Original Article

Hylonome

Mechanical assessment of effects of grape seed proanthocyanidins extract on tibial bone diaphysis in rats

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Abstract

We studied the effects of grape seed proanthocyanidins extract (GSPE) given as a ratio of 3 mg in 100 g in a standard diet, on the tibial bone diaphysis in low-calcium fed rats. Measurements of bone density, mineral content, geometry, and bone strength using peripheral quantitative computed tomography (pQCT). Further, the whole tibia bones were tested for mechanical resistance using a material-testing machine, and mineral elements were also determined. Forty male Wistar rats, 5 weeks old, were divided into control (Co), low-calcium diet (LC), low-calcium diet · standard diet (LCS), and low-calcium diet · standard diet with supplementary GSPE (LCSG) groups. We found no significant differences in body weight among the 4 groups, whereas all of the bone parameters in LC were significantly lower than those in Co ($p<0.01$, except in periosteal perimeter (Peri) $p<0.05$). The cortical bone mineral content (CtBMC), cortical bone density (CtvBMD) and Peri in LCSG were significantly higher than those in LCS ($p<0.01$; $p<0.01$; $p<0.05$, respectively). All bone parameters in LCSG were significantly higher than those in LC ($p<0.01$, except in Peri, and stress strain index to reference axis x (xSSI) $p<0.05$). In addition, Ca, P, and Zn contents in LCSG were significantly higher than those in LCS ($p<0.01$; $p<0.01$; $p<0.05$, respectively). Our results suggest that GSPE included in a diet mixture with calcium has a beneficial effect on bone formation and bone strength for the treatment of bone debility caused by a low level of calcium.

Keywords: Calcium, Grape Seed Proanthocyanidins Extract, Dietary Therapy, Tibial Bone Diaphysis, Rats

Introduction

Several studies have reported that the intake of calcium along with flavonoids, such as ipriflavone, which is abundant in beans and inhibits bone resorption as an inducer of isoflavone, have important effects on bone formation[1-5]. Recently, the effect of grape seed proanthocyanidins extract (GSPE), which is a kind of flavonoid present in plants, has received attention, while several experimental and clinical studies have shown that proanthocyanidins have a cholesterol-lowering effect[6], cytotoxic effects toward human cancer cells[7], and cardioprotective properties[8], and also stimulate

angiogenesis in dermal wound healing[9], without inducing significant toxicological effects[10].

Low-calcium intake has been shown to increase bone resorption and decrease bone mass, which increases the risk of osteoporosis in both rats and humans[11,12]. A strong bone structure in young adulthood is likely one of the most important factors to be considered in the prevention of osteoporosis and associated fractures later in life[12], and inadequate dietary calcium during that time may result in a failure to reach peak bone mass[13]. Although it is generally recognized that calcium is best obtained from food sources, calcium supplements are destined to become an important source of dietary calcium[14]. Further, interactions between administrations of GSPE with calcium and bone responses in the mandible[15] and mandibular condyle[16] particularly during the critical growth and building periods of bone formation have been reported.

In the present study we examined the effects of combinations of GSPE and calcium on rat tibial diaphysis following low-calcium feeding (30% calcium of standard diet), by measuring bone mineral content, density, geometry and non-invasive bone strength using three-dimensional peripheral quantitative computed tomography (pQCT), as well as inva-

sive bone strength with a three-point bending test of mechanical resistance to failure. Further, we also determined the contents of the mineral elements.

Materials and methods

Animal and treatments

Five-week-old male Wistar rats ($n=40$) each weighing about 113 g, and maintained by Seiwa Experimental Research Institute, were randomly divided into 4 groups of 10 and housed in small cages individually under similar conditions with a 12-hour light-dark cycle at $22\pm^{\circ}C$. A low-calcium diet was formulated to provide calcium content that was 30% of standard diet (low-calcium diet 144 mg/100 g; standard diet 480 mg/100 g). GSPE was obtained in a powder form (Tokiwa Phytochemical Co Ltd, Japan) and 3 mg was added to 100 g of the standard diet to form the supplementary GSPE diet. All diets were prepared by Oriental Yeast (Tokyo, Japan) and blended at our laboratory, with the components of each presented in Tables 1a-1b. In the control group (Co), rats were fed a standard diet and given tap water freely for 6 weeks. In the low-calcium diet group (LC), rats were fed the low-calcium diet and given distilled water for 6 weeks. In the low-calcium diet·standard diet group (LCS), rats were fed the low-calcium diet and given distilled water freely for 3 weeks, and then the standard diet and tap water for the next 3 weeks. In the low-calcium diet·standard diet with supplementary GSPE group (LCSG), rats were fed the low-calcium diet and given distilled water freely for 3 weeks, and then fed the standard diet with supplementary GSPE and tap water for the next 3 weeks. At the end of the 6-week experimental period, each rat was killed using thiopental sodium under deep anesthesia with diethyl ether, after which the tibial bones were taken 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

The rats were weighed at the beginning of the experiment and once a week during the study.

