Element analysis in femur of diabetic osteoporosis model by SRXRF microprobe

Yurong Fei a, Min Zhang a, Ming Li a, Yuying Huang b, Wei He b, Wenjun Ding a, Jianhong Yang a,*

a Department of Biology, Graduate University of Chinese Academy of Sciences, 19 Yuquan Road, Beijing 100049, PR China
b Beijing Synchrotron Radiation Facility, Institute of High Energy of Physics, Chinese Academy of Sciences,
19 Yuquan Road, Beijing 100049, PR China

Received 2 August 2006; accepted 11 September 2006

Abstract
Diabetes mellitus affects bone metabolism and leads to osteopenia and osteoporosis, but its pathogenic mechanism remains unknown. To address this problem, mineral element of bone was analyzed in experimental diabetic osteoporosis model. Male Wistar rats were randomly divided into streptozotocin (STZ)-induced diabetic group (n = 5) and control group (n = 5). The experiment lasted 68 days and at the end of the experiment, femoral bone mineral density (BMD) was measured by dual-energy X-ray absorptiometry and element content in femur of animals was determined by synchrotron radiation X-ray fluorescence (SRXRF) microprobe analysis technique. Results showed that femoral BMD in diabetic group was significantly lower than that in control (P < 0.01). Relative mineral content of calcium (Ca), phosphorus (P) and zinc (Zn) in diabetic femurs decreased significantly compared to controls. And strontium (Sr) in diabetics reduced 11% (P = 0.09). Relative content of sulfur (S) in average was statistically higher (P < 0.01) in diabetics than that in controls. But no obvious difference was observed in relative content of chromium (Cr), iron (Fe), copper (Cu), and lead (Pb) between the two groups. Statistical analysis revealed that Ca correlated positively with P (R = 0.85 and P < 0.001), with Sr (R = 0.38 and P < 0.05) and with Zn (R = 0.37 and P < 0.05). Whereas, Zn correlated negatively with S (R = −0.40 and P < 0.05). Our results reveal that loss of minerals accounts for the BMD reduction in diabetics.

© 2006 Elsevier Ltd. All rights reserved.

Keywords: Osteoporosis; Diabetes; Element analysis; Synchrotron radiation; X-ray fluorescence microbe

1. Introduction
Bone is connective tissue mainly composed of organic protein collagen, providing soft framework and inorganic hydroxypatite, adding strength and hardening the framework. Moreover, bone is mineral reservoir for calcium, phosphorus, zinc, strontium and many other minerals. Abnormalities in bone metabolism could cause osteoporosis, a disease in which bones become fragile and more likely to break. Osteopenia or osteoporosis is found in diabetic women, as well as in diabetic men (Inzerillo and Epstein, 2004; Thrailkill et al., 2005; Bridges et al., 2005). And studies have proved that diabetes can bring about abnormalities in element homeostasis (Cooper et al., 2005; Zargar et al., 1998), which are likely related to bone metabolism. However, the exact role of bone minerals in BMD of diabetics is still not clear right now.

SRXRF microprobe is a valuable technique for bone element analysis due to its advantages of high sensitivity, non-destructing, multi-elemental data and relative easy procedure. Several studies have demonstrated that SRXRF microprobe is a very appropriate tool for the study of element content and its distribution in biological samples (Zhang et al., 2005; Shi et al., 2004; Abraham et al., 2005). Zhang et al. (2005) has studied element content distribution in femoral head slice with osteoporosis and found remarkably lower concentration of Ca, P in spongy and cartilage zones. Therefore, we propose that loss of minerals probably leads to significant reduction in BMD of diabetics. To explore the cause of BMD reduction in diabetics, here we investigate relative element content in femur of experimental diabetic osteoporosis model by SRXRF microprobe. Moreover, we will also discuss the correlation between important elements.
2. Materials and methods

2.1. Animals

Eight-week old Wistar rats (Department of Laboratory Animal Science, Peking University Health Science Center, Beijing, PR China) weighing 240 ± 10 g were selected and studied in this study. The animals were kept under standard conditions of temperature, light and were free to standard diet and water. Replacement of cages, daily replacement of tap water and other routine hygiene procedures were done by a licensed technician in the animal lab of Institute of High Energy Physics, Chinese Academy of Sciences. All animal procedures in this study were carried out according to the standards of the guide for the Care and Use of Laboratory Animals.

