Fundamental Toxicological Sciences (Fundam. Toxicol. Sci.)
Vol.6, No.7, 253-258, 2019

The Japanese Society of Toxicology

Fundamental Toxicological Sciences
URL : http://www.fundtoxicolsci.org/index_e.html

Letter

Impact of dietary calcium and phosphorus levels on an ovariectomized Sprague-Dawley rat model of osteoporosis

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(Received September 27, 2019; Accepted October 3, 2019)

ABSTRACT — Ovariectomized rats were used as an animal model of osteoporosis in this study. They were given *ad libitum* access to a diet with high or low calcium to phosphorus ratio, or a normal diet, for a period of 10 weeks and effects on osteoporosis were assessed. Our results showed that the experimental diets affect urine levels of calcium and phosphorus, but do not affect the levels of biochemical, histopathological, or bone-related biomarkers. These results indicate that the administration of feed with different ratios of calcium to phosphorus (the phosphorus content was kept constant and the calcium content varied from 0.2× to 5× relative to the phosphorus content) over a period of 10 weeks does not accelerate the development of osteoporosis in ovariectomized rats with normal renal function to maintain homeostasis.

Key words: Osteoporosis, Calcium, Sprague-Dawley Rat, Bone biomarker, Parathyroid hormone, Postmenopausal

INTRODUCTION

Several factors, such as aging, female menopause, genetic factors, vitamin deficiency, lack of exercise, and stress, are involved in the development of osteoporosis. However, calcium plays a significant role in the prevention of osteoporosis. In adults, 99% of calcium is found in the bones and teeth as hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$), while most of the remaining 1% is distributed in cells, leaving only 0.1% in the blood (Matsumoto, 1992). Approximately 40% of calcium in the serum is bound to albumin or other proteins and is not available for diffusion into the tissues. Meanwhile, chelated calcium ions—calcium phosphate and calcium oxalate (9% of calcium in the serum)— are absorbed and transported to tissues, and the remaining free calcium (51% and 0.051% of calcium in the serum and body, respectively) is used to maintain normal physiological functions (Conceição *et al.*, 2016; Arazi *et al.*, 2016). Ionized calcium is required to regulate muscle contraction, enzyme phosphorylation (protein kinases), nerve conduction, hormone release, blood coagulation, and other metabolic processes (Gerlinger *et al.*, 2019; Stewart and Davis, 2019; Food and Nutrition Board, 1997; Pearce and Thakker, 1997).

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Calcium plays important physiological functions, and its blood levels are tightly regulated by the endocrine system. For example, the calcium-sensing proteins in the parathyroid gland send signals to induce the secretion of parathyroid hormone (PTH) in response to low blood calcium levels caused by insufficient calcium intake (Demontiero *et al.*, 2012). In the kidney, PTH converts vitamin D into its active form calcitriol, which increases the absorption of calcium from the small intestine. PTH-induced calcitriol synthesis activates osteoclasts (cells that reabsorb bone), stimulates calcium release from the bone, increases reabsorption of calcium in the kidney, and reduces urine calcium excretion.

Consequently, the endocrine regulation of blood calcium levels plays an important role in bone metabolism. PTH promotes bone resorption, whereas calcitonin suppresses it. In particular, aging significantly reduces calcitonin secretion and promotes bone resorption. Estrogen promotes bone formation; however, the decrease in estrogen levels at menopause results in reduced bone mass in women. Together, these factors cause the blood calcium levels to drop in elderly individuals, and induce constant release of calcium from the bone into the blood, ultimately resulting in osteoporosis (Raisz, 2005; Azuma, 2007). Excessive dietary intake of calcium results in its accumulation as hydroxyapatite in the bone and decreases the blood levels of calcium and phosphorus. Conversely, low calcium intake can trigger the release of calcium from the bones and increase the blood levels of calcium and phosphorus. Consequently, dietary calcium levels play an important role in calcium metabolism and in the development and progression of osteoporosis. However, only limited studies have been conducted so far. Therefore, the present study was conducted to determine the potential effects of different calcium levels in feed on the development of osteoporosis in ovariectomized (OVX) rats, a well-known animal model of osteoporosis.

