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Mechanical evaluation of debilitated tibia diaphysis in rats during the growth period —Combination therapy with high-calcium diet and grape seed proanthocyanidin extract—

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Abstract The main ingredient of grape seed proanthocyanidin extract (GSPE), is proanthocyanidin, a kind of flavonoid. Proanthocyanidins are known to have a variety of biological regulatory activities, such as antioxidant effects; however, little is known the effects on bone. In the present study, we performed mechanical analysis using three-dimensional peripheral quantitative computed tomography (pQCT), bone strength tests and quantitative analysis of a combination therapy composed of high-calcium diet and GSPE fed to rats during their growth. Forty male Wistar rats, 5 weeks old, were divided randomly into control (A), low-calcium diet (B), low-calcium/high-calcium diet (C) and low-calcium/high-calcium diet with supplementary GSPE (D) groups. After 6 weeks, the tibias were removed and cortical bone density, cross-sectional area, mineral content, cortical bone thickness, periosteal perimeters, endosteal perimeters, and stress strain index (SSI) were measured. Invasive three-point bending tests, and quantitative analysis of Ca, P, Mg and Zn using inductively coupled plasma (ICP) spectral analysis were performed. Body weights were not significantly different in the 4 groups. Cortical bone density and mineral content in group D were significantly higher than those in group C ($P<0.01$) as was the periosteal perimeter ($P<0.05$). SSI and three-point bending test results for group D were not significantly different than those for group C, but recovery in group D was similar to that in the control group. As for the quantitative analysis, Ca, P, and Zn levels in group D were significantly higher than those in group C ($P<0.01$). Our findings suggest that a combined therapy with a high-calcium diet and GSPE can improve the quality and strength of rat tibias during their growth.

Key words
Calcium,
Grape seed proanthocyanidins extract,
Mechanical evaluation,
Tibia bone,
Rat

Introduction

The growth is important, as that is when the development of bone mass, is a major determinant of bone strength. Unfortunately, the current levels of calcium intake by infants in Japan are low and chronic calcium deficiency is often encountered, originating the risk of osteoporosis. Calcium dietary therapy has usually been used for infants with chronic calcium deficiency[1–4].

Significant effects were observed in our series of studies in rats that ingested high levels of a calcium supplement (UNICAL®) for the treatment of debilitated bones[5–8], but, it is difficult to obtain optimal bone mass by calcium supplementation alone. Therefore, we have also studied the effect of grape seed proanthocyanidin extract (GSPE), a flavonoid derivative similar to ipriflavone that has been reported to inhibit bone resorption directly and

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Table 1-1 Composition of experimental diets (%)

Ingredients	Standard diet (Ca 480 mg/100 g)	Low-calcium diet (Ca 144 mg/100 g)
β-corn starch	38.00	37.64
Vitamin-free casein	25.00	25.00
α-potato starch	10.00	10.00
Cellulose powder	8.00	8.00
Soy bean oil	6.00	6.00
Mineral mixture	6.00◆	6.00◇
Granulated sugar	5.00	5.00
Vitamin mixture	2.00	2.00
$CaCO_3$	—	0.36
	100.00	100.00

Table 1-2 Composition of experimental diets (%)

	High-calcium diet	High-calcium diet + GSPE
Standard diet	74.00	74.00
UNICAL®	26.00	26.00
GSPE	—	0.003

Table 1-3 Mineral mixture of standard diet (g/100 g)

$CaHPO_4 \cdot 2H_2O$	14.56
KH_2PO_4	25.72
NaH_2PO_4	9.35
NaCl	4.66
Ca-lactate	35.09
Fe-citrate	3.18
$MgSO_4$	7.17
$ZnCO_3$	0.11
$MnSO_4 \cdot 4 \sim 5H_2O$	0.12
$CuSO_4 \cdot 5H_2O$	0.03
KI	0.01

Adjusted to 100 g with cellulose powder

Table 1-4 Mineral mixture of low-calcium deit (g/100 g)

