Eur J Nutr (2007) 46:125–132
DOI 10.1007/s00394-006-0640-9

ORIGINAL CONTRIBUTION

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Soy isoflavones reduce electronegative low-density lipoprotein (LDL^-) and anti-LDL^- autoantibodies in experimental atherosclerosis

Received: 17 May 2006
Accepted: 12 December 2006
Published online: 30 January 2007

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Abstract *Background* Isoflavones present in soybean may contribute to soy atheroprotectective effects. *Aim of the study* To investigate the effect of soy isoflavones supplementation on the formation of electronegative LDL (LDL^-) and its autoantibodies in blood plasma and aortic atheromas of rabbits fed an atherogenic casein-based diet enriched with isoflavones. *Methods* New Zealand male rabbits (n = 15) were fed an atherogenic diet (27% casein) supplemented with isoflavones (0.73 or 7.3 mg of isoflavones/kg/day, Low and High Iso groups, respectively) for 180 days. Monthly, blood samples were collected after 12–15 h fasting and at 180 days of treatment all animals were sacrificed. Isoflavones were analyzed in plasma and urine samples by HPLC. LDL^- in plasma and atheromas was detected by ELISA and immunohistochemistry, respectively, with a monoclonal antibody reactive to LDL^-. Autoantibodies reactive to LDL^- were analyzed in plasma and aorta by ELISA. *Results* Low and High Iso groups had decreased LDL-cholesterol, increased HDL-cholesterol and lower levels of LDL^- in blood plasma and aortic atherosclerotic lesions than the non-supplemented Control group. IgG autoantibodies reactive to LDL^- were higher in plasma of the Control group in comparison with the High and Low Iso groups. In contrast, the aortas from animals that consumed isoflavones showed higher levels of IgG reactive to LDL^- than the Control group. *Conclusion* Soy isoflavones showed hypolipidemic effects and decreased the pro-inflammatory LDL^- subfraction in blood plasma and aorta of hypercholesterolemic rabbits.

Key words minimally modified LDL – oxidized LDL – hypercholesterolemia

Introduction

Atherosclerosis is the major disease of the artery wall in Western populations induced by a wide range of environmental and genetic factors [1]. Several studies have shown that components present in abundance in diet of Asian populations apparently contribute to reduce the incidence of coronary heart disease [2]. This diet-protective role has been associated to soy-derived substances [3]. Among them, isoflavones have demonstrated in vitro [4] and in vivo [5] beneficial properties. Different mechanisms may contribute to the protective effect of isoflavones intake that include improvement of lipid profile [6,

7] and antiproliferative effect on cells of vessel wall [8]. The estrogenic actions of isoflavones may also be protective as both estrogen receptors α and β are expressed in the arteries [9]. Accordingly, the cholesterol-lowering effect of soybean has been strongly associated to the estrogenic activity of isoflavones [10]. Moreover, it has been confirmed that the presence of isoflavones in soy protein is essential for reduction of cholesterol levels and atherosclerotic plaque development in primates [11]. In addition, soy isoflavones can prevent lipid peroxidation by scavenging lipid-derived peroxyl radicals [12] and inhibit copper-dependent LDL oxidation [13]. In fact, dietary supplementation with products containing isoflavones enhances LDL resistance to copper-dependent oxidation [14]. Therefore, considering that oxidized LDL is important for atherogenesis [13, 14], isoflavones consumption can be important for subjects with high risk for atherosclerosis whose LDL has reduced resistance to oxidation, as well as, increased levels of autoantibodies reactive to oxidized LDL [15]. LDL particles can be oxidatively modified in vivo giving rise to different modified particles including a more electronegative LDL subfraction [16]. The electronegative LDL (LDL^-) found in blood plasma has proinflammatory properties [17] and shows oxidative changes, such as, high content of conjugated dienes and lipid hydroperoxides and a reduced amount of α-tocopherol in comparison with native LDL [18]. In the present study, we have investigated the influence of soy isoflavones supplementation on generation of LDL^- and its reactive autoantibodies in blood plasma and atherosclerotic lesions of rabbits fed casein-based hypercholesterolemic diet.

