

## Increased bioavailability of hesperetin-7-glucoside compared with hesperidin results in more efficient prevention of bone loss in adult ovariectomised rats

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Hesperidin (Hp), a citrus flavonoid predominantly found in oranges, shows bone-sparing effects in ovariectomised (OVX) animals. In human subjects, the bioavailability of Hp can be improved by the removal of the rhamnose group to yield hesperetin-7-glucoside (H-7-glc). The aim of the present work was to test whether H-7-glc was more bioavailable and therefore more effective than Hp in the prevention of bone loss in the OVX rat. Adult 6-month-old female Wistar rats were sham operated or OVX, then pair fed for 90 d a casein-based diet supplemented or not with freeze-dried orange juice enriched with Hp or H-7-glc at two dose equivalents of the hesperetin aglycone (0.25 and 0.5 %). In the rats fed 0.5 %, a reduction in OVX-induced bone loss was observed regarding total bone mineral density (BMD): +7.0 % in OVX rats treated with Hp (HpOVX) and +6.6 % in OVX rats treated with H-7-glc (H-7-glcOVX) v. OVX controls ( $P < 0.05$ ). In the rats fed 0.25 % hesperetin equivalents, the H-7-glcOVX group showed a 6.6 % improvement in total femoral BMD v. the OVX controls ( $P < 0.05$ ), whereas the Hp diet had no effect at this dose. The BMD of rats fed 0.25 % H-7-glc was equal to that of those given 0.5 % Hp, but was not further increased at 0.5 % H-7-glc. Plasma hesperetin levels and relative urinary excretion were significantly enhanced in the H-7-glc v. Hp groups, and the metabolite profile showed the absence of eriodictyol metabolites and increased levels of hesperetin sulphates. Taken together, improved bioavailability of H-7-glc may explain the more efficient bone protection of this compound.

### Bioavailability: Bone mineral density: Hesperidin: Flavanones: Rats

Nutrition plays an important role in the dietary management and prevention of osteoporosis, a major public health problem. While it is established that Ca, vitamin D and micronutrients are essential for bone health, other compounds such as polyphenols, found abundantly in fruit and vegetables, show potential for bone-protective effects. For example, rodent studies have shown that quercetin and quercetin-3-rhamnoglucoside (rutin) from onions<sup>(1,2)</sup>, resveratrol from red wine<sup>(3)</sup> and isoflavones from soya<sup>(4)</sup> inhibit ovariectomised (OVX)-induced bone loss. Until now, most of the human intervention studies have been carried out with high levels of soya isoflavones (80–90 mg), some studies showing prevention of bone loss in postmenopausal women<sup>(5,6)</sup>, while others not<sup>(7,8)</sup>. The level of consumption of soya products is rather low in Western countries compared with Asian ones, whose daily intakes average 20–40 mg<sup>(9)</sup>. Therefore, it would be of interest to identify commonly consumed polyphenols with bone-protective effects, as soya is not used as a staple in the Western diet.

Hesperidin (Hp), a monomethoxylated flavanone found abundantly in citrus fruits such as oranges<sup>(10)</sup>, is highly consumed in Western countries. Indeed, in Finland, Hp consumption was estimated to be 28 mg/d, contributing to 50 % of total flavonoid intake<sup>(11)</sup>. Hp and metabolites show promising health benefits due to their antioxidant, anti-inflammatory and lipid-lowering properties demonstrated in preclinical models<sup>(12–14)</sup>. Furthermore, Hp shows bone-sparing effects in OVX mice and rats of various ages, when provided in the diet at 0.5 %<sup>(15,16)</sup>, and citrus juice consumption has been shown to prevent bone loss in male orchidectomised rats<sup>(17)</sup>.

Flavonoids are commonly conjugated to glycosides when present in plants. Hp is one such conjugated glycoside consisting of the aglycone hesperetin (4'-methoxy-3',5,7-trihydroxyflavanone) bound to rutinose (i.e. one molecule of rhamnose and one molecule of glucose) in the C<sub>7</sub>-position<sup>(18)</sup>. Hp has limited bioavailability in human subjects, due to the delayed action of the colonic microflora needed to release the rutinose

**Abbreviations:** BMD, bone mineral density; D-BMD, diaphyseal BMD; dM-BMD, distal metaphyseal BMD; DPD, deoxyypyridinoline; H-7-glc, hesperetin-7-glucoside; Hp, hesperidin; M-BMD, metaphyseal BMD; OC, osteocalcin; OVX, ovariectomy; pM-BMD, proximal BMD; T-BMD, total BMD.

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moiety of Hp before absorption of its aglycone hesperetin. Indeed, consumption of 1 litre of orange juice containing 450 mg Hp led to circulating plasma concentrations of hesperetin of approximately 1  $\mu\text{M}$  with a peak at 5 h post-ingestion<sup>(19)</sup>. Monoglucuronides of hesperetin were the major forms present in plasma after the ingestion of orange juice<sup>(19)</sup>. We have recently demonstrated that enzymatic removal of the rhamnose sugar from Hp to yield hesperetin-7-glucoside (H-7-glc) improves its bioavailability by 3-fold in human subjects<sup>(20)</sup>. The removal of the terminal rhamnose sugar changes the absorption site from the colon to the small intestine, as it has been previously demonstrated with rutin and quercetin-4-glucoside in onions<sup>(21)</sup>.

The aim of the present study was to test whether H-7-glc was more bioavailable than Hp in the OVX rat (as in human subjects) and whether this correlated with improved efficiency in prevention of bone loss.

