Mechanical Evaluation of Effect of Grape Seed Proanthocyanidins Extract on Debilitated Mandibles in Rats

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Grape seed proanthocyanidins extract (GSPE), whose principal ingredient is proanthocyanidins, shows many activities such as cholesterol lowering effects, antioxidant effects, anti-tumor effects, cardioprotective effects, and protection against ultra-violet rays. However, reports of the effects of GSPE on bone are rare. We performed a mechanical analysis of the effect of GSPE on the interior structure of rat mandibular bone in the growth period, using three-dimensional peripheral quantitative computed tomography (pQCT). A low-calcium /high-calcium diet with supplementary GSPE was compared to a low-calcium/high-calcium diet in rats with debilitated mandibular bones. The group who received added GSPE showed a significant increase in cortical bone density, cross-sectional area, and trabecular bone mineral content (p<0.05). A significant increase was also seen in the results of a non-invasive stress strain index (SSI) (p<0.01) in the added GSPE. Our findings suggest that GSPE can increase bone quality and bone strength of rat mandibles in the growth period.

Key words : GSPE, pQCT, Rat mandible

INTRODUCTION

The peak development of bone mass occurs in the growth and building period from infancy to adolescence, and is a major determinant of bone mass later in life. During this period, insufficient calcium causes debilitated bone, which results in a failure to reach peak bone mass. Thereafter, bone mass decreases with aging and calcium has an influence on the prevention and prognosis of osteoporosis. In conventional therapy, high-calcium supplementation is used to treat debilitated bone[1-4], however, sufficient bone mass in debilitated bone can not be achieved by added calcium alone. Grape seed proanthocyanidins extract (GSPE) is a flavonoid derivative with proanthocyanidins as the principal ingredient. Ipriflavone, which participates in the calcification of bone and has been used to inhibit bone resorption[5-9], has been reported to belong to the same flavonoid group. GSPE is known to have many activities, such as a cholesterol lowering effect[10], a cytotoxic effect on human cancer cells[11], cardioprotective properties[12], stimulation of angiogenesis in dermal wound healing[13], and protection against ultraviolet rays[14]. However, there have been few reports of its effects on bone metabolism, especially in the growth period. In the present study, we performed mechanical analysis of experimentally debilitated mandibular bones in rats to investigate the effect of GSPE with a high-calcium (UNICAL®) diet[15] using three-dimensional peripheral quantitative computed tomography (pQCT) to separately measure trabecular and cortical bone, bone density, mineral content, cross-sectional area, and non-invasive bone strength (with a stress strain index, SSI).

MATERIALS AND METHODS

Animals and treatments
Forty 5-weeks-old male Wistar rats, each weighing about 115 g, were used. They were divided at random into 4 groups and housed in small cages individually under similar conditions at 22±1℃ with a 12-hour illumination time (8 : 00 am to 8 : 00 pm). Group A rats were fed a standard diet (Oriental Combination A diet, Oriental Yeast) and given tap water freely for 6 weeks. Group B rats were given the low-calcium diet (Oriental Combination A variant diet, calcium content 30% of Oriental Combination A diet) and distilled water for 6 weeks. Group C rats were fed the low-calcium diet and given distilled water for 3 weeks, and then a high-calcium diet (74% Oriental Combination A diet, 26% UNICAL) with distilled water for 3 weeks. Group D rats were given the low-calcium diet and distilled water for 3 weeks, and then the same high-calcium diet as in Group C, along with supplementary 0.003% GSPE (TOKIWA PHYTOCHEMICAL CO., LTD) and distilled water for 3 weeks. The components of the individual diets are presented in Tables 1~3. Following the 6-week experimental period, all rats were killed with thiopental sodium (Ravonal (r) ; Tanabe Seiyaku CO., LTD) under deep anesthesia with diethyl ether. The mandibles were immediately removed and fixed in 10% neutral buffered formalin. All procedures were approved by the Committee for the Use and Care of Laboratory Animals of Kyushu Dental College, Japan.

