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The effects of *Cudrania tricuspidata* extract on bone metabolism in ovariectomized rats

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Abstract

The effects of *Cudrania tricuspidata* (*CT*) extract on markers of osteoporosis were examined in ovariectomized rats. We classified 26 rats into five groups and provided a pellet chow diet and tap water throughout the 27-wk experimental period. During the last 15 wk, we added oral injections to each group as follows: sham-operated (SHAM, n=4) and ovariectomized-control (OVX, n=5) with distilled water, alendronate with 10 mg/kg/d of alendronate sodium (ALEN, n=5), CT (CT100, n=6) with 100 mg/kg/d of CT, and CT (CT300, n=6) with 300 mg/kg/d of CT. After the experimental period, blood, urine, and micro-CT images were assessed. The CT100 and OVX groups did not show any significant differences in urinary n-terminal telopeptide (NTx) ($p<0.05$), but with increases in CT concentration, the NTx level was slightly reduced. Serum osteocalcin was significantly higher in the CT groups than in all other groups ($p<0.05$). Notably, the serum calcium levels of all groups were within the normal range, but urinary calcium levels in the CT groups were significantly lower than the OVX group ($p<0.05$). In addition, the CT groups exhibited higher trabecular BMD than the OVX groups while showing similar BMD to the ALEN group ($p<0.05$). The Tb.Th of the ALEN group was lower than all other groups. Based on the overall analysis of results, CT prevented bone loss by inhibiting bone resorption and enhancing bone formation. Although alendronate showed a similar effect in preventing bone loss, it did so by solely inhibiting bone resorption, and its long-term use reportedly causes paradoxical effects such as hip fractures. Thus, for osteoporosis induced by ovariectomy, we conclude that CT extract is an effective natural treatment without severe side effects.

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Introduction

Osteoporosis is known as a “silent thief” whose symptoms do not manifest until fractures occur unexpectedly, often from minimal trauma(Kalu, 1991; Lauritzen, 1996; Melton and Cummings, 1987). When osteoporotic conditions persist, the risk of fractures in the femoral neck, spine, or distal radius, among other areas, increases(Kalu, 1991; Lauritzen, 1996;

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Melton and Cummings, 1987). Although osteoporosis is on the rise as a societal problem in Western countries, the lower diagnosis and treatment rates in Asia seem to reflect a lack of awareness about its severity among Asians(Choi *et al.,* 2012). Since the size and mean age of the elderly population are rising in Asia, the number of hip fractures is on the rise as well(Lim *et al.,* 2009; Kanis and McCloskey, 1996). Therefore, quick recognition of osteoporosis with effective treatment is vital.

In normal adults, bone goes through a process of continuous remodeling, consisting of periods of formation and destruction. Bone remodeling is mediated by osteoclasts, which are cells that resorb bone, and osteoblasts, which are cells that form bone. In bone remodeling, if osteoclastic activity predominates, bone formation decreases and bone remodeling cannot be maintained. As a result, underdeveloped bone cells become prevalent, bones become porous and fragile, and osteoporosis develops.

Risk factors for osteoporosis include female sex, advanced age, low calcium and vitamin D intake, genetic factors, smoking, alcohol abuse, low BMD, low body weight, recurrent falls, personal history of fracture, race or ethnic background, and insufficient physical activity. Despite the wide range of risk factors, the fact that postmenopausal, middle-aged women make up most of osteoporotic population suggests that estrogen deficiency may be the leading cause of osteoporosis(Lim *et al.,* 2009; Li *et al.,* 2013). In fact, estrogen plays an important role in regulating bone metabolism by directly affecting osteoblasts and osteoclasts and by indirectly influencing intestinal calcium absorption, renal calcium excretion, secretion of PTH, and production of $1.25(OH)_2D$ (Lim *et al.,* 2009; KalaiSelvi *et al.,* 2013). Thus, in estrogen-deficient, postmenopausal women, osteoclasts increase in number, bone resorption increases relative to formation, bones become porous and fragile, and calcium is readily wasted.