MATERIALS AND METHODS

Animals and housing conditions

Five-week-old female Slc:Sprague-Dawley (SD) rats were bred at Japan SLC Inc. (Hamamatsu-Shi, Japan), and ovariectomized at the age of 8 weeks. The rats were housed individually in polycarbonate cages with stainless steel wire lid (W25 × D40 × H18 cm) throughout the study period. During the one-week preliminary rearing period, the animals were kept in a room maintained at 20–26°C and 40–80% humidity in a 12/12 hr light/dark cycle, and fed *ad libitum* on Labo MR Stock (Nosan Corp., Yokohama, Japan). The rats were given *ad libitum* access to different TestDiet® feeds (Animal Specialties And Provisions, LLC, PA, USA) and sodium hypochlorite-supplemented well water (free residual chlorine levels were 1–2 mg/L) in bottles, starting at the age of 6 weeks.

Table 1. Feed content (calcium and phosphorus) and group structure.

	Calcium (%)	Phosphorus (%)	Number of Rats
Normal	0.61	0.61	10
0.5× Ca Diet Food	0.305	0.61	10
0.2× Ca Diet Food	0.122	0.61	10
2× Ca Diet Food	1.22	0.61	10
5× Ca Diet Food	3.05	0.61	10

This study was conducted at Japan SLC Inc., an institution certified by the Japanese Society for Laboratory Animal Resources (JSLAR), in compliance with the Act on Welfare and Management of Animals and the Japan SLC Code for Laboratory Animal Welfare, and in accordance with the study protocol reviewed by the study site's laboratory animal welfare committee (the Japan SLC Laboratory Animal Care and Use Committee).

Experimental design

A total of 50 female Slc:SD OVX rats were divided into five groups, each consisting of 10 rats and fed on the following diets *ad libitum* for a period of 10 weeks: normal (TestDiet, 5885); calcium (Ca)-deficient (0.5× normal level; TestDiet, LT429); Ca-deficient (0.2× normal level; TestDiet, LT428); Ca-excessive (2× normal level; TestDiet, LT430); and Ca-excessive (5× normal level; TestDiet, LT431) (Table 1).

Clinical signs

The rats were observed for clinical signs once daily, starting on the day before the start of the feeding study (baseline) and ending on the day of autopsy.

Body weight

The rats were weighed at the time of receipt, group assignment, ovariectomy, and then once every week.

Food consumption

Food consumption was measured once every week using an electronic scale GX-3000 (A&D Co., Tokyo, Japan), and calculated based on the ratio of the amount of feed given to the remaining amount.

Hematology

At the completion of the study, venous blood samples were collected from all rats and analyzed for red blood

cells (RBC), white blood cells (WBC), platelets (PLT), hemoglobin, hematocrit, WBC differential, reticulocyte, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC), using ADVIA2120 (Siemens Healthcare Diagnostics K.K., Tokyo, Japan).

Blood biochemistry

At the completion of the study, serum samples prepared from venous blood collected from all rats were analyzed for serum protein, albumin, alubumin/globulin (A/G) ratio, glucose, cholesterol, triglycerides, phospholipids, bilirubin, urea nitrogen, creatinine, transaminases (AST and ALT), alkaline phosphatase, electrolytes (sodium, potassium and chlorine), Ca, and inorganic phosphorus (iP), using the autoanalyzer 8180 (Hitachi Ltd., Tokyo, Japan).

PTH levels

At the completion of the study, serum samples prepared from venous blood collected from all rats were analyzed for PTH levels using ELISA.

Urinalysis

At the age of 18 weeks, the rats were placed in metabolic cages, and urine was collected for approximately 16 hr to measure urine volume. The collected urine samples were further analyzed for urine Ca and iP levels, using the autoanalyzer 8180.