2.2. Inducement of diabetes

After 1 week of adaptation to the laboratory environment, rats were randomly separated into two groups: control group (\( n = 5 \)) and diabetic group. In the beginning of the experiment, on day 0, rats received a single intravenous administration of freshly prepared streptozotocin (STZ) (Sigma, St. Louis, MO, USA) dissolved in 0.1 M citrate buffer (pH 4.5) at a dose of 55 mg/kg body weight. Equal volume of vehicle was injected into the control rats. After 1 week of administration, five rats with fasting blood glucose (12 h) higher than 12 mmol/L were selected and studied in this research. The experiment lasted 68 days after the onset of diabetes.

2.3. Blood glucose and body weight

Glucose was determined immediately by a glucose analyzer (ACCU-CHEK, Roche Diagnostic GmbH, D-68298 Mannheim, Germany) after blood was collected from a cut at the tail vein of 12 h fasted rats in both diabetic group and control group on days 0, 13, 22, 30, 40, 50, 60, and 68, respectively. Body weight was monitored before glucose determination each time.

2.4. Measurement of bone mineral density

At the end of experiment on day 68, the fasted rats were anesthetized with barbital. Then right femurs in both diabetic group and control group were immediately dissected, cleaned of adherent soft tissue. And bone samples were stored in 10% formalin until analysis.

The total area of bone mineral density (BMD), bone mineral content and bone mineral area of the right femurs were measured by dual-energy X-ray absorptiometry (Excellplus, Norland, USA) with the small animal total body option in the osteoporosis measurement.

2.5. Element analysis

The element determination was carried out at the X-ray fluorescence (XRF) microprobe station at Beijing synchrotron radiation facility (BSRF), Institute of High Energy Physics, Chinese Academy of Sciences. The X-ray light source came from the 4W1B beam line at BSRF. This beam line could provide multi-chromatic X-rays (white light), which energy ranged from 4 to 30 keV. The electron energy in the storage ring is 2.2 GeV, with a current range from 60 to 106 mA. At present, the beam of the microprobe was focused on 150 μm × 150 μm. The emission X-ray spectrum was recorded with the counting live time of 200 s. The femoral sample was fixed on a micro-stage with 0.5 cm/step by a computer-controlled motor. Five points in each sample were selected and scanned as shown in Fig. 1. XRF spectra were collected by a PGT Si (Li) detector, positioned at 90° to the beam line, 4.5 cm from the target. The collected spectra were analyzed by an AXIL program.

Three of five femur samples in each group were randomly selected and were scanned by SRXRF microprobe for element analysis. Data were deleted if they were less than 3 times of the corresponding standard deviation. To correct the effect of synchrotron radiation beam flux variation, the elemental peak area was normalized to the peak counts of Ar, which exists only in air with a constant proportion. The normalized peak areas were used for estimating the relative contents of elements.

2.6. Statistical analysis

Data analysis was performed by SPSS (version 10.0). All results were presented as means ± S.E.M. Comparisons between groups were made by independent-samples \( t \)-test.

![Fig. 1. A sketch of femur sample and five points were selected and scanned by SRXRF microprobe in this experiment.](image-url)
Significant difference was defined at either $P < 0.05$ or $P < 0.01$. A linear correlation test was made by origin 6.1.

3. Results

3.1. Changes on blood glucose

Changes on glucose were monitored throughout the study (Fig. 2). Glucose in diabetic group had been significantly higher than that in control group since the onset of diabetes in the experiment, indicating successful model of STZ-induced diabetes mellitus.

3.2. Changes on body weight

Body weights of experimental animals throughout the study were present in Fig. 3. Average body weight in diabetic group was remarkably lighter than that in control group since the onset of diabetes in the experiment. Particularly, body weight of diabetic rats at the end of the experiment decreased 5% while those in control group increased 49.8% in average compared with initial body weights. Besides, high consumption of diet and water were also observed in diabetic rats (data not shown), consisting with the most of common symptoms of diabetes (e.g., polyphagia, polydipsia and polyuria).

3.3. Femoral BMD of diabetic and control rats

Femoral bone mineral content and bone mineral area of diabetic rats were statistically lower than those of controls (Table 1). Consequently, BMD of diabetic rats was significantly lower than that of controls after the onset of diabetes ($P < 0.01$; Fig. 4).

