Estrogen deficiency and low calcium diet increased bone loss and urinary calcium excretion, but did not alter arterial stiffness in young female rats.

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Abstract Many epidemiological studies have reported that the severity of arterial diseases such as arterial calcification and stiffness is inversely related to bone loss, *i.e.* osteoporosis. However, the nature of this relationship is unclear. The purpose of the present study was to examine the influences of estrogen deficiency and/or low calcium diet (0.1% Ca) on bone metabolism and calcium balance, as well as aortic wall composition and stiffness in young female rats. Twenty eight 6 week-old female rats were randomized into four groups: OVX-Low calcium (OL) and OVX-Normal calcium groups (ON) were ovariectomized, and Sham-Low calcium (SL) and Sham-Normal calcium groups (SN) were sham-operated. After 12 weeks, the bone mineral density of the lumbar spine and tibial proximal metaphysis were significantly lower in ON than in SN, and also significantly lower in OL than in ON. Additionally, OL rats had significant higher (vs. SN and SL) urinary deoxypyridinoline, but not urinary calcium, excretion at 4 weeks after ovariectomy. However, at 12 weeks after ovariectomy, urinary calcium excretion was significantly higher in OL than in SL, with corresponding increases in two bone turnover markers, bone type alkaline phosphatase and tartrate-resistant acid phosphatase. Neither estrogen deficiency nor low calcium diet affected aortic stiffness or elastin degeneration and calcium deposition over the course of the present study, although changes of bone metabolism occurred rapidly. Taken together, these results show that bone loss and arterial stiffness did not progress simultaneously in the present experimental protocol.

Introduction

A rapid demographic shift toward aging societies has resulted in the increased prevalence of age-related diseases such as osteoporosis and cardiovascular disease, in addition to morbidity and disability in elderly patients. Such chronic diseases have increased healthcare costs not only in Japan but also in other developed countries [1-4].

The most common type of osteoporosis is the postmenopausal bone loss associated with ovarian hormone deficiency and dietary low calcium intake [5-7]. Rapid bone loss is associated with a rise in plasma calcium levels, and many studies have suggested that calcium loss via urinary excretion may predispose patients to osteoporosis [8, 9]. Although the influence of ovariectomy on urinary calcium excretion has been extensively studied [10, 11], the combined influence of estrogen deficiency and low calcium diet have not been well examined. Therefore, we were interested in investigating the combined influence of estrogen deficiency and low dietary calcium on bone loss and urinary calcium excretion, given the assumption that postmenopausal women tend to have a fairly low calcium intake [12, 13] and are in negative calcium balance [11,14].

Many epidemiological studies have reported that the severity of arterial diseases such as arterial calcification and stiffness is inversely related to bone loss, osteoporosis [15-18]. Although osteoporosis and arterial disease seem to be associated, the nature of this relationship is controversial. Women lose about 5% of their trabecular bone every year and about 15% of their total bone in the first 5 years after menopause [19, 20]. The incidence of cardiovascular disease in women before menopause is significantly lower

than in males [21]. However, within a few years after menopause, the incidence of cardiovascular disease in women equals that of males in the same age range [22, 23]. Estrogen replacement therapies reduce the risk of rapid bone loss [24] and arterial disease [25] in postmenopausal women. Therefore, estrogen deficiency seems to relate to the parallel progression of bone loss and aortic disease.

Although benefits of long-term estrogen therapy for age-related decreases in systemic arterial compliance have been described in postmenopausal women [26, 27], the effects of estrogen on arterial stiffness are now contradictory [26-28]. Elastin degeneration and calcium deposition increase arterial stiffness [29] and induce the migration of vascular smooth muscle cells into the endothelium [30], which is considered to be a risk factor for atherosclerosis [31]. Estrogen has been reported to inhibit the proliferation of vascular smooth muscle cells [32] and may also exert a Ca^{2+} channel blocker-like effect, suggesting that estrogen treatment could inhibit progression of arterial calcification [33]. However, it is unclear whether rapidly induced estrogen deficiency affects aortic degeneration and elastin calcium deposition. In the present study, we postulated that increased elastin calcium content and rapid degenerative changes in ovariectomized rats might induce an increase in aortic stiffness.

The purposes of this study were: 1) To investigate how estrogen deficiency and/or low calcium diet affects bone loss and urinary calcium excretion. 2) To assess the alteration of elastin components and arterial stiffness induced by estrogen deficiency. 3) To determine whether the degree of bone loss due to estrogen deficiency and low calcium diet is related to the extent of arterial degeneration, elastin calcium deposition,

and aortic stiffening.