One of the most commonly used methods for anti-resorptive therapy is alendronate, a bisphosphonate class drug that effectively treats osteoporosis by inhibiting osteoclastic activity (Imai, 2013). Alendronate inhibits farnesyl diphosphate synthase (FDPS), an enzyme necessary for the formation of the cytoskeleton in osteoclasts. When FDPS is inhibited, protein geranyl-geranylation is suppressed (Imai, 2013; Goffinet *et al.,* 2006; Rodan, 1998), and consequently, osteoclastic activity, which initiates bone resorption, is suppressed as well (Goffinet *et al.,* 2006; Rodan, 1998). However, even alendronate carries a long list of side effects, which include nausea, vomiting, abdominal pain, diarrhea, dysphagia, abdominal distension, gastrointestinal adverse events, and esophageal cancer (Green *et al.,* 2010; Epstein *et al.,* 2006). Moreover, alendronate may produce paradoxical effects, with one study showing that it over-suppresses bone turnover and increases susceptibility to and delays healing of non-spinal fractures (Green *et al.,* 2010; Odvina *et al.,* 2005). Despite its known side effects and paradoxical effects, alendronate is still one of the most common treatments for osteoporosis, as there are no alternatives whose positive effects have been established and verified. Current studies on the side effects of alendronate advocate for serious research on other natural supplements that can treat and prevent osteoporosis.

The author reported that silk fibroin hydrolysates might be a new candidate for the prevention and treatment of osteoporosis in patients (Kweon *et al.,* 2015). In this study, *Cudrania tricuspidata* (CT) was evaluated as a potential medical herb. Rich in minerals, ascorbic acid, and polyphenol, CT's supplementary effects on various diseases such as obesity, colorectal adenocarcinoma, and arthritis have been established and verified (Kim *et al.,* 2009). Numerous studies have confirmed the effects of minerals, ascorbic acid, and polyphenol (Tranquilli *et al.,* 1994; Arslan *et al.,* 2011; Chen *et al.,* 2011), all of which are present in CT, on osteoporosis, but how CT affects osteoporosis has yet to be verified. We hypothesize that CT acts as a natural antioxidant and mineral supplement to delay bone deterioration induced by osteoporosis. Therefore, this study examined the effects of CT on bone loss induced by ovariectomy and evaluated whether CT could be used as an effective natural supplement to substitute alendronate.

Materials and Methods

Cudrania tricuspidata

Cudrania tricuspidata (CT) leaves used in this experiment were obtained in Rural Development Administration based in Suwon. CT was extracted using a Sonicator (Branson 3210, USA) in four stages. First, CT was mixed with alcohol (80%) in

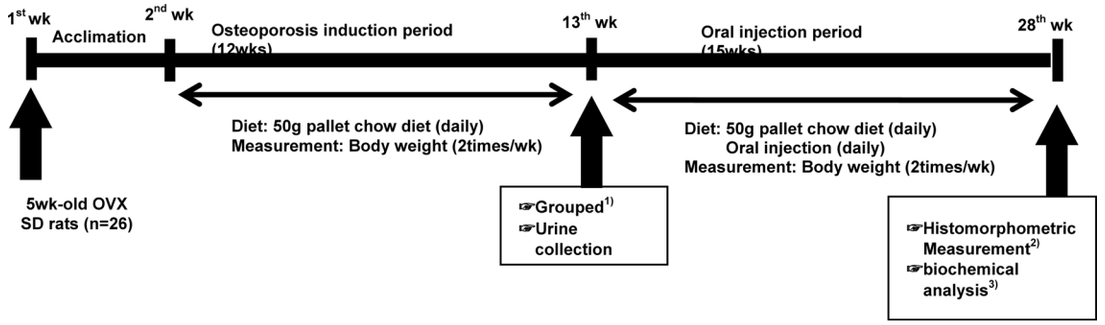

Fig. 1. Study design and analyses.

a 1:20 ratio in an Erlenmeyer flask at 60°C, to which 60kHZ of ultrasound was applied for one h. Then, the mixture was filtered using vacuum filtration. The whole process was repeated three times using the leftover CT. Afterwards, the leftover CT was mixed with distilled water in a 1:20 ratio in an Erlenmeyer flask at 60°C, to which 60 kHz of ultrasound was applied for one h. Finally, the collected extract was concentrated using a Rotatory evaporator (N-1N, EYELY Sunil), and the extract was frozen and freeze-dried using a freeze dryer (FD 8512, Ilshin, Korea).

