Oral Diseases (2004) 10, 27–31

http://www.blackwellmunksgaard.com

Experimental Oral Pathology

Effects of grape seed proanthocyanidins extract on mandibles in developing rats

Y Kamitani, K Maki, I Tofani, Y Nishikawa, K Tsukamoto, M Kimura

Department of Pediatric Dentistry, Kyushu Dental College, Kokurakita-ku, Kitakyushu, Japan

OBJECTIVE: Grape seed proanthocyanidins extract (GSPE), a flavonoid, has a beneficial effect on physical health, which may include the health of bone. The purpose of the present study was to investigate the effects of GSPE on mandibular bone by examining trabecular and cortical bone density, mineral content, and non-invasive bone strength in low-calcium diet rats.

MATERIALS AND METHODS: Wistar male rats at 5 weeks old ($n = 40$) were divided into control (A), low-calcium diet (B), low-calcium diet plus standard diet (C), and low-calcium diet plus standard diet with supplementary GSPE (D) groups. Following 3 weeks of a calcium-restricted diet, group D rats were given 3 mg of GSPE as supplement in 100 g of a standard diet for the next 3 weeks. Following the 6-week experimental period, mandibular bones were examined using peripheral quantitative computed tomography (pQCT).

RESULTS: There were no significant differences in body weight or trabecular bone area among the four groups. Trabecular bone density, and trabecular bone mineral content, cortical bone density, cortical bone cross-sectional area, and cortical bone mineral content were significantly higher in group D than in C. Further, Stress–strain index (SSI) values of xSSI and ySSI in group D were significantly higher than in C, although there was no significant difference in pSSI value between those two groups.

CONCLUSION: Our results suggest that GSPE treatment caused an increase in both bone formation and bone strength in rat mandibles.

Oral Diseases (2004) **10**, 27–31

Keywords: calcium; grape seed proanthocyanidins extract; dietary therapy; mandible; rats

Correspondence: Yoshiyuki Kamitani, Department of Pediatric Dentistry, Kyushu Dental College, 2-6-1 Manazuru, Kokurakita-ku, Kitakyushu 803-8580, Japan. Tel: 81-93-582-1131 (ext. 1906), Fax: 81-93-583-5383, E-mail: kimura-m@kyu-dent.ac.jp
Received 25 April 2003; revised 19 August 2003; accepted 25 August 2003

Introduction

Many studies have explored the role of grape seed proanthocyanidins extract (GSPE), a plant-based flavonoid derivative, on bone. The effect of calcium intake combined with a variety of flavonoids, such as ipriflavone supplement as an inducer of isoflavone, on bone formation have also been investigated experimentally, and used as an inhibitor of bone resorption and a non-hormonal drug for osteoporosis (Gennari *et al*, 1998; Fujita *et al*, 1999; Kimura *et al*, 2002; Ross and Kasum, 2002). Recently, several experimental and clinical studies have shown that proanthocyanidins also have many effects, such as a cholesterol-lowering effect (Preuss *et al*, 2000), cytotoxic effects on human cancer cells (Ye *et al*, 1999), cardioprotective properties (Sato *et al*, 2001), and stimulation of angiogenesis in dermal wound healing (Khanna *et al*, 2001). In other studies, proanthocyanidins did not induce any significant toxicological effects (Wren *et al*, 2002), however they caused a selective depletion of calcium ion (Kosasi *et al*, 1989).

Inadequate dietary calcium may result in a failure to reach peak bone mass (Recker *et al*, 1992), while optimum calcium intake is best obtained from food sources, although calcium supplements are destined to become an important source of dietary calcium (Fujita, 1996). Further, a dietary mixture of calcium and GSPE has been considered as a potential health-food ingredient. However, the effect of a dietary mixture of calcium and GSPE administration on bone have not been well established, especially in mandibular bone.

In the present study, we attempted to clarify the extent of recovery by rat mandibles in a fragile condition due to a low-calcium diet during the formative period, by comparing a standard diet to one containing GSPE. We examined the potential use of GSPE on experimentally debilitated mandibular bones in rats, using three-dimensional peripheral quantitative computed tomography (pQCT), to measure trabecular and cortical bone separately, as well as assess non-invasive bone strength.