NaCl	4.68
$MgSO_4$	7.18
NaH_2PO_4	9.38
KH_2PO_4	28.333
K_2HPO_4	9.55
Fe-citrate	3.187
KI	0.0055
$ZnCl_2$	0.10425
$CuSO_4 \cdot 5H_2O$	0.03275
$MnSO_4 \cdot 4 \sim 5H_2O$	0.12817

Adjusted to 100 g with cellulose powder

Table 1-5 Vitamin mixture in 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	mg

Adjusted to 100 g with cellulose powder

hasten calcification by osteoblasts[9–12]. It is well known that proanthocyanidins, the main ingredient of GSPE, have biological regulatory effects such as antioxidant activities[13–16]. However, there have been few reports of the effects of proanthocyanidins on bone. In the present study, we performed mechanical analysis of experimentally debilitated tibia bones from rats to investigate the effect of GSPE in combination with a high-calcium diet using three-dimensional peripheral quantitative computed tomography (pQCT) to measure cortical bone density, cross-sectional area, mineral content, cortical bone thickness, periosteal perimeters, endosteal perimeters, and noninvasive bone strength (by a stress strain index, SSI). We also performed invasive three-point bending tests, and quantitative analysis of Ca, P, Mg and Zn using inductively coupled plasma (ICP) spectral analysis.

Materials and methods

Animals and treatment

Forty Wistar male rats, 5 weeks old and each weighing approximately 115 g, were used. The rats were divided into 4 groups and housed individually in cages under similar conditions at 22 ± 1°C, with a 12-hour day/night light schedule. All diets were provided by Oriental Yeast (Tokyo, Japan) and mixed in our laboratory, components of which are shown in Tables 1-1–1-5.

Rats in the control group (group A) were given

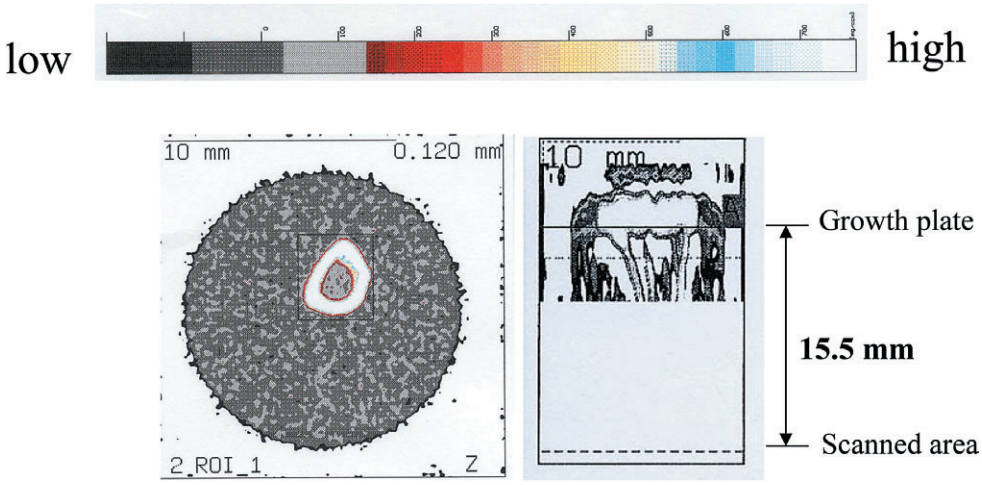

Fig. 1 pQCT scan

Representative diaphysis tibia bone was scanned 15.5 mm distal from the growth plate (right). Bone slice from representative tomographic scan (left).

Oriental Combination A diet and tap water for 6 weeks. Rats in the low-calcium diet group (group B) were fed a low-calcium variant of Oriental Combination A (calcium content: 30% of that is in the standard diet) and distilled water for 6 weeks. Rats in the low-calcium/high-calcium diet group (group C) were given the low-calcium diet and distilled water for 3 weeks and then a high-calcium diet (74% Oriental Combination A, 26% UNICAL) with tap water for 3 weeks. Rats in the low-calcium/high-calcium diet with supplementary GSPE group (group D) were given the low-calcium diet and distilled water for 3 weeks and then the same high-calcium diet as that given to rats in group C together with supplementary 0.003% GSPE and tap water for 3 weeks. Following the 6-week experimental period, all rats in each group were killed with thiopental sodium (Ravonal; Tanabe) under deep anesthesia with diethyl ether. The tibias 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.

