Relationship between arterial calcification and bone loss in a new combined model rat by ovariectomy and vitamin D_3 plus nicotine

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Abstract

Epidemiological studies have reported an association between arterial calcification and bone loss after menopause. However, the underlying mechanism of the association remains unclear. Therefore, to explore the possible mechanisms of the association, we tried to develop a new combined model rat of Ovariectomy (OVX; an animal model of osteoporosis) and Vitamin D_3 plus Nicotine (VDN rat; an animal model of arterial calcification). We tested them by using Sham-operated Control rats (SC), Ovariectomized Control rats (OC), and OVX plus VDN treated rats (OVN). Dissections were performed twice at 4 (4SC, 4OC, and 4OVN) and 8 (8SC, 8OC, and 8OVN) weeks after treatment. 8OVN showed bone loss and arterial calcification, although 8OC showed only bone loss. Moreover, arterial calcium content was associated with indexes of bone loss at 8 weeks. Thus, the OVN rat is considered a good model to examine the relationship of the two disorders after menopause. Additionally, the arterial endothelin-1 (ET-1: a potent regulator of arterial calcification) levels increased in both 4OVN and 8OVN, and the level was associated with arterial calcium content at 8 weeks. Furthermore, the arterial eNOS protein which is an enzyme that produces nitric oxide (NO: an antiatherosclerotic substance), was significantly reduced in only 8OVN. Estrogens affect the alterations of the eNOS and ET-1 proteins. Therefore, we suggest that impairment of the ET-1 and NO producing system in arterial tissue during periods of rapid bone loss by estrogen deficiency might be a possible mechanism of the relationship between the two disorders seen in postmenopausal women.

Key words: Osteoporosis, Vascular calcification, a model rat

Introduction

Aging enhances the development of risk factors for cardiovascular disease and osteoporosis [1, 2]. For women before menopause, the incidence of cardiovascular disease is significantly less than that for males. However, after menopause, the incidence of cardiovascular disease in women becomes close to that in males within a few years [3, 4]. In addition, women lose about 5% of trabecular bone per year and about 15% of their total bone in the first 5 years after menopause [5, 6]. Estrogens reduce the risk of cardiovascular disease and bone loss in postmenopausal women, suggesting that this hormone may participate in the progression of both diseases [7, 8]. Recently, several epidemiological studies have demonstrated that there are positive correlations between vascular disorders, such as increases in arterial calcification or arterial stiffness, and bone loss, i.e., decreases in bone mineral density (BMD) or bone strength [7, 9, 10]. These studies have suggested that bone loss may be an index of the progression of vascular disease in postmenopausal women. However, the underlying mechanism of the association between these two diseases remains unclear.

Ovariectomy (OVX) in animal models is an accepted method to mimic postmenopausal osteoporosis [11]. OVX rats show decreased plasma estrogen levels followed by rapid bone loss (i.e., decreases of bone strength and bone mineral density) [12]. On the other hand, we and other researchers previously reported that OVX rats do not show a clear increase of arterial stiffness or calcification [13, 14]. In case of rats, calcium deposition of aortic wall is very slow whereas the rise in humans by the end of life is about 50-100 times faster [15]. Thus, OVX model rats may be inappropriate for examining the pathologies of vascular calcification or stiffness after menopause in humans. Additionally, vitamin D_3 plus nicotine (VDN) treatment causes calcification in

the elastic arteries of young male rats and it has been proposed as an animal model of arterial stiffness or calcification and vascular pathology associated with advancing age [16, 17]. However, in our preliminary study using young female rats, VDN treatment did not cause arterial calcification. Based on these results, we considered that higher circulating estrogen level in young female rats compared with that in male rats might prevent the progression of arterial calcification by VDN treatment. Thus, combined model of OVX and VDN treatment in female rats may be effective to clarify the epidemiological links between osteoporosis and arterial diseases; however, no investigation using this model has been done.