Table 1 Composition of all diets

(%)

Ingredients	Standard diet	Low-calcium diet	High-calcium diet	High-calcium diet +GSPE
β-corn starch	38.00	37.64	28.12	28.12
Vitamin-free casein	25.00	25.00	18.50	18.50
α-potato starch	10.00	10.00	7.40	7.40
Cellulose powder	8.00	8.00	5.92	5.92
Soy bean oil	6.00	6.00	4.44	4.44
Mineral mixture of standard diet	6.00	—	4.44	4.44
Mineral mixture of low-calcium diet	—	6.00	—	—
Granulated sugar	5.00	5.00	3.70	3.70
Vitamin mixture	2.00	2.00	1.48	1.48
$CaCO_3$	—	0.36	—	—
UNICAL®	—	—	26.00	26.00
GSPE	—	—	—	0.003
	100.00	100.00	100.00	100.00

Table 2 Mineral mixture of standard diet and low-calcium diet

(g)

Ingredients	Standard diet	Low-calcium diet
Ca-lactate	35.09	—
KH_2PO_4	25.72	28.33
$CaHPO_4 \cdot 2H_2O$	14.56	—
K_2HPO_4	—	9.55
NaH_2PO_4	9.35	9.38
$MgSO_4$	7.17	7.18
NaCl	4.66	4.68
Fe-citrate	3.18	3.19
$MnSO_4 \cdot 4 \sim 5H_2O$	0.12	0.13
$ZnCO_3$	0.11	—
$ZnCl_2$	—	0.10
$CuSO_4 \cdot 5H_2O$	0.03	0.03
Kl	0.01	0.01

*It adjusts to 100 g with the cellulose

Table 3 Vitamin mixture of all diets

Vitamin A·acetate	50,000	IU
Vitamin D3	10,000	IU
Vitamin E·acetate	500	mg
Vitamin K3	520	mg
Vitamin B1·hydrochloride	120	mg
Vitamin B2	400	mg
Vitamin B6·hydrochloride	80	mg
Vitamin B12	0.05	mg
Vitamin C	3,000	mg
D-biotin	2	mg
Folic acid	20	mg
Calcium pantothenate	500	mg
Para-aminobenzoic acid	500	mg
Niacin	600	mg
Inositol	600	mg
Corrin chloride	20	g

*It adjusts to 100 g with the cellulose powder

Body weight
During the experimental period, body weight was recorded once a week.

Bone density, cross-sectional area and bone mineral content
For the pQCT (XCT Research SA model. Stratec-Medizintechnik GmbH, Pfozheim, Germany) examinations, the bone samples were centrally located between the source of the scanner unit and detector with the aid of a support. To produce a scout-view that was an image of the bone slice, the tomographic scan was displayed on the screen (Fig. 1). The mandibular bone was scanned around the center of the mandibular first molar mesial root at 3 different positions with an interval of 0.1 mm. The slices, which consisted of trabecular and cortical components, were measured with a voxel size of 0.08 mm and height of 0.26 mm. Using this procedure, the cortical region was determined using cortical mode 1 at a threshold value of 690 mg/cm³, and then cortical bone density (mg/cm³), cortical bone cross-sectional area (mm²), and cortical bone mineral content (mg/mm) were measured. In the same way, we measured trabecular bone density (mg/cm³), trabecular cross-sectional area (mm²), and trabecular bone mineral content (mg/mm) using peel mode 2 at a threshold value of 395 mg/cm³ [16-18].

