassemia [4], sicklemia [5,6], hemochromatosis [7,8], and cessation of menstruation [9]. An increasing number of *in vivo* or *in vitro* experimental studies also strongly suggest the association between iron overload and abnormal bone phenotype, which characterized by low bone mass, osteoporosis/osteopenia, altered microarchitecture and biomechanics, and increased incidence of fractures [10–12]. Moreover, some authors have claimed that iron overload is a risk factor for osteoporosis [13]. However, whether iron overload as an independent risk factor for bone lesion needs to ascertain further.

Bone metabolism is a dynamic process of bone formation and bone resorption. Bone loss produces when bone resorption exceeds bone formation. The mechanisms of bone loss are multiple and complex, and not well understood at present. Certainly, it is still unclear about how iron overload affects bone metabolism. Oxidative stress is generated from an imbalance in the production and elimination of reactive oxygen species (ROS). Increased ROS has been found in the pathogenesis of bone loss-related diseases [14–16]. In addition, excess iron has been evidenced to increase ROS [17,18]. Therefore, ROS may play an important role in iron overload-induced bone loss [19–22].

Receptor activator of nuclear factor kappa B ligand (RANKL), an essential factor for osteoclast differentiation, acts by binding to its receptor, RANK, stimulating the differentiation of osteoclasts [23]. The action of RANKL is prevented by osteoprotegerin (OPG), a soluble

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https://doi.org/10.1016/j.cbi.2017.11.005
Received 26 June 2017; Received in revised form 16 October 2017; Accepted 3 November 2017
Available online 06 November 2017
0009-2797/

decoy receptor expressed by osteoblasts that competes with RANK for binding to RANKL [24]. RANKL has been recently identified as an important mediator of pathogenesis of osteoporosis [25]. Study indicates that ROS stimulates RANKL expression [16]. RANKL may play an important role in the process of iron -induced bone loss [14,16,26].

Therefore, in this study, young male rats were selected to observe the effect of iron-loading on bone metabolism independently. At the same time, explore the role of oxidative stress and RANKL in this pathological process.

2. Materials and methods

2.1. Animal preparation

Experimental animals were provided by the Laboratory Animal Center, Xi'an Jiaotong University Health Science Center. In view of the effect of age and estrogen on bone loss, young male rats were selected for this study to observe the clearer effect of iron on bone. Eighteen male specific-pathogen-free Sprague-Dawley rats (200–220 g, 42 days old) were randomly assigned to two groups: iron-loading group (n = 12) and control group (n = 6). All rats were housed under the normal conditions with unlimited access to water and food. Fe-dextran (250 mg/kg of body weight, Nanjing Xinbai Pharmaceutical Co., Ltd.) was injected intraperitoneally (i.p.) into the rats from iron-loading group, every other day for 5 weeks. Control rats were sham-injected i.p. with the same dose of physiological saline solution at the same time. This study was approved by the Medical Research Animal Ethics Committee of Xi'an Jiaotong University.

2.2. Blood and tissue sampling

At the end of Week 5, all rats were anesthetized with 10% hydrated chlorine aldehyde and blood samples were drawn from rat hearts. The blood sample were centrifuged by 1000r/min and the resulting sera were stored in metal-free Eppendorf tubes at −70 °C. The left femurs of rats were separated immediately with RNase-free instruments. After bone marrow being flushed out thoroughly, the femurs were cut into pieces and snap-frozen in liquid nitrogen and stored at −70 °C. The right femurs were separated for the detection of bone mineral density (BMD). Then the right femurs and the vertebrae were defleshed and fixed in 4% paraformaldehyde. The right tibias of rats were kept in 0.9% saline for their length measurement. The left tibias of rats were dissected and defleshed completely. After the bone marrow was flushed out thoroughly, the tibia tissues were cut into pieces and stored at −70 °C for the detection of Fe, Ca, and P contents of bone. Small pieces of liver (about 1g) and the whole spleen tissues were dissected, rinsed repeatedly with cold phosphate-buffered saline, and stored at −70 °C.

2.3. Atomic absorption and ultraviolet spectrophotometric analysis

Samples of liver (200.0 mg) and spleen (200.0 mg) and tibia (350.0 mg) from each rat were subjected to wet digestion respectively. The total Fe contents in serum, liver, spleen, and bone tissue and the contents of Ca in serum and bone tissue were determined by flame atomic absorption spectrometry (HITACHI Z—8000, Japan), following standard procedures. The content of P in serum and bone tissues was measured at 610 nm wavelength by ultraviolet spectrophotometry (UV—7504, Shanghai XinMao Instrument Co., Ltd., China). All standard curves were prepared using commercially available standards.

