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RESEARCH ARTICLE

Efficacy of phytosterols and fish-oil supplemented high-oleic-sunflower oil rich diets in hypercholesterolemic growing rats

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ABSTRACT

Phytosterols (P) and fish-oil (F) efficacy on high-oleic-sunflower oil (HOSO) diets were assessed in hypercholesterolemic growing rats. Controls (C) received a standard diet for 8 weeks; experimental rats were fed an atherogenic diet (AT) for 3 weeks, thereafter were divided into four groups fed for 5 weeks a monounsaturated fatty acid diet (MUFA) containing either: extra virgin olive oil (OO), HOSO or HOSO supplemented with P or F. The diets did not alter body weight or growth. HOSO-P and HOSO-F rats showed reduced total cholesterol (T-chol), non-high-density lipoprotein-cholesterol (non-HDL-chol) and triglycerides and increased HDL-chol levels, comparably to the OO rats. Total body fat (%) was similar among all rats; but HOSO-F showed the lowest intestinal, epididymal and perirenal fat. However, bone mineral content and density, and bone yield stress and modulus of elasticity were unchanged. Growing hypercholesterolemic rats fed HOSO with P or F improved serum lipids and fat distribution, but did not influence material bone quality.

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Body fat; bone quality; fish oil; high-oleic-sunflower oil; lipid profile; phytosterol; weight and growth gain

Introduction

It is well known that populations consuming a "Mediterranean diet" present a reduced cardiovascular mortality (Arós & Estruch 2013; Ros et al. 2013; Michas et al. 2014). High intake of olive oil (OO) is considered a feature of the traditional Mediterranean diet, resulting in high intake of monounsaturated fatty acids (MUFA) and lower intake of saturated fatty acids. Replacement of saturated fat with monounsaturated lipids greatly reduces coronary heart disease risk (Carrillo Fernández et al. 2011). Furthermore, cardiovascular disease, especially atherosclerosis, coexists in patients with osteoporosis (Graham et al. 2010) and altered bone strength (Pirih et al. 2012). Accumulation of lipids within the bones of animals with osteoporosis has been reported (Tintut et al. 2004; Griffith et al. 2005). It has been shown that animals fed a high-fat diet develop atherosclerosis and osteopenia (Parhami et al. 2001; Macri et al. 2012).

Most of the cardio-protective effects of OO, in the context of the Mediterranean diet, have been attributed to its high MUFA. OO has high quantities of MUFA and contains minor components with biological properties, i.e. the unsaponificable compounds (squalene, sitosterols, triterpenes, pigments, etc.) and the phenolic compounds that contain antioxidant properties (Psaltopoulou et al. 2004; Fitó et al. 2007). In addition, other constituents of the Mediterranean diet, such as fish rich in omega-3 polyunsaturated fatty acids (PUFA), have been shown to reduce cardiovascular morbidity and mortality and contribute to cardio protective effects with improved lipid profiles, reduced inflammation and oxidation (Widmer et al. 2015). Furthermore, several studies have also shown a potentially beneficial effect of fish intake against osteoporosis and fracture occurrence (Calderon-Garcia et al. 2012; Paunescu et al. 2013).

However, we have shown that feeding high-oleic-sunflower oil rich diets (HOSO) to growing healthy (Macri et al. 2012) or hypercholesterolemic rats (Macri et al. 2015) did not demonstrate the benefits on hyperlipidemia and bone mineral content (BMC) that are observed with feedings of diets with OO. Since HOSO is completely devoid of polyphenols, we can infer that the fatty acids in OO convey protective effects by their content of natural antioxidants. Therefore, the goal of this study was to evaluate, in an experimental model of

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Table 1. Diet composition.

	Diets					
Ingredients (g/100 g)	C	AT	OO	HOSO	HOSO-P	HOSO-F
Carbohydrates	49.5	33.3	34.8	34.8	34.8	34.8
Protein (mix of corn, wheat, soybean, fish and meat flour)	23.0	14.3	14.90	14.9	14.9	14.9
Fat (corn oil)	5.0	3.1	3.25	3.25	3.25	3.25
Butter (80% fat)		16				
OO			13			
HOSO				13	13	6.5
Fish oil						6.5
Cholesterol		2	2	2	2	2
Phytosterols					1	
Fiber	6.0	3.7	3.9	3.9	3.9	3.9
Ash	7.5	4.6	4.9	4.9	4.9	4.9
Vitamin mixture	1.0	0.62	0.65	0.65	0.65	0.65
Water	8.0	22.38	22.6	22.6	21.6	22.6
Total kcal	335	335.3	345.0	345.0	345.0	345.0

C: Standard diet consisted of commercially available pellets (Purina chow). The remainder diets were prepared by grinding the C pellets and utilizing 62–65 g to which the desired oils, cholesterol, phytosterols and/or fish oil were added to attain the concentrations listed on the table for each diet: AT: atherogenic diet; OO: extra virgin olive oil; HOSO: High-oleic-sunflower oil; HOSO-P: High-oleic-sunflower oil supplemented with phytosterols; HOSO-F: High-oleic-sunflower oil supplemented with fish-oil.

growing hypercholesterolemic rats, the significance of two components of the Mediterranean diet, phytosterols and n3PUFA, when these are given as supplements in a HOSO diet. The effect of replacing an atherogenic diet (AT) by a HOSO diet supplemented with either phytosterols (P) or fish oil (F) was studied on zoometrics, serum lipoprotein profiles, body fat content and distribution and bone quality.

Materials and methods

Animals

Sixty male weanling Wistar rats (aged 21 ± 2 days), initial body weight $= 50.0 \pm 1.3$ g (mean $\pm$ SD) were obtained from the animal laboratory of the Department of Biochemistry, Faculty of Dentistry, University of Buenos Aires, Argentina. Animals were housed in galvanized cages with meshed floors in order to maintain hygienic conditions and to avoid coprophagy. Rats were kept in individual cages and exposed to a 12-h light/dark cycle throughout the study. Room temperature was maintained at $21 \pm 1\,^{\circ}C$ with a humidity of 50–60%.

Ethics

The rats were maintained in accordance with the USA National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. The protocol for the experiments was approved by the Ethics Committee of the University of Buenos Aires (UBACyT 20020130100506, N° 019/14). The study was part of the doctoral thesis of the lead author of the paper (EA).