Nitric oxide (NO), which is produced by the endothelial isoform of NO synthase (eNOS) in vascular endothelial cells, is a potent vasodilator and has antiatherosclerotic properties [18, 19]. By contrast, endothelin-1 (ET-1), which is a potent vasoconstrictor peptide that is produced by vascular endothelial cells, induces the proliferation of vascular smooth muscle cells [20, 21] and its overexpression may augment calcium uptake into aorta tissue of the VDN male rat and also increases calcium content in vascular smooth muscle cells [22]. ET-1 has negative effects on eNOS protein expression and activity in the endothelial cells [23], and the impairment of ET-1 and NO producing system may induce endothelial dysfunction [24, 25], resulting in further arterial stiffness [26, 27, 28]. Additionally, higher bone turnover and rapid bone loss are seen during the menopausal transition [6], corresponding to a transient increase in excessive calcium circulation. Therefore, as a causal mechanism of the relationship between rapidly progressive arterial diseases and osteoporosis after menopause in females, arterial calcification may be accelerated by impairment of ET-1 and NO producing system during periods of rapid change in bone metabolism.

Accordingly, the aim of the present study was to develop a new combined model of OVX and VDN in female rats which shows arterial calcification and bone loss, and additionally, to explore possible mechanisms of relationship between arterial calcification or stiffness and bone loss. In the present study, we examined whether OVX plus VDN rats induce arterial calcification with alterations of eNOS or ET-1 levels and bone loss, i.e., the decreases in BMD and bone strength. The present study also confirmed associations between arterial calcium content and various indexes of bone loss or arterial tissue protein levels. Furthermore, estrogens affect eNOS expression through binding to estrogen receptor α and β [31, 32]. Thus, we examined whether the estrogen receptor α and β are also altered in OVX plus VDN rats.

Materials and methods

Animals and protocol. Female Sprague-Dawley rats, 6 week-old, were obtained (CLEA Experimental Animals Supply Co. Ltd., Japan) and cared for according to the "Guiding Principles for the Care and Use of Animals" based on the Helsinki Declaration of 1964. The rats were kept in individual cages 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 Committee on Animal Research at the University of Tsukuba. Forty-six female rats were randomly divided into three groups. Two groups of rats (n=32) were ovariectomized (OVX) and the third group of rats (n=14) received sham operation (Sham) under ether anesthesia. The preparation for vascular calcification was as originally described by Niederhoffer et al. [16] with minor modifications. After a week

recovery period from ovariectomy operation, one group of ovariectomized rats received Vitamin D_3 (300,000IU/kg, intramuscular (i.m.); cholecalciferol, Sigma Chemical Co.) and nicotine (25mg/kg, 5ml/kg, per os (p.o.); nicotine hydrogen tartrate, Sigma Chemical Co.) at 9 a.m. (OVX with VDN treatment; OVN, n=16). After that, no treatment was performed until finishing the experimental period, i.e. the VDN is a 1-time only treatment. Ovariectomized control (OVX Control; OC, n=16) rats and sham-operated (Sham Control; SC, n=14) rats received an injection of 0.15 M NaCl (i.m.) and a gavage of distilled water (p.o.). Each seven rats from SC rats and each eight rats from OC and OVN rats were killed at 4 weeks (4SC, 4OC, and 4OVN) and 8 weeks (8SC, 8OC, and 8OVN) after treatment (i.e. dissections were held twice in order to look at the influence of time since VDN treatment). At the end of 4 and 8 weeks after treatment, all the rats were deprived of food overnight. After measurement of body weight, under ether anesthesia, blood samples were obtained from the abdominal aorta. Then, the aortas were isolated and rinsed with cold saline, and stored at -80℃ for measurement of calcium content and stainings and for determination of protein levels of ET-1, eNOS, and ER α and β by sandwich-enzyme immunoassay and/or electrophoresis and immunoblot analysis. Blood samples were drawn and mixed with EDTA-Na_2, aprotinin (500 kallikrein inactivator units/ml). Plasma was separated by centrifugation at 1000 × g for 10 min at 4℃ and stored at -80℃ for measurement of calcium concentration, and for determination of estradiol concentration by enzyme-linked immunosorbent assay. The left and right tibiae of each rat were isolated and freed from any muscle and connective tissue, and immersed in 70% ethanol solution for measurement of BMD. The femur samples were also collected, freed from adhering connective tissues, and immediately tested by measuring the mechanical strength.

Afterward, the femur samples were dried and burnt to ash for measurement of dry and ash weights.

Calcium and estradiol concentrations in plasma. Plasma calcium concentration was measured by the Inductively Coupled Plasma Atomic Emission Spectroscopy (ICAP−AES−575vNippon Jarrell-Ash). Plasma estradiol concentration was determined using enzyme-linked immunosorbent assay (ELISA) kit (EnviroChemicals Ltd., Japan) in accordance with the manufacturer's protocol.