Animals

The experimental protocol was approved by The Animal Care and Use Review Committee (IACUC) of Kyung Hee University. (KHUASP (SU) 13-01). Twenty-six Sprague-Dawley rats, all five weeks old, were purchased from SLC, Inc. (Shizuoka, Japan). Twenty-two rats underwent ovariectomy (ligated and excised the ovaries), during which isoflurane inhalation (3% dissolved in oxygen) was used as anesthesia. The remaining four rats underwent sham operations, the purpose of which was to inflict similar levels of stress to the control rats as the ovariectomized ones. Rats were cage-paired in temperature-controlled rooms (22±2°C) with a relative humidity of 55±5% and a 12-h light/dark cycle.

Study design

The experimental period lasted 28 wk (Fig. 1). During the first week, a pellet chow diet and tap water were provided *ad libitum* to help the rats adapt to the new environment. Then, the rats went through the osteoporosis induction period (12 wk), during which a 50 g pellet chow diet was given every day. After the induction period, we assigned the sham-operated rats to the SHAM group (n=4) and randomly separated the ovariectomized rats into four groups: ovariectomized (OVX n=5), alendronate (ALEN n=5), CT100 (n=6), and CT300 (n=6).

Starting the 14th week of the experimental period, 50 g of pellet chow diet was administered to all groups, and we applied an experimental oral injection to each group until the 28th week. SHAM and OVX groups were orally injected with 2 mL of distilled water. The ALEN group was orally injected with 10mg of alendronate sodium per kilogram of rat once a day. The CT100 group was orally injected with 100 mg of CT per kilogram of rat once a day, and the CT300 group was orally injected with 300 mg of CT per kilogram of rat once a day.

Following the initial week of acclimation, body weights of the rats were measured twice a week. The unconsumed pellet chow was collected and measured at the end of each day. After the end of the experiment period, following 12 h of overnight fasting, the rats were administered intraperitoneal anesthesia with zoletil (0.1 cc/100 g) and rumpun (0.03 cc/100 g), inhalational anesthesia with ethyl ether, and were then sacrificed.

Blood sampling method

Blood samples were collected using cardiac puncture for serum collection. Serum was isolated by centrifugation (PK121R, Milan, Italy) at 3000 rpm for 15 min at 4°C and stored immediately at -70°C until analyzed. Serum calcium (Ca) was measured using a chemistry auto-analyzer (ADVIA 1650, Bayer, Tokyo, Japan), and serum osteocalcin was measured using the MILLOPLEX bone hormone panel (Millipore, Billerica, MA, USA).

Urine sampling method

Urine was collected on the 14th week (basal) and the 28th week (final) of the experimental period for comparison. Before the collection, the metabolic cages were cleaned with 0.1N of hydrogen chloride (HCL). During the 24-h urine collection period, we limited the rats' water source to distilled water in order to prevent any contamination of the urine. Collected urine samples were centrifuged at 2,000 rpm for 15 min at room temperature, and the top layer was collected and stored at -70℃ until future analysis.

The urinary calcium and inorganic phosphorus levels were measured with a chemistry auto-analyzer (ADVIA 1650, Bayer). N-terminal telopeptide (NTx) was measured by ELISA using commercial kits (Osteomark, Wampole Laboratories, Princeton, NJ, USA).

Histomorphometric study

Bone histomorphometric parameters were determined using a Micro-computed tomographic system (μ-CT or micro-CT). At the end of the experimental period, the left femurs of the rats were scanned using micro-CT. Before the scan, the rats were anesthetized with intraperitoneal injections of zoletil 50 (Zoletil 50, Virbac) and rumpun (Rompun, Bayer). After the images were gathered, the cortical and trabecular microstructure were used for 3D reconstruction at 50 kV, 200 μA, at a rotation step of 0.4°. Analysis of the reconstructed scans was conducted using NRecon cone-beam algorithm software (SkyScan), and for three-dimensional analysis and image printing, the files were transferred to the CTan program (SkyScan). Histomorphometric parameters included trabecular bone volume (TRA_BV), trabecular thicknesses (Tb.Th), trabecular number (Tb.N), trabecular separation (Tb.Sp), structure model index (SMI), and trabecular bone mineral density (TRA_BMD).

