Effects of Long-Term Calcium Supplementation on Rats Bone Mineral Density and Cardiovascular Based on Metabonomics

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(Received September 25, 2018)

Summary Calcium supplements were necessary for those people with low calcium intake and high risk of osteoporosis. Recent cohort studies have shown that long-term calcium supplements may raise the risk of cardiovascular disease, but its mechanism is still unclear. In this study, metabonomics were employed to evaluate the changes of metabolism in rats with long-term calcium supplementation and further seek the potential markers of cardiovascular risk. SD rats were divided into two groups including normal control group (calcium intake, 0.50 g/kg bw) and high calcium supplement group (calcium intake, 2.50 g/kg bw). After 6 mo, the cardiovascular system and bone mineral density were observed. UPLC-MS was used to analyze serum metabonomics in rats. The results showed that the contents of total cholesterol and low-density lipoprotein cholesterol in the high calcium group were significantly higher than those in normal control group (p<0.05). The interventricular septum thickness (IVS), left ventricular mass (LVM), left ventricular posterior wall thickness (LVPW) in the high calcium group were higher than those in normal control group (p<0.05). Serum metabonomics analysis showed that there were persistent changes in many metabolites such as sphingosine and its derivatives (p<0.01) in the comparison between the high calcium group and the normal group. These results indicated that long term calcium supplementation can lead to dyslipidemia in rats, such as the rise of cholesterol and low-density lipoprotein, which might induce myocardial hypertrophy. Long-term calcium supplementation can cause the changes of the amount of sphingosine and its derivatives in the body, which may have potential risk to cardiovascular diseases such as myocardial hypertrophy and atherosclerosis.

Key Words bone metabolism, osteoporosis, myocardial hypertrophy, biomarkers, sphingosine

Calcium intake, bone growth and bone metabolism are present in the whole human life. Calcium also has important physiological functions such as cell information transmission and maintenance of neural activity. However, calcium deficiency is a global problem (1). In China, the average calcium intake of all age groups was lower than the dietary reference intakes. The incidence of osteoporosis caused by calcium deficiency in the elder population in China is as high as 56%, of which women account for 60–70% (2). In Guangzhou, dietary calcium intake, of 72.2% young people at 12–17 y of age, was less than 1,000 mg/d, the appropriate intake of AI standard, of which 83.4% were female (3). In Hongkong, the actual calcium intake of adults was less than 700 mg/d, while in some inland areas of China was only 400 mg/d (4). Therefore, it is necessary to perform long-term calcium supplementation. Community nutrition monitoring, blood biochemical analysis, epidemiological investigation and many other methods were used to evaluate the nutritional status of calcium deficiency, but there is little use of these methods to the comprehensive evaluation of calcium supplementation on bone and cardiovascular disease risk.

At present, researchers have realized that high serum calcium may increase the incidence of cardiovascular diseases. Calcium supplementation is an independent risk factor for myocardial infarction (5). It has been reported that the 1 g calcium intake daily for 5 y could lead to the dysfunction of pyrophosphate and calcium sensing receptor (CaSR) (6). This might be one of the reasons for the increased risk of cardiovascular disease.

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Abbreviations: BCC, bone calcium content; BMD, bone mineral density; CVD, cardiovascular disease; FGF, fibroblast growth factor; HC, high calcium; HDL-C, high density lipoprotein-cholesterol; IVS, interventricular septal thickness; LDL-C, low density lipoprotein-cholesterol; LVM, left ventricular mass; LVPW, left ventricular posterior wall; NC, normal calcium; PCA, principal component analysis; PLS-DA, partial least-squares discriminant analysis; QC, quantity control; TC, Total cholesterol; TG, triglyceride; UPLC-MS, ultra performance liquid chromatography–mass spectrometer; VIP, variable importance project.
In the study, calcium intake was increased to induce fibroblast growth factor (FGF), which may lead to myocardial hypertrophy by interaction of inflammatory reaction and vascular endothelial cell dysfunction (7).