3.4. Element analysis

The typical energy spectrum of P, S, Ca, Cr, Fe, Cu, Zn, Sr, and Pb in femur was investigated by SRXRF microprobe in this experiment (Fig. 5). As shown in Table 2, Ca and P were of the macro-elements of bone; S, Cr, Fe, Cu, Zn, Sr, and Pb were among the trace elements of bone (Table 2).

Relative content of element Ca in diabetic group was significantly lower than that in control group ($P < 0.01$). Moreover, P and Zn in diabetics also reduced markedly ($P < 0.05$). Strontium in femur of diabetic rats reduced 11% ($P = 0.09$). However, sulfur in diabetes was obviously higher than that in controls ($P < 0.01$). For Cr, Fe, Cu, or Pb, no significant difference was observed between the two groups.

Statistical analysis showed that Ca had a strong correlation with P ($R = 0.85$ and $P < 0.001$) (Fig. 6A). Calcium also
correlated positively with Sr ($R = 0.38$ and $P < 0.05$) (Fig. 6B) and with Zn ($R = 0.37$ and $P < 0.05$) (Fig. 6C). But element Zn correlated negatively with S ($R = -0.40$ and $P < 0.01$) (Fig. 6D).

4. Discussion

So far, there has been no report on element analysis with SRXRF microprobe to study BMD reduction in experimental diabetic osteoporosis model. Our results showed that abnormalities in bone minerals, especially in Ca, P, Sr, S and Zn, closely related to BMD reduction in diabetics. Other elements including Cr, Fe, Cu, and Pb were also analyzed in this study and no statistic difference was observed in relative content of these elements between diabetics and controls.

<table>
<thead>
<tr>
<th></th>
<th>P</th>
<th>S</th>
<th>Ca</th>
<th>Cr</th>
<th>Fe</th>
<th>Cu</th>
<th>Zn</th>
<th>Sr</th>
<th>Pb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>0.70 ± 0.03</td>
<td>0.24 ± 0.01</td>
<td>205.75 ± 5.40</td>
<td>0.05 ± 0.00</td>
<td>0.69 ± 0.04</td>
<td>0.21 ± 0.01</td>
<td>11.64 ± 0.49</td>
<td>1.74 ± 0.09</td>
<td>0.20 ± 0.02</td>
</tr>
<tr>
<td>Numbera</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>7</td>
</tr>
<tr>
<td>Diabetes</td>
<td>0.61 ± 0.03</td>
<td>0.53 ± 0.05</td>
<td>171.76 ± 9.20</td>
<td>0.06 ± 0.01</td>
<td>0.64 ± 0.02</td>
<td>0.21 ± 0.02</td>
<td>9.67 ± 0.47</td>
<td>1.55 ± 0.06</td>
<td>0.20 ± 0.02</td>
</tr>
<tr>
<td>Numbera</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>14</td>
</tr>
</tbody>
</table>

* $P < 0.05$, ** $P < 0.01$ vs. control group.

* The valid points scanned in diabetic or control group after data were analyzed.

Living and growing tissue, bone is made of organic component (primarily the collagen protein) and mineral substance—hydroxyapatite. There are two main functions of the bone mineral substance. A biomechanical role is the stability of the skeleton and a metabolic one is reservoir for many ions, control mineral homeostasis. Approximately 80–90% of bone mineral content is composed of Ca and P (Ilich and Kerstetter, 2000) and more than 99% of the body’s Ca is contained in the bones and teeth (National Institute of Health Osteoporosis and Related Bone Diseases, 2005). Extracellular Ca is one of the main factors regulating the process of bone remodeling, by means of a multi-organ cross-signaling cascade (Purroy and Spurr, 2002). And the availability, storage and disposal of Ca are regulated by a systemic mechanism partly through PTH and Vitamin D (Purroy and Spurr, 2002).
Numerous studies have proved that Ca plays a key role in bone structure (Ilich and Kerstetter, 2000). For example, it has been reported that calcitriol improves STZ-induced diabetes and recovers bone mineral density in diabetic rats (Del Pino-Montes et al., 2004). Furthermore, statistical analysis in our research showed that Ca has a strong correlation with P ($R = 0.85$ and $P < 0.001$) (Fig. 6A), consistent with others’ reports (Zhang et al., 2005). Phosphorus, an inorganic element, is second to Ca in abundance in the human body with 85% bound to the skeleton (Ilich and Kerstetter, 2000). Results in this study showed significant decrease in relative content of Ca and P in femur of diabetic rats than those in controls, although there were no changes in Ca mineral concentration between diabetic and control group by Instrumental Neutron Activation Analysis (Facchini et al., 2006). Rats in their study were 9- to 12-month old and were female but rats in ours were 2-month old and were male. Thus, the experimental animal and analysis method possibly lead to the different result. However, Einhorn et al. (1988) found decreased ash content of Ca and P. Furthermore, also applying the SRXRF microprobe for bone element scanning analysis, Zhang et al. reported that the concentration of Ca and P was obviously low in both spongy and cartilage zones for the osteoporosis patient slice of the femoral head, but there was no marked difference in the compact zone (Zhang et al., 2005). Therefore, we believe that significant loss of mineral Ca and P in bone accounts for the marked reduction in BMD of diabetics.