Bone strength (non-invasive)
We also evaluated bone strength as a stress strain index (SSI) [19-21], which was determined using pQCT as a non-invasive assessment of mechanical properties at a threshold of 464 mg/cm³. This threshold value was determined to reduce a partial volume effect. The equation SSI=CBD·Z/NCBD (CBD: cortical bone density (mg/cm³), Z: section modulus (mm³), NCBD: normal value of cortical bone density 1200 mg/cm³)
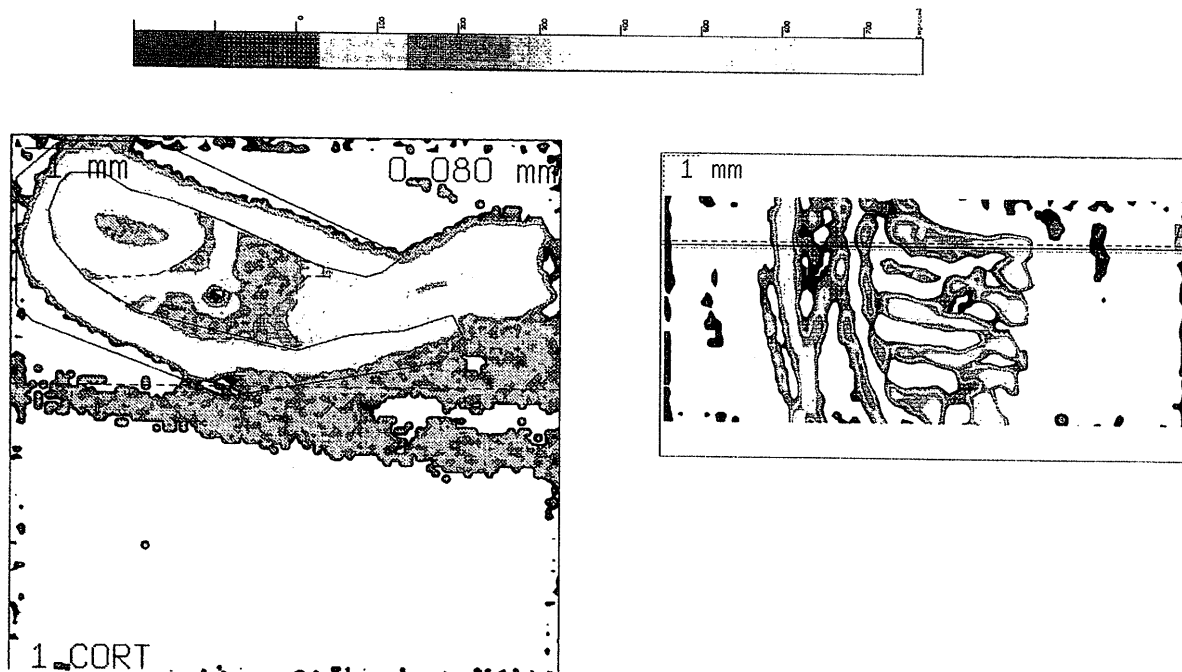
Fig. 1 pQCT slices. Left: Bone slice from tomographic scan. Right: Wister rat mandibular bones were scanned around the center of the mandibular first molar mesial root at 3 different positions with an interval of 0.1 mm.

was used.

Statistical analysis

Data are expressed as the mean±SD for the effect of GSPE. Statistical differences were analyzed using an analysis of variance (ANOVA) at $\alpha = 0.05$.

RESULTS

Body weight

At the beginning of the study, the initial body weights were 110.35±2.02 g, 111.26±2.07 g, 109.65± 1.12 g, and 114.66±5.07 g in Group A, B, C, and D, respectively. At the end of the 6-week experimental period the final body weights were 386.80±51.23 g, 384.02±28.71 g, 390.33±16.35 g, and 380.55±13.26 g, respectively, in the 4 groups, which were not significantly different (Fig. 2).

Bone density, cross-sectional area, and mineral content

As for the cortical bone, bone density and the cross-sectional area in Group D were significantly greater than in Group C ($p<0.05$), while mineral content in Group D was also significantly higher than in Group C ($p<0.01$). Further, the cross-sectional area in Group D was significantly larger than in Group A ($p<0.05$) (Fig. 3).

