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Evaluation of anti-osteoporosis in ovariectomized Wistar rats treated with antler blood by synchrotron radiation X-ray fluorescence microprobe

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Abstract. To evaluate the anti-osteoporotic effect of antler blood, the relationship between the change of element content and the bone mineral density (BMD) increase in femur was investigated. Female Wistar rats were randomly divided into three groups: sham-operated group (SHAM, $n = 5$), ovariectomized group (OVX, $n = 5$) and ovariectomized group with antler blood treatment ($n = 5$). The femoral BMD was analyzed by dual-energy X-ray absorptiometry (DXA) and the element relative content was determined by synchrotron radiation X-ray fluorescence (SRXRF) microprobe. The results showed that the femoral BMD in ovariectomized rats was significantly lower than that of sham-operated rats ($p < 0.05$) but reversed by antler blood treatment ($p < 0.05$). A further study demonstrated that the relative contents of phosphorus (P), calcium (Ca), zinc (Zn) and strontium (Sr) were obviously lower in ovariectomized rats compared to sham-operated rats but only the relative contents of P, Ca and Zn were normalized by antler blood treatment ($p < 0.05$). Our experiments revealed that loss of element Ca, P, Zn and Sr was closely related to the BMD reduction in ovariectomized rats and the anti-osteoporotic effect of antler blood was mediated by increasing the contents of P, Ca and Zn.

Keywords: Element analysis, BMD, osteoporosis, femur, SRXRF

1. Introduction

The human skeleton is composed of organic matter, inorganic matter and water. Inorganic matter includes the macroelements Ca, P, Na, K and the trace elements Mg, Mn, Fe, Cr, Ni, Cu, Zn, Se, Sr and so on. These elements are enveloped in organic protein collagen, influence the process of remodeling by affecting bone mineral crystal size, density and solubility and closely related to the bone structure [1,2]. Studies show that the decrease of bone mineral density (BMD) is a major risk factor leading to osteoporosis [3]. Osteoporosis is a disease characterized by low bone mass and microarchitectural deterioration of bone tissue, leading to increase bone fragility and fracture risk, and can be prevented by

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anti-osteoporotic drugs such as estrogen replacement therapy, parathyroid hormone and so on. Antler blood is a traditional Chinese herb medicine and our experiment showed that antler blood treatment could increase the BMD in ovariectomized rats which is considered to be a standard osteoporosis model similar to human postmenopausal osteoporosis due to estrogen deficiency. But little is known about the relationship between the change of element content and BMD increase in antler blood-treated ovariectomized rats.

Synchrotron radiation X-ray fluorescence (SRXRF) technique is being extensively applied in the fields of life science and medical science because it's more sensitive and less injurious to cell, and also has excellent characteristics such as very high brightness, collimation, polarization and lower bremsstrahlung which is the electromagnetic radiation produced by an accelerated electrically charged subatomic particle [4]. Using SRXRF technique, Zhang et al. [5] found that Ca, P and K were the major elements of bone composition and obviously low in both spongy and cartilage zones of the femoral head slice in osteoporotic patient. The statistical analysis showed that Ca had good correlation with P, Zn, Sr and K. Fei et al. [6] reported that the relative mineral content of Ca, P and Zn in streptozotocin (STZ)-induced diabetic femurs decreased significantly compared to controls ($p < 0.01$) and Ca correlated positively with P, with Sr, with Zn. The aim of our study is that the mineral content of femur is analyzed by SRXRF microprobe to assess its correlation with the BMD in ovariectomized rats treated with antler blood.

2. Materials and methods

2.1. Sample preparation

Fifteen 8-weeks-old female Wistar rats (180 ± 10 g) were purchased from Vital River experimental animal technology Inc. (Beijing, China). Animals were divided into 3 groups randomly, acclimatized for one week before use, and had free access to feed and water. One group was sham operated (SHAM, group 1, $n = 5$), and two groups were subjected to bilateral ovariectomy (OVX, groups 2 and 3, $n = 10$). After 4 weeks, one of OVX groups treated with antler blood (4000 μl/kg daily, group 3, $n = 5$) orally for 10 weeks. At the end of the experiment, all the rats were sacrificed. The left femur was dissected out carefully, cleaned of soft tissue and stored in 10% formalin.