2.4. Enzyme-linked immunosorbent assay (ELISA)

The concentrations of serum ferritin, superoxide dismutase (SOD), and malondialdehyde (MDA) were detected by ELISA (AMEKO, Shanghai Yueyan Biological Technology Co., Ltd, China), according to the manufacturer's instructions. All samples and standards were assayed in duplicate. The results were analyzed with an enzyme microplate reader (Thermo Fisher Scientific, USA).

2.5. Bone densitometry

The BMD of excised bones was measured. The right femurs, kept in 0.9% physiological saline, were placed on the measuring table after water absorption. The BMD of the neck of femur was determined by dual energy X-ray absorptiometry (DPX-L, version 4.6D, Lunar Corp., Madison, WI).

2.6. Tibia length measure

The total lengths of tibias and the transverse maximal diameter of their proximal ends were measured with a vernier caliper.

2.7. Hematoxylin and eosin (HE) staining

The femurs and vertebrae were fixed in 4% paraformaldehyde for 48 h. After being washed with phosphate-buffered saline, the tissues were decalcified with 10% EDTA solution (pH 7.4). After decalcification, the bone samples were dehydrated in increasing concentrations of alcohol, infiltrated, and embedded in paraffin. For HE staining, the paraffin embedded tissue specimens were sectioned (5 μm) and stained with hematoxylin and eosin for 5min and 2min, respectively.

2.8. Real-time fluorescence quantitative PCR

Total RNA was extracted from femurs using liquid nitrogen and TRIzol reagent (Invitrogen Inc., Canada). About 500 mg femoral tissues were snap-frozen with liquid nitrogen and ground using mortar and pestle. TRIzol reagent was added to the grated tissue, according to the manufacturer's instructions. The total RNA was quantified with a spectrophotometer (ND-1000, NanoDrop, Delaware, USA). The purity of the samples was assessed using OD values (260:280 nm) and 1% agarose gel electrophoresis. Total RNA (500 ng) was used for cDNA synthesis with RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific), according to the manufacturer's instructions.

cDNA was subjected to real-time quantitative PCR using the iCycler IQ5 (BIO-RAD, USA), with a SYBR green detection platform. The sequences of the primers were as follows: osteocalcin (OC), 5′-TCTCTG CTCACTCTGCTG-3′(forward) and 5′-GTCTATTCACCACCTTAC TGC-3′(reverse); OPG, 5′-CGAGTGTGCGAATGTGAG-3′(forward) and 5′-ACCTGAGAAGAACCCATCC-3′(reverse); RANKL, 5′-AGAGCGAAGA CACAGAAGCACTAC-3′(forward) and 5′-AGCCACGAACCTTCCATCAT AGC-3′(reverse); interleukin (IL)-6, 5′-CTTCCAGCCAGTTGCCTTC TTG-3′(forward) and 5′-TGGTCTGTTGTGGGTGGTATCC-3′(reverse); and β-actin, 5′-CTATCGGCAATGAGCGGTTCC-3′(forward) and 5′-TGT GTTGGCATAGAGGTCTTTACG-3′(reverse).

PCR was performed on a total reaction volume of 25 μL per tube and run in triplicates in microcapillaries. The PCR reaction mixture consisted of 12.5 μL SYBR Green Real-time PCR Master Mix (Aidlab Biotechnologies Co., Ltd, Beijing, China), 0.5 μL each of forward and reverse 10 μM primers, 9.5 μL H_2O, and 2.0 μL cDNA. PCR thermal cycling was initiated with denaturation at 94 °C for 3min, followed by 40 cycles of denaturation at 94 °C for10s, annealing at 58 or 60 °C for 20s, and extension at 72 °C for 30s, with a final step at 72 °C for 10min. Melting curve analysis and 2% agarose gel shift assay of PCR products were used to distinguish between actual signals from primer dimers and non-specific amplifications.

2.9. Statistical analysis

Statistical analysis was performed using SPSS statistics package. The concentrations of Fe, Ca, and P in serum and bone tissue, the concentrations of serum ferritin, SOD, and MDA, and the BMD of rats are