Bone mineral content, bone density and bone geometry

We used a pQCT (XCT Research SA+, Stratec-Medizintechnik GmbH, Pforzheim, Germany) to measure various parameters in the tibiae. The bone samples were centrally located between the scanner unit source and detector with the aid of a support, then a scout-view image of the bone was produced and the tomographic scan was shown on the display monitor (Figure 1). Each tibial bone diaphysis was scanned at a point 15.5 mm distal from the proximal growth plate, which contained cortical components, with a voxel size of 0.12 x 0.12 x 0.46 mm. The cortical region was determined using cortical mode 1 with a threshold value of

		(g/100g)
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	$\diamond$ 6.00	$\blacklozenge$ 6.00
Mineral mixture	6.00	6.00
Granulated sugar	5.00	5.00
Vitamin mixture	2.00	2.00
$CaCO_3$	0.00	0.36

$\diamond$ $\blacklozenge$ = Mineral mixture (in Table 1b)

Table 1a. Composition of experimental diets.

		(g/100g)
	Standard diet	Low-calcium diet
NaCl	4.66	4.68
Kl	0.01	$5.50 \cdot 10^{-3}$
KH_2PO_4	25.72	$283.33 \cdot 10^{-1}$
NaH_2PO_4	9.35	9.38
K_2HPO_4	0.00	9.55
$MgSO_4$	7.17	$71.87 \cdot 10^{-1}$
$CaHPO_4$	14.56	0.00
Fe-citrate	3.18	$31.87 \cdot 10^{-1}$
$MnSO_4 \cdot 4 \sim 5H_2O$	0.12	$128.17 \cdot 10^{-3}$
$CuSO_4 \cdot 5H_2O$	0.03	$32.75 \cdot 10^{-3}$
$ZnCO_3$	0.11	0.00
Ca-lactate	35.09	0.00
$ZnCl_2$	0.00	$104.25 \cdot 10^{-3}$
Cellulose powder	0.00	$37412.33 \cdot 10^{-3}$

Table 1b. Mineral mixture.

690 mg/cm^3, then cortical bone mineral content (*CtBMC*, mg/mm) and cortical bone density (*CtvBMD*, mg/cm^3) were measured. Further, the bone geometrical parameters periosteal perimeter (*Peri*, mm) and cortical thickness (*CtThc*, mm) were also determined.

Stress strain index (SSI) was also measured with the pQCT as a non-invasive indicator of diaphyseal structural stiffness, and potentially strength, too[17,18] using a threshold of 464 mg/cm^3 and the following formula: The stress strain index to the reference axis x (*xSSI*) was determined by (SSI)=CBD·Z/NCBD. [CBD: cortical bone density (mg/cm^3); Z: section modulus (mm^3); NCBD: normal value of cortical bone density 1200mg/cm^3].
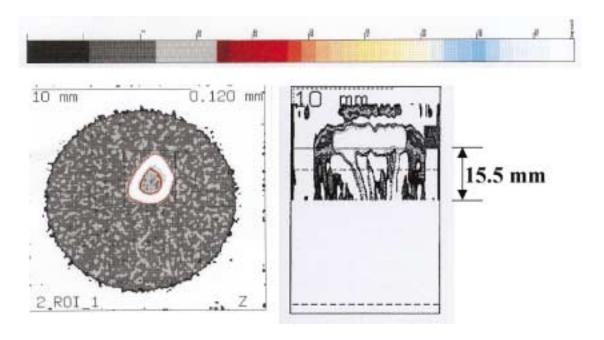
Figure 1. Bone slice from representative tomographic scan (left). Representative diaphysis tibia bone scanned 15.5 mm distal from the proximal growth plate (right).

Mechanical test

The structural stiffness of the mid-shaft of tibia was determined invasively by a three-point bending test, with a material testing machine (Maruto, Testing Machine Co., model MZ-500 S., Tokyo, Japan). The whole tibia was placed between the test rigs on a support, so that the axial load was applied perpendicular to the mid point of the bone to provide an accurately measured span. The three-point bending test was performed to measure the *stiffness* (N/cm) at a constant loading speed rate of 20 mm/minute and a load cell of 50 kgf, with a specimen span of 20 mm.