2.2. Bone density

Before the experiment, each left femur was picked out of 10% formalin and made it dry. The bone mineral density was determined by DXA (Excellplus, Norland Co., USA).

2.3. Element analysis

The scanning X-ray fluorescence microprobe analysis of left femur was performed using synchrotron radiation from the BEPC storage ring (2.2 GeV, 45–90 mA) at Institute of High Energy Physics, CAS. A special set of adjustable slits was used to change the size of the excited X-ray beam. In our experiment, three of five femur samples in each group were randomly selected and fixed on the sample holder. Five points on each femur were scanned by SRXRF microprobe for element analysis (Fig. 1). The size of the microprobe was confined to 20×20 μm^2. The element image of profile was performed point by point.

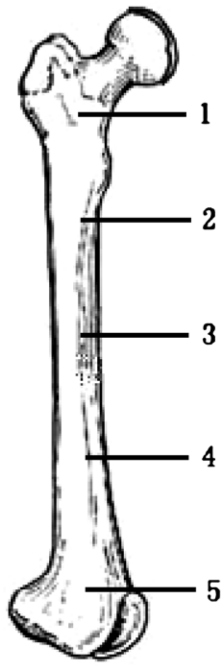

Fig. 1. The left femur. Five points were selected and scanned by SRXRF microprobe.

An ionization monitor chamber filled with nitrogen under normal pressure near the collimation system was used to measure the exciting radiation intensity. The exposure time was 100 s at a photon energy of 3.5–35 keV for each point, and the length of scanning step in the experiments was 0.6 cm. The scanner was driven by a motor driver, which was controlled by a computer. The Si (Li) detector worked under liquid nitrogen and was placed 4 cm from the samples with energy resolution about 165 eV (HWFM) Mn K alpha 5.9 keV. The detector was located at 90° with respect to the beam direction. Because the synchrotron radiation was plane polarized with the electric vector in the plane of the electron orbits in the storage ring, there was a minimum Compton scattering background when the detector was located at 90°. The associated electronics was composed of a pulsed optical preamplifier, spectroscopy amplifier and pulse pile-up rejector. Then, the signals were connected to an Oxford multichannel analyzer for data acquisition and analysis. Data of spectra were processed by using the program AXIL and the relative intensity was obtained by comparing the element X-ray fluorescence (XRF) intensity of the sample with that of Ar. The normalized intensity was for estimating the relative content of elements. A typical energy spectrum of the femur sample was shown in Fig. 2.

2.4. *Statistical analysis*

Statistical analysis was carried out using SPSS program (Version 10.0). Results were expressed as mean $\pm$ SD (standard deviation). Comparisons between groups were made by One-Way ANOVA. Pearson's correlation test was used for correlation analysis. The statistical significance was set if the p-value was less than 0.05 or 0.01.

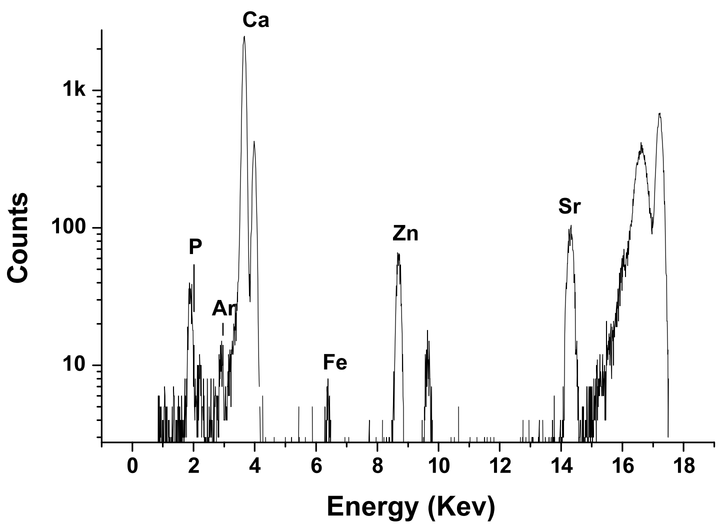

Fig. 2. A typical energy spectrum of the left femur sample is shown.