Materials and methods

Animals and treatments

Wistar male rats at 5 weeks old ($n = 40$) and weighing approximately 115 g, were maintained by Seiwa Experimental Research Institute and used in the present study. They were randomly divided into four groups of 10 and housed in small cages individually under similar conditions with a 12-h light–dark cycle at 22 ± 1°C. Rat food was provided by Oriental Yeast (Tokyo, Japan). In the control group (A), rats were fed a standard diet for 6 weeks, and given tap water freely. In the low-calcium diet group (B), rats were given a low-calcium diet (calcium content 30% of standard diet) and distilled water for 6 weeks. In the low-calcium plus standard diet group (C), rats were given a low-calcium diet and distilled water freely for 3 weeks, and then switched to a standard diet and tap water for the next 3 weeks. In the low-calcium diet plus standard diet with supplementary GSPE group (D), rats were fed a low-calcium diet and given distilled water freely for 3 weeks, and then switched to a standard diet with supplementary GSPE and tap water for the next 3 weeks. The components of the diets are presented in Table 1 (each diet mixture was prepared in our laboratory). After being fed for 6 weeks, the rats were killed using thiopental sodium (Ravonal; Tanabe, Osaka, Japan) under deep anesthesia with diethyl ether. The mandibles were removed and separated into two parts, left and right, and then fixed in 10% neutral buffered formalin.

All procedures were approved by the committee for the use of laboratory animals of Kyushu Dental College, Japan.

Body weight

Body weight was recorded once each week.

Table 1 Composition of experimental diets (%)

Ingredients	*Standard diet (Ca 480 mg 100 g^{-1})*	*Low-calcium diet (Ca 144 mg 100 g^{-1})*
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[a]	6.00[b]
Granulated sugar	5.00	5.00
Vitamin mixture	2.00	2.00
$CaCO_3$	0.00	0.36
Total	100.00	100.00

Standard diet (100.00) with supplementary GSPE (0.003) = 100.003.
[a]Mineral mixture of standard diet (g 100 g^{-1}): NaCl, 4.66 g; KI, 0.01 g; KH_2PO_4, 25.72 g; NaH_2PO_4, 9.35 g; $MgSO_4$, 7.17 g; $CaHPO_4$, 14.56 g; Fe-citrate, 3.18 g; $MnSO_4 \cdot 4$–$5H_2O$, 0.12 g; $CuSO_4 \cdot 5H_2O$, 0.03 g; $ZnCO_3$, 0.11 g; Ca-lactate, 35.09 g.
[b]Mineral mixture of low-calcium diet (g 100 g^{-1}): NaCl, 4.680 g; KI, 0.0055 g; KH_2PO_4, 28.333 g; NaH_2PO_4, 9.380 g; K_2HPO_4, 9.550 g; $MgSO_4$, 7.187 g; Fe-citrate, 3.187 g; $MnSO_4 \cdot 4$–$5H_2O$, 0.12817 g; $CuSO_4 \cdot 5H_2O$, 0.03275 g; $ZnCl_2$, 0.10425 g.

Bone density, cross-sectional area and bone mineral content

For the pQCT (XCT Research SA model; Stratec-Medizintechnik GmbH, Pforzheim, Germany) examinations, the bone samples were centrally located between the scanner unit source and the detector with the aid of a support, to produce a scout-view and a tomographic scan, which were displayed on a monitor (Figure 1). Each mandibular bone was scanned around the center of the mandibular first molar mesial root at three different positions with intervals of 0.1 mm. Three slices with a voxel size of 0.08 mm and height of 0.26 mm, each consisting of trabecular and cortical components, were measured. The trabecular region was defined manually. Using this procedure, we measured trabecular bone density (mg cm^{-3}), trabecular cross-sectional area (mm^2), and trabecular bone mineral content (mg mm^{-1}). The cortical region was determined at contour mode 2, cortical mode 1, and peel mode 2 using a threshold value of 690 mg cm^{-3}, which measured the cortical bone density (mg cm^{-3}), cortical bone cross-sectional area (mm^2), and cortical bone mineral content (mg mm^{-1}).

Bone strength (non-invasive)

Stress–strain index (SSI) was measured by using pQCT as a non-invasive assessment of mechanical properties at a contour mode 2, cortical mode 1, and peel mode 2 using a threshold of 464 mg cm^{-3}. The SSI equation was: $SSI = CBD \cdot Z/NCBD$, where CBD is the cortical bone density (mg cm^{-3}), Z the section modulus (m^3), and NCBD is the normal value of cortical bone density (1200 mg cm^{-3}).

Statistical analysis

Data are expressed as mean ± s.d. for the effect of GSPE. Statistical differences were analyzed using Student's *t*-test.