Measurement of bone calcium, phosphate, magnesium and zinc

The tibiae were cut at 2.5 mm and 2.5 mm from the mid-shaft center to obtain a 5-mm-long diaphyseal bone section, which essentially consisted of cortical bone. After washing with ethyl alcohol, each 5-mm piece of tibial diaphysis was dried at 60°C for 1 hour and weighed. Then, 5 ml of hydrochloric acid and 3 ml of nitric acid was added, dissociation by heating was performed with a sandburst, and the mixture was dissolved in 50 ml of purified water. Next, a 10-fold dilution was performed and 3 standard solutions were prepared (calcium: 0, 8, and 40 ppm; phosphate: 0, 8, and 40 ppm; magnesium: 0, 0.4, and 2 ppm; and zinc: 0, 0.02, and 0.1 ppm, respectively). The contents of each sample were determined using a sequential plasma spectrometer (Shimadzu, ICPS-8000, Japan) and the ratios of: calcium (*Ca*), phosphate (*P*), magnesium (*Mg*) and zinc (*Zn*) contents to dry bone weight were expressed as percentages.

Statistical analysis

Data are expressed as mean ±SD. Statistical differences were analyzed using one-way analysis of variances (ANOVA) followed by a *post hoc* test. Probability values of less than 0.05 were considered statistically significant.

Results

During the 6-week experimental period, all animals survived and no significant abnormal signs were observed.
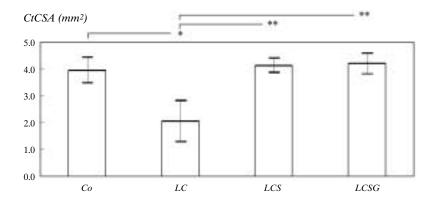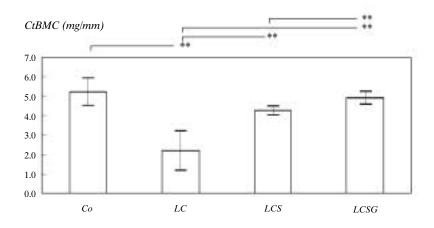
Body weight

After starting the study, no drop in body weight was noted in any of the groups. Further, initial and final body weights were not significantly different among the 4 groups.

Bone mineral content, bone density and bone geometry

All bone parameters in the LC group were significantly lower than those in Co (p<0.01 in all instances but in *Peri*, p<0.05). Whereas, compared to the LC group, all bone parameters in the LCSG were significantly higher (p<0.01 in all instances but in Peri, p<0.05) as shown in Figures 2-4.

Further, *CtBMC* and *CtvBMD* in LCSG were significantly higher than those in LCS (p<0.01) (Figure 2). *Peri* in LCSG was significantly higher than that in LCS (p<0.05), while CtThc was not significantly different (Figure 3).
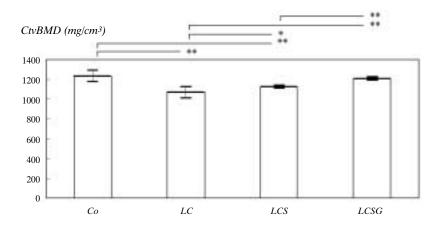
Figure 2. All bone parameters in the LC group were significantly lower than those in Co ($p<0.01$), except for cortical cross-sectional area (*CtCSA*) $p<0.05$. Significant differences were noted in cortical bone mineral content (*CtBMC*) and cortical bone density (*CtvBMD*) between the LCSG and LCS ($p<0.01$) groups. *:$p<0.05$ **:$p<0.01$.

Bone strength

xSSI and stiffness values in LCSG were significantly higher than those in LC ($p<0.05$ and $p<0.01$, respectively), however, those in LCSG were not significantly different from those in LCS (Figure 4).

Calcium restriction alone impaired significantly cortical bone mass (as assessed by CtBMC), volumetric mineral density (CtvBMD), geometry (as assessed by Peri and CtThc) and stiffness, and the tomographic indicator of strength (SSI).