Statistical analysis

The means and standard error of measurements (SEM) of each group were calculated using SPSS software (Version 20.0, SPSS Inc, Chicago, IL, USA). Duncan's multiple range tests followed by ANOVA were performed using $p<0.05$ to determine the significant differences between groups.

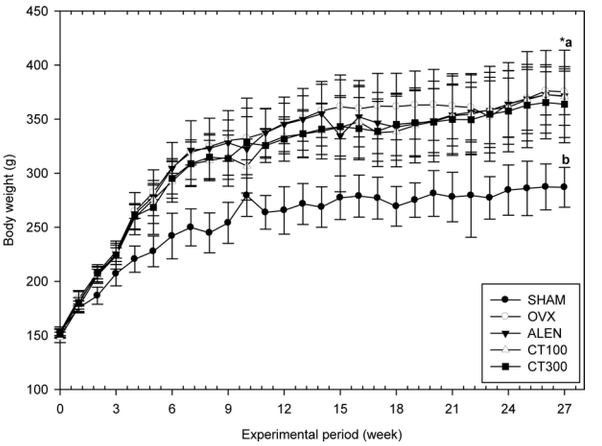

Fig. 2. Comparison of body weights in the experimental groups.

Results

Body weight, dietary intake, and food efficiency ratio (FER)

All groups had a similar initial mean body weight ($p < 0.05$), as shown in Fig. 2. However, the body weight of the SHAM group showed a slight difference from the other groups starting the 3rd week and continuing onward. At the end of the experimental period, ovariectomized groups collectively showed significantly increased body weights compared to the SHAM group ($p < 0.05$).

No significant difference in dietary intake was observed across all groups ($p < 0.05$). However, the CT100 and CT300 groups demonstrated significantly lower FER values than did the OVX and ALEN groups ($p < 0.05$). (Table 1)

Organ weight

The mean liver weights of the SHAM, CT100, and CT300 groups were significantly lower than that of the OVX group ($p < 0.05$). The uterine weight of the SHAM group was significantly higher than the other groups. The spleen weight of SHAM group was significant lower than that of the ALEN group ($p < 0.05$). No significant differences in kidney weight were observed across groups. (Table 1)

Biochemical markers of bone turnover

The basal NTx level of the CT100 group was not significantly

Table 1. Changes in body weight, weight gain, food efficiency rate, and organ weight in experimental rats

	SHAM[1)]	OVX[2)]	ALEN[3)]	CT100[4)]	CT300[5)]
Dietary intake(g/d)	158.83±22.09	143.42±57.83	145.38±51.53	171.36±26.89	169.66±23.02
Initial body weight(g)	152.75±2.87	153.20±4.60	151.80±4.44	148.16±4.88	152.00±3.63
Final body weight(g)	286.92±18.43^{b}	375.28±21.11^{a}	371.44±27.16^{a}	370.98±42.82^{a}	363.75±30.64^{a}
FER(%)[6)]	0.60^{c}	1.10^{a}	1.08^{a}	0.93^{b}	0.89^{b}
Organ weights(g/bw)					
Liver(g)	6.78±0.32^{b}	8.00±0.53^{a}	7.29±0.74^{ab}	6.69±0.75^{b}	6.76±0.27^{b}
Spleen(g)	0.43±0.04^{b}	0.51±0.05^{ab}	0.54±0.06^{a}	0.52±0.09^{ab}	0.49±0.05^{ab}
Kidney(g)	1.8±0.20	1.77±0.12	1.7±0.16	1.68±0.11	1.75±0.10
Uterine(g)	0.73±0.10^{a}	0.14±0.08^{b}	0.11±0.03^{b}	0.10±0.01^{b}	0.13±0.03^{b}