Materials and methods

Experimental animals and feeding protocol

Twenty-eight female Sprague-Dawley rats, 6 week-old, were randomized into four groups: the OVX-Low calcium (0.1% dietary Ca, 0.6% dietary P) group (OL, n = 6) and OVX-Normal calcium (0.6% dietary Ca, 0.6% dietary P) group (ON, n = 6) were ovariectomized via the dorsal route, and the Sham-Low calcium group (SL, n = 8) and the Sham-Normal calcium group (SN, n = 8) were operated on without the ovaries being removed. The experimental period was 12 weeks. The rats were kept in individual cages (15×25×19.5cm) and allowed access to food and distilled water *ad libitum*. Food consumption and body weight gain were measured every second day. Room temperature was kept at 24±1℃, humidity at 50±5%. Fluorescent lights were on from 8:00 a.m. to 8:00 p.m.. Animal care and experimental procedure were approved by the Animal Experimental Committee of the University of Tsukuba.

Serum calcium, phosphorus, bone turnover markers, and 1,25-$(OH)_2D_3$

At the end of the experimental period, all the rats were deprived of food overnight. Under ether anesthesia, animals were killed by exsanguinations from the abdominal aorta. The blood samples were centrifuged at 2,500 rpm for 15 min to extract the serum. The level of serum Ca was measured by the Inductively Coupled Plasma Atomic

Emission Spectroscopy (ICAP−AES – 575 v Nippon Jarrell-Ash) and phosphorus was determined by the Fiske-Subarrow method [34]. The Bone type Alkaline Phosphatase (BAP) and the Tartrate-resistant Acid phosphatase (TRAP) activities were measured as previously reported [35]. Serum 1,25-$(OH)_2D_3$ was detected using 1,25-$(OH)_2D_3$ Radio Immunoassay (RIA) kit (Immunodiagnostic Systems, Inc., USA).

Measurement of bone mineral density

The lumbar spine, and left and right tibiae of each rat were isolated by dissection, and freed from any muscle and connective tissue. Thereafter, bone mineral density (BMD) values for the L3-L6 lumbar spine and the whole tibiae were measured by Dual - energy X-ray Absorptiometry (DXA; Aloka DCS-600R instrument). The analysis of the tibial BMD was carried out as previously reported [36]. Briefly, proximal one-fifth of the tibia, including the epimetaphyseal region representing the trabecular sites, and middle one-fifth of the tibia representing the cortical diaphyseal region.

Femoral weights and mechanical breaking test

At each dissection, femur samples were collected, freed from adhering connective tissues. Thereafter, the bone strength at the middle diaphysis of the femur was tested by measuring the mechanical strength, with an Iio DYN-1255 instrument as previously reported [37]. The force necessary to produce a break at the center of the femur was measured under the following conditions: the sample space was 1.0 cm, the plunger speed was 100.0 mm/min, the load range was 50.0 kg, and the chart speed was 120.0 cm/min. Afterwards, the femurs were dried at 95℃ for 24 hr to measure their dry

weight. The bones were dry-ashed at temperatures from 150℃ to 550℃, with 50℃ increments at each 4 hr and at 600℃ for 24 hr and then the ash weight was measured.

Calcium and deoxypyridinoline excretions in urine

Animals were placed in individual metabolic cages (24×20×18 cm^3). The first phase was carried out on the 3rd and 4th day after starting the experimental diets period (Phase Ⅰ). The next phase (Phase Ⅱ) was carried out on the 31st and 32nd day. The third phase (Phase Ⅲ) was on the 56th and 57th day. The final phase (Phase Ⅳ) took place on the 80th and 81st day, just before the end of the experimental diets period. At each phase, urine was collected over two 24 hr periods. Urine was collected under acidic conditions using 2ml 2N hydrochloric acid. All urine was centrifuged at 2,500 rpm for 15 min to eliminate refuse. The urinary Ca was measured using the same method as that for the biochemical assay of the serum. The bone resorption marker deoxypyridinoline (Dpd, bone-specific Type 1 collagen degradation product) was measured in 24 hr urine samples from all four collection phases, using commercially available kits (Metra Dpd EIA Kit, Quidel USA).

Aortic biochemical studies

The descending thoracic aorta was divided longitudinally into two parts. Each sample was dried, and its dry weight was determined. One half of the aorta from each rat was designated for the measurement of the content of aortic calcium. The other half

of the aorta was boiled with 0.1 N NaOH for fifty minutes, according to the method of Lansing et al, for the determinations of elastin content (alkali-resistant elastin preparation) [38], and calcium content in elastin. The elastin content and calcium content in elastin were also determined from the aortic arches. The calcium content was measured using the same method as that for the biochemical assay of the serum.