However, some studies did not support the conclusion that calcium supplements may increase cardiovascular risk. Bauer’s study found that people who took calcium supplements overlapped with those at high risk of cardiovascular diseases (8). Shin and Kim’s study also suggested that this conclusion could not be drawn from low calcium intake in Asian populations, and low calcium intake is beneficial to the production of collagen fibers and polysaccharide matrix, and promoting bone mineral density (BMD) (9). Huang et al’s study indicated that the imbalance of bone metabolic, but not long-term calcium supplementation, is a risk factor for cardiovascular diseases (10). About the cardiovascular safety of calcium supplementation, more studies should be further carried out.

Metabonomics is the science of the metabolism of endogenous metabolites and their change rules. By means of nuclear magnetic resonance (NMR), mass spectrometry (MS) and other methods, the data were collected and analyzed to found the difference of metabolites. Metabonomics had ever been used to evaluate the disadvantage of low calcium by analyzing rat urinary metabolites and further confirmed the corrective effect of calcium supplements (11). At present, there is no study on excess calcium intake by metabonomics.

**MATERIALS AND METHODS**

**Experimental animals and calcium delivery.** Twenty male SD rats (SPF grade, weight 65–75 g) were ordered from Liaoning Changsheng Biotechnology Co. Ltd. Rats were randomly divided into two groups according to body weight and fed with low calcium diet. There were ten rats in each group. The normal calcium group (0.50 g/kg bw/d) and high calcium group (2.50 g/kg bw/d) was gavaged once a day with 1% kg of body weight of CaCO₃ for a period of 6 mo. The body weight was measured every week and the volume of gastric lavage was adjusted. The animal use was approved by the Animal Care and Use Committee of Harbin Medical University. The approve number is HMUIRB20190002.

**Ultrasound, pathological examination and ultra high performance liquid chromatography–mass spectrometry.** At the end of the experiment, rats were anesthetized and the heart function was measured by small animal ultrasound imaging system; The samples of rat heart and aortic arch were placed in formalin solution for pathological section examination. By embedding, cooling, solidification, the heart tissues were cut into thin slices and were finally stained with hematoxylin and eosin, and examined under a light microscope (Olympus, Japan). The rat femurs were taken for detecting bone calcium content (BCC) and bone mineral density (BMD). Blood samples were taken from the rat’s abdominal aorta and centrifuged. These serum samples were used for blood biochemical analysis and metabonomics analysis by using ultra high performance liquid chromatography–mass spectrometry. First of all, the serum samples including quality control samples were detected by metabonomics. Secondly, through quantitative, qualitative, identification, identification and software data analysis, the sample datas were identified by pattern recognition. The PCA diagram was used to observe the aggregation of different samples. These data were further imported into the PLS-DA mode, cleared the overlap and reduced the dimension conversion. Differences among groups can be more effectively observed. Finally, using the metabonomics database HMBD preliminary screening of potential biological markers.

**Experimental conditions of metabonomics.** Chromatographic condition: The chromatographic column was ACQUITY UPLC™ BEH C18 (2.1 mm×100 mm, 1.7 μm, Waters, USA). The mobile phase A is ultra pure water (containing 0.1% formic acid solution), and the mobile phase B is pure acetonitrile. The flow rate is 0.35 mL/min. The sampling quantity is 5 μL. The column temperature is about 35°C, and the temperature of the automatic sampling device is maintained at a temperature of 4°C.

Mass spectrum conditions: using ESI ion source, in the positive and negative ion mode, to collect data in Centorid mode. Capillary voltage 3,000 V (ESI+)/2,800 V (ESI−) 35 V extraction; cone voltage 3 V; ion source temperature of 110°C; desolvation gas temperature of 320°C; desolvation gas flow rate 600 L/h; cone gas flow 15 L/h; ion energy 1 V; collision energy 6 V; scanning time 0.4 s; scanning time interval is 0.1 s.

Data acquisition range from m/z 50 to 1,000 and the retention time is between 0 and 16 min.