[1)] SHAM : non ovariectomized rat
[2)] OVX : ovariectomized rat
[3)] ALEN : ovariectomized rat + Alendronate acid (10mg/kg bw/d)
[4)] CT100 : ovariectomized rat + Cudrania tricuspidata (100mg/kg bw/d)
[5)] CT300 : ovariectomized rat + Cudrania tricuspidata (300mg/kg bw/d)
[6)] FER : Food efficiency ratio=[weight gain(g)/day]/[food consumed(g)/d]
Values are mean± SEM (n= 6) except SHAM n=4, OVX n=5
Different superscript letters within a row indicate significant differences by Duncan's multiple range test (p<0.05).

different than that of the CT300 group, but for the final NTx level measurement, CT intake resulted in a dose-dependent decrease in NTx, with the CT300 group demonstrating a relatively lower NTx level than the CT100 group. The ALEN group showed a reduced level of NTx compared to the SHAM group, and the ALEN group also showed a significantly lower NTx level compared to the OVX group ($p < 0.05$). (Fig. 3)

The osteocalcin (OC) levels in the CT100 and CT300 groups were significantly higher than that of any other group ($p < 0.05$), and the ALEN group demonstrated a significantly lower level of OC than any other group ($p < 0.05$). In addition, the SHAM and OVX groups did not show any difference in OC level. (Fig. 3)

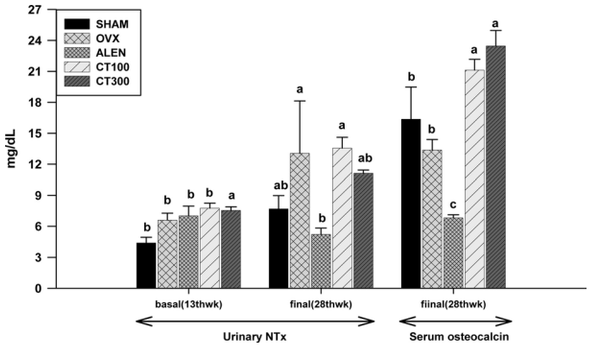

Fig. 3. Effect of ***Cudrania tricuspidata*** on primary biochemical markers of bone turnover

Calcium Homeostasis

Although showing some significant differences between groups ($p < 0.05$), the serum calcium levels of all groups were within the normal range. (Fig. 4)

According to the basal (14th week) urinary calcium measurements, no experimental groups showed significant differences. However, upon the final (28th week) urinary calcium level measurement, the CT100, CT300, and ALEN groups had significantly lower urinary calcium levels than the SHAM and

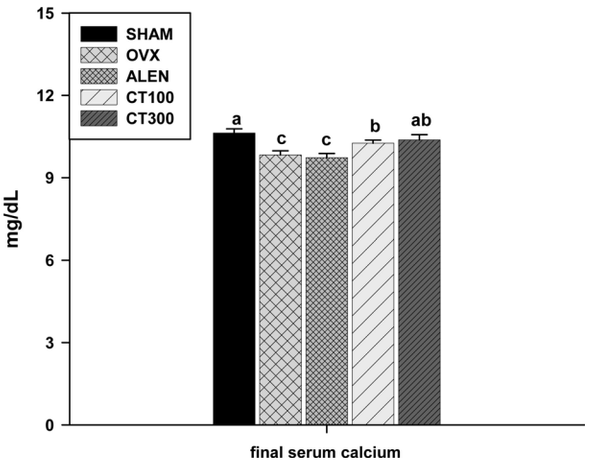

Fig. 4. Effect of ***Cudrania tricuspidata*** on serum calcium level of experimental rats