Diets

The composition of the diets utilized is shown in Tables 1 and 2. The control (C) diet was a standard diet (Purina chow; Gilardoni SA, Buenos Aires, Argentina). The atherogenic (AT) diet provided butter as the main source of fat (80% fat) and contained 16.1% fat/100 g diet; it was rich in saturated fatty acids and cholesterol (2 g/100 g diet). The OO diet provided this oil instead of butter; it contained 16.25% fat/100 g diet and 2% cholesterol. OO was extra virgin olive oil with a composition of: saturated fat 12.0%, monounsaturated fat 81.9%, polyunsaturated fat 6.10%, trans-fatty acids (TFA) 0%, phenolic compounds 1328 mg/kg. The sterol composition (expressed as % of desmethylsterols among total sterols) was as follows: cholesterol ≤ 0.5; brassicasterol ≤ 0.1; campesterol ≤ 4.0; stigmasterol $<$ campesterol; Delta-7-stigmastenol ≤ 0.5. Beta-sitosterol + Delta-5-avenasterol + Delta-5-23-stigmastadienol + chlerosterol + sitostanol + Delta5-24-stigmastadienol $\geq 93.0\%$.

High-oleic-sunflower oil (HOSO) was the substitute of butter; this diet contained 16.25% fat/100 g diet and 2% cholesterol. HOSO composition was: saturated fat 7.82%, monounsaturated fat 87.11% n-9; polyunsaturated fat 4.75%, cholesterol 0%, TFA 0%. (Tocopherol: 54 mg%. Ecoop, Cooperativa Obrera, Bahía Blanca, Argentina). HOSO supplemented with phytosterols (HOSO-P) contained 16.25% fat/100 g diet, 2% cholesterol and 1% mixed phytosterols (β-sitosterol 45% with 18–27% campesterol, 21–35% stigmasterol and other sterols 0–3%, [SAPORITI SA, Buenos Aires, Argentina]). HOSO-F diet had same total fat content (16.25% fat/100 g diet) but had different fat sources, HOSO (6.5%) was supplemented with deodorized refined fish oil (6.5%); it contained 2% cholesterol and

Table 2. Fatty acids composition and content (g/100 g) from the different lipid sources given to the rats.

Fatty acid	Butter (g/%)	OO (g/%)	HOSO (g/%)	Fish oil (g/%)	Standard diet (g/%)
10:0	2.30	–	–	–	
12:0	2.95	–	–	0.16	
14:0	11.12	–	–	7.00	
16:0	30.87	9.40	3.62	17.33	
18:0	14.58	2.60	3.06	3.50	
20:0	–	–	Tr.	–	
22:0	–	–	0.84	–	
24:0	–	–	0.30	–	
∑SFA	61.82	12.0	7.82	28.24	20.00
16:1	2.52	0.30	–	7.96	
18:1	34.13	81.60	87.11	18.11	
20:1	–	–	Tr	1.84	
∑MUFA	36.65	81.90	87.11	27.91	40.4
18:2	1.53	6.10	4.75	1.26	28.0
18:3	–	–	Tr.	1.16	11.6
20:5 n3	–	–	–	23.98	
22.5 n3	–	–	–	2.23	
22:6 n3	–	–	–	15.22	
∑PUFA	1.53	6.10	4.75	43.85	39.6
Total fat	100	100	99.68	100	100

OO: extra virgin olive oil, HOSO: High-oleic-sunflower oil; ∑SFA: total saturated fatty acids; ∑MUFA: total monounsaturated fatty acids; ∑PUFA n3: total polyunsaturated fatty acids.

42.6% $n-3$ (Parafarm, Saporiti SACIFIA, Buenos Aires, Argentina).

Diets were prepared every 2 days and stored at −4 °C until fed. Fresh diets were offered daily and food containers were cleaned before being refilled. Food cups were refilled once a day, and food consumption was measured with a Mettler scale PC 4000 (accuracy ± 1 mg). Daily food intake was recorded as kcal/100 g of body weight per day (kcal/100 g W/day).

Experimental design

Rats were randomly assigned to the control group (C, $n=12$) and to the experimental group (AT, $n=48$). The control group was fed with a C diet for eight weeks and the AT group received an AT diet for 3 weeks. Then AT rats were randomly assigned to one of four different groups according to dietary intake: OO, HOSO, HOSO-P or HOSO-F. All rats were fed *ad libitum* throughout the experiment. At the end of 8-week experimental period, zoometrics and DXA analyses were performed under light anesthesia (a mixture of 2% xylazine hydrochloride (0.5 mg/100 g i.p. Konig Laboratories. Buenos Aires. Argentina) and 5% ketamine hydrochloride (5 mg/100 g i.p. Holliday–Scott SA. Buenos Aires. Argentina). Then animals were euthanized under anesthesia: 0.1 ml of ketamin hydrochloride (100 mg/ml, Holliday Lab.)/100 g body weight was mixed with 0.02 ml of xylazine (100 mg/ml, Konig Lab.)/100 g body weight by intramuscular injection. Immediately, blood was drawn by cardiac puncture, allowed to clot and the serum was stored at −20 °C until biochemical assays were performed. Biochemical determinations, hepatic index, body fat content and biomechanical assessment were performed in all rats.

Zoometrics

Body weight (W) and body length (L) were measured weekly after a fasting period of 2–4 h which allowed sufficient time to determine all zoometric and other parameters (Vijay et al. 2009). A Mettler PC 4000 scale (accuracy ± 0.001 g) was used to measure W; L was determined in slightly anesthetized rats with a scaled ruler in mm from the nose tip to the hairline of the tail.

Bone mass assessment

At the end of the study, total skeleton bone mineral content (BMC) and bone mineral density (BMD) were assessed *in vivo* under light anesthesia, using a total body scanner with software designed specifically for small animals (DPX Alpha 8034, Small Animal Software, Lunar Radiation Corp, Madison, WI), as previously described (Zeni et al. 2002). All rats were scanned using the procedure reported by Zeni et al. (2002). The coefficients of variation (CV) evaluated by repeated measurements of five subjects of similar age and gender on 5 consecutive days were 3.0% and 0.9% for total skeleton BMC and BMD, respectively. To avoid inter-assay error the same operator performed all DXA measurements.