3. Results

3.1. Bone density

The femoral BMD in OVX group (group 2) was significantly lower than that in SHAM group (group 1) ($p < 0.05$) but reversed by antler blood treatment (group 3) ($p < 0.05$) (Fig. 3).

3.2. Element analysis

Using SRXRF microprobe, the results showed that the relative contents of P, Ca, Zn and Sr on femur in OVX group (group 2) were obviously lower than that in SHAM group (group 1) ($p < 0.05$). However, the relative contents of P, Ca and Zn could be reserved by antler blood treatment (group 3) (Figs 4–7).

The correlations between the element relative contents in SHAM group were shown in Table 1. There were very good positive correlation between P and Ca ($R = 0.985$, $p < 0.01$), P and Zn ($R = 0.918$, $p < 0.05$), P and Sr ($R = 0.899$, $p < 0.05$), Ca and Zn ($R = 0.964$, $p < 0.01$), Ca and Sr ($R = 0.936$, $p < 0.05$), Zn and Sr ($R = 0.983$, $p < 0.01$) in sham operated group. But there were no obviously positive correlation between element contents in other two groups.

4. Discussion

At present, many anti-osteoporotic drugs can increase the BMD, but little is known about the relationship between the BMD increase and the change of element content after treating with them. Our results showed that the low BMD ($p < 0.05$) in OVX rats was accompanied by decrease of Ca, P, Zn and Sr contents ($p < 0.05$). However, the BMD could be reserved along with the increase of the relative contents of Ca, P and Zn after antler blood treatment ($p < 0.05$). Furthermore, statistical analysis in SHAM group showed that there were highly positive correlations between Ca and P, Zn and Sr contents.

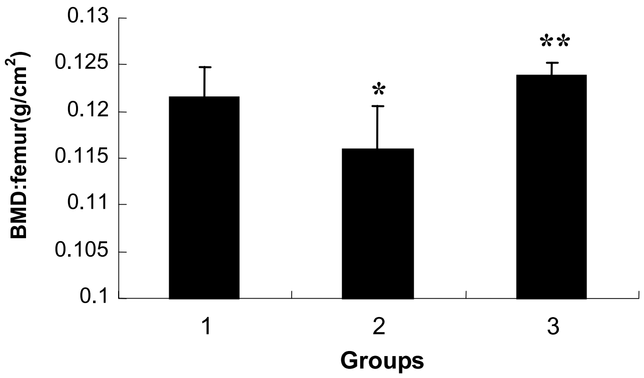

Fig. 3. Bone mineral density of femur. Group 1 is SHAM. Group 2 is OVX (*$p < 0.05$, vs. group 1). Group 3 is OVX treated with antler blood (**$p < 0.05$, vs. group 2). BMD: bone mineral density.

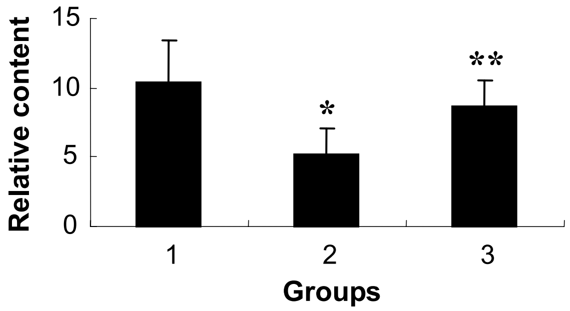

Fig. 4. The relative content of P by SRXRF. Group 1 is SHAM. Group 2 is OVX (*$p < 0.01$, vs. group 1). Group 3 is OVX treated with antler blood (**$p < 0.05$, vs. group 2).