**Metabonomics data preprocessing and multivariate statistical analysis.** The original data (raw data material) of rat serum samples were detected by UPLC-MS. Then the data were analyzed by Progenesis QI software data alignment, peak detection, deconvolution, compound quantification, compound identification, statistical analysis pretreatment (retained p<0.05 and max fold change >2). Then import EZinfo for model analysis. In this experiment, using mean-centered and Pareto (EZinfo software function) to correct the draw of unsupervised principal component analysis (PCA) method, such as high absolute value and high variability. The distribution of samples as accurately observed and further eliminate noise interference. Under the condition of large sample quantity analysis, in order to prevent the PLS-DA mode from over fitting and ensure the robustness of the analysis model, PLS-DA was used to set the VIP value and the variable selection criteria.

**Statistical analysis.** Data were analyzed using SPSS statistics 17.0 software. The difference between groups was analyzed by one way ANOVA and p<0.05 was considered as statistically significant differences. The experimental data are expressed as mean±SD.

**RESULTS**

**Rat body weight, BMD, BCC and blood biochemistry.** During the experiment, the body weight of rats was...
Fig. 1. Rat body weight, BMD, BMC and blood biochemistry. (A) Rat body weight. (B) Rat BMD and BMC. (C) Rat blood biochemistry. NC, normal calcium. HC, high calcium. Ten rats in each group. *p<0.05 HC vs NC. **p<0.01 HC vs NC.

Fig. 2. Cardiovascular ultrasound in rats. (A) left ventricular. (B) Aortic arch. (C) Cardiac index value. NC, normal calcium. HC, high calcium. Ten rats in each group. *p<0.05 HC vs NC. **p<0.01 HC vs NC.
measured every week. Based on this, the dosage of intragastric administration was adjusted and the body weight of each rat was summarized every month. As shown in Fig. 1A, there was no significant difference between normal calcium group and high calcium group during the experimental periods of 6 mo ($p>0.05$). BMD and BCC were measured in both groups of rats. As shown in Fig. 1B, there was no difference in BMD and BCC between normal calcium group and high calcium group ($p>0.05$). Biochemical analysis of serum calcium and serum lipids were also performed in both groups of rats. As shown in Fig. 1C, total cholesterol and low density lipoprotein cholesterol in high calcium group were significantly higher than those in normal calcium group ($p<0.05$). However, there was no significant difference on serum calcium between the two groups ($p>0.05$).

Cardiovascular ultrasound in rats

After anesthesia, the left ventricular and aortic arch were detected by using a small animal ultrasound detector. The left ventricle inner diameters of rats in high calcium group is obviously lower than normal calcium group (Fig. 2A). In addition, aortic arch and its three branches of the brachiocephalic artery, left common carotid artery and left subclavian artery vascular imaging were clear; and there were no obvious ectopic calcium deposition or atherosclerotic thrombotic tendency (Fig. 2B). Rat cardiac indexes were also obtained by the ultrasonic detector after data analysis. As shown in Fig. 2C, diastolic and systolic rat ventricular septal thickness (IVS), left ventricular posterior wall thickness (LVPW) of rats in normal calcium group was significantly lower than that in high calcium group ($p<0.05$). Left ventricular mass of rats in normal calcium group were significantly lower than that in high calcium group ($p<0.05$).

Pathological analysis

At 100-fold magnification, there is no differences on the morphology of myocardial cells and tissues of rats in both groups (Fig. 3A). At 400-fold magnification, the myocardial cells of rats in high calcium group tends to be hypertrophy, compared to normal calcium group (Fig. 3B).

Rat serum metabonomics

Evaluation of the stability of UPLC-MS serum metabonomics platform. The instrument stability, sample quality and column pressure temperature of the UPLC-MS in the course of metabolomics experiments was strictly controlled by Waters dual spray ion source Lock spray correction solution (200 pg/mL leucine brain peptide solution) and QC. After each 5 serum samples were determined, 1 mixed samples were extracted. In order to ensure that the samples in different groups are not contaminated with each other and do not interfere with the experimental results. The chromatographic column was cleaned with a pure acetonitrile. Mixed samples and
blank samples were dispersed in the whole process of rat serum metabonomics. The PCA score of the rat serum samples and the quality control serum samples of 10 rats as shown in Fig. 4A. It can be seen from this figure that the serum of the rat and the quality control serum are gathered in each district, and the distance is larger. It shows that the system is stable.