Biochemical determinations

At week 8, blood samples were drawn from the heart after 4–6 h fasting to determine lipid parameters

(Vijay et al. 2009). Serum was isolated and immediately stored at −20 °C for biochemical analysis. Total serum cholesterol (T-chol; mg/dL of serum), high-density lipoprotein-cholesterol (HDL-chol; mg/dL of serum) and triglycerides (TG; mg/dL of serum) were determined by standardized methods (Roche Diagnostics GmbH, Mannheim, Germany) in a Hitachi 917 auto-analyser (Hitachi, Tokyo, Japan). Circulating serum HDL-chol levels in rats is usually higher than the low-density lipoprotein-cholesterol (LDL-chol; mg/dL of serum), the measurement of this parameter loses sensitivity – therefore, non-HDL cholesterol (mg/dL of serum) was calculated as the difference between the T-chol and HDL-chol. Non-HDL-chol represents the set of atherogenic lipoproteins rich in apo B that constitute the criteria for human intervention and treatment (Zago et al. 2010). TG/HDL-chol ratio was calculated to estimate cardiovascular disease (CVD) risk.

Hepatic index

The liver was removed immediately after death to avoid dehydration, and weighed with an electronic analytical scale. Data were used to calculate the hepatic index, expressed as liver weight/total body weight × 100 (% LiW/TBW); the liver of each rat was normalized to the percentage of TBW to minimize the individual differences in body size. This index was used to determine enlargement of the liver (hepatomegaly) (Wang et al. 2002; Qin & Tian 2010).

Body fat tissue

Fat content from epididymal, intestinal and perirenal areas were identified, removed and weighed in order to evaluate visceral adiposity. Chemical carcass analysis was performed as described elsewhere (ATP III, NCEP 2001]. Total body fat content was determined by Soxhlet extraction method (AOAC 1990). Data were expressed as percent of body weight.

Biomechanical tests on femur

The femurs were submitted to a three-point bending test in a computerized Instron Universal Testing Machine (model 4442; Canton, MA). In this procedure of bone breaking, the force was delivered to the mid-shaft by a crosshead at a constant speed. The breaking force was applied perpendicularly to the long axis of the bone at mid-shaft. Bones were placed lying horizontally with the anterior aspect facing down on two supports equidistant from their ends, separated by a constant distance and loaded (50 Newton = N) centrally at a speed of 5 mm/min. Femurs were broken from the anterior to posterior plane. The plots of load *v*. deformation (W – d) obtained were analyzed to determine bone mechanical properties. These plots recorded both the elastic (linear) and plastic components, separated by the yield point. The following structural (whole-bone) mechanical properties were determined: (1) Load-bearing capacity (Wf) or fracture load (N), which expresses bone strength in a broad sense. (2) Yielding load (Wy), load at the yield point (N), an expression of bone maximum elastic strength. (3) Yielding deformation (dy), arrow of the arch formed by the bending bone at the yield point (mm), an index of maximum elastic deformation. (4) Bone stiffness in elastic conditions or load-to-deformation ratio (Wy/dy), determined graphically at any point over the linear portion of the curve (N/mm). Because bone segments between the supports were closely comparable to hollow elliptically shaped cylinders, the micromorphometry of the horizontal and vertical external (H and V) and internal (h and v) diameters of the fracture sections enabled us to calculate the following geometric properties: (1) Cross-sectional bone area, CSA: π/4.V.H. (mm^2), (2) Medular area, $MA = \pi/4.v.h$ (mm^2), (3) Cross-sectional cortical bone area, $A = CSA - MA$ (mm^2), (3) Second moment of inertia of the cross-section in relation to the horizontal axis, $Ix = \pi\ (V^3H - v^3h)/64$ (mm^4). A stress–strain curve was determined from the previous force-deformation curve using engineering formulae (Turner & Burr 1993). Material properties of the bone tissue were calculated as follows: (1) Yield stress, the force per unit cross-sectional area at the yield point, $sy = WyLB = 8Ix$ (N/mm^2), where L is the distance between the supports, an estimation of tissue strength and (2) Young's modulus of elasticity, $E = WyL3 = 48dyIx$ (N/mm^2), represented by the slope of the stress–strain curve within the elastic region, which is an estimator of tissue intrinsic stiffness.

Statistical analysis

The results were expressed as mean values with their standard deviations (SD). One-way analysis of variance (ANOVA) was used to compare data among groups. When a statistically significant difference was encountered a Student–Newman–Keul's test or Dunn (not parametric test) was performed. In all the analyses, Bartlett's test for homogeneous variances was done. The Kolmogorov–Smirnov test was used to verify whether data had normal distribution. Significance was set at the $p < 0.05$ level (Glantz 1997). The Statistical Product and Service Solutions for Windows 14.0

Table 3. Zoometrics and Energy intake of the different groups.

	Initial Body Weight (g)	Final Body Weight (g)	Initial Body Length (cm)	Final Body Length (cm)	Body Weight Gain (g/100 g rat/day)	Energy Intake (kcal/100 gW/day)
C	46.38 ± 5.01	305.74 ± 54.49	13.09 ± 0.60	23.05 ± 1.16	2.43 ± 0.21	34.76 ± 6.74
OO	50.51 ± 5.74	294.63 ± 20.33	12.90 ± 0.53	22.82 ± 0.32	2.36 ± 0.12	40.91 ± 11.05
HOSO	47.80 ± 5.96	278.65 ± 28.35	13.07 ± 0.59	22.64 ± 0.91	2.35 ± 0.14	38.57 ± 8.11
HOSO-P	50.97 ± 5.27	278.21 ± 25.27	13.03 ± 0.34	22.75 ± 0.58	2.30 ± 0.08	38.98 ± 7.62
HOSO-F	47.30 ± 5.90	276.15 ± 39.22	12.82 ± 0.48	22.82 ± 0.93	2.34 ± 0.18	35.7 ± 7.53
P	0.121	0.200	0.654	0.805	0.345	0.338

C: Control group; OO: extra virgin olive oil group; HOSO: High-oleic-sunflower oil group; HOSO-P: High-oleic-sunflower oil supplemented with phytosterols group; HOSO-F: High-oleic-sunflower oil supplemented with fish-oil group. Data are means ± SD.