Body weight

During the experimental period, body weights were recorded once each week.

Bone density, cross-sectional area, bone mineral content, and bone geometric index

For pQCT (XCT Research SA models, Stratec-Medizintechnik GmbH, Pforzheim, Germany) tests, the bone samples were centrally placed between the source of the scanner unit and the detector with the aid of a support. The tibia diaphysis was scanned at a point 15.5 mm from the proximal growth plate and measured with a voxel size of 0.12 mm, height of 0.46 mm and distance of 100 mm (Fig. 1). The cortical region was determined using cortical mode 1 at a threshold value of 690 mg/cm^3, and then cortical bone density (CtBD, mg/cm^3), cross-sectional area (CtCSA, mm^2), and mineral content (CtBMC, mg/mm) were measured and cortical bone thickness (CtThc, mm), periosteal perimeters (Peri, mm), and endosteal perimeters (Endo, mm) were determined for a geometric index.

Bone strength (noninvasive assessment)

Bone strength was evaluated to determine a stress strain index (SSI), using a pQCT for noninvasive assessment of mechanical properties, with a threshold of 464 mg/cm^3 for reducing the partial volume effect. SSI was calculated by the equation SSI = CBD•Z/NCBD, where CBD is cortical bone density (mg/cm^3), Z is section modulus (mm^3), and NCBD is the normal value of cortical bone density (1200 mg/cm^3).

Bone strength (invasive assessment)

Tibia diaphysis bone strength was also determined by a three-point bending test using a material testing machine (Model MZ-500 S, Maruto Testing

Table 1-6 Measurements of elements for quantitative analysis

Elements	Wavelength (nm)	Grating	ATT	AMP	Accumulation time (sec)	Measurement condition
MG	279.553	2M	3	×10	5	1
P	178.287	1M	9	×10	5	1
Ca	393.336	2M	3	×1	5	1
Zn	213.856	1M	12	×10	5	1

ATT: attenuation, AMP: amplification

Table 2 Body weight changes during the study period (g)

	A	B	C	D
Initial BW	115.05 ± 2.77	112.72 ± 1.48	110.20 ± 2.30	113.54 ± 5.39
Final BW	385.48 ± 30.45	382.82 ± 32.22	388.24 ± 29.45	381.63 ± 24.59

Data are shown as means ± SD; BW: body weight

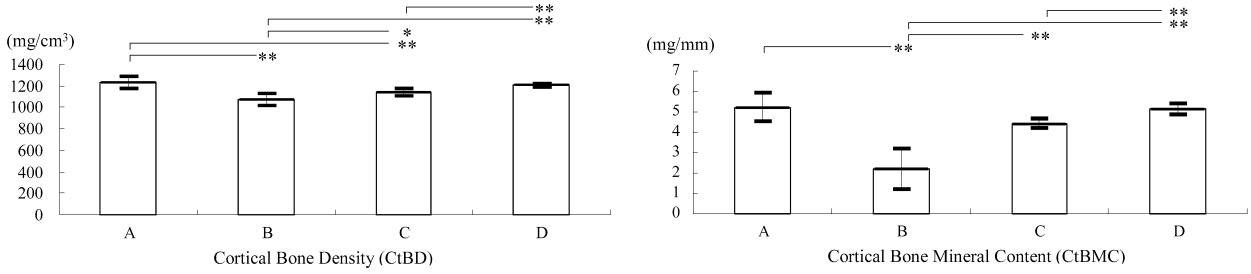

Fig. 2 CtBD and CtBMC

Significant differences were noted in cortical bone density (CtBD) and cortical bone mineral content (CtBMC) between group C and D ($P<0.01$). *: $P<0.05$, **: $P<0.01$

Machine Co., Tokyo, Japan). The whole tibia bone was placed perpendicular to the midpoint of the tibia to give an accurately measured span. To measure stiffness (N/cm), a three-point bending test was then performed at a constant loading speed rate of 20 mm/minute and with a load cell of 50 kgf and specimen span of 20 mm.