PCA and PLS-DA models of serum samples from high calcium group and normal control group. As shown in Fig. 4B, each of the red or black samples in the PCA chart represents a rat serum sample. The detected substances are gathered around the samples, and the high calcium group is significantly partitioned with the normal calcium control group. As shown in Fig. 4C (PLS-DA diagram), the difference of serum sample metabolism between normal calcium control group and high calcium group was more distinct by further noise removal and data conversion. PCA and PLS-DA two plots showed that the normal calcium control group and the high calcium group produced metabolic differences under 6 mo different calcium intake interventions.

Determination and identification of markers in serum

In PLS-DA diagram, a large amount of differential metabolites can be obtained by comparing the two groups. Through the EZinfo software VIP key, these differential metabolites were arranged. Differential metab-
olites with VIP less than 1 are removed by exclude and fit key. The value of the remaining differential metabolites were transferred back to Progenesis QI software, and combined with statistical p values (screening p<0.05), preliminary biomarkers may be identified. These metabolites are searched in the metabolomics database. Combining the metabolites description about structure, components and fragments of Progenesis QI software, we access the possibility of these metabolites as a biomarker for present experiment. Not only do the metabolic differences have statistical significance, but they also have biological implications for routine monitoring. The results are derived from software to obtain the corresponding compound information, including biomarkers of retention time, name, change trend, quality of chemical etc. we finally identified sphingosine and its derivatives in positive and negative ion mode as shown in Table 1. These substances was significantly larger than in the high calcium group compared with the normal control group.

**DISCUSSION**

As the most abundant mineral elements in the body, adequate calcium had significant effects on body weight, body hair, growth and development (12). However, high calcium intake had no further effect on body weight and BMD compared with normal calcium intake. These results are consistent with previous studies, and an increase in calcium intake does not have a positive effect on the body (13). This may be related to the development of bone, and the absorption and reuse of calcium in the body. The appropriate amount of calcium and other nutrients in the gastrointestinal tract is digested and absorbed (14). According to the needs of the body, calcium is balanced and regulated in the tissues and organs. The excessive amount of calcium intake in the body’s digestion and absorption presented in different ways and results. For example, a low dose of calcium supplements could also formed within a short period of time critical hypercalcemia in the human body, and kept for several hours (15). In this study, rats blood calcium determination reflected the long-term effects of calcium supplementation on the body. There is no difference between the serum calcium values of different calcium intake groups, possibly due to regulation of calcitonin, 1,25(OH)2D3, parathyroid hormone and calcium balance within the body. The stability of serum calcium values implied the damage of calcium deficiency to the bone and long term calcium supplementation might increase the load of calcium homeostasis.

On the basis of epidemiological investigation of osteoporosis prevention in postmenopausal women, long-term calcium supplementation can affect the serum cholesterol of female population, and eventually lead to calcium deposition in the carotid artery (16). In this study, high calcium intake significantly increased rat serum levels of TC, TG, LDL-C and HDL-C. Particularly, the increasing effect of long term high dose calcium supplementation on LDL-C significantly, which is closely related to atherosclerosis. The possible mechanism was considered as that long-term calcium supplementation converted the smooth muscle cells into osteoblast like cells, which the formation of matrix vesicles to secrete mineralized crystals, leading to smooth muscle cell apoptosis (17). We believe that the discovery of the markers of lipid change is more significant than the exploration of the mechanism. Further experiments on mechanisms will be carried out by our laboratory through metabonomics or other methods.

To confirm the effect of long-term calcium supplementation on the cardiovascular system, the left ventricular myocardial growth was observed by ultrasound and pathological sections. The results showed that long-term calcium supplementation in rats, left ventricular diameter data became small, ventricular septal thickness (IVS), left ventricular mass (LVM), left ventricular posterior wall thickness (LVPW) in both myocardial systole or diastole were increased. In the clinical diagnosis, asymmetric septal thickening is an important criteria for the diagnosis of hypertrophic cardiomyopathy of patients. Ratio of ventricular septum and posterior wall thickness ratio should be greater than or equal to 1.3 (18). However, the rat has no diagnostic criteria for reference. In fact, myocardial hypertrophy is a slow and effective compensatory mechanism, which may be a common pathological change before and during the course of cardiovascular diseases. Some researchers have found that the daily calcium intake can be combined with oxidative stress and lipid levels as potential biomarkers for cardiovascular disease risk monitoring (19).