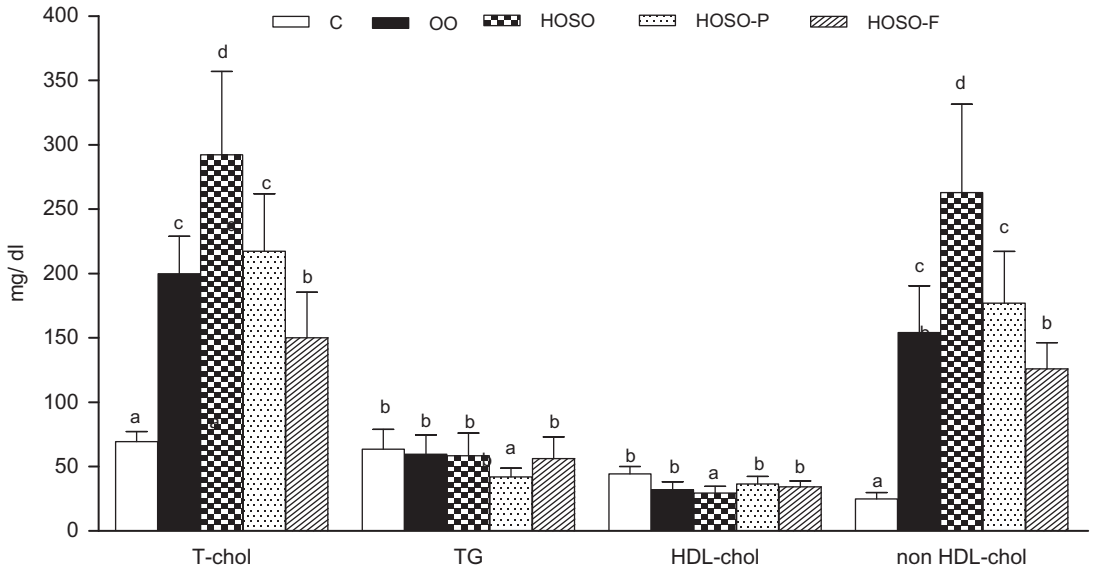

Figure 1. Serum lipid lipoprotein profile. Total cholesterol (T-Chol), Triglycerides (TG), High density lipoprotein (HDL-chol) and non-HDL-Chol (non HDL-chol). C: control group; OO: extra virgin olive oil group; HOSO: High-oleic-sunflower oil group; HOSO-P: High-oleic-sunflower oil supplemented with phytosterols group; HOSO-F: High-oleic-sunflower oil supplemented with fish-oil group. Values with different superscript letters are significantly different ($p < 0.05$) among groups. Data are means ± SD.

(SPSS, Inc., Chicago, IL) and Graphpad Prism (version 5.0) statistical package (Graphpad) were used for statistical analyses.

Results

Effects on zoometrics and energy intake

The final body weight and body length of the rats, as well as the food consumption of the four experimental groups and the C rats are shown in Table 3. All the animals in the five groups had similar body weights and lengths in the beginning of the experiment. The type and amount of fat in the diets did not alter final body weight ($p = 0.200$) or linear body growth ($p = 0.805$) over the duration of the experiment. All the groups of rats attained similar body weights and size at the end of the experimental period. Rats fed MUFA diets containing either extra OO or HOSO did not show significant differences in energy intake as compared with C group ($p = 0.338$), despite some differences in energy content among the diets. Also phytosterols and fish oil supplementation did not alter food consumption or energy intake.

Effects on serum lipid profile

Serum lipid profiles of the five groups of rats are shown in Figure 1. All the rats fed MUFA diets rich in cholesterol showed differences among them and with the C group. HOSO rats showed the highest serum T-chol and non-HDL-chol levels; and had the lowest HDL-C concentrations ($p < 0.001$). The supplementation of P (HOSO-P) diminished the T-chol and the non-HDL-chol levels to values that were similar to those attained by the OO group. However, with the supplementation of fish oil (HOSO-F) the rats attained significantly reduced levels of these two serum lipoproteins, below OO rats, though the serum TG levels were not improved. Both HOSO-P and HOSO-F intake increased serum HDL-chol levels; both of these groups attained a similar concentration of HDL-chol as that of the OO rats. TG/HDL-chol index was significantly lower in supplemented groups rather than in HOSO

fed rats; HOSO-P: 1.18 ± 0.30; HOSO-F: 1.64 ± 0.48; HOSO: 2.04 ± 0.42 ($p = 0.0001$).

Effects on total body fat and body fat distribution

The percentages of total body fat and final body fat distribution at the end of the experimental period are shown in Figure 2. The percentage of total body fat was not different among the rats fed the various diets ($p = 0.11$). Rats consuming the HOSO-F diet showed intestinal fat levels that were similar to the C group; these were significantly lower as compared with the OO, HOSO and HOSO-P rats ($p < 0.001$). The percentages of epididymal fat were higher in HOSO rats ($p < 0.05$); the perirenal fat was similar in the C and the experimental groups ($p = 0.05$). However, epididymal fat and perirenal fat in HOSO-F rats showed a tendency to lower levels. The hepatic index was approximately 74% higher in all MUFA diets as compared with the C rats. The mean ± SD hepatic index was similar among the OO (6.69 ± 0.42), HOSO (6.38 ± 0.48), HOSO-F (6.47 ± 1.17) and HOSO-P (6.32 ± 0.76) rats; $p = 0.721$. However, all MUFA fed rats had a hepatic index that was significantly increased as compared with the C group (3.7 ± 0.21) ($p = 0.001$).

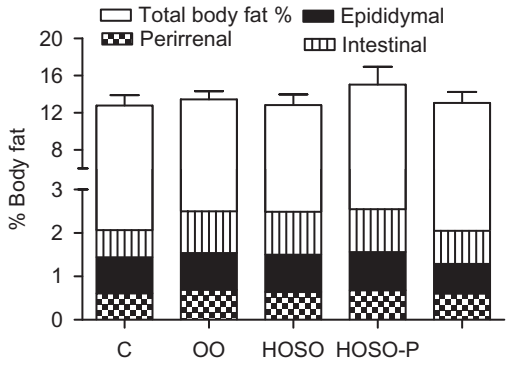

Figure 2. Percentage of total body fat and body fat distribution. C: control group; OO: extra virgin olive oil group; HOSO: High-oleic-sunflower oil group; HOSO-P: High-oleic-sunflower oil supplemented with phytosterols group; HOSO-F: High-oleic-sunflower oil supplemented with fish-oil group. Data are means ± SD. Percentage of total body fat: no SD among groups ($p = 0.11$). Percentage of intestinal fat: HOSO-F and C < OO, HOSO and HOSO-P ($p < 0.001$). Percentage of Epididymal fat: HOSO > C, OO, HOSO-P and HOSO-F ($p < 0.05$). Percentage of perirenal fat: no SD among groups.

