Reversal of Osteoporotic Changes of Mineral Composition in Femurs of Diabetic Rats by Insulin

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Abstract Insulin plays an important role in bone prevention of diabetic osteoporosis, but little is known about the relation between the bone mineral density (BMD) increase and the change of mineral content after treatment with insulin. To address this problem, male Wistar rats were randomly divided into three groups: normal group (n=6), streptozotocin-induced diabetic group (n=5), and streptozotocin-induced diabetic group with insulin treatment (n=5). The femoral BMD was measured by dual energy X-ray absorptiometry, and the element content was determined by inductively coupled plasma atomic emission spectrometry (ICP-AES). The results showed that the femoral BMD in diabetic group was significantly lower than that in normal group (P<0.01) but restored by insulin treatment (P<0.01 vs diabetic group). ICP-AES analysis revealed that the element content of calcium (Ca), phosphorous (P), magnesium (Mg), strontium (Sr), and potassium (K) in diabetic group were remarkably lower than those in normal group (P<0.01) but only Ca, P, and Mg content were significantly increased compared with diabetic group (P<0.05) after insulin treatment. However, no significant differences were observed in element zinc (Zn) content among three groups. Our findings suggested that the loss of Ca, P, Mg, Sr, and K content accounted for the lower BMD in streptozotocin-induced diabetic rats, insulin treatment could restore BMD by increasing the content of Ca, P, and Mg.

Keywords Element analysis · Bone mineral density · Insulin · Diabetes · ICP-AES

Introduction

Osteoporosis is a kind of metabolic disease of bone, in which there is a decrease in bone mass and an increase in sensitive to bone fracture, compared with normal bone [1]. The
decrease of bone mineral density (BMD) is a major predictor for osteoporosis. Many factors can cause osteoporosis. Nowadays, it is increasingly recognized that alterations in bone metabolism have been associated with diabetes mellitus [1–3]. Type 1 diabetic mellitus (T1DM) is a chronic disease, which is characterized by a lack of insulin production and high serum glucose levels. One of the long-term complications of T1DM is diabetic osteopenia and osteoporosis. Observational studies [4–6] and animal models [7, 8] suggest that T1DM is associated with modest reductions in BMD, leading to increased fracture rate [9, 10] and delayed fracture healing [11, 12].

Although the pathogenetic mechanism accounting for diabetic bone loss remains unclear, insulin may play a key role in type 1 diabetic osteoporosis. Insulin has been proposed to be an anabolic agent in bone [13]. Studies have already proved that insulin receptors present on osteoblasts [6–8]. Insulin could promote osteoblast proliferation, collagen synthesis, and alkaline phosphatase production [7, 14–16]. Besides direct effects of insulin on bone cells, insulin could also have effect on bone metabolism through other factors, such as insulin-like growth factor 1. Several T1DM rodent models revealed that insulin treatment could normalize BMD and markers of bone turnover [17, 18] and reverse the histomorphometric, biomechanical, and biochemical abnormalities and improve bone strength and ameliorate impaired diabetic bone fracture healing [19, 20].

Up to now, there were few studies on the correlation between restored BMD and changes of elements after insulin treatment in type 1 diabetic osteoporosis rat model. Some animal experiments revealed that low content of Ca, P, and Mg associated with the BMD decrease in diabetic osteoporosis [21, 22], but it is not clear how the bone mineral element changes after insulin treatment. The aim of this study is to assess the correlation between the BMD and the mineral content after insulin treatment in STZ-induced diabetic rats. The BMD of right femurs in three groups were measured by dual energy X-ray absorptiometry (DEXA), and quantification of the elements was performed by inductively coupled plasma atomic emission spectrometry (ICP-AES).

Materials and Methods

Animals

Male Wistar rats (240–260 g, Weitonglihua Animal Center, Beijing, People's Republic of China) were acclimatized for 1 week and randomly divided into three groups: control group, diabetic group, and insulin-treated diabetic group. Rats were rendered diabetic by an intravenous injection of streptozotocin (STZ, 50 mg/kg body weight; Sigma, MO, USA), freshly dissolved in citrate buffer (0.1 mol/l, pH 4.5), through the tail vein. The control group (n=6) were injected with citrate buffer only. After 1 week of administration, rats with fasting blood glucose (12 h) higher than 12 mmol/l were selected and divided into two subgroups: diabetic group (n=5) and insulin-treated diabetic group (n=5). The insulin-treated diabetic group received subcutaneously injected insulin (1.8–2.2 U/animal, Protamine Zinc Insulin Injection, Wanbang Biochemical Pharmaceutical, China) at 8:30 a.m. each day. To verify insulin action in the diabetic subjects, urine glucose levels were measured by Urine Glucose Reagent Paper 24 h after the injection of insulin (Guilin Zhongxing Biotechnology Dev., China). The experiment lasted 32 days after the onset of diabetes. All animal procedures in this study were carried out according to the standards of the Guide for the Care and Use of Laboratory Animals.