Quantitative analysis

Quantitative analysis using inductively coupled plasma (ICP) spectral analysis of Ca, P, Mg and Zn was performed using a sequential plasma spectrometer (ICPS-8000, Shimadzu, Kyoto, Japan). Sample tibias each of 5 mm in width were collected from the region 20 mm from the epiphysis. Each sample was irrigated with ethyl alcohol and dried at 60°C for 1 hour. After the samples had been weighed, 5 m*l* of hydrochloric acid and 3 m*l* of nitric acid were added to each sample and the samples were hydrolyzed in a sand bath. Next, deionized water was added to make a total volume 50 m*l*, and the solution was diluted 10 times. A calibration curve was set up using a standard solution (Mg: 0, 0.4 and 2 ppm; P: 0, 8 and 40 ppm; Ca: 0, 8 and 40 ppm; Zn: 0, 0.02 and 0.1 ppm) and analysis was performed. Measurement conditions of elements for

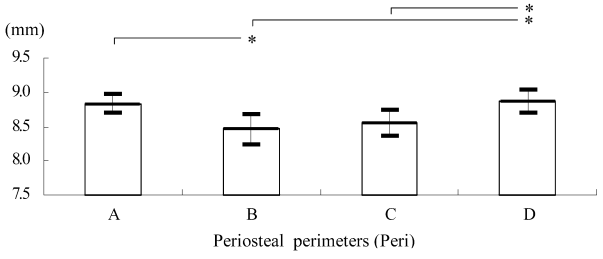

Fig. 3 Periosteal perimeter

Periosteal perimeter (Peri) in group D was significantly higher than in group C ($P<0.05$). *: $P<0.05$

Table 3 Cortical cross-sectional area, thickness and endosteum

	A	B	C	D
CtCSA (mm^2)	3.96 ± 0.47	1.07 ± 0.10**	1.20 ± 0.12	1.39 ± 0.13*
CtThc (mm)	0.59 ± 0.06	799.09 ± 39.54**	909.82 ± 21.02	940.80 ± 31.84
Endo (mm)	4.86 ± 0.43	1.12 ± 0.08**	1.25 ± 0.15	1.41 ± 0.12

Data are means ± SD;
*: Compared with control group (group A), $P<0.05$, **: Compared with control group (group A), $P<0.01$

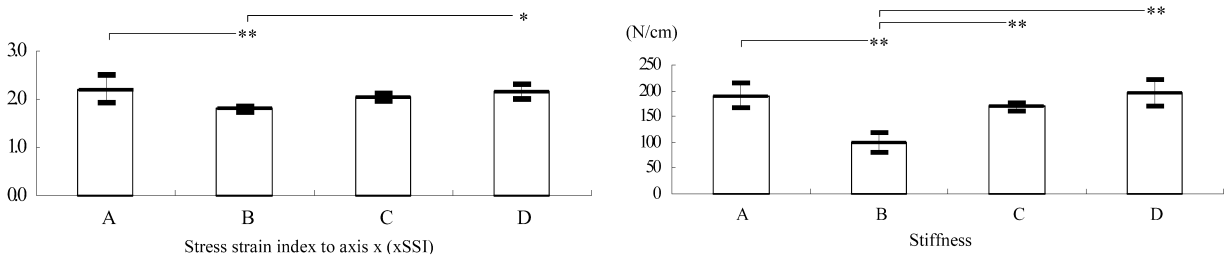

Fig. 4 SSI and stiffness

Values of stress strain index to the reference axis x (xSSI) and stiffness in group D were significantly higher than those in group B ($P<0.05$ and $P<0.01$, respectively), however, no differences were seen between group D and group C. *: $P<0.05$, **: $P<0.01$