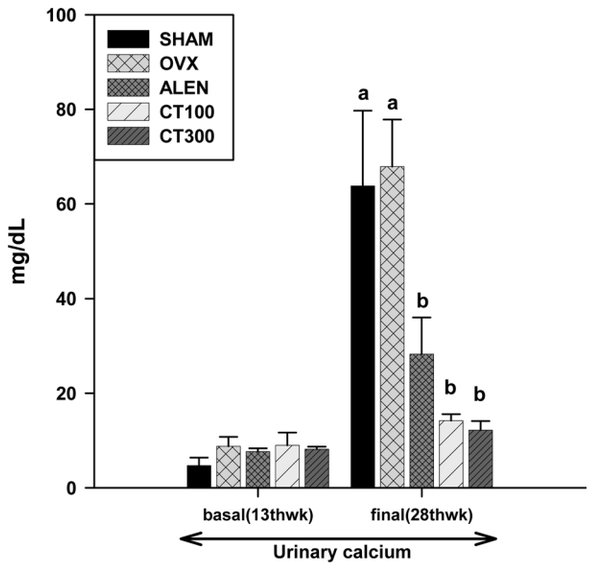

Fig. 5. Effect of ***Cudrania tricuspidata*** on urinary calcium level of experimental rats

OVX groups ($p < 0.05$). (Fig. 5)

Histomorphometric parameters of trabecular bone

Compared to other groups, the SHAM group showed significant differences in structure model index (SMI), bone surface (BS), bone volume (BV), trabecular number (Tb.N), and trabecular separation (Tb.Sp) ($p < 0.05$). In addition, the trabecular thickness (Tb.Th) of the ALEN group was significantly lower than that of the SHAM group ($p < 0.05$) and was also decreased compared to the OVX group. (Table 2)

BMD of trabecular bone and cortical bone

As seen in Fig.6c, SHAM group manifested significantly higher trabecular bone mineral density (TRA BMD) than the four other groups ($p < 0.05$). Additionally, the ALEN and CT-fed groups demonstrated significantly higher TRA BMD than the OVX group ($p < 0.05$). Such difference could be observed in the 3D images of the trabecular bone (Fig.6a). Cortical bone mineral density (COR BMD) of the five groups depicted in Fig.6d showed no significant difference. The 3D images of the cortical bone appear to illustrate as such (Fig.6b).

Discussion

In this study, CT prevented bone loss by inhibiting bone resorption and enhancing bone formation. As a result, CT

Table 2. Effect of Cudrania tricuspidata on trabecular histomorphometric measurement

	SHAM[1)]	OVX[2)]	ALEN[3)]	CT100[4)]	CT300[5)]
Tb.Th (mm)[6)]	0.132±0.013^{a}	0.123±0.006^{b}	0.120±0.000^{ab}	0.130±0.010^{ab}	0.130±0.010^{ab}
BS(mm)[7)]	23.15±2.46^{a}	31.40±2.60^{b}	33.14±0.60^{b}	30.68±3.01^{b}	29.25±1.42^{b}
BV(mm)[8)]	49.88±6.58^{a}	17.93±0.81^{b}	20.81±7.63^{b}	19.27±3.58^{b}	21.97±4.57^{b}
Tb.Sp (mm)[9)]	0.142±0.024^{a}	0.426±0.074^{b}	0.383±0.196^{b}	0.437±0109^{b}	0.380±0.079^{b}
Tb.N (mm)[10)]	3.927±0.435^{b}	1.366±0.051^{a}	1.620±0.579^{a}	1.453±0.278^{a}	1.633±0.427^{a}
SMI [11)]	0.782±0.789^{a}	2.256±0.300^{b}	2.186±0.219^{b}	2.143±0.116^{b}	2.053±0.031^{b}

[1)]SHAM : non ovariectomized rat
[2)]OVX : ovariectomized rat
[3)]ALEN : ovariectomized rat + Alendronate acid (10mg/kg bw/d)
[4)]CT100 : ovariectomized rat + Cudrania tricuspidata (100mg/kg bw/d)
[5)]CT300 : ovariectomized rat + Cudrania tricuspidata (300mg/kg bw/d)
[6)]Tb.Th : trabecular thickness
[7)]BS : bone surface
[8)]BV : bone volume
[9)]Tb.Sp : trabecular separation
[10)]Tb.N : trabecular number
[11)]SMI : structure model index
Values are mean± SEM (n= 6) except SHAM n=4, OVX n=5
Different superscript letters within a row indicate significant differences by Duncan's multiple range test ($p<0.05$).