Blood Glucose and Body Weight

Glucose was determined immediately by a glucose kit (Biosino Bio-technology and Science, China) after blood was collected from a cut at the tail vein of 12-h fasted rats in three groups on days 0, 4, 8, 12, 16, 20, 24, 28, and 32, respectively.

Body weight was monitored before glucose determination each time.

Determination of Bone Mineral Density

At the end of the experiment, all animals were killed, and the right femurs were separated and cleaned of soft tissues. The femurs were fixed in 10% formalin. The total area of BMD of right femurs of the rats were measured by dual energy X-ray absorptiometry (Excellplus, Norland, USA) with small animal software.

Sample Treatment and Element Analysis

Chemical Reagents and Materials HNO₃ and H₂O₂ were of MOS grade, purchased from Beijing Chemical Reagents (Beijing). Deionized distilled water was prepared by a Milli-Q water purification system (Millipore, Bedford, MA, USA). All chemical wares and polytetrafluoroethylene (PTFE) vessels were soaked in 50% HNO₃ for 24 h and rinsed with deionized distilled water before used.

The femurs were dried for 48 h at 80°C until the weight did not change. Sample digestion was undertaken following the literature [23, 24], and the method was improved. In this case, each sample was taken into a PTFE Teflon beaker, predigested respectively in a mixture of HNO₃ and H₂O₂ (4:1 mixed, 5 ml per femur) overnight at room temperature to decouple organic component. Then, the sample was heated on a hot plate in sequence at 75°C for 0.5 h, 130°C for 0.5 h, and 200°C for 2 h. In this process, 2% HNO₃ was added continuously to prevent the solution drying until it was clear. After cooling, the samples were made up to 20 ml with 2% HNO₃. This solution is hereafter referred to as the analysis solution and was subjected to the determination of elements by ICP-AES.

An ICP-AES instrument (Kechuang Haiduang, Beijing, China) was used to quantify the mineral content of Ca, P, Mg, Zn, Sr, and K in the acid-digested samples. External calibration for each element was used throughout the analysis and the HNO₃ concentration in the calibration solutions was the same as that in the final sample solution.

Statistical Analysis

Data analysis and a linear correlation test were performed by SPSS (version 13.0). Results were presented as the mean±standard deviation (SD). Significant difference was defined at either of P<0.01 or P<0.05 by t test.

Results

Blood Glucose

As shown in Fig. 1, blood glucose in diabetic group was significantly higher than that in control group (P<0.01) during the experimental period. However, insulin treatment could normalize the levels of glucose in diabetic rats manifestly (P<0.01).
Body Weight

Average body weight in diabetic group was remarkably lower than that in controls through the study (P<0.01). However, the body weight of insulin treated group was significantly rescued by insulin treatment (P<0.01; Fig. 2).

Bone Mineral Density

The BMD of right femur was shown in Fig. 3. Femoral BMD of diabetic rats was significantly lower than that of controls (P<0.01). The levels of BMD in insulin-treated group were restored notably by insulin treatment (P<0.01).

Dry Weight of Femur

Dry weight of femur was significantly decreased in diabetic group compared to the control group but reversed obviously by insulin treatment (P<0.01; Table 1).

Determination the Contents of Ca, P, Mg, K, Sr, and Zn by ICP-AES

The element analysis by ICP-AES was shown in Fig. 4. The contents of Ca, P, Mg, K, and Sr in diabetic group were significantly lower than those in control group (P<0.01). After treated with insulin, the levels of Ca, P, and Mg were remarkably increased compared to diabetic group (P<0.05). However, the levels of K and Sr in insulin-treated group did not have any change (P>0.05). For Zn, no significant differences were observed among three groups.

The Correlation Among Mineral Elements

The correlation coefficients among mineral elements in control group were shown in Table 2. The statistical analysis showed that there were highly positive correlation (r) between Ca and P (r=0.968, P=0.001), Ca and Mg(r=0.651, P=0.081), and P and Mg(r=0.718, P=0.054), respectively. In addition, the correlation between K and Ca (r=0.628, P=0.091), Zn and Ca (r=0.794, P=0.03), Zn and P (r=0.857, P=0.015), Zn and Mg(r=0.773, P=0.036), Sr and K (r=0.913, P=0.006) were also very high.

Furthermore, statistical analysis in STZ-induced diabetic group and insulin-treated group showed high correlation between Ca and P, Ca and Mg, and P and Mg contents.