Material and methods

Animals and diet

A 15-days-old male New Zealand rabbits weighing 1.4 kg purchased from PROCRIA (Suzano, Sao Paulo, Brazil) were divided into three groups: Control ($n = 5$), rabbits fed a diet containing 27% casein (ROSTER, São Paulo, Brazil); Low Iso group ($n = 5$), rabbits fed 27% casein diet plus 0.73 mg of isoflavones/kg body weight/day; and High Iso group ($n = 5$), rabbits fed 27% casein diet plus 7.3 mg of isoflavones/kg body weight/day. At the end of experimental time (6 months) the rabbits were killed under anesthesia (50.0 mg/kg, Ketalar®, Parke-Davis) by exsanguination. The experimental protocol was according to the ethical guidelines of the institution for studies with animals. The chow chemical composition (Table 1) was analyzed according to the Association of Official Analytical Chemists [19] and isoflavones content and composition were determined as described by Franke et al. [20]. The diet consumption and body weight variation were monitored monthly (Table 2).

Table 1 Chemical composition of chow administrated to Control, Low Iso and High Iso groups during the experimental time

Nutrients[a]	mg/kg	g/kg
Casein		270
Dextrose		585
Cellulose		50
Corn extract (50%, v/v)		30
Lipids		10
Vitamin mixture		15
Mineral mixture		40
Isoflavones in Low Iso group	0.73	
Isoflavones in High Iso group	7.30	

Nutrient concentrations were based on the National Research Council (1985) Report and Reborts et al. (1981). The isoflavones content was determined as described by Franke et al. (1998). All the experimental groups were mantained with the standard diet described above (Control group) plus 0.73 mg isoflavones (Low Iso group) or 7.3 mg isoflavones (High Iso group). Diet formulated was supplied by Roster®, São Paulo, Brasil

[a]The concentrations of protein, lipid, fiber, minerals, and water were analyzed after chow preparation. Analyses were done in triplicates, according to the Association of Official Analytical Chemists (1980)

Isoflavones purification and quatitation

Isoflavones were extracted from soy molasses and purified as previously described [20–22]. The isoflavones from chow, plasma and urine were analyzed according to Ref. [22, 23].

Table 2 Evaluation of body weight variation, chow consumption and coefficient of alimentary efficacy (CAE) and concentration of isoflavones in plasma and urine in the studied groups

	Control	Low Iso	High Iso
Chow intake (kg)[1]	0.07 ± 0.02^a	0.06 ± 0.01^a	0.06 ± 0.60^a
Δ Weight (kg)[2]	1.72 ± 0.33^a	2.21 ± 0.14^b	2.39 ± 0.29^{bc}
CAE[3]	23.57 ± 2.01^a	40.89 ± 9.81^b	44.06 ± 11.64^{bc}
Plasma (uM)			
Total isoflavones	0.27 ± 0.04^a	2.30 ± 0.38^b	2.78 ± 0.27^b
Genistein	0.03 ± 0.00^a	0.28 ± 0.05^b	0.18 ± 0.05^b
Daidzein	0.09 ± 0.00^a	0.64 ± 0.15^b	0.44 ± 0.32^b
Equol	0.15 ± 0.04^a	1.38 ± 0.33^b	1.89 ± 0.17^b
Urine 24 h (uM)			
Total isoflavones	3.01 ± 0.63^a	6.36 ± 1.76^b	33.33 ± 1.76^c
Genistein	0.24 ± 0.08^a	0.71 ± 0.21^b	3.51 ± 0.15^c
Daidzein	0.91 ± 0.31^a	1.46 ± 0.34^b	4.38 ± 0.31^c
Equol	1.58 ± 0.59^a	4.08 ± 1.36^b	24.56 ± 1.36^c

Values are presented as mean ± standard deviation. [a]Control group; [b]Low Iso group and; [c]High Iso group. Different characteres indicate significant differences among the groups ($P < 0.05$) by Tukey's test. [1]Mean values of chow ingestion/day monitored montly (10 days/month). [2]Mean of body weight variation during the experimental time (6 months). [3]Values obtained by the chow ingestion/body weight ratio

Sample collection

Blood was collected in 1.0 mg/ml EDTA after 12–15 h fasting at basal, 30, 60, 90, 120, 150, and 180 days of feeding. Plasma was immediately separated and treated according to Ref. [24].