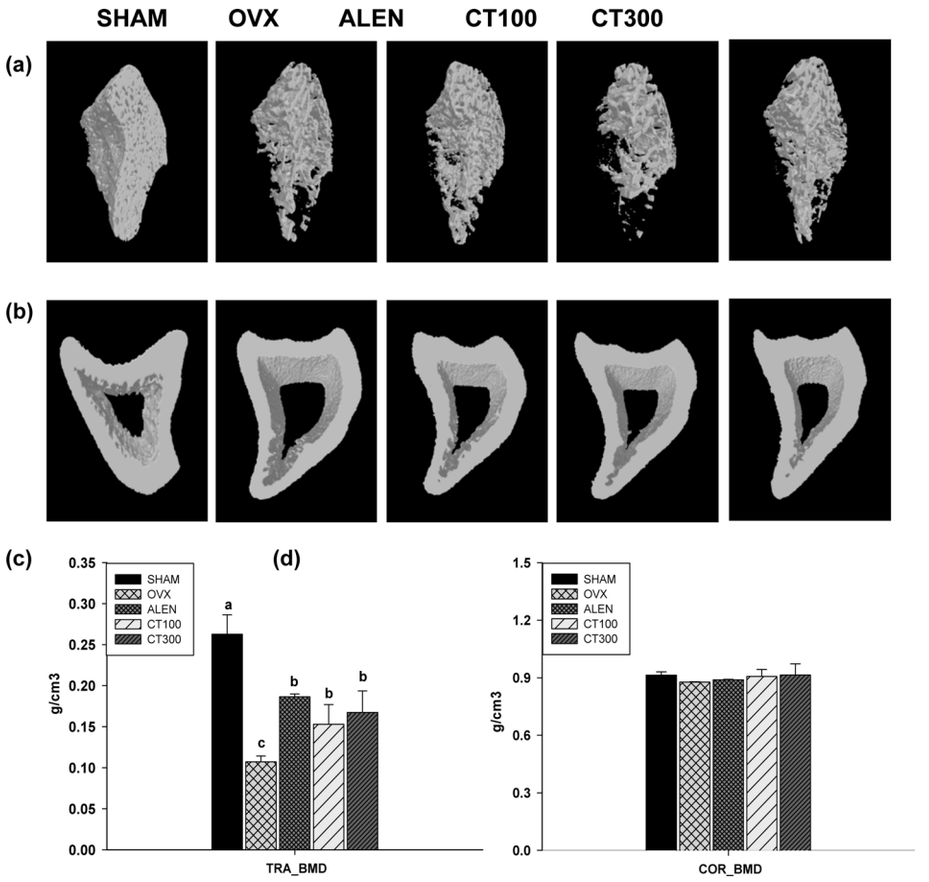

Fig. 6. 3D images of trabecular (a) and cortical bones using microtomorphy (b) and bone mineral density of trabecular bone (c) bone mineral density of cortical bone (d)

increased bone health as indicated by biochemical, radiological, and histomorphometric measurements. Therefore, CT extract can be considered as natural treatment for osteoporosis induced by ovariectomy.

As a sensitive biomarker of osteoclastic activity, urinary n-terminal telopeptide (NTx) accurately reflects bone resorption (Ataoğlu *et al.,* 2013). Although the CT100 group exhibited similar NTx levels to the OVX group, the CT300 group demonstrated slightly reduced NTx levels compared to the OVX group. These differences in the NTx level suggest that with increasing concentration of CT, osteoclastic activity can be proportionally inhibited, and these results also suggest that CT has an anti-resorptive effect. We speculate that the anti-resorptive effect of CT can be explained by its flavonoid content, as a previous study showed that flavonoid appears to inhibit osteoclastic activity (Chen *et al.,* 2011).

Our data confirmed the effective inhibition of alendronate on osteoclastic activity (Mundy, 1991; Halasy-Nagy *et al.,* 2001). However, the lower NTx level of the ALEN group compared to the SHAM group suggests a possibility of over-suppression of bone resorption. In fact, T. Dandinoğlu *et al*. reported that long-term use of bisphosphonates causes over-suppression of bone turnover and thereby inflicts microdamage, reduces bone strength, and increases the fracture risk (Dandinoğlu *et al.,* 2013). Although alendronate is generally safe and effective, this reported complication implies that long-term administration of alendronate should be cautiously followed (Odvina *et al.,* 2005).