Calcium replacement in the diet completely prevented the restriction-induced impairments in CtBMC, CtThc, and
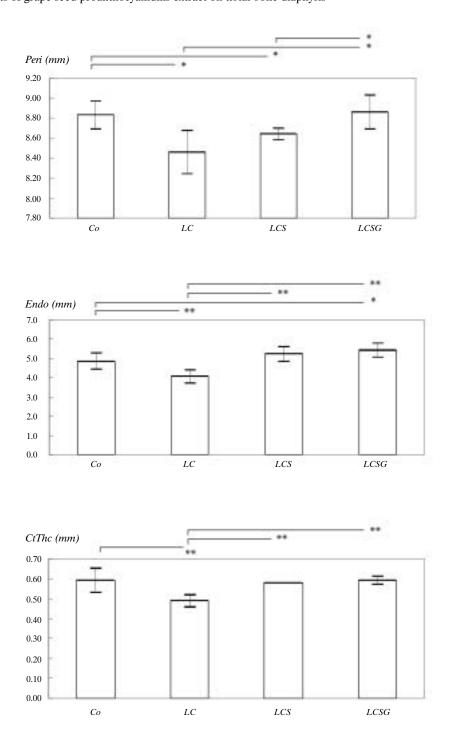
Figure 3. Periosteal perimeter (*Peri*) in the LCSG group was significantly higher than in LCS (p<0.05). Compared to the LC group, all bone parameters in LCSG, those are endosteal perimeter (*Endo*) and cortical thickness (*CtThc*), were significantly different (p<0.01), except for *Peri* p<0.05). *:p<0.05, **:p<0.01.

structural stiffness, and incompletely those in CtvBMD and Peri. The xSSI data were inconclusive in this regard (not significantly different from either Co or LCSG).

Addition of GSPE to the standard calcium diet produced some additive protective effects to those induced by calcium replacement in CtBMC, CtBMD and Peri.

Ca, P, Mg, and Zn contents

The bone mineral content of calcium, phosphate, magnesium and zinc was expressed as percentages and summarized in Table 2. All bone mineral contents in LC were significantly lower than those in Co (p<0.01), while some were
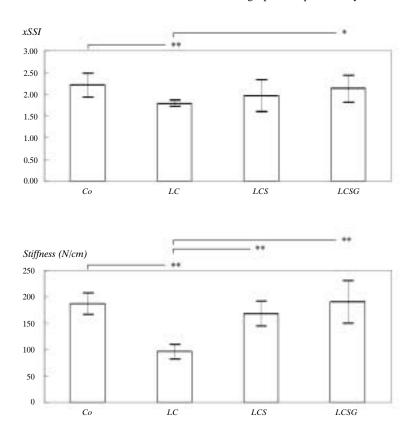
Figure 4. Values of stress strain index to the reference axis x (xSSI) and *stiffness* in the LCSG group were significantly higher than those in LC ($p < 0.05$ and $p < 0.01$, respectively), however, no differences were seen between LCSG and LCS. *: $p < 0.05$, **: $p < 0.01$.

augmented by GSPE supplementation. Furthermore, Ca and P in LCSG were significantly higher than those in LCS ($p < 0.01$) as was Zn ($p < 0.05$), whereas no differences were seen for Mg.

Discussion

GSPE belongs to a flavonoid group, and that used in the present study was a natural extract from the seeds of Vitis vinifera grapes[10,19], and contained 90% proanthocyanidins. The 6 major subclasses of flavonoids include flavones, flavonols, flavanones, catechins, anthocyanidins, and isoflavones[2,20], while proanthocyanidins are a polymer of catechin. Although beneficial bone responses to flavonoid therapy are well reported in studies using ipriflavone, which is abundant in beans and has been used to inhibit bone resorption as an inducer of isoflavone[1-5], the effects of proanthocyanidins in those responses have not been well established. Proanthocyanidins are safe[10] to consume and known to possess a broad spectrum of pharmacological, medicinal, and therapeutic properties[21]. Further, several GSPE mixtures have been reported, including a niacin-bound chromium and GSPE mixture[6], as well as a pancreatic enzyme and GSPE mixture, that was used as an analgesic[22]. In the present study, GSPE was added to a standard diet and given to rats. An increase in food consumption was observed, though it was not accompanied by an increase in body weight[10]. In our study, over a 6-week experimental period, there were no significant differences in body weight among the 4 groups.

