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# Hesperidin inhibits ovariectomized-induced osteopenia and shows differential effects on bone mass and strength in young and adult intact rats

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<sup>1</sup>Institut National de la Recherche Agronomique Clermont-Ferrand/Theix, Human Nutrition Unit UMR 1019, St-Genès Champanelle, France; <sup>2</sup>Centro de Edafología y Biología Aplicada del Segura-Consejo Superior de Investigaciones Científicas (CEBAS-CSIC), Departamento de Ciencia y Tecnología de Alimentos, Espinardo, Spain; <sup>3</sup>Department of Nutrition and Dietetics, Faculty of Allied Health Sciences, National University of Malaysia, Jalan Raja Muda Abdul Aziz, Kuala Lumpur, Malaysia; <sup>4</sup>Nestlé Research Center, Nutrition and Health Department, Lausanne, Switzerland

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Horcajada MN, Habauzit V, Trzeciakiewicz A, Morand C, Gil-Izquierdo A, Mardon J, Lebecque P, Davicco MJ, Chee WS, Coxam V, Offord E. Hesperidin inhibits ovariectomized-induced osteopenia and shows differential effects on bone mass and strength in young and adult intact rats. J Appl Physiol 104: 648-654, 2008. First published January 3, 2008; doi:10.1152/japplphysiol.00441.2007.-The main aim of this study was to investigate the bone-sparing effect of hesperidin, one of the main flavonoid present in oranges, in two age groups of ovariectomized female rats, compared with their intact controls. Young (3 mo) and adult (6 mo) female Wistar rats were sham operated (SH) or ovariectomized (OVX) and then pair-fed for 90 days a casein-based diet supplemented or not with 0.5% hesperidin (Hp; n = 10/group). In older rats, Hp intake led to a partial inhibition of OVX-induced bone loss, whereas a complete inhibition was obtained in younger animals. At both ages, while plasma osteocalcin concentrations were unchanged, urinary excretion of deoxypyridinoline was reduced by Hp intake, suggesting that Hp was able to slow down bone resorption. Unexpectedly, in intact young rats, Hp consumption resulted in a significant increase in bone mineral density (BMD). Indeed, 6-mo-old HpSH rats had a similar BMD to 9-mo-old nontreated SH adult rats, suggesting an accelerated bone mass gain in the young rats. In contrast, in intact adult rats, Hp did not further increase BMD but did improve their bone strength. The results of this study show a protective effect of Hp on bone loss in OVX rats of both ages without uterine stimulation and accompanied by a lipid-lowering effect. The unexpected and intriguing findings obtained in intact rats showing improved BMD in young rats and improved femoral load in adult rats merit further investigation. The bone and lipid benefits of hesperidin make it an attractive dietary agent for the management of the health of postmenopausal women.

bone growth; bone sparing effect; bone mineral density; polyphenols

A NUMBER OF POPULATION-BASED studies support a positive link between high consumption of fruit and vegetables and improved bone mineral density, an effect postulated to be due to their acid-base buffering capacity, although not yet proven in human intervention studies (26). Experimental rodent studies have shown positive effects on bone with individual polyphenolic components of fruits and vegetables such as quercetin and rutin (a quercetin glycoside) from onions (15, 24), resveratrol from red wine (18), and isoflavones from soy (36) that inhibit ovariectomy-induced bone loss in rats by inhibition of bone resorption, independent of the acid-base composition (23, 24). Some human intervention studies with high levels of soy isoflavones (80–90 mg/day) have shown prevention of bone loss in postmenopausal women (5, 30), whereas others have not (4, 21). Nevertheless, in practice, consumption of soy products is relatively low in Western countries compared with the Asian population, whose average daily intake is 20–40 mg/day (34).

Hesperidin is a monomethoxylated flavanone very abundant in citrus fruits such as oranges (13) and highly consumed in Western countries. For example, in Finland, consumption of hesperidin was estimated to be 28.3 mg/day and contributed to 50% of total intake of flavonoids (17). The structure of hesperidin corresponds to hesperetin (4'-methoxy-3',5,7-trihydroxyflavanone) bound to rutinose (composed of one molecule of rhamnose and one of glucose) in the  $C_7$  position (14). Hesperetin is absorbed in the colon following release of the rutinose moiety of hesperidin by the gut microflora (20). Human bioavailability studies have shown that flavanones metabolites reached a peak between 5 and 7 h postingestion (20, 27). In the study of Manach et al. (20), the peak plasma concentration of hesperetin was found to be close to  $1.3 \ \mu M$ following consumption of 1 liter of orange juice, which provided  $\sim$ 450 mg of hesperidin. In the study of Nielsen et al. (27), in which healthy volunteers consumed hesperetin equivalents supplied as fortified orange juice (corresponding to an ingested dose of  $192 \pm 30$  mg), the peak plasma concentration of hesperetin was  $1.05 \pm 0.25 \ \mu$ M.