Results

Body weight

All groups gained weight during the study period. The average initial body weight was 114.05 ± 2.10, 112.13 ± 1.18, 113.04 ± 2.21 and 114.23 ± 1.23 g, final body weight was 385.71 ± 50.03, 386.2 ± 24.82, 396.31 ± 29.61 and 389.62 ± 30.09 g in groups A, B, C, and D, respectively. There were no significant differences in body weight among the four groups (Figure 2).

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

Results of the measurements of bone density, cross-sectional area and bone mineral content of cortical, and trabecular bone in the mandible are summarized in Table 2.

Trabecular bone density and trabecular bone mineral content in group D were significantly higher than group C ($P < 0.05$).

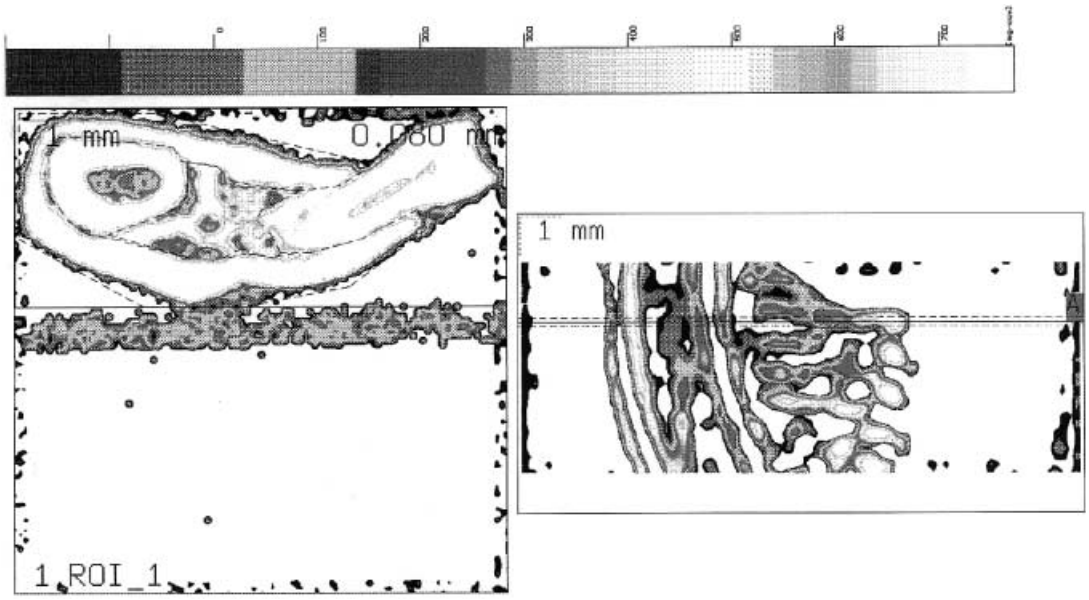

Figure 1 pQCT slices. Representative bone slice from tomographic scanning (left); mandibular bones of Wistar rats were scanned around the center of the mandibular first molar mesial root at three different positions with an interval of 0.1 mm (right)

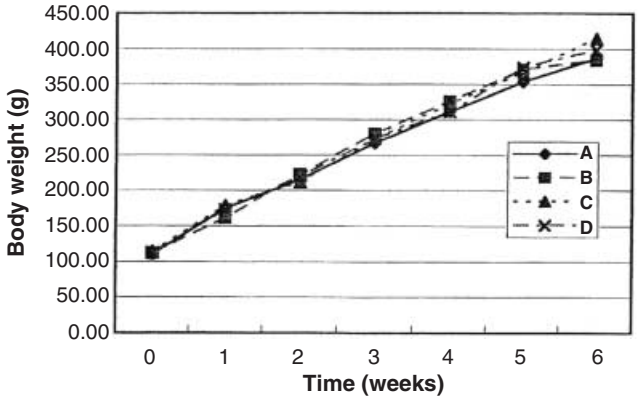

Figure 2 Body weight during the study period. There were no significant differences in body weight among the four groups. A, control group; B, low-calcium diet group; C, low-calcium diet plus standard diet group; D, low-calcium diet plus standard diet with supplementary GSPE group

Further, cortical bone density, cortical bone cross-sectional area, and cortical bone mineral content in group D were also significantly higher than in group C ($P < 0.05$, $P < 0.01$, $P < 0.05$, respectively).

Bone strength (non-invasive)

Stress–strain index to the reference axis x (xSSI), axis y (ySSI) and to the axis z (pSSI) are summarized in Table 3.