Regarding the trabecular bone, the cross-sectional area and mineral content in Group D were significantly greater than in Group C ($p<0.05$),
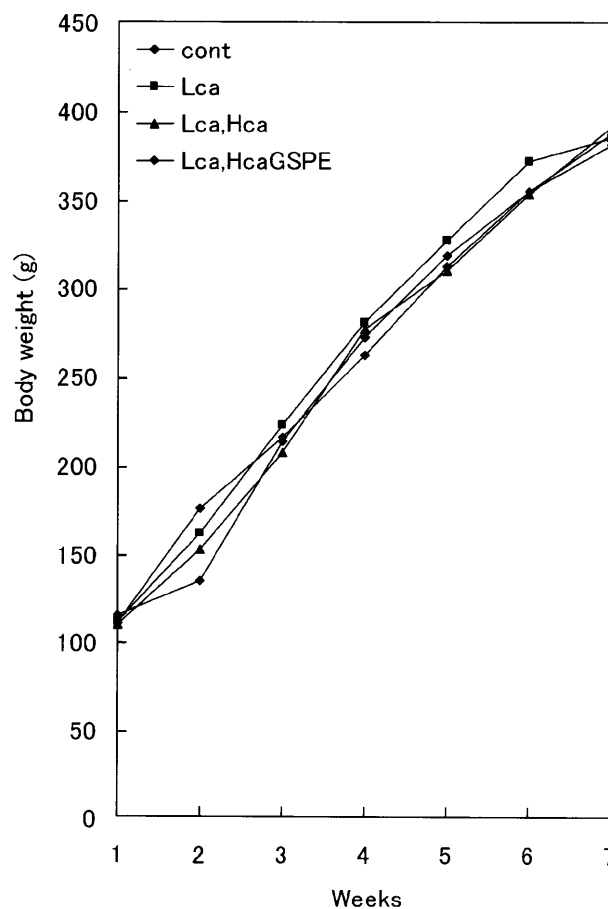

Fig. 2 Body weight of rats throughout the study period.
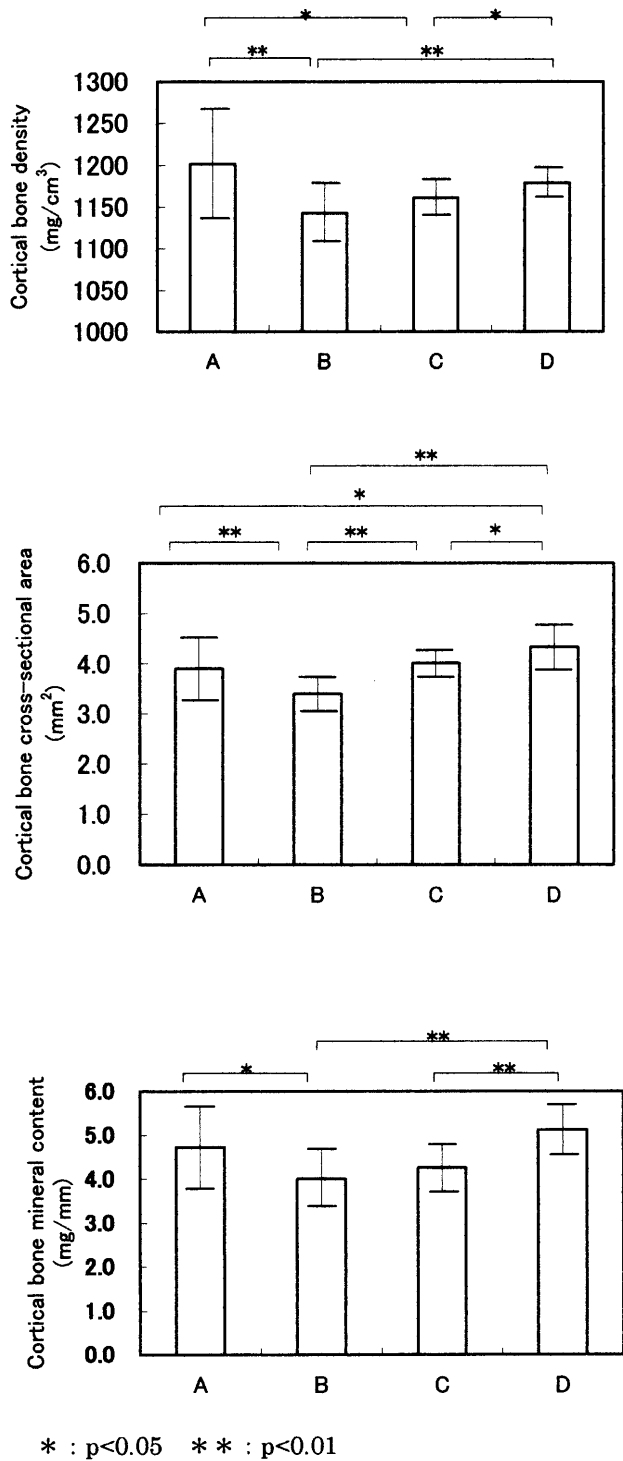
* : p<0.05　＊＊ : p<0.01