Hesperetin and metabolites have been shown to exert several biological activities in preclinical studies, including antioxidant, anti-inflammatory, analgesic, vasorelaxing, antineoplastic, and lipid-lowering effects (10, 11, 22). In terms of bone health, hesperidin has been shown to inhibit bone loss in 2-mo-old ovariectomized mice following consumption of 0.5% hesperidin in the diet (6) and to prevent bone loss in male orchidectomized rats following citrus juice consumption (8).

In the work presented here, we primarily investigated the ability of hesperidin to prevent ovariectomy-induced bone loss in two age groups of female rats (3-6 mo and 6-9 mo). The effect of this citrus flavanone on the sham-operated counterparts was also checked. As a secondary outcome, the lipid modulating effect of hesperidin was measured due to its relevance to postmenopausal women's health.

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#### MATERIALS AND METHODS

#### Animals and Diets

The study was carried out in accordance with the recommendations of the Ethical Committee of the French National Institute for Agronomical Research (Institut National de la Recherche Agronomique) and according to the current legislation on animal experimentation in France (order no. 87-848 modified in 2001). Two groups of 40 young 3-mo-old ( $266.2 \pm 1.2$  g) and adult 6-mo-old ( $312.5 \pm 3.2$  g) virgin female Wistar rats were purchased from INRA (Institut National de la Recherche Agronomique, Clermont-Ferrand/Theix, France). Half of the rats in each group were ovariectomized (OVX) and the other half were sham operated (SH) under anesthesia using chloral hydrate (Fluka Chemie, Buchs, Switzerland; 80 g/l in saline solution; 0.4 ml/100 g body wt ip). The animals were housed individually in plastic cages allowing separation and collection of urine and feces at 21°C with relative humidity of 55% and under a 12:12-h light/dark cycle.

After an adaptation period of 1 wk with a semipurified standard diet devoid of any soy proteins (which were replaced by casein; INRA, Jouy-en-Josas, France), the animals were fed the same diet, which contains 0.4% calcium (Ca) and 0.3% phosporus (P), but supplemented or not with hesperidin. Hesperidin (from Sigma, L'Isle d'Abeau, Chesnes, France) was added to the diet (5 g/kg of diet) of 20 sham-operated (HpSH) and 20 ovariectomized rats (HpOVX; Table 1). Diets were prepared every week and stored at 4°C. To prevent hyperphagia induced by castration, the rats were pair-fed. The animals had free access to water during the entire experiment, and their body weights and food intake were measured weekly. The daily mean consumption within each group and during the whole experimental period (90 days) was 17.9  $\pm$  0.14 g. Urine of each animal was collected over a 24-h period on day 0 and the day before slaughtering to measure urinary excretion of deoxypyridinoline (DPD), a marker for bone resorption (32).

Forty-eight hours before death, the body composition was estimated by dual energy X-ray absorptiometry (DEXA; 33). At necropsy, on *day 90*, blood samples were collected into ice-cooled heparinized plastic tubes containing 200 peptidase inhibitory units of aprotinin (Iniprol, Choay, Paris, France) per milliliter blood, and centrifuged immediately (3,500 g for 5 min at 4°C). Then plasmas were frozen at  $-20^{\circ}$ C until measurements of osteocalcin, a marker of osteoblastic activity (12). Previously, plasma dedicated to hesperetin (the aglycone form of hesperidin) detection was acidified with acetic acid (0.1 M, 1%). Uterine horns were removed and immediately weighed. Femurs were separated from adjacent tissue, cleaned, and used for physical measurements (mechanical testing and bone mineral density).