## Experimental methods

### Animals and diets

The study was carried out in accordance with the recommendations of the Regional Ethical Committee on animal experimentation in France. Fifty-eight adult, 6-month-old virgin female Wistar rats were purchased from Institut National de la Recherche Agronomique (Clermont-Ferrand/Theix, St-Genès Champanelle, France). Five groups of ten rats were surgically OVX and one group of eight rats was sham operated (SH) under anaesthesia, using chloral hydrate (Fluka Chemie AG, Buchs, Switzerland; 80 g/l in saline solution; 0.4 ml/100 g body weight intraperitoneally). The animals were housed individually in plastic cages allowing separation and collection of urine and faeces at 21°C with relative humidity of 55% and under a 12 h:12 h light:dark cycle.

The animals were fed a semipurified standard diet devoid of any soya proteins (Institut National de la Recherche Agronomique, Jouy en Josas, France). The control diet contained 150 g casein/kg, 50 g rapeseed oil/kg, 16.8 g  $\text{Ca}_3\text{PO}_4(2\text{H}_2\text{O})/\text{kg}$ , 6.5 g NaCl/kg, 11.4 g potassium citrate/kg, 5 g  $\text{MgSO}_4(7\text{H}_2\text{O})/\text{kg}$ , 10 g trace elements mix/kg, 10 g vitamin mix/kg and wheat starch *quantum satis* to achieve 1 kg (here 740 g/kg). The SH group and one OVX group were fed this non-supplemented control diet. The four remaining OVX groups were given the supplemented diets prepared on the same basis as the control diet by adding 2.5 g/kg or 5 g/kg of Hp or H-7-glc, respectively, at the expense of wheat starch. Hp and H-7-glc were prepared by fortification of Nestlé Thailand orange juice with 9.2 g/l of orange bioflavonoid complex (Nutrafur, Alcantarilla, Spain) containing 90% Hp, incubation with or without  $\alpha$ -rhamnosidase (Hesperidinase Amans concentrated, Amano Enzymes, Chipping Norton, Oxfordshire, UK) and freeze drying according to Nielsen *et al.*<sup>(20)</sup>. The freeze-dried juice-based product obtained without  $\alpha$ -rhamnosidase contained 33 mg/g of Hp and the product obtained with  $\alpha$ -rhamnosidase contained corresponding amounts of H-7-glc. Four diets containing 0.25% w/w or 0.5% w/w of Hp or H-7-glc, respectively, were prepared every week and stored at 4°C until use.

During the whole experimental period and in order to prevent well-known castration-induced hyperphagia, the quantity

of diet given to each rat per day was adjusted to the mean food intake registered in SH animals (pair feeding). The animals had free access to water during the entire study, and their body weights and food intake were measured weekly. Urine of each animal was collected over a 24-h period on day 0 and the day before killing, to measure urinary excretion of deoxypyridinoline (DPD), a marker for bone resorption.

At killing, on day 90, blood samples were collected into ice-cooled heparinised plastic tubes containing 200 peptidase inhibitory units of aprotinin (Iniprol, Choay, Paris, France) per millilitre blood, and centrifuged immediately (3500 g for 5 min at 4°C). Then plasma samples were frozen at  $-20^\circ\text{C}$  until measurements of osteocalcin (OC), a marker of osteoblastic activity. Before freezing at  $-20^\circ\text{C}$ , plasma samples dedicated to the analysis of hesperetin metabolites were acidified with acetic acid (0.1 M, 1%) in order to protect the structure of these metabolites. 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 (BMD)).

### Bone measurements

**Bone mineral density.** BMD was assessed by dual-energy X-ray absorptiometry, 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 metaphyseal zone (M-BMD), rich in cancellous bone, and the other to the diaphyseal zone (D-BMD), rich in cortical bone, were determined<sup>(22)</sup>. In each femur scan, distal (dM-BMD) and proximal (pM-BMD) 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  $\text{g}/\text{cm}^2$ .

**Femoral mechanical testing.** Immediately after collection in NaCl (9 g/l), the length of the left femur and the mean diameter of the diaphysis (i.e. the mean of the greatest and the smallest diaphysis diameters) were measured using a precision caliper (Mitutoyo, Shropshire, UK). Femoral failure load was determined using a three-point bending test with a Universal Testing Machine (Instron 4501; Instron, Canton, MA, USA). The load at rupture (N) was automatically determined and recorded by Instron 4501 software. This method was previously validated by using Plexiglas standard probes<sup>(23)</sup>.

**Bone biomarkers analyses.** Plasma OC concentrations (ng/ml) were measured by RIA, using rat  $^{125}\text{I}$ -labelled OC, goat anti-rat OC antibody and donkey anti-goat second antibody (Biochemical Technologies kit, Stoughton, MA, USA). The sensitivity was 0.01 ng/ml. The intra- and interassay precisions were 6.8 and 8.9%, respectively.

DPD was measured in urine by competitive RIA (Pyrilinks D kit; Metra Biosystems, Mountain View, CA, USA). The assay requires a rat monoclonal antibody against DPD, which is coated to the inner surface of a polystyrene tube and  $^{125}\text{I}$ -labelled DPD. In the present experimental conditions, the sensitivity was 2 nmol/l, and the intra- and interassay variations were 4.0 and 6.0%, respectively. Results are expressed as nmol of DPD/mmol of creatinine to take into account

interindividual differences of urine concentration<sup>(24)</sup>. The creatinine assay kit (BioMérieux, Marcy l'étoile, France) is based on a modified Jaffe's method in which picric acid forms a yellow compound in presence of creatinine<sup>(25)</sup>.

#### Leptin assay

Plasma leptin concentration was assessed by RIA using anti-rat leptin antibody and rat leptin as a standard (Rat Leptin RIA kit; Linco Research, Inc., St Charles, MO, USA). The lowest limit of sensitivity was 0.5 ng/ml, and the intra- and interassay variations were 1.5 and 2.5 %, respectively.