Fig. 3　Cortical bone density, cross-sectional area, and mineral content.
　　　　Group A: standard diet
　　　　Group B: low-calcium diet
　　　　Group C: low-calcium/high-calcium diet
　　　　Group D: low-calcium/high-calcium diet with supplementary GSPE
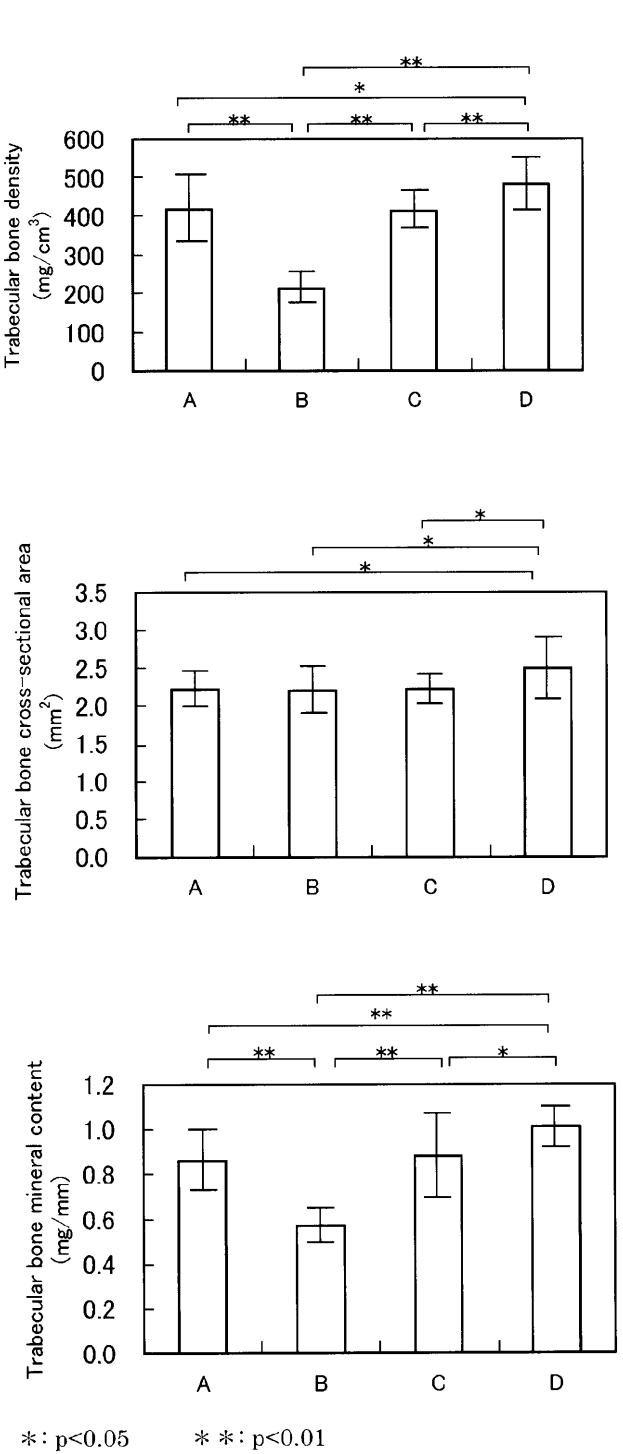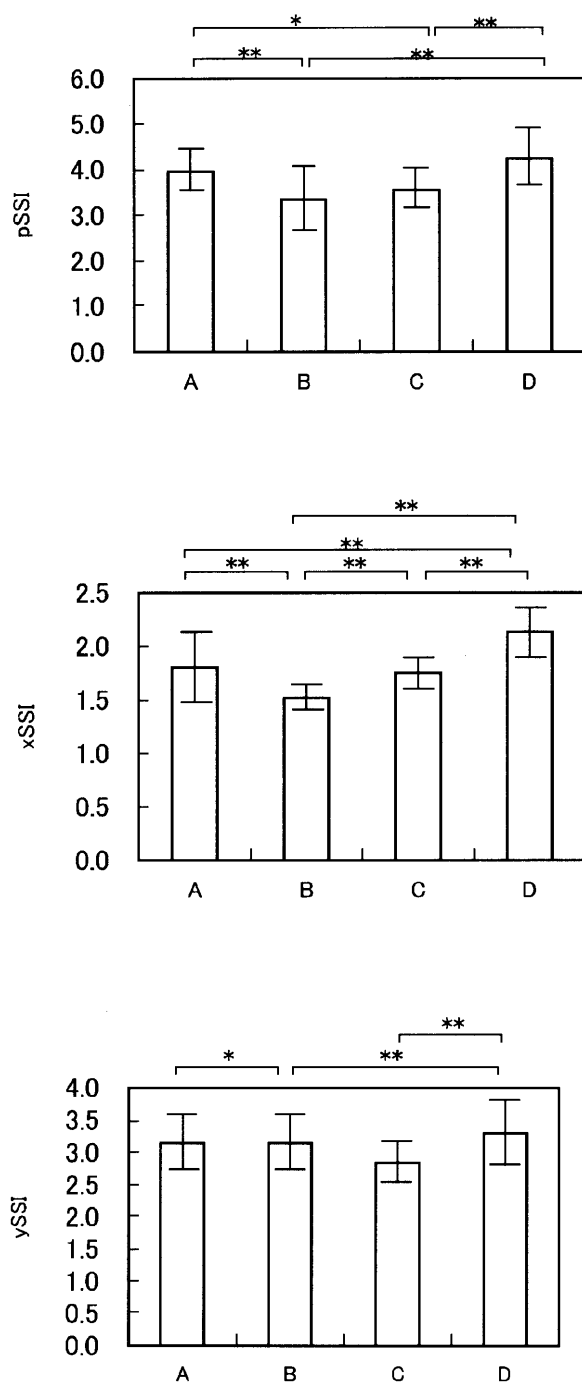
＊: p<0.05　　＊＊: p<0.01