#### Physical Analysis

Whole body composition. Rats were anesthetized by intraperitoneal injection of chloral hydrate (80 g/l in saline solution; 0.4 ml/100 g

#### Table 1. Composition of diets

	Control Diet	Hesperidin Diet		
Casein	15	15		
Rapeseed oil	5	5		
Wheat starch	74.0	73.5		
Calcium phosphate (2H <sub>2</sub> O)	1.68	1.68		
Sodium chloride	0.65	0.65		
Potassium citrate	1.14	1.14		
Magnesium sulfate	0.5	0.5		
Trace elements mix	1	1		
Vitamin mix	1	1		
Hesperidin	_	0.5		

Values are shown as g/100 g dry food.

body wt). They were scanned with DEXA using Hologic ODR-4500

*Bone mineral density.* Bone mineral density (BMD) was assessed by DEXA, with the Hologic QDR-4500 A X-ray bone densitometer. The total right femur BMD (T-BMD), as well as the BMD of two subregions, one corresponding to the distal femur metaphyseal zone (M-BMD), rich in cancellous bone, and the other to the diaphyseal zone (D-BMD), rich in cortical bone, were determined (28). In each femur scan, distal and proximal metaphyseal regions of interest were positioned at the same distance from the distal and proximal femur extremity, respectively, and with the same height. The region of interest between the two preliminary delimited metaphyseal zones corresponds to the diaphyseal subregion. Results are given in grams per square centimeter.

*Femoral mechanical testing.* Immediately after collection in NaCl (9 g/l), the length of the right femur and the mean diameter of the diaphysis were measured using a precision caliper (Mitutoyo, Shroppshire, UK). Because of the irregular shape of the femoral diaphysis, the diameter used in the calculation was the mean of the greatest and the smallest diaphysis diameters. Femoral failure load was determined using a three-point bending test. Each bone was secured on the two lower supports (diameter, 4 mm; separation, 20 mm) of the anvil of a Universal Testing Machine (Instron 4501; Instron, Canton, MA). An upper crosshead roller (diameter, 6 mm) was applied in front of the middle of the shaft of the bone and advanced at 0.5 mm/min. The load at rupture (Newtons) was automatically determined and recorded by Instron 4501 software. This test method was previously validated by using Plexiglas standard probes (38).

#### **Biochemical Analysis**

*Marker of osteoblastic activity.* Osteocalcin (OC) in plasma was measured by RIA, using rat <sup>125</sup>I-labeled OC, goat anti-rat OC antibody, and donkey anti-goat second antibody (Biochemical Technologies kit, Stoughton, MA). The sensitivity was 0.01 nmol/l. The intraand interassay precisions were 6.8 and 8.9%, respectively.

*Marker of bone resorption.* Deoxypyridinoline (DPD) was measured in urine by competitive RIA (Pyrilinks D kit; Metra Biosystems, Mountain View, CA). The assay requires a rat monoclonal antibody against DPD, which is coated to the inner surface of a polystyrene tube and <sup>125</sup>I-labeled DPD. In our experimental conditions, the sensitivity was 2 nmol/l, and the intra- and interassay variation was 4 and 6%, respectively. Results are expressed as nanomoles DPD per millimole creatinine to take into account interindividual differences of urine concentration (32). The creatinine assay (kit BioMérieux, Marcy l'Etoile, France) is based on a modified Jaffé's method in which picric acid forms a yellow compound with creatinine presence (7).

*Plasma lipids.* Plasma total cholesterol and plasma triglycerides concentrations were enzymatically determined using ready-to-use kits purchased from BioMérieux (Marcy l'Etoile, France). After incubation with reagents during 10 min at room temperature, the intensity of the coloration, which is proportional to the cholesterol or triglycerides content, was measured at 490 nm.

*Plasma hesperetin levels.* Plasma samples (180 μl) were acidified with acetic acid to pH 4.9 and incubated 5 h at 37°C in the presence of 1,000 units of β-glucuronidase and 45 units of sulphatase (from helix pomatia, Sigma G0876, L'Isle d'Abeau, Chesnes, France). Samples were then mixed with 4 volumes of methanol:HCl (200 mmol/l) and centrifuged 5 min at 12,500 g. The supernatant was analyzed by HPLC. HPLC analysis was performed using a system consisting of two pumps (model 580, ESA) for high pressure gradient, a temperature-controlled autosampler (Gilson, Villiers-le-Bel, France), a 150×4.6 mm Hypersil BDS C<sub>18</sub>-5µ column (Touzard et Matignon, Les Ulis, France), a thermostatic chamber and eight-channel Coull-Array detector (model 5600, Eurosep, Cergy, France). Mobile phases consisted of a 30 mmol/l NaH<sub>3</sub>PO<sub>4</sub> buffer (pH 3) containing 20%