Discussion

At present, there were few studies on the relation between restored BMD and the change of element content after insulin treatment in type 1 diabetic osteoporosis rat model. Our present experiment revealed that the low BMD (P<0.01) in diabetic group was accompanied by decrease of Ca, P, Mg, K, and Sr content (P<0.01). However, BMD was normalized, and the content of element Ca, P, and Mg was also increased significantly by insulin treatment (P<0.05). Furthermore, statistical analysis in three groups showed that there were highly positive correlations between Ca and P, Ca and Mg, and P and Mg contents.

As we known, bone is a tissue that is made of organic component, mainly the collagen protein and inorganic compound, hydroxyapatite. There are two main functions of the bone mineral substance: one is a biomechanical role, which is the strength stability of the skeleton, and the other is a metabolic one, which is a reservoir for many ions, controlling mineral homeostasis. Approximately 80–90% of bone mineral content is comprised of calcium and phosphorus [25]. Ca is a major component of mineralized tissues, and almost 99% of body Ca is found in bone, where it serves a key structural role as a component of hydroxyapatite. Ca is required for normal growth, development and maintenance of the skeleton, where it provides strength and structure [25–27], and calcium (Ca) deficiency is a major risk factor for osteoporosis. Furthermore, bone acts as a major metabolic reservoir of Ca for the maintenance of extracellular homeostasis [28]. Extracellular Ca is one of the

Table 1 Dry Weight of the Femurs in Three Groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Dry weight of femur (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=6)</td>
<td>0.6823±0.05167</td>
</tr>
<tr>
<td>STZ (n=5)</td>
<td>0.4484±0.04738</td>
</tr>
<tr>
<td>STZ + insulin (n=5)</td>
<td>0.59264±0.06537</td>
</tr>
</tbody>
</table>

*Significantly different from diabetic group, by independent sample t test (SPSS), P<0.01.
main factors regulating the process of bone remodeling, by means of a multi-organ cross-signaling cascade [29]. Epidemiological evidences [30, 31] and animal experiments [32] show that Ca intake was positively correlated with BMD. On the other hand, P is second to Ca in abundance in the human body with 85% of the body’s P bound to the skeleton [33]. P regulates the bone formation, inhibits the bone resorption, and acts as one of anti-osteoporosis nutrients [34]. Study revealed that P also affected the regulation of calcium metabolism [35]. Supply with Ca and P was already considered as a treatment of osteoporosis [36]. Our experiment also showed that the BMD and the content of Ca and P in diabetic group are significantly lower than those in control group ($P<0.01$). Consequently, the drop in the contents of Ca and P may account for the decrease in BMD in our diabetic rats, just like other diabetic animal models [17, 21, 41]. However, the BMD and the content of Ca and P increased after insulin treatment remarkably ($P<0.05$ vs diabetes). Furthermore, there was a positive correlation between Ca and P in control group ($r=0.968, P=0.001$), diabetic group ($r=0.758, P=0.069$), and insulin-treated group ($r=0.619, P=0.133$). Therefore, insulin treatment could increase the contents of Ca and P and finally restore the BMD in diabetic rats.

Besides Ca and P, Mg is also an important element that influences the process of bone remodeling. Mg is the most abundant intracellular cation, which has a fundamental role as a co-factor in more than 300 enzymatic reactions involving energy metabolism and nucleic acid synthesis. Moreover, it is also involved in several processes like hormone-receptor binding, gating of calcium channels, and other processes [37, 38]. Bone is one of the main Mg pools in the body because about half the total Mg of the body is existed in bone. It is also reported that Mg deficiency induced a decrease in osteoblasts number, an increase in osteoclasts number, a drop in trabecular bone volume, and a decrease in BMD in rats [39]. However, dietary Mg supplementation improved bone formation, bone resorption, and bone strength in ovariectomized rats and in rats fed a high phosphorus diet [40]. Epidemiologic studies have demonstrated a positive correlation between Mg intake and BMD [33]. Some other studies also showed positive correlations between balance of Mg and balances of both Ca and P [41]. Therefore, Mg could be a nutrient for bone, and the loss of Mg could also partly contribute to diabetic bone loss. Our results also showed that the femoral BMD and the Mg content decreased significantly ($P<0.01$) in diabetic group just like the Simockeya’s report [22]. However, it is important that the BMD and the content of Mg restored after insulin treatment ($P<0.01$ vs diabetes). Furthermore, we found that Mg had a good correlation with Ca and P in control group, and this result suggested that Mg could regulate Ca and P metabolism in bone and have some effects on BMD.

Classically, K is the principal cation in the fluid inside of cells, and the beneficial role of K in bone metabolism is that K may have a function as a buffering agent to lighten the acid load for bone and influence calcium excretion directly [42, 43]. In addition, K is a cofactor for some enzymes, such as Na+, K+-ATPase, and pyruvate kinase. Granda et al. [44] reported that the STZ-induced diabetic rats had an obvious increase in K excreted, and insulin treatment was effective for restoring urinary output but K loss remained high. Meanwhile, epidemiological research also showed a positive relation between dietary supplemental K and BMD [45, 46] indicating that K is beneficial to maintenance of BMD. Therefore, the decrease of K content in femur might partly contribute to the low BMD just like our study on diabetic osteoporosis and Zhang’s report about the femoral head slice with osteoporosis [47]. However, the insulin treatment could not restore the concentration of K in femur of STZ-induced diabetic rats in our study.