Fig. 4　Trabecular bone density, cross-sectional area, and mineral content.
　　　　Group A: standard diet
　　　　Group B: low-calcium diet
　　　　Group C: low-calcium/high-calcium diet
　　　　Group D: low-calcium/high-calcium diet with supplementary GSPE

* : p<0.05　＊＊ : p<0.01

Fig. 5　Stress strain index (SSI).
Group A: standard diet
Group B: low-calcium diet
Group C: low-calcium/high-calcium diet
Group D: low-calcium/high-calcium diet with supplementary GSPE

while bone density in Group D was also significantly higher than in Group C (p<0.01). Further, bone density and the cross-sectional area in Group D were significantly higher than in Group A (p<0.05), while mineral content in Group D was also significantly higher than in Group A (p<0.01) (Fig. 4).

Non-invasive bone strength

SSI was demonstrated as the x (xSSI), y (ySSI), and z (pSSI) axes. xSSI, ySSI, and pSSI of Group D were significantly greater than those of Group C (p<0.05), and xSSI of Group D was significantly higher than that of Group A (p<0.05) (Fig. 5).

DISCUSSION

In recent years, with the rapid aging of Japanese society, concern regarding osteoporosis has increased, and the establishment of prevention and treatment modalities is considered to be a serious problem. Risk factors for the onset of osteoporosis are divided into internal factors such as hormones, aging, and genetic inheritance, and external factors including nutrition, exercise, and lifestyle. The former factors have a variety of congenital aspects, making adjustment difficult, however, the latter may be alterable by voluntary changes. The most important factor of osteoporosis development is bone density. Bone density is primarily a hereditary factor, however, it also can be influenced by nutrition and exercise. In adolescence, bone density reaches peak bone mass, and then it decreases in postmenopausal women and with aging in men. Prevention and treatment of osteoporosis is typically drug treatment and exercise therapy. As for drug therapy, high-calcium supplements, vitamin D metabolites, ipriflavone, bisphosphonates, calcitonin, estrogen, and combined therapy[22-27] are often given. However, these are symptomatic treatments and show various difficulties including degree of symptoms, patient basal disease, sensitivity to calcium or other drugs, and side effects.