Table 4 Ca, P, Mg and Zn mineral contents in diaphysis of Wistar rat tibia (%)

	A	B	C	D
Ca	28.31 ± 0.313	20.52 ± 0.371 [a]	25.05 ± 0.139 [ab]	29.17 ± 0.182 [abc]
P	14.14 ± 0.115	10.73 ± 0.368 [a]	13.04 ± 0.120 [ab]	14.78 ± 0.186 [abc]
Mg	0.516 ± 0.013	0.405 ± 0.011 [a]	0.503 ± 0.011 [b]	0.542 ± 0.010 [abc]
Zn	0.0156 ± 0.001	0.0213 ± 0.001 [a]	0.0252 ± 0.001 [ab]	0.0370 ± 0.007 [abc]

Data are shown as means ± SD; vs. A: [a]$P<0.01$, vs. B: [b]$P<0.01$, vs. C: [c]$P<0.01$

quantitative analysis are shown in Table 1-6.

Statistical analysis

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

Results

Body weight

The body weights in groups A, B, C and D at the beginning of the study were 111.05 ± 2.77 g, 112.72 ± 1.48 g, 110.20 ± 2.30 g and 113.54 ± 5.39 g, respectively. At the end of the 6-week experimental period, the final body weights in groups A, B, C and D were 385.48 ± 30.45 g, 382.82 ± 32.22 g, 388.24 ± 29.45 g and 381.63 ± 24.59 g, respectively, which were not significantly different (Table 2).

Bone density, cross-sectional area, bone mineral content, and geometric index

Cortical bone density (CtBD) and mineral content (CtBMC) in group D were significantly higher than those in group C ($P<0.01$) (Fig. 2), and the periosteal perimeter (Peri) in group D was also significantly greater than that in group C ($P<0.05$) (Fig. 3). In contrast, there were no significant differences between groups D and C in cortical bone cross-sectional area (CtCSA), cortical bone thickness (CtThc) and endosteal perimeters (Endo) (Table 3).

Bone strength (invasive and noninvasive assessments)

SSI and Stiffness in group D were not significantly different than those in group C (Fig. 4).

Quantitative analysis

Ca, P, Mg and Zn levels in group D were significantly higher than those in group C ($P<0.01$) (Table 4).

Discussion

The average calcium intake in Japan is lower than the levels in other industrialized countries and is less than the minimum of 600 mg/day recommended by the Japanese Ministry of Health and Welfare. A decrease in bone mass due to a chronic lack of calcium is one of the causes of osteoporosis, which was defined at a consensus development conference on osteoporosis in 1991[17] as "a disease characterized by low bone mass and microarchitectural deterioration of bone tissue, leading to enhanced bone fragility and a consequent increase in fracture risk". As a result of the aging society in Japan, bone fracture has become the second-most frequent cause of becoming bedridden in older Japanese people. Osteoporosis is a retrograde lesion that progresses with aging, but it is difficult to differentiate from a normal condition. Therefore, osteoporosis is often undetected in the early stages, and its rate of prevalence is high.

Calcium supplements, hormones, independent bisphosphonates, and combined therapy[18–21] or kinesiotherapy[22,23] are generally used for treatment of osteoporosis. However, when improvement is delayed, the patient's quality of life is hampered by pain. Therefore, there is great interest in diagnosis and prevention of osteoporosis.

As for prevention, it is known that calcium intake and bone mass are related. In adolescence, which is a period of rapid growth and development, attempts have been made to prevent later osteoporosis by increasing peak bone mass to reach that an adult. Calcium intake is important in adolescence since calcification of bone is approximately 400–500 mg/day and calcium absorption is greater than 40% in the intestine. However, in addition to a chronic lack of calcium, the acquisition of bone mass alone is not enough to lessen the problems associated with infants, diet and eating disorders (cibophobia)[24,25]. Therefore, it has been suggested that osteoporosis is not a problem only for the aged. Calcium dietary therapy has been used for treating debilitated bone[1–4]. In a study by Johnston *et al.*[26] on monozygotic twins, it was found that calcium supplementation significantly increased bone mass, though a significant increase was not seen in post-adolescent subjects. It was, therefore, suggested that calcium supplementation during adolescence is important.