acetonitrile (A) or 40% acetonitrile (B) (37). Separation was achieved using a gradient elution ( $35^{\circ}$ C, 0.8 ml/min): 0–3 min, 100% A; 3–30 min, linear gradient from 100% A to 100% B; 30–35 min, 100% B; 35.01–45 min, 100% A. Potentials were set at 50-350-480-550-700-760-820-850 mV (Pd as reference). Hesperetin was quantified using the sums of height obtained on the electrodes 2, 3 and 4, 5, 6, respectively (20).

#### Statistical Analysis

Results are expressed as means  $\pm$  SE. All data were analyzed using XLSTAT software (Addinsoft, Paris, France). The femoral BMD and biomechanical variables were subjected to a three-way analysis of covariance with body weight as the covariate. Other parameters were analyzed using a three-way ANOVA, testing for any difference among groups. Thus the main effects assessed were age, dietary treatment (Hp or control), and operation (SH or OVX). If a result was found significant (P < 0.05), the Student-Newman-Keul's multiple comparisons test was used to determine the specific differences between means. Parametric ANOVA was performed when data were normally distributed with equal variance. If not, nonparametric methods were selected. Thus a Kruskall-Wallis test was first performed. If it indicated a significant difference among groups (P < 0.05), the Mann-Whitney U test (with Bonferroni adjustment to account for multiple comparisons) was used to determine specific differences. The level of significance was set at P < 0.05 for all statistical tests.

#### RESULTS

#### Uterine Weight and Body Composition

The mean uterine weight was lower in all the ovariectomized groups compared with sham-operated groups (P < 0.01) at both ages of rats. Thus accurate ovariectomy was confirmed by a marked atrophy of uterine horns. In our experimental conditions, uterine weight was unaffected by hesperidin treatment (Table 2).

The animals continued growing throughout the experimental period, and the same pattern of body weight evolution was observed in each age group between *day 0* and *day 90*. At baseline (*D0*), 6-mo-old rats had significantly higher body weight (P < 0.05) than 3-mo-old animals and this was maintained until the end of the study (*D90*). Despite pair-feeding, the ovariectomized groups had a significantly (P < 0.01) higher body weight than sham-operated groups for both the young and older rats. Hesperidin treatment did not influence the body weights of the animals.

As observed for total body weight, the mean percentage of fat mass measured in the older animals was significantly higher (P < 0.01) than in the younger ones and hesperidin consumption had no effect on this parameter (Table 2).

#### BMD

In the ovariectomized control groups at both ages, ovariectomy induced a significant decrease in total BMD (T-BMD: -5.2%, P < 0.05 in 6 mo old; -11%, P < 0.01 in 9 mo old) as well as in metaphyseal BMD (M-BMD: -6.2%, P < 0.05 in 6 mo old; -13.3%, P < 0.01 in 9 mo old). Diaphyseal BMD was unaffected by ovariectomy in 6-mo-old animals but was significantly decreased in 9-mo-old animals (D-BMD for OVX vs. SH: -6.6%, P < 0.01; Fig. 1).

Total and metaphyseal bone loss was totally inhibited in 6-mo-old ovariectomized rats fed with hesperidin (HpOVX vs. OVX: +14.9% T-BMD, +10.7% M-BMD; P < 0.01), whereas a more partial inhibition was observed in 9-mo-old rats (HpOVX vs. OVX: +5.6% T-BMD, +7.6% M-BMD; P < 0.05; Fig. 1, *A* and *B*). Diaphyseal BMD, although not reduced by ovariectomy in 6-mo-old rats, was nevertheless improved by hesperidin consumption (HpOVX vs. OVX: +12.7%; P < 0.01). Moreover, there appears to be a protection by hesperidin of D-BMD loss in the 9-mo-old ovariectomized rats (HpOVX vs. OVX: +6.9%; P < 0.01; Fig. 1*C*).

Unexpectedly, in the sham-operated group of 6-mo-old rats, BMD was significantly improved at all sites (total, metaphyseal, and diaphyseal) by hesperidin consumption (HpSH vs. SH: T-BMD +10.6%, M-BMD +11.5%, D-BMD +11.8%; P < 0.01; Fig. 1).