Aortic biomechanical studies

To obtain a static stress-strain curve, each ring specimen of thoracic aorta was mounted as an intact loop between two smooth rods attached to an Instron-type tensile testing machine (Toyo Baldwin) as previously described [39,40]. Briefly, 2 mm ring specimens for tensile testing were excised from the proximal portion of each descending thoracic aorta, and were immersed in saline solution at 4℃. The incremental elastic modulus (a measure of arterial stiffness) of the aorta at an extension ratio of 1.5 was defined as the ratio of the incremental stress to the incremental strain of 0.1 across the extension ration of 1.5 which is comparable to the aortic wall distension at an arterial blood pressure of 100 mmHg. The ultimate tensile stress (tensile strength) and ultimate tensile strain were obtained from the stress-strain curves as the values at the breaking point of the specimen.

Statistical analysis

All the data are expressed as the mean ± SE. One-way and two-way analysis of

variance were used to test for statistically significant differences between groups. Analysis of effects of ovariectomy, dietary calcium, and interaction between those factors as grouping variables was performed and the significance between individual groups was determined using *post hoc* PLSD test. Statistical comparisons of calcium and Dpd excretions in urine among four groups over time were performed by a mixed design two-way ANOVA with repeated measures. After significant interactions, one-way ANOVA were employed each phase (4 phases) to compare and contrast the effect of groups. If a significant difference was detected, these were further evaluated by *post hoc* PLSD test. Association between bone turnover markers and urinary calcium excretion was performed by Pearson correlation test. A significant level of $p < 0.05$ was used for all comparisons. All statistical treatments were done using the Stat View 5.01 software (SAS Institute Inc. Cary, NC, USA, 2000-2001).

Results

Body weight, food intake, and food efficiency

Table 1 shows body weight, food intake, and food efficiency. Initial body weight did not differ among the four groups. Two-way ANOVA analysis showed that ovariectomy significantly altered the final body weight, body weight gain, food intake, and food efficiency ($p < 0.0001$), but dietary calcium did not significantly influence them in both Sham-operated groups and OVX groups.

BMD of lumbar spine and tibia

The BMD of the lumbar spine and tibia are shown in Fig 1. The BMD of the lumbar spine, which contains cancellous-rich region, was significantly lower in OL than in ON and Sham-operated groups (SN and SL). The BMD of the tibial proximal metaphysis, which contains cancellous-rich region, showed the same trend as those of the lumbar spine. Both ovariectomy ($p < 0.0001$) and dietary calcium ($p < 0.0001$) had significant effects on the cancellous BMD of the lumbar spine and tibial proximal metaphysis, and the interaction was highly significant for the BMD of the tibial proximal metaphysis ($p = 0.0479$). Low calcium diet significantly reduced the BMD of the tibial diaphysis, which contains little or no cancellous bone (cortical-abundant region), in both Sham-operated groups and OVX groups ($p < 0.001$), but estrogen deficiency alone did not reduce the BMD values.

Serum calcium, phosphorus, bone turnover markers, and serum PTH and 1,25 $(OH)_2D_3$ levels

Table 2 shows serum calcium, phosphorus, BAP, and TRAP levels. It did not observe any significant differences in serum calcium and phosphorus among the groups. Two-way ANOVA analysis showed that the interaction between ovariectomy and dietary calcium was significant for serum calcium ($p = 0.0017$), but the values were physiologically within normal range. The combined influence of estrogen deficiency and low calcium diet (OL) produced significant increases in BAP levels compared with the other groups, and also showed significant increases in TRAP levels compared with SL. BAP and TRAP levels in OVX groups (ON and OL) were correlated with urinary

calcium excretion at 12 weeks from OVX operation (Pearson r = 0.565, $p < 0.05$ for BAP and Pearson r = 0.592, $p < 0.05$ for TRAP), but those in sham groups (SN and SL) were not observed. Low calcium diet significantly increased serum 1,25 $(OH)_2D_3$ level in both Sham-operated groups ($p < 0.001$) and OVX groups ($p < 0.01$). The serum 1,25 $(OH)_2D_3$ level was significantly higher in ON than in SN. Two-way ANOVA analysis showed that ovariectomy tended to alter the serum 1,25 $(OH)_2D_3$ level ($p = 0.0643$), but dietary calcium significantly influenced the level ($p < 0.001$).