Arterial calcium content. Each sample of aorta was dried, and its dry weight was determined. Then they were burnt to ash at 550 ~ 600℃ for 15 h, and the ash was dissolved in 1N nitric acid. The arterial calcium content was measured using the same method as that for plasma calcium concentration [33].

Von Kossa and elastica van Gieson stainings in aorta. Von Kossa staining was performed to observe calcium deposit in the medial layer of aorta with minor modifications [34]. Serial sections of six-micrometer thickness were cut from frozen aorta of each rat and attached to slide glasses. Briefly, the slides were treated with 5% $AgNO_3$ for 30 min. Then, specimens were then counterstained with safranine and examined under a light microscope (original magnification 40×). Elastic fiber network in the medial layer of aorta was observed by elastica van Gieson staining [35,36]. Briefly, the slides (Serial sections of twelve-micrometer thickness cut from frozen aorta)

treated with the chemical reagents of resorcinol fuchsin, iron hematoxylin, and van gieson. After washing and dehydrating, specimens were examined under a light microscope (original magnification 40×).

Bone mineral density of tibial proximal metaphysis. BMD values of the tibia were measured by Dual - energy X-ray Absorptiometry (DXA; Aloka DCS-600R instrument). The BMD of the tibial proximal metaphysis was assessed in proximal one-fifth of the tibia, including the epimetaphyseal region representing the trabecular sites [37].

Dry weight, ash weight, and mechanical breaking test of femur. 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 dry weight. The bones were burnt to ash at temperatures from 150℃ to 550℃, with 50℃ increments at 4 hr intervals and at 600℃ for 24 hr. Then, ash weight was measured. The ash weight reflects the mineral weight and includes about 40 % of calcium [38].

Sandwich-enzyme immunoassay in aorta. ET-1 level in the aorta tissue extracts was determined using a sandwich-enzyme immunoassay Kit (Immuno-Biological

Laboratories Co. Ltd., Japan). The reported cross-reactivity of the antibody was≤0.1% for all big ETs,≤0.1% for ET -3, and 3.3% for ET-2. The assay procedure was carried out as previously described [39, 40].

Electrophoresis and immunoblot analysis in aorta. Western blot analysis of eNOS and ER α, β proteins in the aorta tissue was performed according to our previous paper [41] with minor modifications. The aorta tissue was homogenized with 10 volumes of 10 mM Tris-HCL (pH 7.8), 1mM EDTA, 150mM NaCl, 1% NP 40, 1mM phenylmethylsulphonyl fluoride on ice using a potter tissue homogenizer (model PT10SK/35; Kinematica) and rotated for 90 min at 4 ℃. The homogenate was centrifuged at 10,000 × g for 30 min at 4 ℃. The resulting supernatant (cytosolic and membrane fraction) was stored at - 80℃ until eNOS and ER α, β proteins assays. Protein concentrations were determined by the bicinchoninic acid protein assay reagents (Pierce, Rockford, IL) with BSA as a standard. The samples (15μg protein) were followed by heat denaturation at 96 ℃ for 5 min with β- mercaptoethanol and sodium dodecylsulphate (SDS) sample buffer (62.5 mM Tris-HCl buffer, pH 6.8, containing 25% glycerol, 2% SDS). Each cytosolic and membrane fraction preparation of the aorta was separated on a SDS-polyacrylamide gel (7.5%) and then transferred to polyvinylidenedifluoride (PVDF; Millipore, Tokyo, Japan) membranes at 2.5 mA/cm^2 for 60 min. After the membrane was treated with blocking buffer 1% skim milk (eNOS), 3% skim milk (ER α), or 4% skim milk (ER β) in phosphate-buffered saline contained 0.05% Tween 20 (PBS-T) for 1 h at room temperature. The membrane was probed with monoclonal anti-eNOS antibody (Transduction Laboratories, Lexington, KY USA; 1:800 dilution with blocking buffer), anti-ER α antibody (Novocastra, Newcastle, UK,

1:300 dilution with blocking buffer), and anti-ER β antibody (Affinity BioReagents, Golden, CO, USA, 1:800 dilution with blocking buffer) for 12 h at 4 ℃, washed with PBS-T three times, and then incubated with a horseradish peroxidase-conjugated secondary antibody, which was an anti-mouse immunoglobulin antibody (eNOS and ER α; 1:2000 dilution with blocking buffer, Amersham Life Science, Buckinghamshire, UK.) and an anti-rabbit immunoglobulin antibody (ER β; 1:4000 dilution with blocking buffer, Amersham Life Science, Buckinghamshire, UK.) for 1 h at room temperature. After this reaction, the membrane was washed with PBS-T five times. Finally, the eNOS and ER α, β were detected by ECL Plus system (Amersham Life Science), and exposed to Hyper film (Amersham Life Science). The photograph was scanned by CanoScan 600 (Canon, Tokyo, Japan), and quantification was performed by a computer with MacBAS software (Fuji Film, Tokyo, Japan).