Trace elements also influence the process of bone remodeling by affecting bone mineral crystal size, density, and solubility, through their roles as metallo-enzymes in the synthesis of collagen and other proteins that form the structure of bone (Reinhold, 1975; Saltman and Strause, 1993). Results in our investigation showed that relative concentrations of Sr and Zn were obviously low in diabetics than in controls. Besides, Ca correlated positively with Sr ($R = 0.38$ and $P < 0.05$) (Fig. 6B) and with Zn ($R = 0.37$ and $P < 0.05$) (Fig. 6C). Intensive studies have been done on Sr for biological interest lately. Strontium ranelate, a novel agent containing two strontium atoms, has been licensed in United Kingdom for the treatment of osteoporosis (Fogelman and Blake, 2005). Recent studies in vitro showed that strontium ranelate acts as an effective anti-osteoporotic drug by inhibiting bone resorption by osteoclasts and promoting osteoblast replication and bone formation (Marie, 2005). Studies have proved that Sr is an agonist of the calcium-sensing receptor with a lower affinity than Ca and that the reported anti-osteoporotic effects of the drug is due to the modulation of the calcium-sensing receptor in bone cells by strontium ranelate (Coulombe et al., 2004), and other mechanisms are to be identified. Here, in the present experiment, we found that relative content of Sr in femur of diabetics reduced 11% ($P = 0.09$) than that of controls and statistical analysis showed that Sr had a good correlation with Ca. The decrease in Sr in diabetics is assumed to be one of the causes leading to diabetic osteoporosis.

An essential element in bone metabolism, Zn acts as a cofactor for several enzymes, such as ALP – necessary for bone mineralization and collagenase – essential for development of the collagenous structure of bone (Beattie and Avenell, 1992). Beneficial effect of Zn supplementation in bone formation is well reported by Gonzalez-Reimers (Gonzalez-Reimers et al., 2005) and other research groups. Besides a positive correlation with Ca, Zn also had a negative correlation with S (Fig. 6D), which increased significantly in diabetics compared to controls (Table 2). Sulfur exists in many substances that are essential to bone metabolism. For example, heparin sulfate is a major co-factor in common sharing with the majority of osteoblast growth promoting elements (Cool and Nurcombe, 2005). And estrone sulfate, which may be a better marker for estrogen status, is the predominant circular estrogen in normal physiological situations (Castracane et al., 2006). The dehydroepiandrosterone sulfate binds strongly to albumin and seems to be a storage form of testosterone (Keles et al., 2006). However, the specific mechanism of S in bone is not outlined right now. Therefore, obvious decrease of Zn in the current study probably contributed to BMD reduction of diabetic animals and increase of S is possibly not beneficial to bone remodeling.

In summary, investigated by SRXRF microprobe in the experimental diabetic osteoporosis, significant decrease was observed in bone mineral content of Ca, P, Zn, Sr and marked increase in relative content of S in diabetic femur than those in controls. These results show that loss of minerals, detrimental to bone metabolism, is an important reason for BMD reduction in diabetics. And our study on element analysis will help to clarify the mechanism of BMD reduction in diabetics.

**Acknowledgements**

This work is supported by the Scientific Research Foundation of Graduate University of Chinese Academy of Sciences (no. 055101FM03) and China National Natural Sciences Foundation (grant no. 20571084). We thank Yanbin Hao and Maocai Zhang for help in statistical analysis.

**References**