#### Bone Size and Ultimate Load

The length (mm) and diameter (mm) of the femur were similar within groups and between ages of animals (Table 2).

Femoral strength was assessed and reported as femoral failure load (Table 2). Ovariectomy significantly decreased this parameter in 9-mo-old control animals (-12.9%; P < 0.05) but not in 6-mo-old rats. Consumption of hesperidin totally inhibited the ovariectomy-induced loss of femoral strength in 9-mo-old animals (HpOVX vs. OVX: +24.3%; P < 0.01) and also improved femoral strength in 6-mo-old rats (HpOVX vs. OVX rats: +9.9%; P < 0.05).

In the sham-operated groups, hesperidin consumption significantly enhanced femoral strength in 9-mo-old animals (HpSH vs. SH: + 23.6%; P < 0.01), whereas no significant increases in femoral strength was observed in intact 6-mo-old animals (Table 2).

#### Bone Turnover

On *day 90*, no differences in plasma OC (ng/ml) levels were observed within 6-mo-old groups with the exception of ovari-

Table 2. Uterine weight, body composition, and femoral ultimate load

	6 mo old			9 mo old				
	SH	OVX	HpSH	HpOVX	SH	OVX	HpSH	HpOVX
Uterus wt, mg	1031.9±82.3	146.6±9.1 <sup>a</sup>	787±67.3	238±22.3ª	607.1±21.1	108.6±3.3 <sup>a</sup>	636.3±37.2	101.5±4.5 <sup>a</sup>
Body wt, g	326±9 <sup>b</sup> *	$363 \pm 7^{a*}$	$335 \pm 15^{b*}$	406±12 <sup>a</sup> *	$401 \pm 11^{b}$	$414 \pm 12^{a}$	$382 \pm 12^{b}$	$423 \pm 7^{a}$
Fat mass, %total body wt	$23.2 \pm 1.1 \ddagger$	27.7±1.6†	25.7±4.4†	$27.7 \pm 1.1 \ddagger$	$33.5 \pm 2.2$	$35.5 \pm 1.6$	$32.7 \pm 1.9$	$34.7 \pm 1.6$
Length, mm	$36.22 \pm 0.36$	$36.35 \pm 0.31$	$35.97 \pm 0.30$	$36.46 \pm 0.30$	$36.87 \pm 0.27$	$36.67 \pm 0.27$	$37.25 \pm 0.26$	$37.30 \pm 0.20$
Diameter, mm	$3.58 \pm 0.06$	$3.48 \pm 0.05$	$3.61 \pm 0.04$	$3.71 \pm 0.05$	$3.74 \pm 0.07$	$3.57 \pm 0.05$	$3.78 \pm 0.06$	$3.80 \pm 0.06$
Ultimate load, N	$109.5 \pm 3.3*$	$102.2 \pm 2.9$	106.3±5.3†	$112.4 \pm 3.4^{h*}$	$116.3 \pm 4.3$	$101.2 \pm 2.9^{cdg}$	$143.8 \pm 6.7^{\mathrm{a}}$	$125.8 \pm 7.4$

Values are means  $\pm$  SE. Uterus weight, body weight, fat mass, femoral length, diameter, and ultimate load measured on *day 90* (D90) in 6- and 9 mo-old animals sham-operated (SH) or ovariectomized (OVX) receiving a standard diet or supplemented with 0.5% hesperidin (HpSH and HpOVX). <sup>a</sup>P < 0.01 vs. SH; <sup>b</sup>P < 0.01 vs. OVX; <sup>c</sup>P < 0.05 vs. SH; <sup>d</sup>P < 0.01 vs. HpSH; <sup>g</sup>P < 0.01 vs. HpOVX; <sup>h</sup>P < 0.05 vs. OVX. \*P < 0.05 and †P < 0.01 vs. 9-mo-old rats.