Statistical analysis. All the data are expressed as the mean ± SE. Statistical analysis was carried out by analysis of ANOVA followed by Fisher`s F-test for multiple comparisons. Also, the relationships between indexes of osteoporosis and arterial calcium content or arterial ET-1 levels and arterial calcium content were examined by Pearson's correlation analysis. 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).

<The position of Table 1>

Results

The final body weight of both 4OC (314.2±7.4 g, $p < 0.001$ vs. 4SC) and 4OVN

(314.7±3.8 g, $p < 0.001$ vs. 4SC) was significantly higher than that of 4SC (247.4±7.5 g), whereas there were no differences between 4OC and 4OVN. The final body weight of both 8OC (361.9±11.0 g, $p < 0.001$ vs. 8SC) and 8OVN (359.6±10.4 g, $p < 0.001$ vs. 8SC) was also significantly higher than that of 8SC (281.9±6.2 g), whereas there were no differences between 8OC and 8OVN.

Plasma calcium and estradiol concentrations are shown in Table 1. There were no differences in plasma calcium concentration among 4SC, 4OC, and 4OVN or among 8SC, 8OC, and 8OVN. Plasma estradiol levels in either 4OC and 4OVN or 8OC and 8OVN were not detected (below 7.8 pg/ml), whereas those in 4SC and 8SC were 20.41±0.88 pg/ml and 18.08±0.44 pg/ml, respectively.

Fig. 1 shows the arterial tissue calcium content, representative images of von Kossa staining for aortic calcification and elastica van Gieson staining for the elastic fiber network in the medial layer of aorta. The arterial calcium content in 8OVN was significantly higher than that in 8SC and 8OC, although there were no differences among 4SC, 4OC, and 4OVN (Fig. 1A). Von Kossa staining in the medial layer of the aorta in 8OVN showed linear, scattered aggregates of calcification (Fig. 1B). The elastica van Gieson staining of the elastic fiber network of the medial layer of the aorta in 8OVN revealed drastic disassociation and fragmentation (Fig. 1B). These results indicated that OVN rats at 8 weeks clearly exhibited arterial calcification, whereas OVN rats after 4 weeks did not yet show this alteration.

<The position of Figure 1>

As shown in Table 2, the BMD of the tibial proximal metaphysis and the femoral breaking force in 4OVN were significantly lower than those in 4SC and 4OC, although there were no differences between 4SC and 4OC. The dry weight and ash weight of the

femur in both 4OC and 4OVN were significantly lower than those in 4SC, whereas there were no differences between 4OC and 4OVN. On the other hand, the BMD of the tibial proximal metaphysis, the femoral breaking force, and dry weight and ash weight of the femur in both 8OC and 8OVN were significantly lower than those in 8SC, whereas they did not differ between 8OC and 8OVN. These results indicate that OVN rats showed clear bone loss from an early period of 4 weeks compared with SC rats, and the bone loss was maintained until 8 weeks, whereas the significant difference between OC and OVN rats disappeared at 8 weeks.

<The position of Table 2>

Because OVN rats after 8 weeks exhibited both arterial calcification and bone loss, we analyzed the associations between arterial tissue calcium content and indexes of bone loss, i.e., BMD of the tibial proximal metaphysis, femoral breaking force, and dry weight and ash weight of the femur, in 8SC and 8OVN (Fig. 2). The BMD of the tibial proximal metaphysis, femoral breaking force, and dry weight and ash weight of the femur were inversely correlated with the arterial calcium content.

<The position of Figure 2>

The arterial tissue ET-1 level was significantly higher in 4OVN than in 4SC and 4OC, and was also higher in 8OVN than in 8SC and 8OC (Fig. 3A). ET-1 increases calcium uptake into aorta tissue and vascular smooth muscle cells and thereby acts as a potent regulator of arterial calcification [22]. The present study showed a significant positive correlation between arterial ET-1 level and calcium content in 8SC and 8OVN (Fig. 3B).