#### Analysis of hesperetin and metabolites in plasma and urine

**Preparation of plasma and urine samples for HPLC analyses.** HPLC analyses were performed directly or after an hydrolysis step for which plasma samples (180 µl) were acidified to pH 4.9 with acetic acid, incubated for 5 h at 37°C with β-glucuronidase (1000 units) and sulphatase (forty-five units; from *Helix pomatia*, Sigma G0876, L'Isle d'Abeau, Chesnes, France), mixed with four volumes of methanol-HCl (0.2 M) and the supernatant analysed after centrifugation (4 min, 14 000 rpm). Urine samples were diluted in acetate buffer (0.1 M, pH 4.9), mixed with four volumes of methanol-HCl (0.2 M) and the supernatant analysed after centrifugation as mentioned earlier.

**Analysis of hesperetin metabolites in plasma by HPLC.** Qualitative analysis of hesperetin metabolites and determination of the respective proportion of hesperetin conjugates (percentage of total flavanones) were performed in non-hydrolysed plasma by HPLC with mass spectrometric detection. Plasma and urine hesperetin concentrations were obtained after the hydrolysis step described earlier and analysis by HPLC with detection by CoulArray. For HPLC with mass spectrometric detection, a YMC-Pack ODS-AM column (250 × 3.0 mm inner diameter, 5 µm particle size) was eluted with water-formic acid (99:1, v/v; solvent A) and acetonitrile-formic acid (99:1, v/v; solvent B): 20 % B for 2 min; linear gradient to 45 % of B over 20 min; 65 % of B from 20 to 30 min. Detection was by a diode array detector and API 2000 mass spectrometer. Liquid chromatography-electrospray ionisation-MS/MS triple quadrupole MS was optimised for hesperetin, hesperetin 7-O-glucuronide and

homoeriodictyol. MS and MS<sup>2</sup> data were acquired in the multiple-reaction monitoring mode, with maximum intensity for the transitions at *m/z* 301:286 (negative mode), 477:301 (negative mode) and 303:151 (positive mode), respectively. Hesperetin 7-O-glucuronide was confirmed using an authentic synthetic standard.

For multichannel electrochemical detection, HPLC analysis was performed on a 150 × 4.6 mm Hypersil BDS C<sub>18</sub>-5 µm column (Touzard et Matignon, Les Ulis, France), with an eight-channel CoulArray detector (model 5600, Eurosep, Cergy, France). Mobile phases were 30 mM NaH<sub>2</sub>PO<sub>4</sub> buffer (pH 3) containing 20 % acetonitrile (A) or 40 % acetonitrile (B). Separation was achieved using a gradient elution (35°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. Hesperetin was quantified using the sums of height obtained on the electrodes 2, 3 and 4, 5 and 6, respectively.

#### Statistical analysis

Results are expressed as means with their standard errors. All data were analysed using the Graphpad InStat software (Microsoft, San Diego, CA, USA). A parametric one-way ANOVA was first performed to test for any significant difference among groups. If the results were significant ( $P < 0.05$ ), the Student–Newman–Keuls multiple comparisons test was used to determine the specific differences between means. Parametric ANOVA was performed when data were sampled from populations with equal variance. If not, a Kruskal–Wallis test followed by the Mann–Whitney *U* test was performed to compare specific differences between groups. The level of significance was set at  $P < 0.05$  for all statistical tests.

## Results

#### Body and uterine weight changes

Successful ovariectomy was confirmed by marked atrophy of uterine horns since the mean uterine weight was lower in the OVX groups compared to sham-operated (SH) groups ( $P < 0.001$ ; Table 1). No uterotrophic effect was elicited by consumption of Hp or H-7-glc, since uterine weight was

**Table 1.** Effects of ovariectomy (OVX), hesperidin (Hp) and hesperetin-7-glucoside (H-7-glc) on food consumption (g/d), body and uterus weights (g) and plasma leptin (ng/ml)

(Mean values with their standard errors for eight rats in sham operated (SH) control group and ten rats per group in all other groups)

	SH		OVX		0.25 HpOVX		0.5 HpOVX		0.25 H-7-glcOVX		0.5 H-7-glcOVX	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Food intake, day 30	16.33	0.51	16.55	0.54	17.16	0.32	17.55	0.02	17.44	0.23	17.34	0.35
Food intake, day 60	15.74	0.62	16.83	0.46	16.93	0.39	17.52	0.11	15.79	1.53	17.06	0.48
Initial body weight	277.4	5.6	274.2	6.1	271.3	5.3	284.2	6.1	278.4	4.8	282.1	6.5
Final body weight	309.6*	11.0	356.2	7.6	351.8	5.7	358.2	9.7	366.2	6.8	371.1	9.9
Uterus weight	0.689	0.056	0.116†	0.011	0.102†	0.006	0.133†	0.030	0.131†	0.017	0.115†	0.013
Plasma leptin	8.07	1.5	11.41	0.96	10.64	0.72	9.50	1.12	11.07	1.08	12.76	0.89

\* Mean values were significantly different from those of all OVX groups ( $P < 0.01$ ).

† Mean values were significantly different from those of SH control group ( $P < 0.001$ ).

unaffected by these treatments at both doses and was similar to that of the OVX control rats (Table 1).

Despite the pair feeding (during the whole experimental period, the daily mean food intake in each group was 16.9 (SEM 0.17) g), a significantly lower weight in SH animals was reported ( $P < 0.01$ ), from day 21 up to the end of the experimental period (day 90). No differences in body weight among the control and treated OVX groups were observed (Table 1). Furthermore, no significant difference in plasma leptin levels between groups was detected at day 90 (Table 1). Thus, either Hp or H-7-glc consumption, at either dose, influenced neither the body weight nor the body composition, as suggested by leptin assay.