Effects on bone mass

The results of total skeleton BMC and total skeleton BMD are shown in Figure 3. Rats fed MUFA diets had decreased BMC and BMD as compared with the C group ($p < 0.001$). However, the BMC and BMD was similar among the OO, HOSO, HOSO-P and HOSO-F rats; $p = 0.881$ and $p = 0.101$, respectively.

Effects on bone biomechanical competence

The load-bearing capacity and stiffness of the femoral diaphysis is shown in Figure 4.

Structural properties, i.e. load-bearing capacity (Wf), yielding load (Wy) and diaphyseal stiffness (Wy/dy), were negatively affected in MUFA rats as compared to the C group ($p < 0.0001$). (Figure 4A–C, respectively).

The geometrical properties of the femur are shown in Figure 5. The cross-sectional bone area (CSA), cross-sectional cortical area (*A*) and moment of inertia of the fracture section (*Ix*), were significantly lower in rats fed MUFA-rich diets than the values found in the C rats ($p = 0.000$) (Figure 5A–C, respectively).

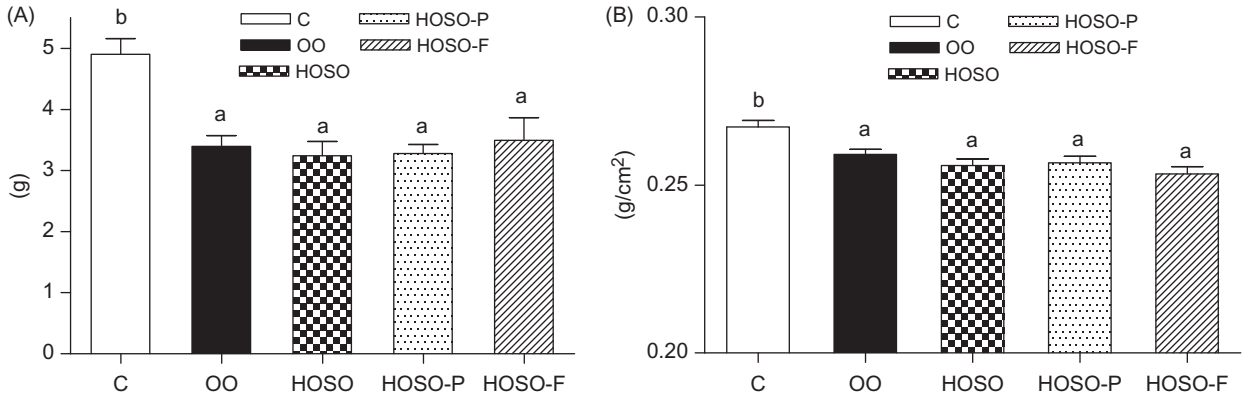

Figure 3. Bone mineral content and Bone mineral density in different groups. (A) Bone mineral content; (B) Bone mineral density. C: control group; OO: extra virgin olive oil group; HOSO: High-oleic-sunflower oil group; HOSO-P: High-oleic-sunflower oil supplemented with phytosterols group; HOSO-F: High-oleic-sunflower oil supplemented with fish-oil group. Data are means ± SD. Values with different superscript letters are significantly different ($p < 0.05$) among groups.

Young's modulus of elasticity that is an estimator of tissue intrinsic stiffness, represented by the slope of the stress–strain curve within the elastic region of the femoral diaphysis, is shown in Figure 6. There were no significant differences among material properties, *E* and sy (Figure 6A–C). The effects of supplementation with phytosterols or fish oil did not influence the material bone quality in terms of yield stress and modulus of elasticity (*E*).

Discussion

The findings of this study confirmed the observations previously made (Macri et al. 2015). We established that substituting saturated fat for MUFA rich diets ameliorated dyslipidemia and improved bone mass in hypercholesterolemic growing rats. However, HOSO rats continued to demonstrate hypercholesterolemia and high non-HDL-chol, low HDL-chol and increased epididymal fat content as compared with OO fed rats (Macri et al. 2015).

In this study, we showed that HOSO supplementation with either phytosterols or fish oil improved serum lipoprotein profiles and body fat distribution. Phytosterols supplemented HOSO showed a lipid profile that resembled that of the rats fed OO – both showing the combination of lower serum T-chol and non-HDL-chol levels, considered positive for atherosclerosis protection. These data are in agreement with others regarding the treatment of hypercholesterolemia