Bone density and bone mineral content

The right femurs of the rats were removed during autopsy and analyzed for bone mineral content and bone density, using the bone mineral analyzer DCS-600EX-III (Aloka Co. Ltd., Mitaka, Tokyo, Japan).

Histopathology

The following organs were weighed during autopsy: brain, heart, lungs, kidneys, spleen, liver, uterus, adrenal glands, thymus, submandibular glands (including the sublingual gland); and pituitary gland. The following organs were fixed in 10% (vol) neutral buffered formalin solution: skin, brain, pituitary gland, thyroid glands (including the parathyroid glands), thymus, trachea, lungs and bronchi, heart, submandibular glands (including the sublingual gland), liver, spleen, kidneys, adrenal glands, pancreas, uterus, vagina, mammary glands, thigh muscle and sciatic nerve, tongue, esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, rectum, gall bladder, lymph nodes (mesenteric nodes), spinal cord, eyeball (including optic nerves), Harderian gland; and bone and bone marrow (sternum and femur). Hematoxylin and eosin (H&E) stained samples were prepared according to standard procedures, and all samples were examined light microscopically.

Statistical analysis

The mean and standard deviation for each study group were determined for body weight, food consumption, blood biochemistry values, organ weights, and other parameters. Multiple comparison test was performed using SAS 9.2 (SAS Institute Japan Ltd., Tokyo, Japan) and Exsus ver. 8.8.1 (CAC Exicare Corp., Tokyo, Japan), a system that works in tandem with the former. The level of significance was set at 5%.

RESULTS

Cage-side observations of the rats did not reveal observable changes. Moreover, no differences were observed in their food consumption, body weight, urine volume, hematological values, bone mineral content, bone density, organ weight (Supplemental data), blood PTH level (Fig. 5), or histopathological values (bone changes; Fig. 6, images 1–3). These results suggested that none of the rats developed osteoporosis.

Hematology and blood biochemistry

The difference in blood Ca or iP levels between the control and experimental groups (Figs. 1 and 2) was not statistically significant.

Urinalysis

The level of urine calcium excretion in the rats that were fed with a high Ca diet was higher than that in the rats fed with a low Ca diet. However, the difference was not significant (Fig. 3). The level of urine iP excretion in rats that were fed with 0.5× Ca diet was significantly higher than that in the rats that were fed with a high Ca diet, with excretion nearly undetectable in the 5× Ca diet group (Fig. 4).

PTH levels

Blood PTH levels remained almost unchanged from the baseline at 10 weeks (Fig. 5).

DISCUSSION

There are roughly 4 types of osteoporosis animal models: bone resorption promoted, bone formation suppressed, modeling animals, and remodeling animals. OVX rats are the most widely used model for osteoporosis. Reduced

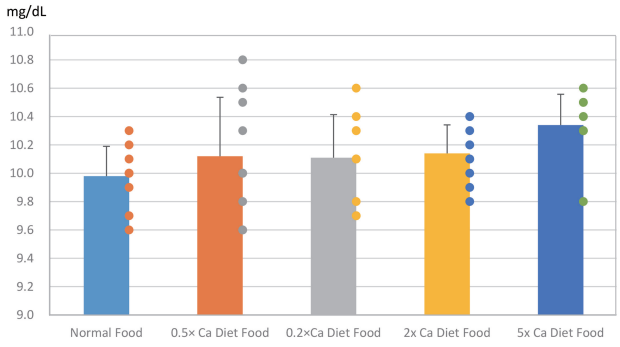

Fig. 1. Change in plasma calcium levels at 10 experimental weeks.