The pQCT used in the present study is accurate and precise, as demonstrated previously by others[17,23], and is able to distinguish cortical from cancellous bone and determine bone density as volumetric density. For the present experiments, the tibial diaphysis, which is essentially pure cortical bone, was selected, because cortical bone response is thought to have a relationship to mechanical stimulus[24]. Further, the net effect of diet is also important, as inadequate dietary calcium during the critical growth and building periods may result in a failure to reach peak bone mass[14]. Since peak bone mass is recognized as a major determinant of bone mass later in life, its increase during the skeletal maturity period is directly related to a decreased risk of osteoporotic fractures[12-13,25-26]. In the present study, cortical bone mineral content and cortical bone density in the LCSG group were significantly higher than those in the LCS and LC groups. These findings indicate that the amount of mineralized tissue was increased.

Further, we measured tibial cortical bone thickness, periosteal circumference, as these variables may help to evaluate modeling-derived changes caused by GSPE, though

	Co	LC	LCS	LCSG
Mineral contents (%) :				
Ca	23.970±0.031	16.830±0.038[a]	21.860±0.135[a,b]	24.600±0.323[a,b,c]
P	12.580±0.123	8.980±0.043[a]	11.180±0.151[a,b]	12.820±0.234[b,c]
Mg	0.467±0.006	0.291±0.08[a]	0.395±0.006[a,b]	0.396±0.004[a,b]
Zn	0.024±0.003	0.011±0.001[a]	0.015±0.001[a,b]	0.020±0.003[b,d]

Data are mean ± SD; vs.Co; [a]p<0.01; vs.LC; [b]p<0.01; vs.LCS; [c]p<0.01; [d]p<0.05

Table 2. Ca, P, Mg and Zn mineral contents.

they do not affect the quality of the architectural design or the mechanical competence of the bone, in contrast to cross-sectional moment of inertia[25]. The much larger deficit in periosteal perimeter in rats in the LCS group as compared to those in LCSG suggests that the impairment in cortical bone modeling induced by calcium restriction was prevented by GSPE. To further evaluate the relative contribution of the effects of GSPE, we also determined the thickness of the shaft. This value was slightly but not significantly higher in LCSG than that in LCS, and significantly higher than in LC, suggesting that GSPE treatment did not add any effect to the positive impact of calcium replacement on cortical bone thickness and bone weakness.

SSI, measured by pQCT, and stiffness, measured with a three-point bending test, are indicators of bone strength[17]. According to Van der Meulen et al.[27] there is no chance to test whole bone strength based solely on geometry or bone mineral content. In contrast, Jamsa et al.[23] stated that both mechanical testing and pQCT measurements are relevant for biomechanical studies of bones in mice. In the present study, the stiffness of the tibial diaphysis was measured using a three-point bending test for mechanical resistance to failure as an invasive assessment, while pQCT was utilized for non-invasive assessment. xSSI was higher in rats receiving GSPE than in the LC group, and similar to that of the LCS and Co rats.

Diaphyseal stiffness in the LCSG group was significantly higher than that in LC rats, though no significant difference was seen as compared with that in LCS animals. These findings indicate that calcium alone or calcium with GSPE increased the structural stiffness of tibial diaphysis, perhaps with some impact on their strength, too.

Calcium restriction seemed to have impaired bone modeling and mineralization during growth in these experimental conditions, with a negative impact on diaphyseal stiffness. The impairment observed in the tomographic xSSI values suggests that this impact could also have affected bone strength, provided that this variable was actually reflected by the non-invasive indicator in the assayed conditions. Calcium replacement in the diet completely prevented the restriction-induced, negative effects on bone mass (as assessed by CtBMC) and structural stiffness, and only partially those observed on bone mineralization (as assessed by CtvBMD) and geometry (as assessed by Peri). No conclusive evidence of any protective effect on the actual bone strength could be derived from the data. Addition of GSPE to the standard calcium diet completed the effects of calcium replacement on bone mineralization and geometry, apparently with no further impact on diaphyseal structural stiffness or strength.

There is ample evidence that Ca, P, Mg, and Zn elements play an important role in bone metabolism, as shown in both clinical[12,13,26], and experimental[11,28-30] reports. In the present study, GSPE in diet produced a satisfactory result regarding alterations in the mineral elements, suggesting a role of those factors in its effects on bone structural properties.

In conclusion, the present data from a 6-week experimental period show that tibial cortical parameters were increased by GSPE treatment, suggesting a potential therapeutic application of this compound for treatment of bone debility. The effects were greater toward periosteal perimeters than cortical bone thickness. Further, bone mass increases seemed to have had a positive impact on diaphyseal stiffness and perhaps strength, too. However, the data do not allow us to derive any conclusive statement concerning any interaction of GSPE with calcium replacement in the diet in this regard.

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