#### Bone mineral density

Concerning BMD, similar patterns were observed for T-BMD, D-BMD, pM-BMD and distal metaphyseal (dM-BMD) femoral mineral densities. As expected, ovariectomy induced a significant decrease in BMD at all femoral sites in OVX control rats (T-BMD: OVX control:  $-7.4\%$  v. SH,  $P < 0.01$ , Fig. 1; data not shown for D-BMD, pM-BMD and dM-BMD).

Consumption of 0.5% Hp led to a significant improvement in T-BMD in the HpOVX group compared with OVX controls (T-BMD:  $+7.0\%$  v. OVX,  $P < 0.05$ ; Fig. 1). In other bone regions, similar levels of improvement in BMD were observed with this dose of Hp (D-BMD:  $+6.7\%$ ; dM-BMD:  $+7.0\%$ ; and pM-BMD:  $+7.9\%$  v. OVX,  $P < 0.05$ ; data not shown). The lower Hp dose (0.25%) failed to prevent the significant ovariectomy-induced loss of BMD (compared with SH control group), as no differences were observed at any of the bone sites compared to OVX control group.

In the H-7-glcOVX groups, T-BMD was significantly improved (compared with OVX control group) at both doses of 0.25% (T-BMD:  $+6.6\%$ ,  $P < 0.05$ ; Fig. 1) and 0.5% (T-BMD:  $+6.6\%$ ,  $P < 0.05$ ; Fig. 1). BMD at diaphyseal and metaphyseal femoral sites was increased to the same extent than T-BMD with dose of 0.25% (D-BMD:  $+7.0\%$ ,  $P < 0.01$ ; dM-BMD:  $+6.9\%$ ; pM-BMD:  $+6.9\%$ ,  $P < 0.05$ ; data not shown) as well as dose of 0.5% (D-BMD:  $+7.8\%$ ,  $P < 0.01$ ; dM-BMD:  $+6.1\%$ ; pM-BMD:  $+7.0\%$ ,  $P < 0.05$ ; data not shown). Actually, compared with OVX controls, the 0.25%

dose H-7-glcOVX group showed an equivalent improvement in BMD to that of the 0.5% dose HpOVX group and achieved the same T-BMD (Fig. 1), D-BMD or M-BMD (data not shown). However, no further increase in T-BMD, D-BMD or M-BMD was seen with the higher dose of H-7-glc (0.5%) compared to the lower one (0.25%). Thus, H-7-glc exhibited similar BMD-sparing effects to Hp but at a lower dose.

#### Bone size and ultimate load

No significant differences among groups were demonstrated for femoral length and femoral diameter at day 90 (Table 2). Femoral strength was assessed by the femoral failure load. Ovariectomy significantly decreased this parameter compared with SH animals (OVX:  $-6.5\%$  v. SH,  $P < 0.05$ ; Table 2). Consumption of Hp at 0.5% inhibited this OVX-induced loss of strength (0.5% HpOVX:  $+10.0\%$  v. OVX,  $P < 0.05$ ; Table 2). The magnitude of the effect (percentage of change) was quite similar for the two groups fed with H-7-glc (0.25% H-7-glcOVX:  $+7.4\%$  v. OVX,  $P < 0.05$ ; 0.5% H-7-glcOVX:  $+14.7\%$  v. OVX,  $P < 0.05$ ; Table 2). In the 0.25% Hp group, there was a trend to increase femoral strength, however, without reaching significance (Table 2). Thus, as shown for BMD mentioned earlier, both Hp and H-7-glc helped to restore the OVX-induced loss of femoral strength, but H-7-glc achieved the same effect as Hp (0.5%) at the lower dose (0.25%).

#### Bone turnover markers

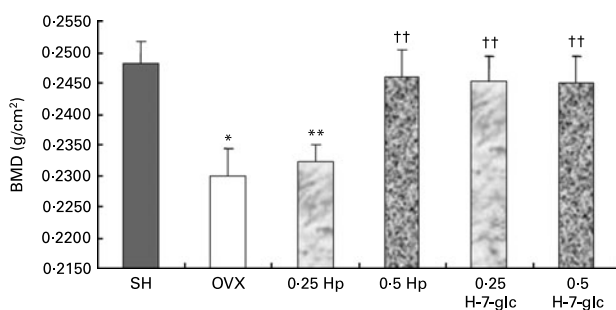
Ovariectomy resulted in a significant increase in OC plasma levels in control rats (OVX control:  $+62.5\%$  v. SH,  $P < 0.001$ ), probably due to enhanced bone turnover following ovariectomy, and this level was unaffected by the consumption of Hp or H-7-glc at either dose (Fig. 2(a)).

An increase in urinary DPD excretion was observed following ovariectomy (OVX control:  $+145\%$  v. SH,  $P < 0.001$ ), which was partially, but significantly, inhibited by Hp and H-7-glc consumption (0.25% HpOVX:  $-33.0\%$  v. OVX,  $P < 0.05$ ; 0.5% HpOVX:  $-41.7\%$  v. OVX,  $P < 0.01$ ; 0.25% H-7-glcOVX:  $-48.4\%$  v. OVX,  $P < 0.01$ ; 0.5% H-7-glcOVX:  $-29.4\%$  v. OVX,  $P < 0.05$ ; Fig. 2(b)).

#### Hesperetin analyses and metabolite profile in plasma and urine

**Plasma and urine concentrations (compared with food intake).** Total hesperetin concentrations in plasma, measured by multi-channel electrochemical detection after an enzymatic hydrolysis, are shown in Fig. 3(a). A dose-dependent increase was observed between 0.25 and 0.5% doses of the individual treatments (i.e.  $1.13\ \mu\text{M}$  from 0.25% Hp;  $2.06\ \mu\text{M}$  from 0.5% Hp;  $2.30\ \mu\text{M}$  from 0.25% H-7-glc; and  $4.91\ \mu\text{M}$  from 0.5% H7glc). However, intake of H-7-glc at either dose led to higher (approximately 2-fold) circulating plasma levels of hesperetin than with equivalent doses of Hp. Indeed, similar plasma hesperetin concentrations were observed with 0.25% H-7-glc ( $2.30\ \mu\text{M}$ ) as 0.5% Hp ( $2.06\ \mu\text{M}$ ; Fig. 3(a)).