Femoral characteristics and biomechanical testing

As shown in table 3, the femoral dry and ash weights were significantly lower in OL than in the other groups. Ovariectomy ($p < 0.0001$) and dietary calcium ($p < 0.0001$) had significant effects on the dry and ash weights. The femoral breaking force was reduced by low calcium diet in both Sham-operated groups and OVX groups, and the femoral breaking energy was significantly lower in OL than in the other groups.

Calcium and Dpd excretions in urine

Calcium and Dpd excretions in urine showed in Fig. 2. Two-way ANOVA with repeated measures for calcium and Dpd excretions in urine showed significant group-by-time interactions ($p < 0.0001$) as well as group ($p < 0.0001$) and time ($p < 0.0001$) effects. Urinary Ca excretion in normal calcium diet groups (SN and ON) were significantly higher than in SL from phase Ⅰ to phase Ⅳ and in OL from phase Ⅰ to phase Ⅱ. With time, the urinary Ca excretion in OL increased and was significantly

higher than in SL from phase Ⅲ to phase Ⅳ. Urinary Ca excretion was significantly higher in ON than in SN only in phase Ⅲ. Urinary Dpd excretion did not differ among the groups at phase Ⅰ, but from phase Ⅱ to phase Ⅳ, it was significantly higher in SL and OVX groups (OL and ON) than in SN. Especially, in the phase Ⅱ, the urinary Dpd excretion was significantly in OL higher than in SN and SL. Urinary Dpd excretion in SL and OVX groups (OL and ON) began with the increase being greatest in earlier period (phase Ⅱ) and it declined with time.

Aortic biochemical results

As shown in table 4, the content of calcium in thoracic aorta did not differ between the four groups. The content of elastin in the thoracic and arch aortas did not differ between the four groups and there was also no significant difference in the elastin calcium content.

Aortic biomechanical results

As shown in table 5, there were no significant differences in incremental elastic modulus, ultimate tensile stress, and ultimate tensile extension ratio between the four groups.

Discussion

We observed that the combined influence of estrogen deficiency and low calcium diet decreased both the BMD of the tibia and the lumbar spine. Additionally, the

combination of estrogen deficiency and low calcium diet resulted in significantly increased urinary calcium excretion and bone turnover marker expression at 12 weeks after ovariectomy. However, estrogen deficiency and/or low calcium diet did not affect aortic stiffness or elastin degeneration and calcium deposition during periods of rapid change in bone metabolism.

The BMD in both cortical (tibial diaphysis) and cancellous (tibial proximal metaphysis and lumbar spine) bones was significantly decreased in OVX rats fed low calcium diet (Fig 1). These results correspond to the reduced dry and ash femur weights observed in OVX rats fed low calcium diet (Table 3). Femoral breaking force and energy were also markedly decreased in OVX rats fed low calcium diet (Table 3), suggesting the increased risk of fracture. Previous studies using OVX rats fed 0.1% low calcium diet have shown decreases in bone mineral density as well as increases in circulating parathyroid hormone and 1,25-$(OH)_2D_3$ [7, 52]. In consistence with the results of the previous studies, the present study showed that serum 1,25-$(OH)_2D_3$ in OVX rats fed low calcium diet clearly increased, but normal calcium diet suppressed the level.

At 4 weeks after ovariectomy, urinary Dpd excretion in OVX rats fed low calcium diet was significantly higher than in sham-operated rats fed low calcium diet (Fig 2). The increased Dpd excretion is suggestive of osteopenia resulting from increased bone resorption [41]. In this study, it is possible that increased bone resorption in OVX rats fed low calcium diet led to higher bone loss at the earlier time point of 4 weeks after ovariectomy. Despite the increase in bone resorption, urinary calcium excretion was

unchanged at 4 weeks (Fig 2). Estrogen receptors modulate tubular calcium reabsorption in the kidneys [42], and urinary calcium excretion significantly increases after menopause, indicating that renal tubular reabsorption of calcium decreases in estrogen deficiency state [53]. It has been generally considered that higher urine calcium excretion after menopause reflects an increase in the filtered load of calcium from kidney, occurring as a result of increased bone resorption [54]. However, in the present study, it is possible that in 4 weeks after ovariectomy, calcium reabsorption in the kidneys might still occur through an alternate mechanism.