The values of xSSI and ySSI for group D were significantly greater than group C ($P < 0.05$).

Discussion

Peak bone mass, which is obtained during childhood and the adolescent growth, is one of the major risk determinants for developing osteoporosis and fracture. In many studies, rats fed a low-calcium diet rat have been used as an animal model for osteoporosis. In the present study, a remarkable decrease in trabecular and cortical bone density in low-calcium diet rats resulted in a condition of bone debility.

Grape seed proanthocyanidins extract is a flavonoid derivative of *Vitis vinifera* (Castillo *et al*, 2000) and proanthocyanidins are oligomers of monomeric flavan-3-ol units that are partially metabolized to lactones and phenolic acids by intestinal microflora. These metabolites are absorbed through the intestinal lumen. In

Table 2 Bone density, cross-sectional area and bone mineral content of mandibular Wistar rats

	Control group	*Low-calcium diet group*	*Low-calcium diet plus standard diet group*	*Low-calcium diet plus standard diet with supplementary GSPE group*
Trabecular bone density (mg cm^{-3})	418.74 ± 85.54	212.67 ± 40.74**	322.93 ± 65.19**	376.48 ± 63.85
Trabecular bone cross-sectional area (mm^2)	2.22 ± 0.23	2.20 ± 0.31	2.15 ± 0.19	2.20 ± 0.22
Trabecular bone mineral content (mg mm^{-1})	0.86 ± 0.14	0.57 ± 0.08**	0.80 ± 0.14	0.94 ± 0.17
Cortical bone density (mg cm^{-3})	1201.26 ± 65.17	1142.92 ± 35.94**	1178.78 ± 15.76	1190.38 ± 15.92
Cortical bone cross-sectional area (mm^2)	3.90 ± 0.63	3.39 ± 0.35**	3.64 ± 0.37	3.99 ± 0.30
Cortical bone mineral content (mg mm^{-1})	4.72 ± 0.95	4.00 ± 0.65*	4.33 ± 0.74	4.74 ± 0.21

Data are given as mean ± s.d. Compared with control group: *$P < 0.05$, **$P < 0.01$.

Table 3 Stress–strain index of mandibular Wistar rats

	Control group	*Low-calcium diet group*	*Low-calcium diet plus standard diet group*	*Low-calcium diet plus standard diet with supplementary GSPE group*
pSSI	3.95 ± 0.47	3.34 ± 0.71**	3.48 ± 0.50*	3.88 ± 0.63
xSSI	1.80 ± 0.32	1.52 ± 0.11**	1.75 ± 0.18	1.93 ± 0.23
ySSI	3.14 ± 0.44	2.73 ± 0.50*	2.75 ± 0.39*	3.18 ± 0.54

Data are given as mean ± s.d. Compared with control group: *$P < 0.05$, **$P < 0.01$. pSSI, stress–strain index to the reference axis *z*; xSSI, stress–strain index to the reference axis *x*; ySSI, stress–strain index to the reference axis *y*.

previous studies, GSPE appeared to increase the insoluble fraction of the diet (Wren *et al*, 2002) and also protect gastric mucosa (Ray *et al*, 2001), while it is considered a potential health-food ingredient because of these beneficial properties (Nakamura and Tonogai, 2002), and does not induce any significant toxicological effects (Wren *et al*, 2002). Niacin-bound chromium and GSPE mixture (Preuss *et al*, 2000), and an analgesic, pancreatic enzyme and GSPE mixture (Banerjee and Bagchi, 2001), have been reported.

Effective prevention strategies are considered helpful to preserve the bone structure, and genetic potential, gender, ethnic origin, and lifestyle factors including nutrition, growth patterns, and physical activity each have an influence on the accretion of bone mineral content during childhood and help to determine peak bone mass. Lifestyle factors such as dietary calcium are modifiable (Recker *et al*, 1992; Miller and Maropis, 1998), and a dietary mixture of calcium and GSPE was administered in the present experiment as a pair-dietary experiment, in which the effects of a mixture of calcium and GSPE on the mandible were assessed in rats with experimentally debilitated bone. In our study, there were no significant differences in body weight among the four groups. This result is similar to that previously reported, which found a significant increase in food consumption without an increases in body weight (Wren *et al*, 2002).