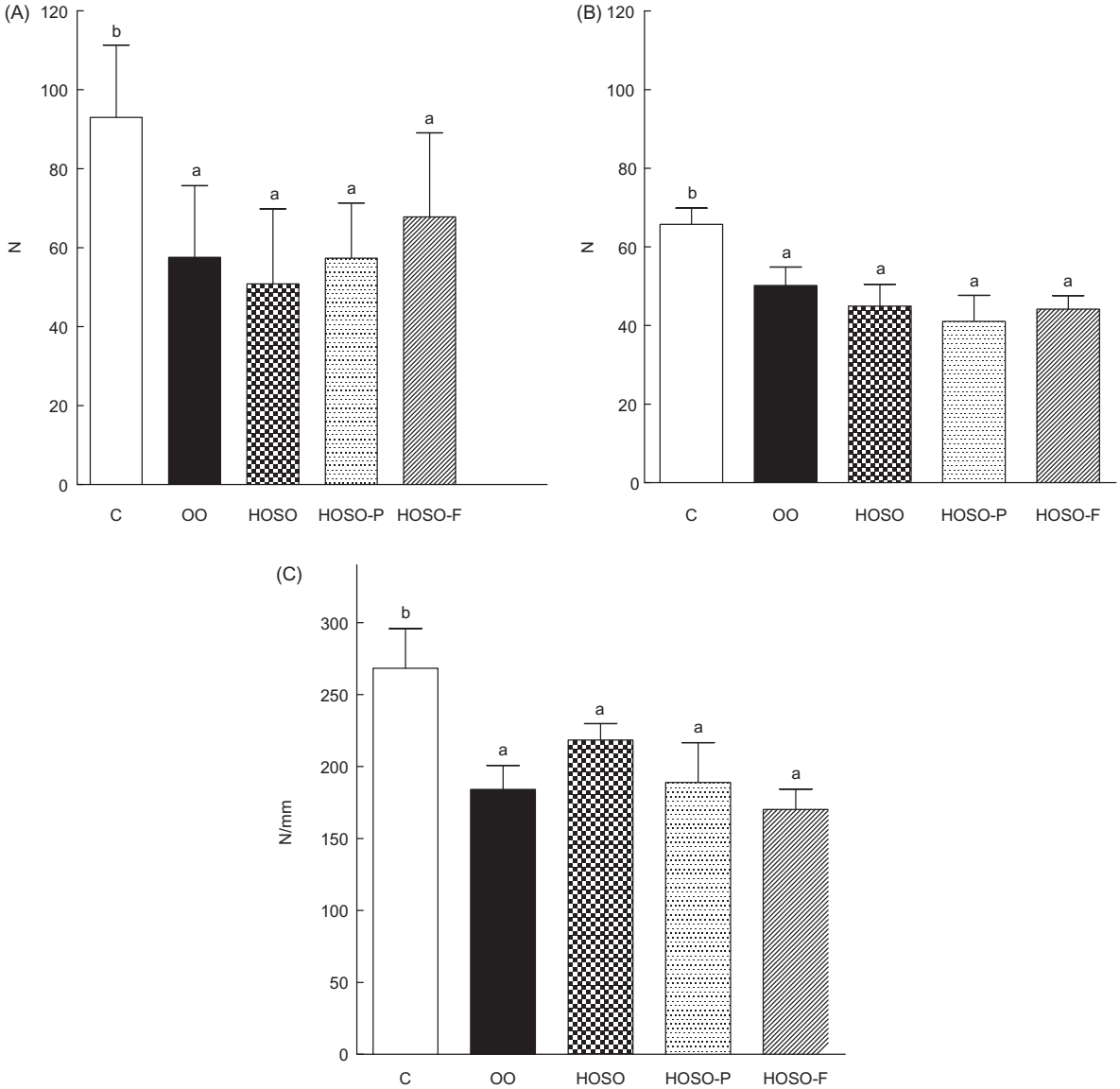

Figure 4. Load-bearing capacity (A), yield load (B) and stiffness in elastic conditions (C) of the femoral diaphysis. Values are means, with standard errors of the mean represented by vertical bars, for ten animals per group. Mean value is significantly different from that of the control at the same time point: $p < 0.0001$. Data are means ± SD. Values with different superscript letters are significantly different ($p < 0.05$) among groups.

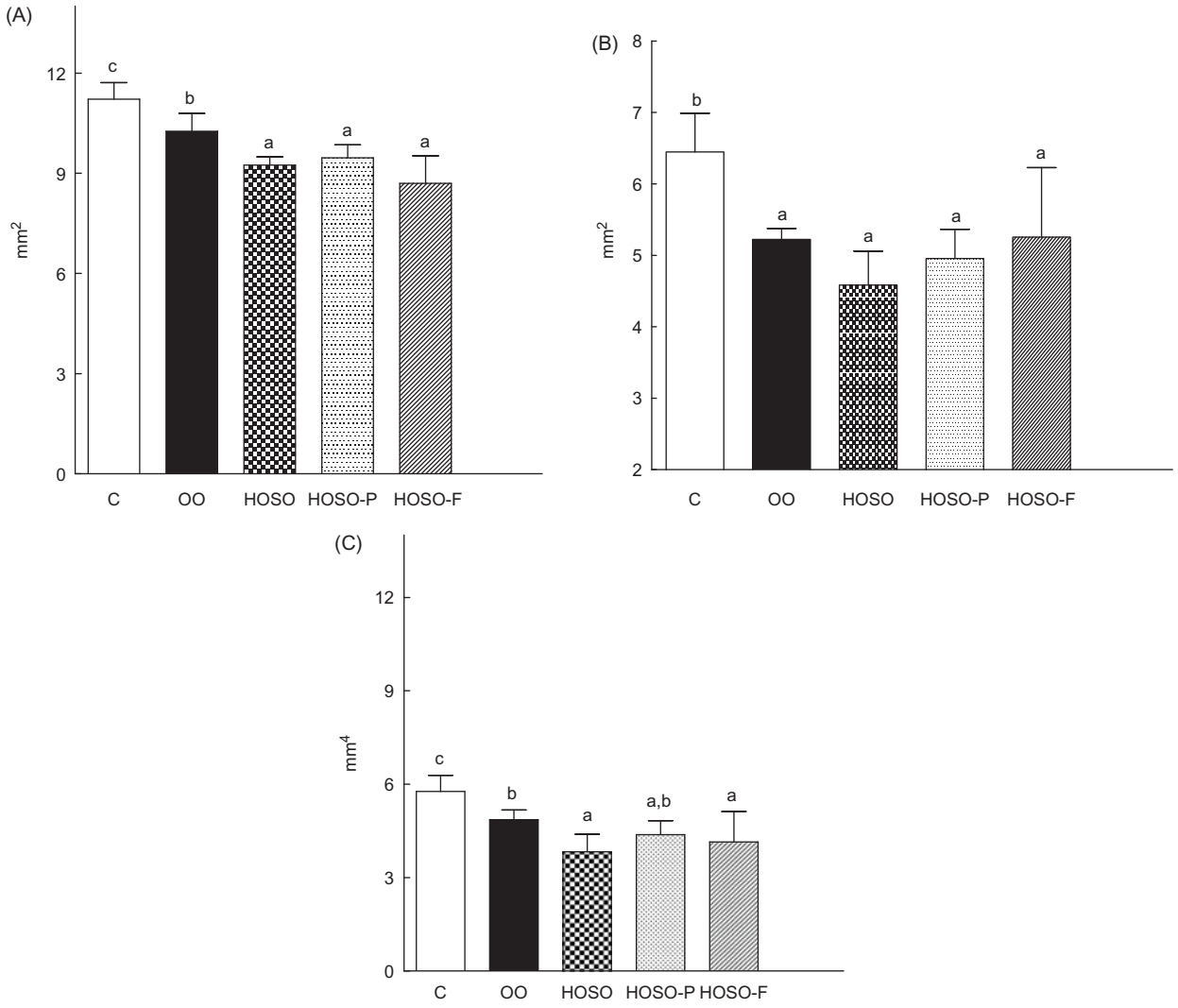

Figure 5. Cross-sectional bone area (A), cross-section cortical area (B) and moment of inertia (C) of the fracture section of the femoral diaphysis Values are means, with standard errors of the mean represented by vertical bars, for ten animals per group. Mean value is significantly different from that of the control at the same time point: $p = 0.001$. Data are means ± SD. Values with different superscript letters are significantly different ($p < 0.05$) among groups.

with diets enriched with phytosterols (Lerman et al. 2008). These authors showed beneficial effects on the lipid profile, possibly due to the effect of dietary phytosterols, which may help reducing blood cholesterol levels through the inhibition of its absorption from the small intestine (Ostlund et al. 2002).