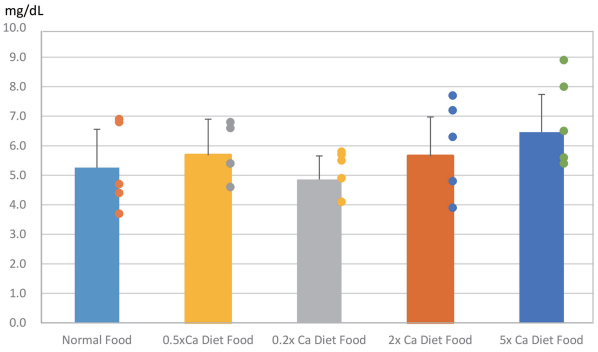

Fig. 2. Change in plasma inorganic phosphorus levels at 10 experimental weeks.

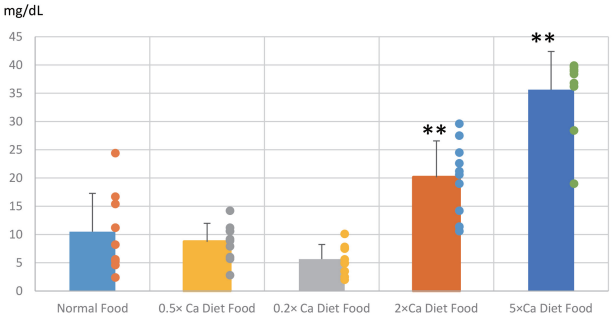

Fig. 3. Change in urine calcium levels at 10 experimental weeks.

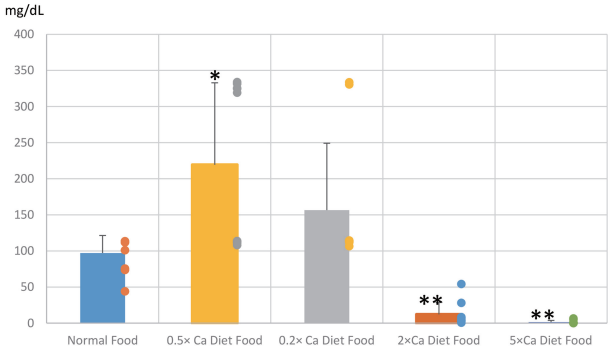

Fig. 4. Change in urine inorganic phosphorus levels at 10 experimental weeks.

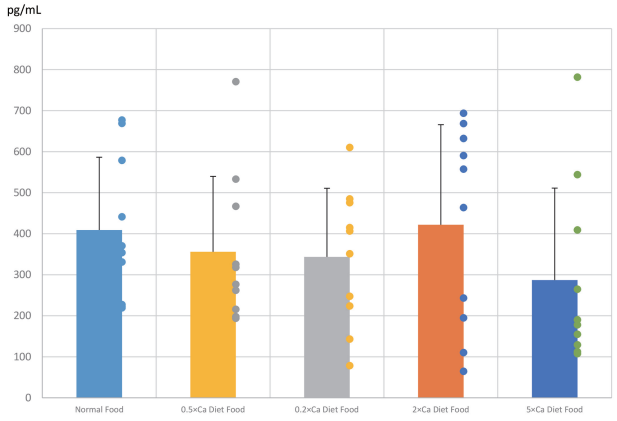

Fig. 5. Change in PTH levels at 10 experimental weeks.

estrogen levels in these rats result in increased bone metabolism and a marked bone loss in the epiphysis of long bones or vertebral cancellous bones (Azuma, 2007). OVX rats fed with a low Ca diet exhibit accelerated bone loss in both cancellous and cortical bones (Azuma, 2007). Nakada *et al.* (2014) showed that OVX rats fed with diets that contained Ca either in an amount approximately twice or half that of iP exhibit differences in their femur bone mass. Taking these results into consideration, we fed ovariectomized female rats with several types of Ca diets, ranging from 0.2× to 5× excess relative to iP content for a period of 10 weeks, and examined changes in the blood and urine levels of Ca, iP, and bone-related markers.