Secreted solely by the bone-building osteoblast cells, osteocalcin (OC) is a convincing marker of osteoblastic activity (Karsenty and Oury, 2013). CT-fed rats showed significantly higher serum osteocalcin (OC) levels, while rats in the ALEN group showed significantly lower OC levels than any other group. This result supports that CT injection stimulates bone formation while alendronate does not affect bone formation because osteoblastic activity reflects bone formation (Karsenty and Oury, 2013). CT's stimulatory effect on osteoblasts may

be explained by the flavonoid in CT, whose effects have been established by previous studies (Li *et al.,* 2013). Moreover, OC is a well-known mediator of bone mineralization and calcium homeostasis (Puchacz *et al.,* 1989; Lee *et al.,* 2007). OC binds to calcium ions and moves calcium to bone where it is needed (Mizuguchi *et al.,* 2001; Kennedy *et al.,* 2013). Thus, from the OC data, we can speculate that CT increases bone mineral content and aids in restoring calcium homeostasis.

Although serum calcium levels varied among the experimental groups, they were all within the normal range (Choi and Kim, 2011). Such results do not convey much information about calcium homeostasis, but that calcium homeostasis was functioning properly. However, it is important to analyze what changes in calcium homeostasis ensued, as slight differences between groups could help distinguish the effects of the experimental treatments on bone resorption. To assess these changes in calcium homeostasis, urinary calcium level was examined.

According to our results, urinary calcium was significantly lower among CT-fed rats than in the OVX group, which indicates that CT intake might have improved calcium reabsorption. Because increased renal calcium reabsorption decreases the amount of necessary calcium released from bone to serum, renal calcium reabsorption is an important biological process that is closely related to bone resorption (Nordin, 1997). In other words, increases in calcium reabsorption reduce the need for osteoclastic activity. One way in which CT is suggested to prevent bone loss is via CT's flavonoid and phosphorus content, which increases calcium and potassium reabsorption and decreases calcium excretion (Chen *et al.,* 2011; Nordin, 1997).

Considering the above evidence, we conceived that CT intake could stabilize bone metabolism and improve bone health. For tangible evidence, we analyzed the radiological and histomorphometric measurements of trabecular and cortical bones. While significant differences were not observed in cortical bone measurements across all groups, trabecular BMD, a primary diagnostic indicator of osteoporosis, was higher among the CT-fed groups than in the OVX group, but it was not significantly different between the CT-fed and ALEN groups. Because cortical bone responds slowly to ovariectomy and treatments, these results are reasonable (Mizughuchi *et al.,* 2001; Arjmahdi *et al.,* 1996).

Previous reports suggest that that BMD is positively correlated to serum osteocalcin and negatively correlated to urinary NTx and calcium excretion (Baeksgaaard *et al.,* 1998; Bharadwaj *et al.,* 2009; Vlasiadis *et al.,* 2008). Consistent with these studies, we observed that as the NTx and urinary calcium levels decreased in the CT-fed groups, their OC and TRA BMD increased. Therefore, we conclude that CT intake enhanced bone formation while, at the same time, limited bone resorption to stabilize bone metabolism, which is contrary to alendronate, which only demonstrated an anti-resorptive effect.

The findings in this study are unique in that CT minimizes bone loss by stimulating bone formation and inhibiting bone resorption. Most drugs for osteoporosis are only purported to inhibit bone resorption (Nordin, 1997), but excessive suppression of bone resorption could result in paradoxical effects, such as increases in the fracture risk and reduction of bone strength (Odvina *et al.,* 2005). Although not definitive, the fact that the Tb.Th level of the ALEN group was lower than that of any other group highlights this potential complication of alendronate therapy (Odvina *et al.,* 2005). Therefore, the benefit of CT as a natural treatment method for osteoporosis with no over-suppressive effects on bone resorption should be properly noted.

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