However, fish oil supplementation of the HOSO diets attained significantly reduced T-chol and non-HDL levels, below OO rats and below the HOSO-P group. Thus, the HOSO-F supplementation data of our studies were in conformity with data showing that fish oil was a better option of cardiovascular disease (CVD) prevention, progression and mortality (Ostlund et al. 2002; He et al. 2004; Lerman et al. 2008; Harris et al. 2008; Lankinen et al. 2014). In addition, HOSO-F improved HDL-chol levels comparably to OO rats. Studies investigating the effects of fish oil on HDL-chol particles subspecies have reported increases in large HDL particles (Burillo et al. 2012) that are inversely associated with CVD (Pirillo et al. 2013).

In this study, there was a lack of a lowering effect on serum triglycerides levels by fish oil. This was likely due to the quantity of fish oil used in our experiments. In fact, some studies that compared different fish oil doses found that the greatest decreases in triglycerides occurred among subjects consuming the highest doses of fish oil. This outcome was dose dependent and this effect could be attributable to a decrease in the hepatic production of triglyceride rich particles and to an increase in fractional clearance rates (Balk et al. 2006).

The diminished serum T-chol and triglycerides levels observed in rats fed the HOSO diet supplemented

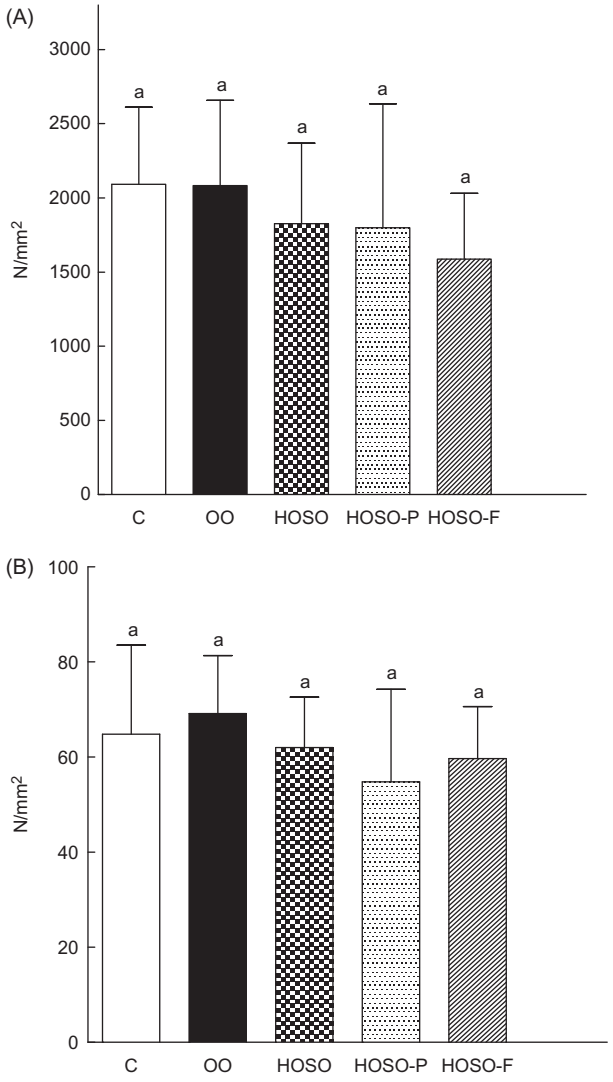

Figure 6. Yield stress (A) and elastic modulus (B) of the femoral diaphysis. Values are means, with standard errors of the mean represented by vertical bars, for ten animals per group. Data are means ± SD. Values with different superscript letters are significantly different ($p < 0.05$) among groups.

with P or F as compared with rats fed saturated fat diet (Macri et al. 2015), exhibited the beneficial effects of the supplementation on serum lipid biomarkers.

Supplementation of HOSO diet with P or F did not modified body fat content as compared with rats fed with HOSO diet as well as in rats fed saturated fat diet (Macri et al. 2015).

The adipose tissue biology in rodents has similar characteristics to other visceral adipose tissues (Yamaguchi et al. 2012). Epididymal fat storage tissue is anatomically indistinguishable from intestinal and kidney fat, metabolically it is considered an intra-abdominal fat type (Altintas et al. 2011). Several studies have consistently shown that excessive visceral fat accumulation is related with an increased CVD risk as well as several inflammatory and metabolic disturbances (Costa et al. 2011; Zaman et al. 2011). The highest increase in the amount of epididymal fat was detected in rats fed the HOSO diet; this may denote a higher risk of CVD. The supplementation with phytosterols and/or fish oil diminished its content and therefore it may be surmised that the CVD risk would also be ameliorated.

Our results evidenced that HOSO diet rich in either P or F diminished visceral fat content as compared with rats fed saturated fat diet (Macri et al. 2015).

Differences in visceral fat content may serve as a metabolic marker of insulin resistance, whereby fat will be deposited in undesirable places such as the liver, intra-abdominally (Després et al. 2008) or bone lipids (Parhami et al. 1999, 2001). Fat accumulation in the liver could cause further metabolic dysfunctions, eventually resulting in liver damage (Qin & Tian, 2010). Our previous studies demonstrated that an atherogenic diet increased liver weights; this was almost entirely at the expense of lipid deposits, observed by histological methods with micro- and macro-vesicular fat deposits (Zago et al. 2010). In this study, the replacement of dietary saturated fat by supplemented HOSO with phytosterols or fish-oil still did not inhibit the pronounced increase in liver weights. Future histological studies will elucidate whether HOSO-P and HOSO-F diets are prone to increase liver weights due to lipid deposit.

In this study, MUFA diets contained 65% of protein, fiber and vitamins respect to control diet; however, no differences on growth parameters among groups were observed besides it is well known the strong relationship between protein and vitamin content in the diet and body growth. Then, we infer that that differences on serum lipids, body fat composition and distribution, could be attributable to the type and amount of fat diet.