Arterial eNOS protein expression was significantly lower in 8OVN than in 8SC and 8OC, but did not differ among 4SC, 4OC and 4OVN (Fig. 4).

<The position of Figure 3>

<The position of Figure 4>

Finally, we examined whether arterial estrogen receptors were changed by VDN treatment after OVX, because estrogen elevates eNOS expression via binding to estrogen receptor. Immunoblotting for ER α and β proteins in the aorta tissue showed that there were no differences in the expression of ER α and β proteins in the aorta among 4SC, 4OC and 4OVN or among 8SC, 8OC and 8OVN (Fig. 5).

<The position of Figure 5>

Discussion

The present study demonstrated that the combined model rat of OVX plus VDN induced bone loss and arterial calcification. Moreover, the present study showed associations between various indexes of bone loss and arterial calcium content. Additionally, the present study showed that the arterial ET-1 level was increased and the arterial eNOS protein was decreased in the combined model rat. Furthermore, the present results showed an association between the ET-1 level and calcium content in the arterial tissue.

At 8 weeks after treatment, OVX plus VDN rats induced bone loss and arterial calcification whereas OVX control rats caused bone loss but not the alteration of calcification in arterial tissue. Furthermore, BMD, breaking force, and dry and ash weights were inversely correlated with arterial calcium content at 8 weeks after treatment. The present study also demonstrated that VDN treatment in OVX rats caused calcification on the medial elastic layer of the aorta and also showed the alterations of arterial structure, such as drastic disassociation of the elastic fiber network. The calcium

deposition in medial elastic layer of artery is a risk factor for increases in arterial stiffness and atherosclerosis [42, 43, 44]. Therefore, we suggest that OVX rat treated by VDN treatment might be an appropriate animal model to examine the relationship of arterial calcification or stiffness and bone loss, which shows similar pathologies to those observed in postmenopausal women.

The present study used only thoracic aorta for analysis since thoracic artery provides a suitable tissue for studying arterial stiffness or calcification in previous studies of VDN model rats. In the present study, we could not obtain calcium content in other tissues except for aortas, being not sure whether the other tissues might be calcified or not. However, Henrion et al. previously reported that VDN treatment to young male rats produced large increases in calcium content of various tissues, e.g., thoracic aorta, abdominal aorta, common carotid artery, tail artery, kidneys, and heart (*Journal of Hypertension, 1991*). We cannot rule out the possibility of VDN treatment-induced changes of calcium content and phenotypes of the various organs in OVX rats, being expected that we could clarify the pathologies of calcification in the organs in estrogen deficiency state.

In the present study, both OVX control rats and OVX plus VDN rats exhibited decreases in the circulating estrogen level at both 4 and 8 weeks after treatment. OVX plus VDN rats showed clear decreases of all indexes of bone content at 4 weeks, whereas OVX control rats did not show altered BMD or bone breaking force. On the other hand, at 8 weeks, both OVX control rats and OVX plus VDN rats showed clear bone loss. It has been reported that VDN treatment in male rat affects bone loss [45]. Accordingly, we consider that VDN treatment has an additive effect on bone loss during estrogen deficiency at an early time point of 4 weeks. It is well established that OVX

rats develop osteopenia in association with increased bone turnover [46]. Therefore, OVX plus VDN rats may suffer rapid bone loss with higher bone turnover from the earlier time point of 4 weeks.

Rapid bone loss in postmenopausal women is associated with a rise in plasma calcium levels [47, 48]. However, in the present study, plasma calcium concentrations did not differ among all groups at both 4 and 8 weeks although rapid bone loss occurred from the earlier period of 4 weeks in OVX-operated groups. The present study also showed that there was no correlation between plasma and tissue calcium concentrations at both 4 and 8 weeks. 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 mentioned in previous epidemiological studies. However, in the present study, plasma calcium concentrations were unchanged during either periods of only rapid bone loss (4 weeks after treatment) or periods of both bone loss and arterial calcification (8 weeks after treatment). Therefore, we consider that only plasma calcium levels could not reflect the progression of aortic calcification and/or bone loss after menopause in females and more other factors might be responsible.