Relative urinary excretion (Fig. 3(b)) increased between the 0.25 and 0.5% Hp doses (relative increase from 2.4 to 3.6%),



**Fig. 1.** Effects of ovariectomy (OVX), hesperidin (Hp) and hesperetin-7-glucoside (H-7-glc) on total femoral BMD (g/cm<sup>2</sup>), measured on day 90. Results are expressed as means with their standard errors per group. \*Mean values were significantly different from those of SH control group ( $P < 0.01$ ). \*\*Mean values were significantly different from those of SH control group ( $P < 0.05$ ). ††Mean values were significantly different from those of OVX control group ( $P < 0.05$ ).

**Table 2.** Effects of ovariectomy (OVX), hesperidin (Hp), hesperetin-7-glucoside (H-7-glc) on femoral length (mm), femoral mean diameter (mm) and femoral failure load (N) measured on day 90

(Mean values with their standard errors for eight rats in sham operated (SH) control group and ten rats per group in all other groups)

	SH		OVX		0.25 HpOVX		0.5 HpOVX		0.25 H-7-glcOVX		0.5 H-7-glcOVX	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Length	35.95	0.30	35.70	0.22	36.33	0.24	36.92	0.27	36.34	0.33	36.89	0.35
Diameter	3.63	0.04	3.61	0.05	3.56	0.03	3.72	0.06	3.72	0.05	3.74	0.05
Femoral failure load	111.3	3.2	104.1*	2.7	108.7	3.1	114.5†	3.9	111.8†	2.4	119.4†	5.1

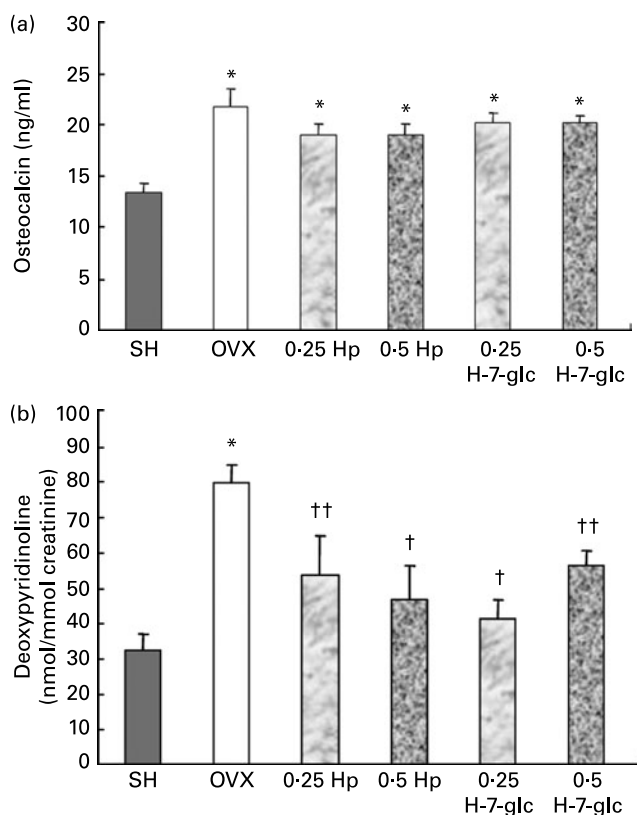
\* Mean values were significantly different from those of SH control group ( $P < 0.05$ ).† Mean values were significantly different from those of OVX control group ( $P < 0.05$ ).

but was significantly higher in the 0.25 % H-7-glc group (10.9 in 0.25 % H-7-glc v. 2.4 in 0.25 % Hp) and did not further change in the 0.5 % H-7-glc group (10.8 %). Comparing equivalent doses of Hp and H-7-glc, it was clear that the  $\alpha$ -rhamnosidase treatment of orange juice converting Hp to H-7-glc clearly resulted in enhanced relative urinary excretion of hesperetin, indicative of increased absorption.

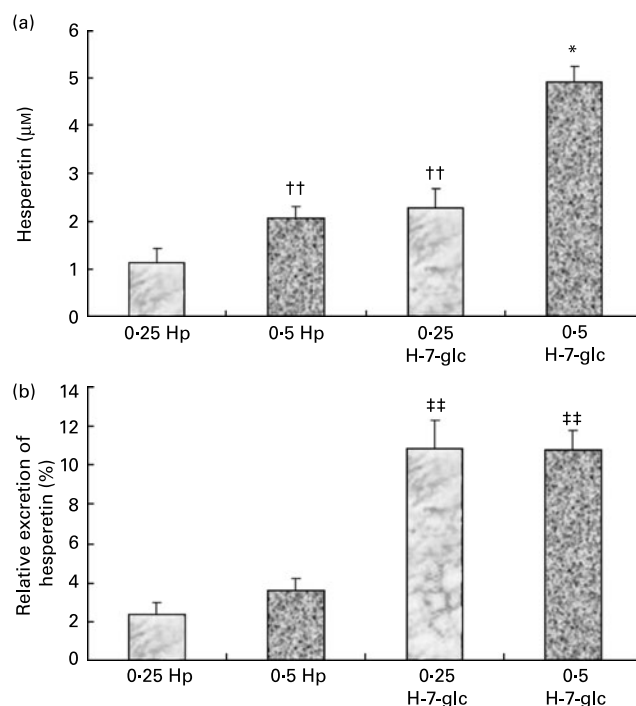
**Metabolic profile.** In all plasma samples, hesperetin-7-*O*-glucuronide and two hesperetin sulphate conjugates were detected (Fig. 4). Quantitatively (results are expressed in

percentage of total flavanone metabolites), hesperetin 7-*O*-glucuronide was the major circulating form in plasma whatever the treatment or dose (54–66 %; Table 3). Conjugates with glycine and glutathione were not present, and free hesperetin aglycone was present only at very low levels in all samples.