Fig. 1. Total (T-BMD; *A*), metaphyseal (M-BMD; *B*), and diaphyseal (D-BMD; *C*), femoral density (g/cm<sup>2</sup>) measured on *day 90* (d90) in 6- and 9-mo-old animals sham-operated (SH) or ovariectomized (OVX) receiving a standard diet or supplemented with 0.5% hesperidin (HpSH and HpOVX). Means  $\pm$  SE. "*P* < 0.01 vs. SH; "*P* < 0.01 vs. OVX; "*P* < 0.05 vs. SH; "*P* < 0.01 vs. HpSH; "*P* < 0.05 vs. HpOVX; "*P* < 0.01 vs. HpSH; "*P* < 0.05 vs. HpOVX; "*P* < 0.01 vs. HpOVX.  $\pm P < 0.01$  vs. HpOVX; "*P* < 0.01 vs. HpOVX.  $\pm P < 0.01$  vs. HpOVX; "*P* < 0.01 vs. HpOVX.  $\pm P < 0.01$  vs. 9-mo-old rats.

ectomized rats fed hesperidin where a significantly higher concentration was measured compared with sham-operated rats (HpOVX: 38.5  $\pm$  2.6 vs. SH: 25.3  $\pm$  1.9; *P* < 0.05). In 9-mo-old animals, ovariectomy enhanced osteocalcin plasma levels in control rats (OVX: 26.6  $\pm$  2 vs. SH: 18.9  $\pm$  1.1; *P* < 0.05) but hesperidin consumption had no effect on this parameter (Table 3).

Urinary DPD excretion (Table 3) was increased in ovariectomized control rats in both ages (P < 0.01). Hesperidin consumption totally inhibited the ovariectomy-induced increase in DPD excretion in 6-mo-old rats (HpOVX vs. OVX: -38%; P < 0.01), whereas a partial inhibition was noted in 9-mo-old animals (HpOVX vs. OVX: -18%; P < 0.05). In intact animals, hesperidin consumption led to a reduction of

Table 3. Plasma osteocalcin, urinary deoxypyridinoline creatinin, total cholesterol, and triglycerides concentrations

		6 mo old			9 mo old			
	SH	OVX	Hp SH	Hp OVX	SH	OVX	Hp SH	Hp OVX
OC, ng/ml DPD, nmol/mmol creatinin TC, g/l TG, g/l	25.3±1.9* 121.8±8.8* 0.64±0.11 2.31±1.14	$33.1\pm2.8*$ 164.9±13.4 <sup>a*</sup> 0.76±0.04 1.77±0.99	$32.6 \pm 3.7^{*}$ $69.9 \pm 7.9^{ab*}$ $0.53 \pm 0.09^{c}$ $1.36 \pm 0.43^{c}$	$\begin{array}{c} 38.5 \pm 2.6^{\text{c}*} \\ 102 \pm 7.3^{\text{b}*} \\ 0.64 \pm 0.07^{\text{b}} \\ 1.24 \pm 0.37^{\text{h}} \end{array}$	$18.9 \pm 1 \\ 54.9 \pm 3.3^{b} \\ 0.68 \pm 0.12 \\ 2.64 \pm 0.75$	26.6±2° 103.3±11.5 0.77±0.08 1.67±0.61	$\begin{array}{c} 22.5 \pm 2.6 \\ 54.7 \pm 4.7^{\rm b} \\ 0.60 \pm 0.11^{\rm c} \\ 1.47 \pm 0.43^{\rm a} \end{array}$	$\begin{array}{c} 29.9 \pm 1.7^{c} \\ 88.8 \pm 4.5^{ch} \\ 0.69 \pm 0.09^{h} \\ 1.23 \pm 0.36^{h} \end{array}$

Values are means  $\pm$  SE. Plasma osteocalcin (OC, ng/ml), urinary deoxypyridinoline (DPD, nmol DPD/mmol creatinin), total cholesterol (TC, g/l), and triglycerides (TG, g/l) concentrations measured on *day 90* in 6- and 9-mo-old SH, OVX, HpSH, and HpOVX animals. <sup>a</sup>P < 0.01 vs. SH; <sup>b</sup>P < 0.01 vs. OVX; <sup>c</sup>P < 0.05 vs. SH; <sup>h</sup>P < 0.05 vs. OVX. \*P < 0.01 vs. 9-mo-old rats.

urinary DPD levels in 6-mo-old rats (HpSH vs. SH: -16%; P < 0.01) but had no effect in 9-mo-old rats.

#### Plasma Lipids and Hesperetin Concentrations

Plasma total cholesterol and triglycerides concentrations (Table 3) were significantly lower in the hesperidin-treated groups compared with the control groups (P < 0.05) at both ages.

Finally, the mean plasma concentration of hesperetin was  $3.57 \pm 0.68 \mu$ M in all rats consuming hesperidin, while it was not detectable in plasma of control rats.