Sr, an essential trace element, is good for bone remodeling. Strontium ranelate, a new compound, was recently shown to act as an effective anti-osteoporotic drug [48]. Animal data with strontium ranelate suggest this compound can both promote bone formation and

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**Table 2** Correlation (r) Between Element Contents in Control Group

<table>
<thead>
<tr>
<th></th>
<th>Ca</th>
<th>Mg</th>
<th>K</th>
<th>Sr</th>
<th>Zn</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca</td>
<td>1</td>
<td>0.968&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.151</td>
<td>0.628</td>
<td>0.523</td>
</tr>
<tr>
<td>P</td>
<td>0.968&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1</td>
<td>0.718</td>
<td>0.477</td>
<td>0.307</td>
</tr>
<tr>
<td>Mg</td>
<td>0.151</td>
<td>0.718</td>
<td>1</td>
<td>0.601</td>
<td>0.241</td>
</tr>
<tr>
<td>K</td>
<td>0.628</td>
<td>0.477</td>
<td>0.601</td>
<td>1</td>
<td>0.913&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Sr</td>
<td>0.523</td>
<td>0.307</td>
<td>0.241</td>
<td>0.913&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1</td>
</tr>
<tr>
<td>Zn</td>
<td>0.794&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.857&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.773</td>
<td>0.408</td>
<td>0.200</td>
</tr>
</tbody>
</table>

<sup>a</sup>Correlation is significant at the 0.01 level.
<sup>b</sup>Correlation is significant at the 0.05 level.
inhibit bone resorption [49]. Studies also 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 this drug is due to the modulation of the calcium-sensing receptor in bone cells [50]. Clinical trials support the use of strontium ranelate as a treatment for postmenopausal osteoporosis [51]. In the present experiment, we found that content of Sr in femur of diabetic mice significantly decreased (P<0.01) than that of controls, in accordance with the report from Fei et al. [52]. The decrease of Sr in diabetics is assumed to be one of the causes leading to diabetic osteoporosis. However, the content of Sr did not been restored after insulin treatment.

In conclusion, the results in our experiment showed that there was a significant decrease in BMD accompanied by marked decrease in bone mineral content of Ca, P, Mg, K, and Sr and a significant increase in BMD accompanied by marked increase in bone mineral content of Ca, P, and Mg after insulin treatment. Our findings revealed that loss of minerals, detrimental to bone metabolism, was an important reason for BMD reduction in diabetics and insulin treatment could restore the BMD by increasing the mineral content of Ca, P, and Mg, and the mechanism that insulin may increase the content of Ca, P, and Mg is still under study.

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Effects of Restraint Stress on Iron, Zinc, Calcium, and Magnesium Whole Blood Levels in Mice

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Abstract

Objective Study the effects of acute and chronic restraint stress on the whole blood concentrations of iron (Fe), zinc (Zn), calcium (Ca), and magnesium (Mg) in mice.

Materials and methods Single or repeat restraints were applied to mice to induce acute or chronic stress. The levels of elements in whole blood were determined by flame atomic absorption spectrometry.

Results The levels of Fe, Zn, Ca, and Mg in blood in the acute-stress group were 351, 5.05, 60, and 44 μg/ml, respectively, and those in the corresponding control group were 391, 5.90, 59, and 45 μg/ml, respectively. The levels of blood Fe, Zn, Ca, and Mg in the chronic-stress group were 291, 3.62, 59, and 40 μg/ml, respectively, and those in the corresponding control group were 393, 4.82, 48, and 43 μg/ml, respectively. The levels of Fe and Zn in the blood of both the acute-stress and the chronic-stress groups were significantly lower (P<0.05) than that in the control groups. The Ca level in whole blood was significantly (P<0.05) higher in the chronic-stress group than that in the control group.

Conclusion Acute and chronic restraint stress can cause changes in blood levels of Fe and Zn in mice.

Keywords Stress · Fe · Zn · Ca · Mg · Whole blood

Introduction

Stress is the sum of physical and mental responses to unacceptable experiences. Mental stress, bacterial and/or viral infections, overwork, and many other factors can induce a stress response in the body. Stress is mainly regulated through the autonomic nervous system and hormones secreted from the hypothalamo-pituitary adrenal axis [1]. Stress is a normal response as part of a self protection mechanism. However, an intense or long-lasting stress response could be harmful to the body because it may induce neural, endocrine, or