The present study demonstrated that arterial calcification was seen at 8 weeks, not at 4 weeks, after VDN treatment in OVX rats. Furthermore, the arterial ET-1 level was significantly increased from the earlier time point of 4 weeks after VDN treatment in OVX rats, and this increase was maintained until 8 weeks. ET-1 antagonists prevent increase in calcium uptake into arterial tissue of VDN male rats and also suppress increase in calcium content in calcified vascular smooth muscle cells [22]. The present results also demonstrated an association between ET-1 and the arterial calcium content. Thus, we cautiously speculate that long-term exposure of arterial tissue to higher ET-1

might partly contribute to the formation of arterial calcification in OVX plus VDN rats. However, contrast to possibility of the role of ET-1 on calcification in arterial tissue, ET-1 might inhibit the mineralization in osteoblastic cells relating to bone formation [49]. Therefore, in the future study, it would be of important to demonstrate if ET-1 would contribute to the underlying processes between arterial calcification and bone loss by using an ET-1 antagonist in OVX plus VDN rats.

It is well known that NO synthesized by eNOS affects endothelial function [18]. In the present study, arterial eNOS protein was decreased at 8 weeks after VDN treatment in OVX rats, whereas arterial ET-1 levels were increased at both 4 and 8 weeks. Because ET-1 downregulates eNOS protein in endothelial cells and isolated arteries [23, 24], it is possible that the increased ET-1 in the OVX plus VDN rats might have caused the reduction of eNOS protein. Additionally, estrogen elevates eNOS expression through binding to estrogen receptor α and β [31, 32]. In OVX plus VDN rats, estrogen production was decreased by ovariectomy; however, the levels of estrogen receptor α and β proteins did not change at 4 or 8 weeks. In our preliminary study using young female rats, VDN treatment did not cause any changes in phenotype and calcification in arterial tissue (data not shown). Thus, rapid loss of circulating estrogen in OVX plus VDN rats might partly contribute to the alteration of eNOS protein or ET-1 level in arterial tissue. The impairment of eNOS protein or ET-1 level in arterial tissue may induce endothelial dysfunction [24, 25], resulting in further arterial stiffness [26, 27, 28]. Taken together, impairment of the ET-1 and NO producing system in arterial tissue during rapid loss of circulating estrogen might be an initiator of progression of arterial stiffness or calcification, resulting in the emergence of relationship between arterial diseases and bone loss after menopause in females.

In the present study, although bone loss occurred at an early stage (4 weeks) after VDN treatment in OVX rats, arterial calcification was observed at a late stage (8 weeks). Therefore, we consider that it might not be necessary to progress bone loss and arterial calcification simultaneously, that is to say, time interval difference might occur in the progression of these two diseases. However, because the present study showed the association between the two diseases at 8 weeks after treatment, we consider that there might be causal and common factors in this process. To clarify causal factors in this process, estrogen replacement, or specific ET-1 and/or NOS inhibitors should be used in the future studies. Furthermore, because many other cytokines and hormonal factors may multiply affect the progression of this process, more works used hormone replacement or antagonists need to be done.

Most prevention studies have examined the effects of pharmacological therapies (e.g., hormone replacement therapy) on either osteoporosis or vascular disease [50, 51]. However, on the basis of the present findings, strategies for the prevention of both the impairment of ET-1 and NO system and the progression of bone loss may be beneficial in postmenopausal women with progressive arterial disease. Therefore, unique therapeutic approaches, e.g., combination of hormone replacement therapies and/or nonpharmacological therapies, may be needed to prevent both disorders.

In conclusion, the present study demonstrated that the combined model rat of OVX plus VDN exhibit both rapid bone loss and arterial calcification. Moreover, the present study showed associations between indexes of bone loss and arterial calcium content. Therefore, OVX rat treated by VDN treatment could be an appropriate animal model to examine the relationships of arterial calcification or stiffness and bone loss, which shows similar pathologies to those observed in postmenopausal women.

Additionally, arterial tissue ET-1 levels were increased at both 4 and 8 weeks after VDN treatment in OVX rats, whereas eNOS protein was decreased at 8 weeks. Furthermore, the present results showed an association between the ET-1 level and calcium content in the aorta tissue. Accordingly, we propose that imbalance of the ET-1 and NO producing system in arterial tissue during periods of rapid bone loss caused by estrogen deficiency may be one of several possible mechanisms underlying the emergence of relationship between arterial calcification or stiffness and bone loss after menopause in females. Further studies need to clarify the causal factors between arterial diseases and osteoporosis.

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