An estrogen-deficient condition, in a postmenopausal osteoporosis rat model, did not have an effect on the bone mineral density of the mandibles (Elovic *et al*, 1995). However, a recent study by Kuroda *et al* (2003) reported that the alveolar and condylar regions of the mandible were clearly affected by exposure to estrogen-deficient conditions, while, a decrease in trabecular bone mineral density of the mandibular bone was detectable, but low, in the molar region, and no difference was seen in cortical bone mineral density. In the present study, the low-calcium diet group model for osteoporosis had cortical bone density that was significantly lower than the control group. Further, bone formation was promoted by GSPE administration, in which there was a significant increase in cortical bone. Cortical bone response is thought to have a relationship with mechanical stimulus (Hagino *et al*, 2001) and functional occlusion has been shown to be crucial in maintaining the structure of the mandible (Spyropoulos and Tsolakis, 1997; Marker *et al*, 2000).

Trabecular bone density, an indicator of apparent bone density, and trabecular bone mineral content, an indicator of bone mass, in the group D reached the level of the control group and then showed improvement. Further, change in trabecular bone density occurred faster than in cortical bone density in those rats. The same result was reported by Gasser (1995), as changes in cortical bone density were much slower than in trabecular bone density. The GSPE compound caused an increase in trabecular bone mineral content value, indicating bone formation, and showing a direct effect of GSPE toward bone condition.

Khanna *et al* (2001) reported that proanthocyanidins facilitate angiogenesis in wound healing. Although the relation of neovascularization to bone formation is well known in ipriflavone-treated bone (Martini *et al*, 1998), the angiogenesis effect of GSPE on bone has not been established. In the present study, the trabecular bone cross-sectional area in each group was not significantly different, however, for a proper biomechanical estimation of bone quality, trabecular bone cross-sectional area is not directly suitable (Ferretti, 2000) while, according to Jamsa *et al* (1998), mechanical tests and pQCT measurements are relevant in biomechanical studies on mouse bones. Further, the elastic modulus of the material of which the structure is made can only be assessed mechanically, however it can be reasonably estimated by apparent mineral density (Ferretti, 2000). In contrast, van der Meulen *et al* (2001) stated that there is no alternative to testing whole bone strength based solely on geometry or bone mineral content. In the present study, the xSSI and ySSI parameters revealed that GSPE-treated bone strength increased linearly with bone mass. Recently, the effectiveness of ipriflavone on osteoblast and osteoclast cells has been clarified (Notoya *et al*, 1993; Benvenuti *et al*, 1994), and in the near future, we hope to clarify the effectiveness of GSPE on these cells in an *in-vitro* examination.

In conclusion, we found that GSPE induced bone formation in mandibular bones of low-calcium diet rats, suggesting a potential therapeutic application of this compound for the treatment of bone debility.

References

Banerjee B, Bagchi D (2001). Beneficial effects of a novel IH636 grape seed proanthocyanidin extract in the treatment of chronic pancreatitis. *Digestion* **63:** 203–206.

Benvenuti S, Petilli M, Frediani U (1994). Binding and bioeffects of Ipriflavone on a human preosteoclastic cell line. *Biochem Biophys Res Commun* **201:** 1084–1089.

Castillo J, Benavente-Garcia O, Lorente J *et al* (2000). Antioxidant activity and radioprotective effects against chromosomal damage induced in vivo by X-rays of flavan-3-ols (Procyanidins) from grape seeds (*Vitis vinifera*): comparative study versus other phenolic and organic compounds. *J Agric Food Chem* **48:** 1738–1745.

Elovic RP, Hipp JA, Hayes WC (1995). Ovariectomy decreases the bone area fraction of the rat mandible. *Calcif Tissue Int* **56:** 305–310.

Ferretti JL (2000) Peripheral quantitative computed tomography for evaluating structural and mechanical properties of small bone. In: Yuehuei HA, Draughn RA, eds. *Mechanical testing of bone and the bone-implant interface*. CRC Press: Washington, DC, pp. 393–395.

Fujita T. (1996). Clinical guidelines for the treatment of osteoporosis in Japan. *Calcif Tissue Int* **59:** S34–S37.

Fujita T, Fujii Y, Miyauchi A, Takagi Y (1999). Comparison of antiresorptive activities of ipriflavone, an isoflavone derivative, and elcatonin, an eel carbocalcitonin. *J Bone Miner Metab* **17:** 289–295.

Gasser JA (1995). Assessing bone quality by pQCT. *Bone* **17:** 145S–154S.

Gennari C, Agnusdei D, Crepaldi G *et al* (1998). Effect of ipriflavone-a synthetic derivative of natural isoflavones on bone mass loss in the early years after menopause. *Menopause Spring* **5:** 9–15.