#### DISCUSSION

In the present work, we investigated the effect of hesperidin, a citrus flavonoid, on bone mineral density and bone metabolism in ovariectomized rats of two ages (3-6 mo and 6-9 mo) compared with their sham-operated intact counterparts.

#### Characteristics of the Two Ages of Rats

In the main strains of laboratory rats, sexual maturity is observed between 4 and 9 mo. Thus we can consider both age groups of rats as "mature" regarding estrogen status. According to Schapira et al. (35), building of bone mass occurs throughout the first year of life in Wistar rats with peaks for parameters of bone formation and mineralization (bone protein content, alkaline phosphatase activity, BMD, bone calcium content) mostly attained by age of 8 mo. Thus we can consider that the younger rats (3–6 mo) still have the capacity to acquire bone mass (confirmed by comparison of total BMD of shamoperated rats from 3 to 6 mo, Fig. 1), whereas the older rats (6–9 mo) have achieved their peak bone mass (no difference in total BMD of sham-operated animals from 6 to 9 mo, Fig. 1).

As expected, ovariectomy, validated by uterine weight, induced a significant decrease in BMD in rats of both ages. Similar to what is observed in postmenopausal women, bone turnover was also increased in ovariectomized rats compared with sham-operated ones as indicated by a higher urinary DPD excretion and an increase in plasma levels of osteocalcin (31).

However, comparing the two ages of rats, ovariectomyinduced osteopenia was more severe in the 6- to 9-mo-old rats than in young 3- to 6-mo-old rats, leading to greater loss of trabecular and cortical bone density as well as femoral bone strength and higher bone turnover in accordance with previously reported results in growing (15) and adult (29) animals. Indeed, the younger rats were remarkably insensitive to changes in bone strength by ovariectomy (Table 2). These results may be explained by the fact that the diaphyseal BMD and femoral failure load are related to the cortical compartment that mainly fulfils mechanical functions and is thus less sensitive to estrogen deficiency than cancellous bone, especially in the growing skeleton (31). Hence, the 6- to 9-mo-old adult rats being highly sensitive to ovariectomy-induced osteopenia with effects on BMD, bone resorption biomarkers, and bone strength may indeed be the better model for postmenopausal bone loss (3).

In terms of body composition changes, although ovariectomized rats showed a higher body weight than intact rats, no difference in fat mass content was observed within animals between both age groups by the end of the 90-day feeding period. Moreover, the differences in body weight and fat mass between young and adult rats at the beginning of the experiment remained stable after 3 mo of treatment, suggesting a normal evolution of both body weight and composition.

#### Effect of Hesperidin on Two Ages of Ovariectomized Rats

Hesperidin intake led to a complete inhibition of ovariectomy-induced bone loss in the younger rats and a partial inhibition in the older rats, where the osteopenia was more severe. In accordance with the BMD results, ovariectomy induced a higher urinary excretion of DPD, compared with values obtained for sham-operated animals. This effect was inhibited by intake of hesperidin in both age groups of rats (Table 3). These results suggest that hesperidin protects against ovariectomy-induced osteopenia, at least in part due to inhibition of bone resorption. Consistent with these results, a previous study by Chiba et al. (6) showed that in 2-mo-old ovariectomized mice fed 0.5% hesperidin (same dose as our study) for 2 mo, trabecular bone loss and thickness were reduced (compared with sham-operated animals) at the femoral metaphysis accompanied by a reduction in the number of osteoclasts. Similarly, Deyhim et al. (8) showed that citrus juice consumption prevented bone loss in male orchidectomized rats accompanied by a decrease in urinary excretion of hydroxyproline, this being consistent with decreased DPD urinary excretion observed in our study and an overall antiresorptive effect.

In terms of bone strength, the older rats were considerably more sensitive than the younger rats to ovariectomy-induced reduced bone strength and hesperidin was able to reverse this loss of strength (Table 2). In the younger rats, ovariectomy did not significantly affect the cortical bone density, i.e., the diaphyseal BMD, nor the femoral bone strength. Yet, surprisingly, hesperidin intake led to a significant increase in femoral load in the HpOVX animals but not in the HpSH animals (Table 2). Taken together, these results indicate that hesperidin primarily affected BMD in young intact rats with an effect on strength (femoral load) only in ovariectomized animals despite

the fact that ovariectomy itself did not result in a lower femoral load. Assuming that effects on strength are linked to changes in the bone microarchitecture, then ovariectomy may have fragilized the bones of the younger rats in some way that was not reflected by the femoral load test and that hesperidin intake was able to counteract the effect of ovariectomy such that an improved femoral load was observed in the HpOVX animals.