For rats fed Hp in untreated orange juice, hesperetin-3'-*O*-glucuronide, homoeriodictyol-4'-*O*-glucuronide and homoeriodictyol-7-*O*-glucuronide (all tentatively identified using MS fragmentation pattern of the aglycone standards) were observed in plasma. These compounds have been detected previously at similar levels<sup>(26)</sup>. Total homoeriodictyol glucuronides (38.0 and 36.9 % for both 0.25 and 0.5 % doses, respectively; Table 3) were the second most abundant metabolite group in rats fed Hp. For rats fed H-7-glc in orange juice



**Fig. 2.** Effects of ovariectomy (OVX), hesperidin (Hp) and hesperetin-7-glucoside (H-7-glc) on (a) plasma osteocalcin (ng/ml) and (b) urinary deoxyypyridinoline (nmol/mmol), measured on day 90. Results are expressed as means with their standard errors per group. \* Mean values were significantly different from those of SH control group ( $P < 0.001$ ). † Mean values were significantly different from those of OVX control group ( $P < 0.01$ ). †† Mean values were significantly different from those of OVX control group ( $P < 0.05$ ).



**Fig. 3.** (a) Plasma hesperetin concentrations ( $\mu\text{M}$ ) and (b) relative urinary excretion of hesperetin (%) measured on day 90 in ovariectomised (OVX) groups supplemented with hesperidin (Hp) or hesperetin-7-glucoside (H-7-glc) at both doses 0.25 and 0.5 %. Results are expressed as means with their standard errors per group. \* Mean values were significantly different from those of all other groups ( $P < 0.01$ ). †† Mean values were significantly different from those of the 0.25 HpOVX group ( $P < 0.05$ ). ††† Mean values were significantly different from those of HpOVX groups ( $P < 0.001$ ).

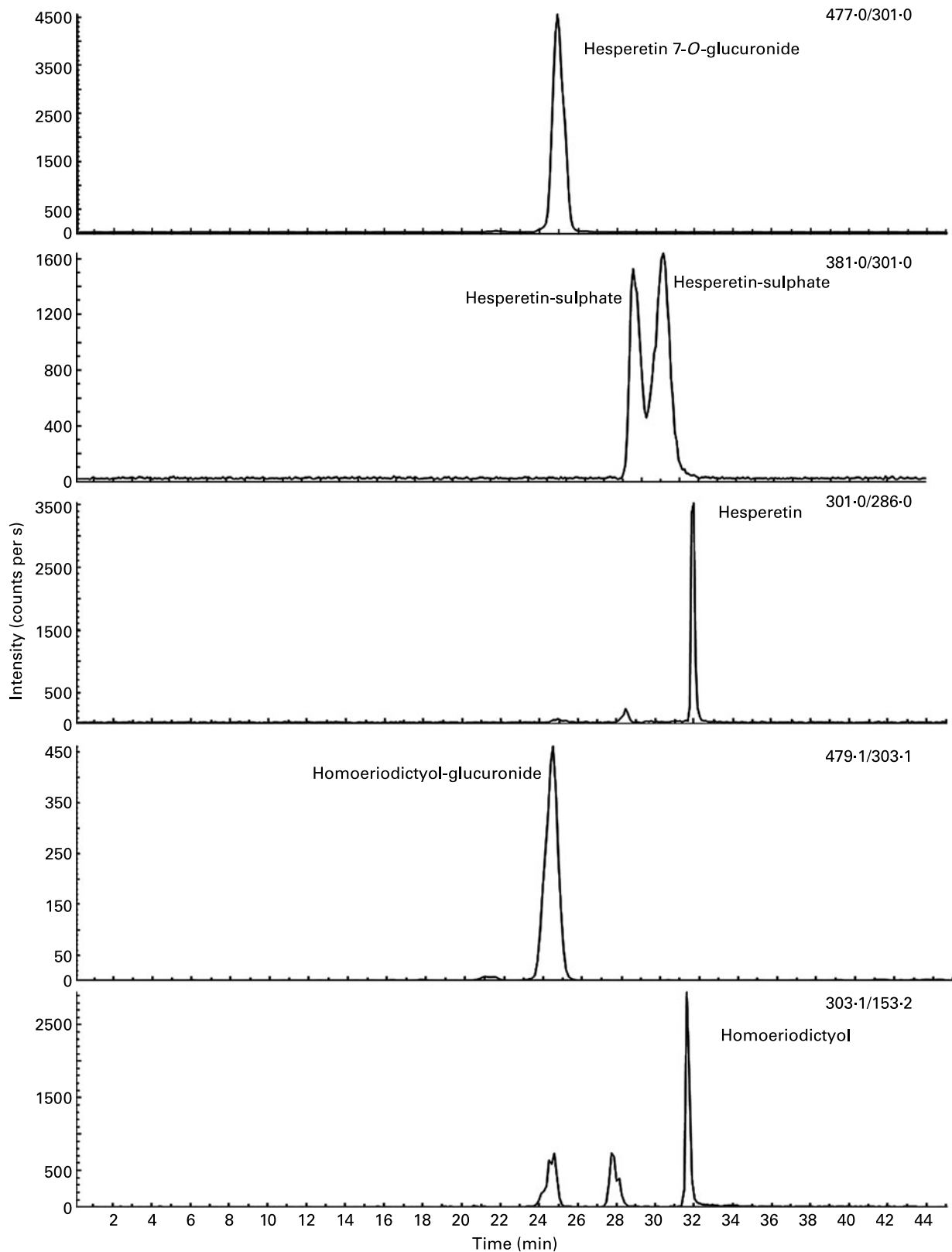


Fig. 4. Liquid chromatography-electrospray ionisation-MS/MS mass spectra for hesperidin (Hp) and hesperetin-7-glucoside (H-7-glc) circulating metabolites.