Urinary calcium excretion in OVX rats fed low calcium diet started to increase 8 weeks after ovariectomy and was significantly higher than that of sham-operated rats fed low calcium diet from 8 weeks to 12 weeks after ovariectomy (Fig 2). At 12 weeks after ovariectomy, positive correlations between urinary calcium excretion and the expression of two bone turnover markers, BAP and TRAP, were observed in the OVX groups only. Morris et al also showed that OVX rats with higher bone turnover lost more urinary calcium when compared to those with lower levels of bone turnover [43]. The positive correlation between urinary calcium excretion and expression of bone turnover markers in the present study was not observed in sham-operated rats regardless of dietary calcium intake at all time points, suggesting that estrogen might influence the renal reabsorption of calcium [42]. Estrogen receptors are present in human bone [44], and increases in urinary calcium excretion during estrogen deficiency are accompanied by increased bone resorption [43]. Therefore, at 12 weeks after ovariectomy, the increased urinary calcium excretion in OVX rats fed low calcium diet may reflect not

only increased bone resorption but also an inhibitory effect on renal tubular calcium reabsorption. In the present study, although bone formation marker, serum BAP, in OVX rats fed low calcium diet was significantly higher than in the other groups, bone resorption marker, serum TRAP, was not so higher. It might be because the bone turnover markers were analyzed using serum sample at the end point of this experiment (i.e., 12 weeks after ovariectomy) when much progression of bone loss had already occurred compared with rapid progression of bone loss at the earlier time point (i.e., 4 weeks after ovariectomy). Actually, at 4 weeks after ovariectomy, urinary Dpd excretion in OVX rats fed low calcium diet was significantly higher than in both sham-operated rats fed low calcium diet and sham-operated rats fed normal calcium diet. Taken together, our results suggest that increased urinary calcium and bone turnover might be risk factors for osteoporosis in postmenopausal women who have low calcium intake.

In the present study, urinary calcium excretion was significantly higher in OVX rats fed normal calcium diet at 8 weeks after ovariectomy when compared to sham-operated rats, whereas no difference was observed at 12 weeks (Fig 2). The effect of ovariectomy on urine calcium excretion is currently controversial, with reports of no effect [7, 45] and also of an increased excretion [10, 11]. However, the previous studies only assessed urinary calcium excretion only at one time point. Therefore, the conflicting findings may have been caused by differences in the age of the rats or the duration of the experiments.

In the present study, we did not observe any changes in aortic stiffness or elastin degeneration and calcium deposition during estrogen deficiency (Table 4, 5). Potential

protective mechanisms of estrogen relating to arterial stiffness include induction of endothelium-dependent vasodilative factors [46, 47] and inhibition of vasocontracting factors [48, 49] (i.e. functional factors). In the present study, to analyze arterial stiffness we used dissected thoracic aorta, which did not have dynamic functional factors and might be influenced by structural factors such as the composition of arterial wall elastin and its calcium content. A previous study showed that the calcification level of the arterial tunica media was positively correlated with antemortem aortic pulse wave velocity [50]. In the present study, estrogen deficiency did not induce aortic elastin degeneration or calcification, which may have in turn caused the observed lack of change in aortic stiffness. We cannot rule out the possibility of the estrogen deficiency-induced increase of arterial stiffness in aged OVX rats, being expected that significant changes in phenotypes of artery relating to arterial stiffness can be observed. However, to our knowledge, little has been reported whether aged OVX rats induce increase in arterial stiffness. Furthermore, structural arterial alterations have been observed to develop within 2 months in a rat hypertension model [51], but there were no changes in the osteoporotic model of young rat in the present study. Therefore, future studies using a combination model rat, e.g. atherosclerotic stimuli in ovariectomized rats or aged ovariectomized rats may help to clarify the epidemiological links between osteoporosis and aortic diseases.

We also did not observe any changes in aortic stiffness or elastin degeneration and calcium deposition when estrogen deficiency was combined with low calcium diet (Table 4, 5). The paradox of bone loss accompanied by aortic calcification, where there

might be a calcium shift from bone to arteries (calcium shift theory), has been reported in previous epidemiological studies. However, in the present study, aortic calcification and stiffness were unchanged during periods of rapid bone loss caused by either estrogen deficiency or the combined influence of estrogen deficiency and low calcium diet. It is possible that the surrounding vascular tissue was insufficient to accept plasma calcium deposition after bone resorption.

In conclusion, we observed that the combined influence of estrogen deficiency and low calcium diet decreased the bone mineral density of both cortical and cancellous bones in the tibia and lumbar spine. The combination of estrogen deficiency and low calcium diet also caused an increase in urinary calcium corresponding to increased bone turnover, 12 weeks after ovariectomy. Although rapid changes were observed in bone metabolism, neither estrogen deficiency nor low calcium diet affected aortic stiffness or elastin degradation and calcium deposition. These data suggest that rapid bone loss and biochemical aortic alterations caused by estrogen deficiency and low calcium diet did not progress simultaneously.

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