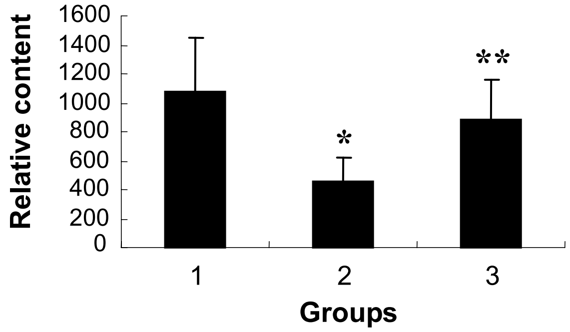

Fig. 5. The relative content of Ca by SRXRF. Group 1 is SHAM. Group 2 is OVX (*$p < 0.05$, vs. group 1). Group 3 is OVX treated with antler blood (**$p < 0.05$, vs. group 2).

As we known, bone is a dynamic tissue constantly remodeled throughout life. The structure of bone consists of a solid mineral substance in close association with an organic compound. The main mineral component of bone is an imperfectly crystalline hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$], which comprises $1/4$ the volume and $1/2$ the mass of the normal adult bone [7]. Therefore Ca is considered the most important nutrient concerning bone health and its deficiency can lead to a decrease in BMD as well as a predisposition to osteoporosis. Epidemiological evidences show that Ca intake was positively correlated

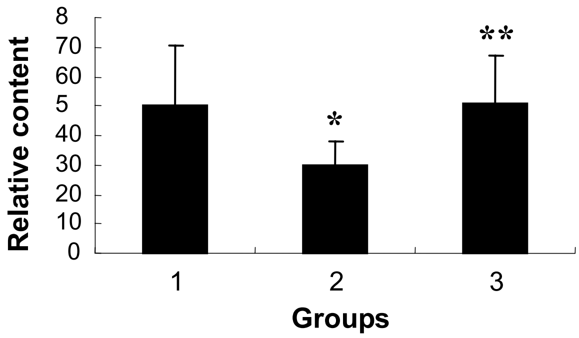

Fig. 6. The relative content of Zn by SRXRF. Group 1 is SHAM. Group 2 is OVX (*$p < 0.05$, vs. group 1). Group 3 is OVX treated with antler blood (**$p < 0.05$, vs. group 2).

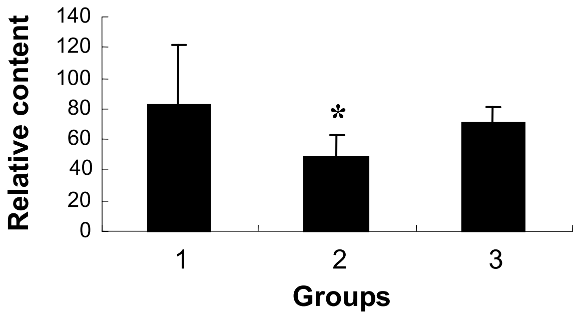

Fig. 7. The relative content of Sr by SRXRF. Group 1 is SHAM. Group 2 is OVX (*$p < 0.05$, vs. group 1). Group 3 is OVX treated with antler blood.