Similarly, the replacement of saturated fat by MUFA-rich diets did not improve bone mass, BMC and BMD as seen previously in rats fed saturated fat diet (Macri et al. 2015), Studies *in vitro have* demonstrated that a minimal oxidation of LDL-chol influenced by the type of dietary oil intake and its oxidation, might affect bone mineralization and bone marrow differentiation (Parhami et al. 1999, 2001). This may be the consequence of inhibition of the differentiation and mineralization of osteoblastic cells. In this study, the reduction in bone mass was more related to the presence of a MUFA-rich diet, rather than to the source of MUFA. Previous studies have also shown that diets with high fat content have an adverse effect on bone mineralization due to lipid oxidation and lipid composition alterations (Covas 2008)

resulting in low bone mass and poor bone quality in growing animals (Lac et al. 2008).

Atherogenic (AT) diets represented a risk factor for bone modeling; rats fed PUFA-rich diets showed altered tibiae static-histomorphometry analysis, low bone alkaline phosphatase activity (Gamba et al. 2009; Macri et al. 2009] and low BMC with an inadequate subendochondral ossification and mineralization (Macri et al. 2009, 2012). The diets and supplements used in this study could not reverse the detrimental effects of the high-saturated animal fat intake; the phytosterols and fish oil additions were not sufficient to ameliorate the consequences of hypercholesterolemia on bone modeling.

The mechanical properties of bone are determined by both geometry (bone architecture) and material properties (bone tissue) (Turner & Burr 1993). Changes in the structural properties of a solid body of bone could be due to changes in mass and its spatial distribution (geometry) and/or intrinsic mechanical quality of its constitutive substance (material properties) (Ferretti 1997). Our results showed that the mechanical strength of femoral diaphysis was negatively affected in response to external loading in animals fed OO or HOSO as compared with C rats. It was also evident that HOSO supplementation with phytosterols or fish oil did not reverse detrimental bone competence. In addition the cross-sectional parameters, CSA, A and Ix, were significantly lower in OO, HOSO, HOSO-P and HOSO-F rats as compared with C animals. However, the material quality of bone (E and sy) (Ferretti et al. 1991) was not affected by MUFA rich diets; this is in agreement with other authors (Boyer et al. 2005; Pintos et al. 2013; Tasat et al. 2014). The lack of difference in material properties, as expressed by modulus of elasticity and yield stress between groups, suggests that the impaired performance of diaphyseal shafts of MUFA rats should be regarded as the result of changes in bone mineral content and spatial distribution of bone material, rather than bone intrinsic quality. Bone with a faster turnover rate (modeling), could reveal potential adverse effects of inappropriate types of fat intake on the skeletal and biomechanical bone properties of a growing animal (Byers et al. 2000).

Comparison between AT-fed rats (Macri et al. 2015) and either HOSO diet alone or HOSO supplemented with P or F, revealed that the replacement of saturated fat by MUFA rich diets did not induce significant changes in bone mass and quality during the experimental period.

In this study, bone mineral content and density as well as bone biomechanical competence were lower in MUFA diet fed rats compared to C group. Since previous studies by us (Macri et al. 2015) showed lower BMC and BMD in rats fed with MUFA diets versus C, besides both containing the same type and concentration of calcium, phosphorus and Vitamin D; therefore, this study suggests that differences observed on bone parameters could be due to MUFA rich diets.

Studies by others, in human and animals, have found that hypercholesterolemia and the resulting deposition and oxidation of lipids in tissues lead to atherosclerosis (Ross 1999; Hmamouchi et al. 2009; Tamaki et al. 2009) and osteopenia (Parhami et al. 2000). A low BMD was used as a clinical marker for coronary artery disease (Barengolts et al. 1998; Tanko et al. 2003). The presence of arterial disease and osteoporosis may denote that the same bioactive lipids, lipoproteins and phospholipids that promote atherosclerosis also adversely affect bone (Tintut & Demer 2014). Atherogenic high-fat diets further increase lipoprotein levels and their oxidative products attenuate osteogenic differentiation *in vitro* and induce osteoclast genesis (Pirih et al. 2012).

Conclusion

The results of this study in growing hypercholesterolemic rats indicate that the replacement of saturated fat by HOSO supplemented with phytosterols or fish oil provides beneficial effects on the cardiovascular system markers, but not sufficiently enough to be reflected on the skeletal system.

It is known that serum lipid abnormalities have been contributed to atherosclerosis, and several epidemiological and cohort studies have established a strong association between T-chol, LDL-chol or low HDL-chol and the incidence of atherosclerosis-related diseases, such as ischemic heart disease, stroke and peripheral vascular disease (da Luz et al. 2008). Moreover, TG/HDL-chol index is predictive of risk of CVD and incidence of ischemic heart disease (Jeppesen et al. 1997). In fact, TG/HDL-chol ratio was chosen as a CVD risk factor, rather than considering LDL-chol or T-chol as individual risk factors, because the combination of both lipid components resembles the effect of diet on serum lipid lipoprotein profile as we demonstrated in previous studies (Lifshitz et al. 2012). Our results evidence the HOSO supplemented diets provide a cardio-protection effect.

The cardio-protective effects that MUFA could confer may be related to other bioactive compounds present in the oil, rather than MUFA *per se*. It is well known that the beneficial effects of OO on CVD risk are related to phenolic components, which reduce oxidative lipid

damage, and not due to MUFA content *per se* (Fitó et al. 2000; Macri et al. 2015). The observed changes on lipid lipoprotein profiles of HOSO intake with phytosterols suggest that the strong antioxidant properties of phenolic components account for the improved lipoprotein markers in OO and HOSO-P, not evident in the rats fed the HOSO diet (Fitó et al. 2000). However, our data also demonstrated that the changes found on serum T-chol, HDL-chol and non-HDL-chol levels and body fat distribution in rats fed HOSO-F diet constituted a more effective option for the treatment of hypercholesterolemia (Lankinen et al. 2014). A variety of international recommendations have urged to replace or eliminate trans-fatty acids from food products (FAO/WHO 2009; Uauy et al. 2009). Therefore, in order to fulfill this requirement food industries have introduced monounsaturated fatty acid (ω-9MUFA) oils based on the observed OO benefits (Macri et al. 2007). HOSO has also been introduced for frying processes and baking (Valenzuela 2008). Although HOSO has a similar composition of ω-9MUFA as compared to OO, it differs in the amount and type of vegetable sterols and other non-nutritive components (Huang & Sumpio 2008). Thus, the replacements introduced to eliminate trans-fatty acids and/or to potentially derive benefits of the substitution of atherogenic products must be fully evaluated.

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Disclosure statement

The authors declare that they have no conflict of interest.

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