#### Effect of Hesperidin in Intact Rats

Unexpectedly, hesperidin intake led to a significant increase in BMD at all three regions in the sham-operated younger rats (Fig. 1). Furthermore, urinary DPD excretion was significantly reduced by hesperidin consumption in the sham-operated rats, indicating a suppression of bone resorption. Whether hesperidin also stimulated bone metabolism in the sham-operated rats contributing to the improved BMD will require further mechanistic studies. Interestingly, hesperidin intake seemed to accelerate achievement of peak bone mass in the younger animals since 6-mo-old HpSH animals had the same peak bone mass as 9-mo-old sham-operated rats (Fig. 1). No further increase in BMD was seen between 6- and 9-mo-old HpSH animals (Fig. 1). Indeed, in the older sham-operated rats (which had already achieved peak bone mass), hesperidin did not improve BMD but did improve strength, once again suggesting an effect of hesperidin on the bone microarchitecture. All together, these surprising effects of hesperidin on improved BMD in young intact rats and improved bone strength in older intact rats require further investigation with more detailed biochemical and histomorphometric analyses.

#### Potential Mode of Action of Hesperidin

Although the molecular action of hesperetin on bone cells is not yet known, some insights into its mode of action are emerging with respect to its action on modulation of central cellular signaling pathways linked to its antioxidant and antiinflammatory properties. An important study by Kim et al. (16) showed that hesperetin fed to 6- and 24-mo-old rats modulated nuclear factor (NF)-kB in their kidneys, a finding that sheds light on the possible antiresorptive action of hesperetin since NF- $\kappa$ B is also a key signaling factor in osteoclast proliferation and differentiation (1). Indeed, Kim et al. found that hesperetin suppressed NF-kB signaling through four signal transduction pathways, NIK/IKK, ERK, p38, and JNK and further showed effects on the redox regulating transcription factors Trx/Ref-1. These results would implicate hesperetin as an overall antiaging agent, acting on many tissues, including antiresorptive effects in bone cells.

Concerning the surprising effect we observed on intact rats (improved BMD in younger rats, improved ultimate load in older rats), we can only speculate at this point on a potential stimulation of bone metabolism by hesperidin. One possibility is that there is a relationship with the lipid-lowering effects of hesperidin [see Table 3 for confirmation of lowering of cholesterol and triglycerides levels in our study, in accordance with previous findings (6, 9)]. It has been shown that statins not only inhibit cholesterol synthesis but also stimulate bone formation via induction of bone morphogenic protein (BMP-2) (25). Whether hesperidin stimulates bone formation in this way remains to be proven. However, in support of an effect on bone formation, we have preliminary data in osteoblast cells show-

ing stimulation of alkaline phosphatase activity after 12 days of culture with 4  $\mu$ M of hesperetin (data not shown), indicative of an effect on osteoblast differentiation as previously shown with simvastatin, on MC3T3-E1 cells (19).

#### Conclusions and Perspectives

Taken together, the results of this study show a clear protective effect of hesperidin on bone loss and strength in ovariectomized rats of two ages without uterine stimulation. The older rats were more sensitive to the ovariectomy-induced bone loss than the younger ones and the main effect of hesperidin was a slowing down of bone resorption. Surprisingly, hesperidin also stimulated in one hand BMD in young intact rats and in the other hand femoral strength in older intact rats that had probably already achieved their peak bone mass at the start of the feeding period (35). Although the mode of action of hesperidin is not yet elucidated, our results are consistent with an antiresorptive effect of hesperetin possibly via altered NF-kB signaling in osteoclast cells. The lipidlowering effect of hesperidin was confirmed, but whether this has any link to a statin-like effect and associated stimulation of BMP-2 and induced bone formation will require further investigation.

The identification of a non-hormonal dietary agent that may help prevent bone loss and stabilize blood lipids opens up perspectives for the dietary management of women's health in relation to menopause or osteopenia, particularly for those women who are not yet on osteoporotic drug treatment. Furthermore, the broader antiaging potential of hesperidin is of interest to explore for further health benefits. Detailed mechanistic studies will help to direct the primary organ and target population in the future that would benefit most from hesperidin.

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