with BMD [8,9]. An adequate intake of Ca determined the development of a maximum bone mass at the end of the adolescence period, and ameliorated the progressive loss of this bone mass with age [10]. In addition, P is second to Ca in abundance in the human body with 85% bound to the skeleton [11]. P regulates the bone formation, inhibits the bone resorption, and acts as one of anti-osteoporosis nutrients [12]. Study revealed that P also affected the regulation of calcium metabolism [13]. Supply with Ca and P was already considered as a treatment of osteoporosis [14]. Zhang et al. and Fei et al. [5,6] also found that the element Ca and P in femur were obviously low in patients and rats with osteoporosis. In our study, the results demonstrated that the relative contents of P and Ca as well as the femoral BMD in ovariectomized rats were significantly lower than that in sham-operated group ($p < 0.05$) but normalized by antler blood treatment ($p < 0.05$) (Figs 3–5). Meanwhile, statistical analysis showed that there was a very good positive correlation between P and Ca ($R = 0.985$, $p < 0.01$) (Table 1), consistent with Zhang's report. Hence, the results revealed that loss of element P and Ca was closely associated with the BMD decrease and it's very important that antler blood treatment could increase the BMD by enhancing the relative contents of P and Ca.

Additionally, other trace minerals have effects on bone biology by affecting bone mineral crystal site, density, and solubility [1,2]. Bone has one of the highest concentrations of zinc of all tissues and zinc is essential for the growth, development, and maintenance of healthy bones [15]. Experimental data suggested that Zn stimulated bone protein synthesis and bone formation by increasing the activity of alkaline phosphatase which was involved in bone mineral deposition and also inhibited bone resorption

Table 1
Correlation between element contents in femur of sham-operated group

	P	Ca	Zn	Sr
P	1	0.985**	0.918*	0.899*
Ca	0.985**	1	0.964**	0.936*
Zn	0.918*	0.964**	1	0.983**
Sr	0.899*	0.936*	0.983**	1

*Correlation is significant at the 0.05 level.

**Correlation is significant at the 0.01 level.

by reducing osteoclast activity [16–18]. Furthermore, Zn regulated secretion of calcitonin from thyroid gland which was presumed to play a role in regulating calcium metabolism and calcium transport [19]. Rossi and colleagues [20] revealed that zinc deficiency in growing rats resulted in reduced bone growth and bone volume. Atik [21] reported that men suffering from senile osteoporosis had lower serum zinc and lower femur zinc compared with control patients. Some studies showed that Zn supplements added to the diet could exert a positive influence on human bone linear growth [22,23]. In our present experiment, statistical analysis showed that there was a very good positive correlation between Ca and Zn ($R = 0.964$, $p < 0.01$) (Table 1). We also found that the relative content of Zn and the femoral BMD decreased in ovariectomized group compared to sham-operated group but reserved by antler blood treatment ($p < 0.05$) (Figs 3 and 6). Furthermore, we concluded that the increase of trace element Zn after antler blood treatment was positively related to high BMD.

Sr is another trace element that could regulate the bone balance and increase in bone mass [24,25]. Strontium was mainly adsorbed on the crystal surface in bone, and only one in ten calcium cations in the apatite crystal was found to be replaced by strontium *in vivo* [26]. Strontium ranelate, a novel agent containing two stable strontium atoms, has been licensed in United Kingdom for the treatment of osteoporosis [27]. Studies *in vivo* showed that strontium ranelate reduced bone resorption by decreasing preosteoclast differentiation and osteoclastic activity [28–30] and increased bone formation by promoting osteoblast replication [31–33] and activity [34]. As an effective anti-osteoporotic drug, strontium ranelate could activate the calcium-sensing receptor in bone cells, resulting in activation of inositol trisphosphate production and mitrogen-activated protein kinases signaling [35,36]. In our experiment, we found that the relative content of Sr and the femoral BMD decreased in ovariectomized group compared to sham-operated group ($p < 0.05$) but the relative content of Sr did not increase obviously after antler blood treatment (Figs 3 and 7) although Sr had very good positive correlation with P, Ca and Zn (Table 1).

In conclusion, using SRXRF technique, we found that loss of the relative contents of Ca, P, Zn and Sr accounted for the BMD decrease in ovariectomized rats. However antler blood treatment could restore the BMD by increasing the mineral contents of Ca, P and Zn. Hence, dietary antler blood supplements may be useful for preventing osteoporosis.

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