Biomarkers play an important role in the research of medicine and nutrition. So far, there is no reports of metabonomics biomarkers on different doses of calcium intake effects to the cardiovascular system. Serum meta-

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**Table 1. Potential risk biomarkers of cardiovascular diseases.**

| Metabolites       | Molecule composition | Scan mode | Retention time (min) | Measured mass (Da) | Mass error (ppm) | Trend |
|-------------------|----------------------|-----------|----------------------|--------------------|------------------|-------|
| Cholesterol sulfate | C27H46O4S           | —         | 10.9472              | 466.3117           | ~7.1936          | ↓     |
| Sphinganine       | C18H39NO2            | +         | 5.234                | 301.5078           | 0.8416           | ↓     |
| Phytosphingosine  | C18H39NO3            | +         | 4.674                | 317.5072           | 0.6539           | ↓     |
| Ceramides         | C12H26NO3            | +         | 11.176               | 539.5277           | 1.5316           | ↓     |
| Ganglioside       | C102H178N4O47        | +         | 3.621                | 2212.5033          | ~0.2639          | ↓     |
Metabonomics on Long-Term Calcium Supplementation

Sphingosine, widely distributed in the body, is a metabolite of sphingolipids in certain conditions, which has a variety of physiological activities, such as sphingosine on myocardial cell membrane involved in calcium intake pump (20). A variety of sphingolipids were derived from the sphingosine backbone (21). Sphingosine itself also plays a unique role in the formation of 1-sphingosine (S1P), which is a marker of atherosclerosis after binding to HDL-C (22). Sphingosine and its derivatives play a role to prevent a variety of lipid deposition, vascular intimal focal fibrosis after combination with HDL-C (23). Because sphingosine is an important structure on the cell membrane, it can protect the vascular endothelial cells to reduce the damage of endothelial cells and delay the progression of atherosclerosis (23). In this study, compared to normal calcium group, the levels of rat serum sphingosine in the high calcium group was decreased significantly, and the free HDL-C levels was increased. These results indicated that long-term calcium supplementation inhibited the binding of S1P and HDL-C.

Except for sphingosine and its derivatives, the amount of cholesteryl sulfate decreased significantly in the high calcium group, compared to normal calcium group. The latest study in 2015 showed that sulfate ions in the blood vessels are important for maintaining endothelial cell health and microvascular permeability (24). After the decrease of sulfate ion, the cholesterol sulfate ester was reduced, making free cholesterol unable to form the atherosclerotic plaque. Intake of foods with high sulfate may prevent progression of atherosclerosis (24). In this study, high calcium intake significantly decreased in vitro synthesis of cholesterol sulfate, which is coincided with the latest research results. However, the mechanism should be further studied.

The purpose of this study is not only to evaluate the different effects of long-term calcium supplements on bone and cardiovascular system of rats, but also to find risk biomarkers of metabolomics for cardiovascular diseases caused by these supplements. The innovation of this study is to find the possible relationship between long-term calcium supplementation and substances, such as sphingosine, through metabolomics. These substances could be used for monitoring the risk of cardiovascular diseases. Our metabolomics experiment is aimed at preliminarily finding differential metabolites. Further experiments should be conducted to provide sufficient data to support these risk markers. In this study, we did not carry out the confirmation of the biomarkers in the susceptible population, such as the long-term calcium supplement or the high incidence of cardiovascular diseases, which should be further investigated in the future studies.

Long-term calcium supplementation could inhibit bone metabolism activity and lead to the increase of total cholesterol, triglyceride and low density lipoprotein cholesterol, which might be used as potential risk biomarkers of cardiovascular diseases induced by long-term calcium supplementation.

Disclosure of state of COI
The authors declare no conflict of interest.

Acknowledgments
This study was supported by grants from the Heilongjiang Province Postdoctoral Science Foundation (LBH-Z12196) and China Postdoctoral Science Foundation (2013MS541416).

Author contributions
Designed the experiments: LY. Performed the experiments: CH, ZY, HY and YS. Wrote the paper: CH and LY.

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