No difference in the bone mass or bone tissue was observed in the rats of the control group, and they did not develop osteoporosis. Moreover, no change was observed in the low-Ca diet (0.5× and 0.2×) groups, suggesting that changing Ca content in the feed did not accelerate the

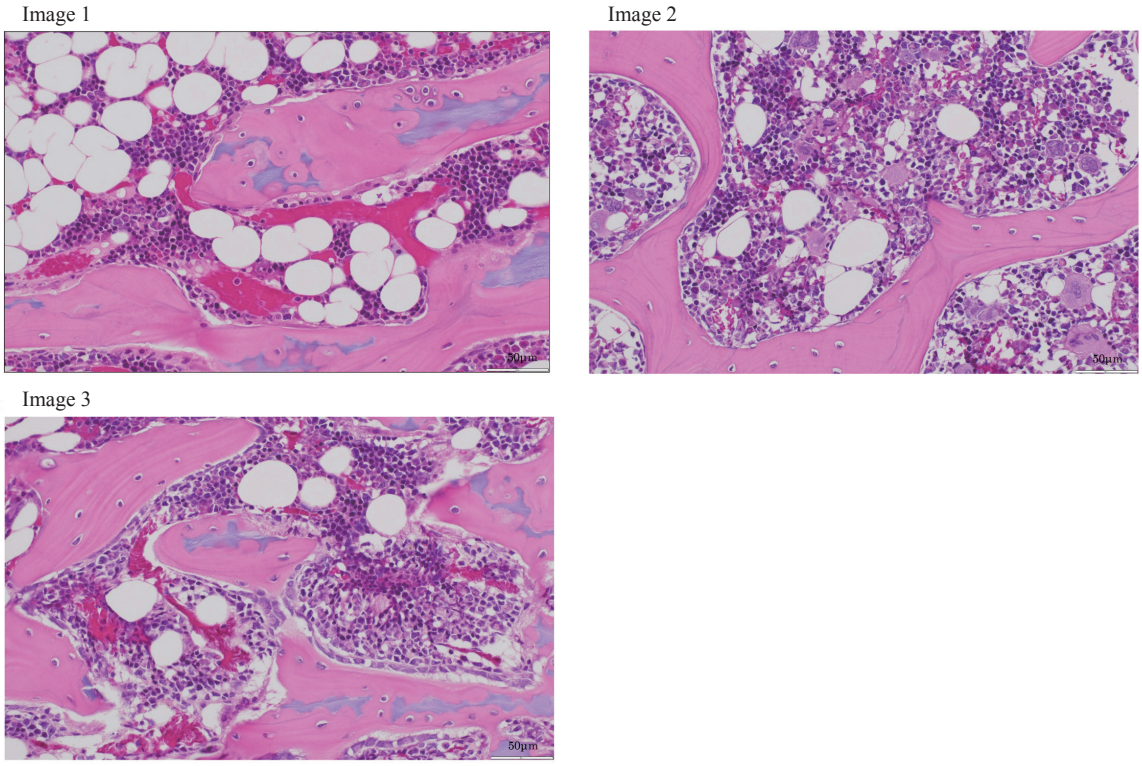

Fig. 6. Image 1: Normal group, lumber, no remarkable change. Scale bar: 50 μm. Image 2: 0.2× Ca diet food group, lumber, no remarkable change. Scale bar: 50 μm. Image 3: 5× Ca diet food group, lumber, no remarkable change. Scale bar: 50 μm.

development of osteoporosis. Hematological analysis did not reveal any changes between any experimental group and the control group for any parameter, including blood Ca and iP levels. Moreover, urinalysis results showed high urine Ca levels and markedly low urine iP levels in the rats of high-Ca, diet groups. In the 5× Ca diet food group, almost all of the urine iP had been reabsorbed to nearly undetectable levels. Conversely, low urine Ca levels were observed in the rats of low-Ca diet groups. However, differences in urine Ca levels between both groups were not significant. Furthermore, urine iP levels were high in the rats of low-Ca diet groups; however, the effect was dose independent. While some individual animals showed high levels and others normal levels, the absence of abnormal hematology results suggested that this difference was within the range of physiological variation in the renal excretory function.