**Table 3.** Identification and quantification of hesperidin (Hp) and hesperetin-7-glucoside (H-7-glc) metabolites in plasma analysed by liquid chromatography-electrospray ionisation-MS/MS

	Respective proportion of metabolites (percentage of total flavanones)				
	Hesperetin	Hesperetin-7- <i>O</i> -glucuronide	Hesperetin sulphate	Homoeriodictyol	Homoeriodictyol glucuronides
0.25 Hp	1.9	54.0	2.7	3.0	38.4
0.5 Hp	3.8	55.0	2.0	2.7	36.5
0.25 H-7-glc	1.5	60.0	38.5	–	–
0.5 H-7-glc	4.7	66.0	29.3	–	–

pretreated with  $\alpha$ -rhamnosidase, plasma hesperetin sulphates (38.5 and 29.3 % for both 0.25 and 0.5 % doses, respectively; Table 3) were much higher than in the groups fed Hp in untreated orange juice. Hesperetin-3'-*O*-glucuronide, homoeriodictyol-4'-*O*-glucuronide and homoeriodictyol-7-*O*-glucuronide were all absent from plasma in the group fed H-7-glc.

## Discussion

Presently, the OVX rat model is the most widely used animal model for studying postmenopausal-induced osteopenia<sup>(27)</sup>. We, and others, have previously reported that OVX-induced osteopenia was more severe in adult (6–9 months old) than growing (3–6-months old) rats, leading to greater loss of trabecular and cortical bone density as well as femoral bone strength<sup>(1,28)</sup>. In the present study, we chose the adult rat as the present model for postmenopausal osteoporosis. As expected, ovariectomy, validated by uterine atrophy (Table 1), induced significant osteopenia at all femoral sites (T-BMD: –7.4 % OVX control *v.* SH; Fig. 1). Both cancellous and cortical bones were affected by bone loss. Femoral mechanical properties, determined by the femoral diaphysis failure load, were impaired by OVX (Table 2). Similar to that observed in postmenopausal women<sup>(29)</sup>, bone turnover was increased following OVX as indicated by a higher urinary DPD excretion and an increase in plasma levels of OC in OVX rats compared with SH (Fig. 2). Thus, the OVX adult rat used in the present study may be considered as an appropriate model for postmenopausal bone loss<sup>(30,31)</sup>.

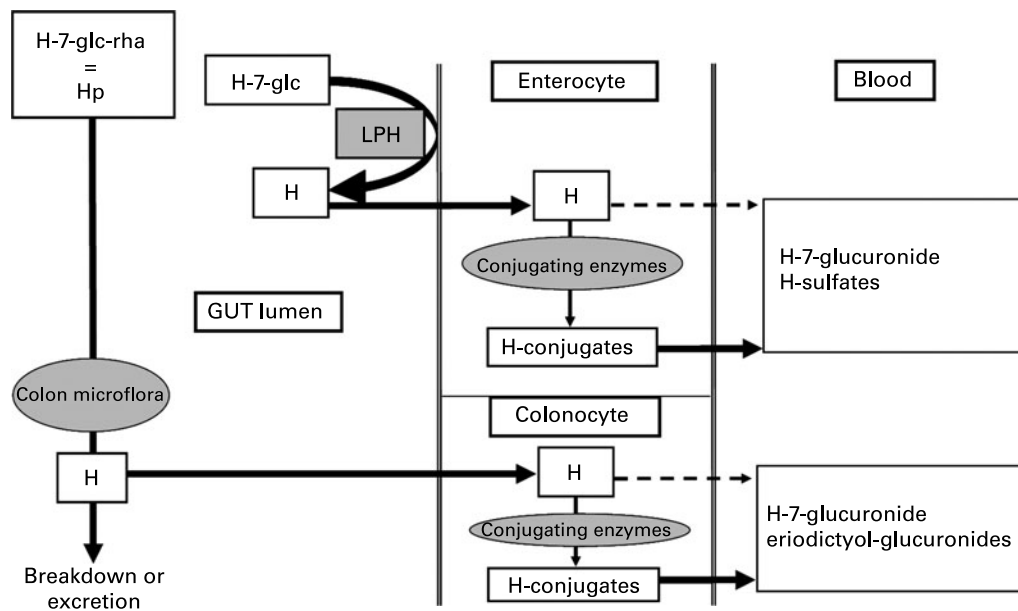
In accordance with our previous study in young and adult OVX rats<sup>(16)</sup>, the data shown here confirm that consumption of Hp at 0.5 % in the diet leads to a prevention of OVX-induced bone loss in adult rats, without a uterotrophic effect and with no effect on body weight. The BMD-sparing effect (T-BMD: +7.0 % *v.* OVX control; Fig. 1) was associated with a partial inhibition of OVX-induced DPD excretion (–41.7 % *v.* OVX; Fig. 2) and a significant improvement in femoral failure load (+10.0 % *v.* OVX; Table 2). These data on the bone-sparing effects of Hp are further supported by the work of Chiba *et al.*<sup>(15)</sup>, who showed that 0.5 % Hp in the diet of young OVX mice was able to inhibit OVX-induced trabecular bone loss and thickness at the femoral metaphysis accompanied by a reduction in the number of osteoclasts, and Deyhim *et al.*<sup>(17)</sup>, who showed that citrus juice prevented bone loss in male orchidectomised senescent rats accompanied by a decrease in urinary excretion of hydroxyproline.