In our department, a significant effect was observed in a series of studies using large amounts of a calcium supplement (UNICAL®) to treat debilitated bones in rats[5–8]. UNICAL calcium is a mixture of calcium carbonate and citrated calcium taken from sea urchin shells, and it is known to improve absorption by the intestine from the addition of chondroitin sulfate and by refining the granularity. However, it is difficult to obtain optimal bone mass by calcium supplementation alone. The main ingredient of GSPE is proanthocyanidin, a condensed tannin that belongs to the catechin family. Further, flavonoids are known to have many effects, such as a cytotoxic effect on human cancer cells, protection against ultraviolet rays, and anti-diabetic effects[27–30]. Ipriflavone has been reported to a subject of isoflavone family, and has been used in clinical practice for bone formation[31–33]. In addition, GSPE has also been reported to have many activities[13–16], but there are few reports of its effects on bone metabolism.

In the present study, we performed mechanical analysis of experimentally debilitated tibia bones in rats to investigate the effect of GSPE with a high-calcium (UNICAL®) diet using three-dimensional pQCT to determine bone strength noninvasively (by a stress strain index, SSI) as well as invasive three-point bending tests and quantitative analysis using inductively coupled plasma (ICP) spectral analysis. Dual energy X-ray absorptiometry (DXA) is currently used method for measurement of bone density[34]. Bone density determination by three-dimensional pQCT, however, enables more accurate measurement. pQCT also enables separate measurements of the densities of cortical bone and trabecular bone. We have used pQCT to estimate vertebral fracture status by cortical bone area and mineral content[35] as well as to evaluate the reaction to medication by determining trabecular bone density[36].

In the present study, we performed mechanical analysis of tibia bones in rats to determine bone density, cross-sectional area, mineral content, cortical bone thickness, periosteal perimeters, and endosteal perimeters in cortical bone. We found that bone density and mineral content in group D were significantly greater than those in group C ($P<0.01$) as were the periosteal perimeters ($P<0.05$). The results were the same when compared with those

for group A, suggesting that the bone masses of debilitated tibias in group D had been restored to levels comparable to those of the control rats. The xSSI and stiffness in group D were not significantly different than those in group C and A, suggesting that bone strength recovered with an improvement in bone quality and bone mass.

Elements contained in bone, such as calcium, vary in composition during growth and under different nutritional conditions. Therefore, we evaluated changes in cortical bone based on variations of the composition of Ca, P, Mg, and Zn, which are involved in calcification of bone. The amount of Ca in group D was significantly greater than the amounts in groups C and group A ($P<0.01$). The Ca/P ratio, which indicates bone quality, in group D was 1.97, and it was 2.00 in group A. The amount of Mg in group D was larger than that in group A, and the amounts of Mg in groups B and C were smaller than that in group A. Magnesium competes with calcium for hydroxyapatite, so it is understandable that the levels in groups B and C were not higher than the level in group A. It is most unlikely that zinc decreases and increases in bone; however, the level of zinc in groups C and D were higher than the level in group A. These results were likely from the demand of alkaline phosphatase, which requires zinc when its level increases with a rise in bone metabolism turnover. Taken together, the results suggest that a combined therapy of high-calcium and GSPE can improve quality and strength in debilitated rat tibias. It has also been reported that ipriflavone inhibits bone resorption and hastens bone osteogenesis; however, it is not clear how GSPE acts on bone. In the future, we hope to report the details of its action mechanism determined by *in vitro* experiments.

Our results are encouraging since GSPE has no toxicity[37] or influence on infants in the growth and development stage, and it may be a safe supplement for various foods.

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