In adolescence during the growth and development period, attempts have been made to prevent osteoporosis by increasing peak bone mass through the intake of calcium[28-30]. Females see a rapid increase of bone density in the growth period, which typically occurs 2 to 3 years later in males. In adolescence, calcification of the bone is approximately 400-500 mg/day and calcium absorption is greater than 40% in the intestinum, therefore, calcium intake is important at this stage. However, current calcium intake by infants is lacking and chronic calcium deficiency is often encountered. Conventionally, calcium dietary therapy is directed against this condition[1-4]. In our department, a significant effect was observed in a series of studies that used high levels calcium (UNICAL®) to treat debilitated bones in rats[31,32]. UNICAL calcium is a mixture of calcium carbonate

and citrated calcium from sea urchin shells and is known to improve absorption by the intestinum from the addition of chondroitin sulfate and by refining the granularity[33]. However, it is difficult to reach optimal bone mass values without continual calcium supplementation.

In the present study, we investigated the effects of GSPE with a high-calcium diet. GSPE is a flavonoid with a vitis vinifera origin[34]. Flavonoids are classified by their structure into 9 groups; flavones, flavonols, isoflavones, flavanes, catechins, flavanones, flavanonol, chalcones, and anthocyanin. The main ingredients of GSPE, proanthocyanidins, are a part of the catechins group and typically are condensed as tannin. They can be divided into procyanidins, prodelphinidins, and profisetinidins by the substitution form of hydroxyl on the basic frame, flavan-3-ols. Flavonoids have biological regulatory activities, such as antioxidant, anti-tumor, antihypertensive, and antidiabetic effects[35-38]. As for the effect on bone, many reports have focused specifically on ipriflavone of the isoflavones group[5-9]. GSPE has variety of biological regulatory effects as do other flavonoids, however, its effect on bone has rarely been reported[10-14]. We gave a high-calcium diet supplemented with GSPE to rats that had debilitated bones as the result of a previous low-calcium diet. We studied on dietetic treatment in rats during the growth and development period, so we used male rats to avoid a factor of hormones such as estrogen.

Of the methods, dual energy X-ray absorptiometry (DXA) has come to be known as the standard[39-41]. However, DXA expresses bone density as the area (g/cm^2) because it only obtains two-dimensional information about the bone. On the other hand, bone density determination by pQCT enables the measurement of density per unit volume. Using a mechanical analysis of mandibles in the growth period with pQCT, we made a more accurate analysis. Further, we were able to separately measure cortical and trabecular bone, as well as determine SSI from a non-invasive mechanical analysis of cortical bone density and modulus of section. As a result, we were able to measure mandibles in a quantitative fashion.

As for cortical bone density, Group D had significantly greater bone density than Group C. Strength of the cortical bone and Young's modulus depends on cortical bone density[42], therefore, an increase of density results in an increase of bone strength, which was also shown in our SSI results. SSI is a reliable measurement, because it showed a high correlation $(r=0.94)$[43] in comparison to a three-point bending test of rat femurs.

Trabecular bone has an 8-10 times greater amount of metabolization as cortical bone, and has been used as an indicator of the reaction of bone to aging and medication[44]. The trabecular bone density in Group D was high, showing that GSPE had a pronounced effect on the rat bones. Surprisingly, trabecular bone density in Group D was higher than that in Group A, which we considered to be a result of a temporary improvement in calcium absorptive ability in the bone. Cortical and trabecular cross-sectional areas in Group D were increased more than in the other groups. Therefore, we concluded that GSPE significantly improved bone growth, because the bone cross-sectional area increases earlier than bone mineral content in childhood[45]. By 0.003% of just a little GSPE, Group D showed significantly improved bone growth compared with Group C. It has been reported that ipriflavone inhibits bone resorption by an effect on osteoclasts[46,47] and hastens bone osteogenesis by an effect on osteoblasts[48,49], however, it is not clear how GSPE acts on bone. In future, we hope to report its action mechanism from *in vitro* results, as basic research with osteoporosis of the mandibula is not enough on account of the fact that it is not clearly seen as a symptom of bone fracture and can not be compared with other types of bone.

Osteoporosis increases the risk of periodontal disease, and bone loss in the mandible has an influence on denture stability, as well as the application and prognosis of implants. It is considered that an increase in peak bone mass by the intake of calcium during the growth period, as well as diagnosis by quantitative and non-invasive estimation using pQCT, is also highly significant for the mandible.

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