These results demonstrated that when animals were fed diets with different Ca/iP ratios, their blood Ca levels remain constant by virtue of renal Ca/iP excretion and reabsorption. Moreover, the animals did not develop osteoporosis, even when they were fed diets with 5× Ca for 10 weeks. The normal renal function in these animals is presumed to substantially reduce iP excretion levels. However, prolonged administration of such feeds can put strain on the kidneys and potentially accelerate the development of osteoporosis.

In conclusion, the rats fed on high-Ca diets exhibited markedly high levels of urine Ca excretion and renal iP reabsorption, indicating the major contribution of the renal regulatory function. On the other hand, the contribution of renal function was minor in the rats fed on low-Ca diets. This suggests that other organs might play a regulatory role in these rats.

ACKNOWLEDGMENTS

This study was supported in part by the research budget of Japan SLC, Inc. Our deepest appreciation goes to Dr. Hisayoshi Takagi (Japan SLC, Inc, Hamamatsu-Shi, Japan) for his technical and financial support and Dr. Laura Kaufman (nonclinicai consultant, New Jersey, USA) for his scientific support.

Conflict of interest---- The authors declare that there is no conflict of interest.

REFERENCES

Arazi, H., Eghbali, E., Saeedi, T. and Moghadam, R. (2016): The relationship of physical activity and anthropometric and physiological characteristics to bone mineral density in postmenopausal women. J. Clin. Densitom., **19**, 382-388.

Azuma, Y. (2007): Animal models of osteoporosis and development of anti-osteoporotic agents. Folia. Pharmacol. Jpn., **130**, 201-205.

Conceição, E.P., Carvalho, J.C., Manhães, A.C., Guarda, D.S., Figueiredo, M.S., Quitete, F.T., Oliveira, E., Moura, E.G. and Lisboa, P.C. (2016): Effect of early overfeeding on palatable food preference and brain dopaminergic reward system at adulthood: role of calcium supplementation. J. Neuroendocrinol., 28.

Demontiero, O., Vidal, C. and Duque, G. (2012): Aging and bone loss: new insights for the clinician. Ther. Adv. Musculoskelet. Dis., **4**, 61-76.

Food and Nutrition Board, Institute of Medicine. Dietary reference intakes for Calcium, Phosphorus, Magnesium, Vitamin D, and Fluoride. Washington (DC): National Academies Press, 71-145.

Gerlinger, C., Oster, M., Borgelt, L., Reyer, H., Muráni, E., Ponsuksili, S., Polley, C., Vollmar, B., Reichel, M., Wolf, P. and Wimmers, K. (2019): Physiological and transcriptional responses in weaned piglets fed diets with varying phosphorus and calcium levels. Nutrients, 11.

Matsumoto, T. (1992): Calcium metabolism regulators. Exp. Med., **10**, 510-515.

Pearce, S.H. and Thakker, R.V. (1997): The calcium-sensing receptor: insights into extracellular calcium homeostasis in health and disease. J. Endocrinol., **154**, 371-378.

Nakada, H., Sakae, T., Watanabe, T., Takahashi, T., Fujita, K., Tanimoto, Y., Teranishi, M., Kato, T. and Kawai, Y. (2014): A new osteoporosis prevention supplements-diet improve bone mineral density in ovariectomized rats on micro-CT. J. Hard Tissue Biol., **23**, 1-8.

Raisz, L.G. (2005): Pathogenesis of osteoporosis: concepts, conflicts, and prospects. J. Clin. Invest., **115**, 3318-3325.

Stewart, T.A. and Davis, F.M. (2019): An element for development: calcium signaling in mammalian reproduction and development. Biochim. Biophys. Acta Mol. Cell Res., **1866**, 1230-1238.