Hagino H, Okano T, Akhter MP, Enokida M, Teshima R (2001). Effect of parathyroid hormone on cortical bone response to in vivo external loading of the rat tibia. *J Bone Miner Metab* **19:** 244–250.

Jamsa T, Jalovaara P, Peng Z, Vaananen HK, Tuukanen J (1998). Comparison of three-point bending test and peripheral quantitative computed tomography analysis in the evaluation of the strength of mouse femur and tibia. *Bone* **33:** 155–161.

Khanna S, Roy S, Bagchi D, Bagchi M, Sen CK (2001). Upregulation of oxidant-induced VEGF expression in cultured keratinocytes by a grape seed proanthocyanidin extract. *Free Radic Biol Med* **31:** 38–42.

Kimura M, Nishida I, Xia B, Maki K, Tofani I (2002). Ultrastructural study on the effect of dietary therapy on bone debility in developing rats – on the enchondral ossification. *Dentistry in Japan* **38:** 109–116.

Kosasi S, Hart LA, van Dijk H *et al* (1989). Inhibitory activity of Jatropha multifida latex on classical complement pathway activity in human serum mediated by a calcium-binding proanthocyanidin. *J Ethnopharmacol* **27:** 81–89.

Kuroda S, Mukohyama H, Kondo H *et al* (2003). Bone mineral density of the mandible in ovariectomized rats: analyses using dual energy X-ray absorptiometry and peripheral quantitative computed tomography. *Oral Dis* **9:** 24–28.

Marker P, Nielsen A, Lehmann BH (2000). Fractures of the mandibular condyle. Part 1: Patterns of distribution of types and causes of fractures in 348 patients. *Br J Oral Maxillofac Surg* **38:** 417–421.

Martini M, Formigli L, Tonelli P *et al* (1998). Effects of ipriflavone on perialveolar bone formation. *Calcif Tissue Int* **63:** 312–319.

van der Meulen MCH, Jepsen KJ, Mikic B (2001). Understanding bone strength: size isn't everything. *Bone* **29:** 101–104.

Miller EC, Maropis CG (1998). Nutrition and diet-related problems. *Prim Care* **25:** 193–210.

Nakamura Y, Tonogai Y (2002). Effects of grape seed polyphenols on serum and hepatic lipid contents and fecal steroid excretion in normal and hypercholesterolemic rats. *J Health Sci* **48:** 570–578.

Notoya K, Yoshida K, Takeomi S (1993). Ipriflavone on osteoclast mediated bone resorption and new osteoclast formation in long-term cultures of mouse unfractionated bone cells. *Calcif Tissue Int* **53:** 206–209.

Preuss HG, Wallerstedt D, Talpur N *et al* (2000). Effects of niacin-bound chromium and grape seed proanthocyanidin extract on the lipid profile of hypercholesterolemic subjects: a pilot study. *J Med* **31:** 227–246.

Ray S, Bagchi D, Lim PM *et al* (2001). Acute and long-term safety evaluation of a novel IH636 grape seed proanthocyanidin extract. *Res Commun Mol Pathol Pharmacol* **109:** 165–197.

Recker RR, Davies KM, Hinders SM, Heaney RP, Stegman MR, Kimmel DB (1992). Bone gain in young adult women. *JAMA* **268:** 2403–2408.

Ross JA, Kasum CM (2002). Dietary flavonoids. Bioavailability, metabolic effects, and safety. *Annu Rev Nutr* **22:** 19–34.

Sato M, Bagchi D, Tosaki A, Das DK (2001). Grape seed proanthocyanidin reduces cardiomyocyte apoptosis by inhibiting ischemia/reperfusion-induced activation of JNK-1 and C-JUN. *Free Radic Biol Med* **31:** 729–737.

Spyropoulos MN, Tsolakis AI (1997). Altered mandibular function and prevention of skeletal asymmetries after unilateral condylectomy in rats. *Eur J Orthod* **19:** 211–218.

Wren AF, Cleary M, Frantz C, Melton S, Norris L (2002). 90-Day oral toxicity study of a grape seed extract (IH636) in rats. *J Agric Food Chem* **50:** 2180–2192.

Ye X, Krohn RL, Liu W *et al* (1999). The cytotoxic effects of a novel IH636 grape seed proanthocyanidin extract on cultured human cancer cells. *Mol Cell Biochem* **196:** 99–108.