The new finding reported here is the greater efficiency of H-7-glc compared with native Hp, in preventing OVX-induced bone loss. Indeed, in terms of hesperetin aglycone equivalents, the doses of 0.25 % H-7-glc and 0.5 % Hp showed similar

protection against OVX-induced bone loss as shown by the improvement of BMD (+6.6 and +7.0 %, respectively, for T-BMD compared with OVX controls,  $P < 0.05$ ; Fig. 1) and femoral failure load (+7.4 and +10.0 %, respectively, compared with OVX controls,  $P < 0.05$ ; Table 2). The higher dose (0.5 %) of H-7-glc did not result in any further improvement in BMD or femoral failure load, suggesting a threshold effect at a certain concentration of hesperetin. The BMD and femoral failure load changes were accompanied by a partial but significant inhibition of the OVX-induced bone resorption (Fig. 2(b)). However, there was not a dose–response relationship of Hp or H-7-glc to this marker, and no significant change was seen in the OVX-induced OC levels.

Fig. 5 summarises the main findings on the metabolism of Hp based on literature data and our new finding reported here. Hp intake by human subjects showed that hesperetin is absorbed in the colon after cleavage of the rhamnoglucoside moiety. Subsequently, the main circulating forms observed are conjugates of hesperetin with glucuronic acid and/or sulphate<sup>(19)</sup>. After enzymatic removal of the rhamnose sugar to give H-7-glc, the level of hesperetin in blood from healthy volunteers was increased<sup>(20)</sup>. The results reported here in rats also show that the nature of the sugars attached to hesperetin is critical in determining the site and extent of absorption, and, in agreement with the human studies, show that more is found in the plasma after consumption of H-7-glc compared with Hp (Fig. 3(a)). Indeed, the circulating plasma levels were approximately 2-fold higher in the H-7-glc groups compared with the Hp groups at each dose, and the 0.25 % H-7-glc dose resulted in the same plasma hesperetin levels as the 0.5 % Hp dose (Fig. 3(a)). These higher plasma levels were accompanied by significantly higher urinary excretion levels of hesperetin in the H-7-glc groups (Fig. 3(b)). Comparing equivalent doses of Hp and H-7-glc, it was clear that the  $\alpha$ -rhamnosidase treatment of orange juice converting Hp to H-7-glc resulted in enhanced relative urinary excretion of hesperetin (Fig. 3(b)). All together, these results show an increased bioavailability of H-7-glc compared with Hp.

In addition to the higher overall levels of hesperetin in plasma after consumption of H-7-glc, the profile of conjugates and metabolites is changed (Table 3). Shifting the absorption from the colon to the small intestine increases the percentage of sulphated metabolites. This could be due to the site of absorption and/or to flavanone-mediated induction of UDP-glucuronosyltransferases (UGT1A1)<sup>(32)</sup>. The change in absorption site from the colon to the small intestine also decreased the formation of eriodictyol conjugates to undetectable levels. The latter are derived from microbial metabolism, consistent with this altered site of absorption, since rat gut microflora is able to transmethylate hesperetin



**Fig. 5.** Differences in metabolism of hesperidin (Hp; hesperetin-7-glucose-rhamnose, H-7-glc-rha) compared with hesperetin-7-glucoside (H-7-glc): conjugation profiles and sites of absorption. H, hesperetin; LPH, lactate phlorizin hydrolase.

to form homoeriodictyol (from  $-\text{OCH}_3$  at 4'-position and  $-\text{OH}$  at 3'-position in hesperetin to  $-\text{OCH}_3$  at 3'-position and  $-\text{OH}$  at 4'-position in homoeriodictyol)<sup>(33)</sup>. Rat colonic microflora can also hydroxylate and/or dehydroxylate and methylate naringenin to hesperetin and isosakuranetin<sup>(34)</sup>.

### Conclusions and perspectives

In conclusion, in the present study, both Hp and H-7-glc were able to elicit protective effects on bone loss in adult animals, probably via an inhibition of bone resorption and without uterotrophic effects. The H-7-glc form achieved the same level of protection as Hp but at the lower dose of 0.25% compared with 0.5% Hp, but showed no further increase in bone protection at 0.5% in the diet, suggesting a threshold effect linked to hesperetin availability. Taken together with the analysis of the hesperetin plasma and urine metabolites, these results may be explained by the difference in bioavailability between these two compounds. The enzymatic conversion of Hp to H-7-glc leading to both a higher level of total hesperetin at the lower dose and an increased level of hesperetin sulphates could contribute to the more efficient bone-sparing effect of H-7-glc. On the other hand, the absence of the homoeriodictyol metabolites following H-7-glc consumption suggests that these metabolites are not involved in the bone-sparing effect of Hp. Hp or H-7-glc metabolites could reach bone tissues, or possibly be deconjugated at the target site, as reported for other conjugated flavonoids in target tissues under stress conditions<sup>(35,36)</sup>.

The exact molecular mechanism of action of hesperetin or its metabolites on bone cells is not yet elucidated, although some insight from its antioxidant and anti-inflammatory properties suggest that at least some of the important pathways include NF- $\kappa$ B and its related signal transduction pathways, NF- $\kappa$ B-inducing kinase/inhibitor of NF- $\kappa$ B kinase, extracellular signal-regulated kinase, p38 and c-Jun N-terminal kinase as well as the redox-regulating transcription factors thioredoxin/redox factor-1<sup>(37)</sup>. As these pathways are also implicated in the

bone resorption process, they may help to explain the antiresorptive effect of Hp. The higher bioavailability of hesperetin from H-7-glc, together with the different metabolite profile, may strongly influence the cellular signalling responses in bone and other tissues and may help to explain the higher efficiency in bone protection by H-7-glc shown here.

The identification of a commonly consumed polyphenol, Hp, together with its more potent metabolite, H-7-glc, which help to prevent OVX bone loss, opens up perspectives for the dietary management of women's health in relation to menopause or osteopenia.

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