Biochemical analysis

The cholesterol in plasma and lipoproteins (VLDL, LDL, and HDL) were monitored by using an enzymatic assay (BioSystem, Barcelona, Spain). Protein was analyzed according to Ref. [25].

Detection of LDL⁻ in plasma and atherosclerotic lesions

Electronegative low density lipoprotein was LDL⁻ was determined in blood plasma by ELISA and in sections (4.0 μm thickness) obtained from cryo-preserved aorta atherosclerotic lesions by immunohistochemistry as previously described by Damasceno et al. [26]. Negative controls for aorta immunohistochemical analysis were done by incubating sections of atherosclerotic and non-atherosclerotic aorta with either polyclonal goat anti-sheep IgG2b plus anti-mouse biotinylated IgG in the absence of anti-LDL⁻ MAb or only with the anti-mouse biotinylated IgG.

Detection of autoantibodies reactive to LDL⁻ in blood plasma

The IgG antibodies reactive to LDL⁻ from blood plasma of rabbits were determined according to Ref. [27] except for the HRP-conjugated dog anti-rabbit IgG polyclonal antibody used here. Results were expressed as IgG equivalent (Abs x titer of LDL⁻ minus Abs × titer of native LDL) in relation to an IgG standard curve (1–2900 μg/ml).

Autoantibodies reactive to LDL⁻ in atherosclerotic lesions

At 180 days, segments of aorta were collected for anti-LDL⁻ autoantibodies analysis. The IgG from aorta was extracted and purified according to Yla-Hertuala et al. (1988) and Owens et al. (1997) [28, 29]. Anti-LDL⁻ IgG was evaluated as described for blood plasma samples.

Statistical analysis

The mean area under the curve was used considering data obtained from zero to 180 days of experimental time for each animal. The homogeneity test was applied (Levene test) and variation between groups was determined by MANOVA. The differences between groups were evaluated by Tukey test ($P < 0.05$) by using SPSS 10.

Results

Diet consumption

No differences were observed among groups in relation to chow consumption (Table 2). Body weight variation and the coefficient of alimentary efficacy (CAE) were higher in the groups supplemented with isoflavones than in Control group.

Isoflavones in plasma and urine

The content of genistein, daidzein, and equol was higher in blood plasma of both groups supplemented with isoflavones in comparison to Control group. Higher amounts of isoflavones were excreted in the urine of High Iso group in relation to the Low Iso group (Table 2).

Lipid profile

The Low Iso and High Iso groups showed decreased LDL-cholesterol and increased HDL-cholesterol levels when compared to the Control group (Table 3). The LDL-cholesterol/HDL-cholesterol and total choles-

Table 3 Cholesterol, triglycerides, and protein concentrations of blood plasma and lipoproteins (VLDL, LDL, and HDL) in the studied groups

Lipid Profile			
Parameter	Control	Low Iso	High Iso
Cholesterol (mg/d/L)			
Plasma (total)	1667 ± 541[a]	1211 ± 222[a]	1457 ± 260[a]
VLDL	294 ± 121[a]	139 ± 44[b]	238 ± 111[ab]
LDL	1216 ± 440[a]	688 ± 115[b]	695 ± 124[b]
HDL	171 ± 37[a]	383 ± 107[b]	563 ± 80[c]
LDL:HDL ratio	7 ± 2[a]	1 ± 0[b]	2 ± 1[c]
Total:HDL ratio	10 ± 3[a]	3 ± 1[b]	3 ± 1[b]
Cholesterol			
(% total lipids)	79 ± 12[a]	68 ± 8[b]	70 ± 3[b]
Protein (mg/ml)			
Plasma	406 ± 13[a]	353 ± 20[b]	381 ± 41[ab]
VLDL	1 ± 1[a]	1 ± 0[a]	1 ± 1[a]
LDL	2 ± 1[a]	2 ± 1[a]	8 ± 4[a]
HDL	5 ± 2[a]	7 ± 2[ab]	12 ± 5[c]

Values are shown as the mean area under the curve ± standard deviation, considering the data from basal to 180 days of experimental time. [a]Control group; [b]Low Iso group and; [c]High Iso group. Different characteres indicate significant differences among the groups ($P < 0.05$) by Tukey's test

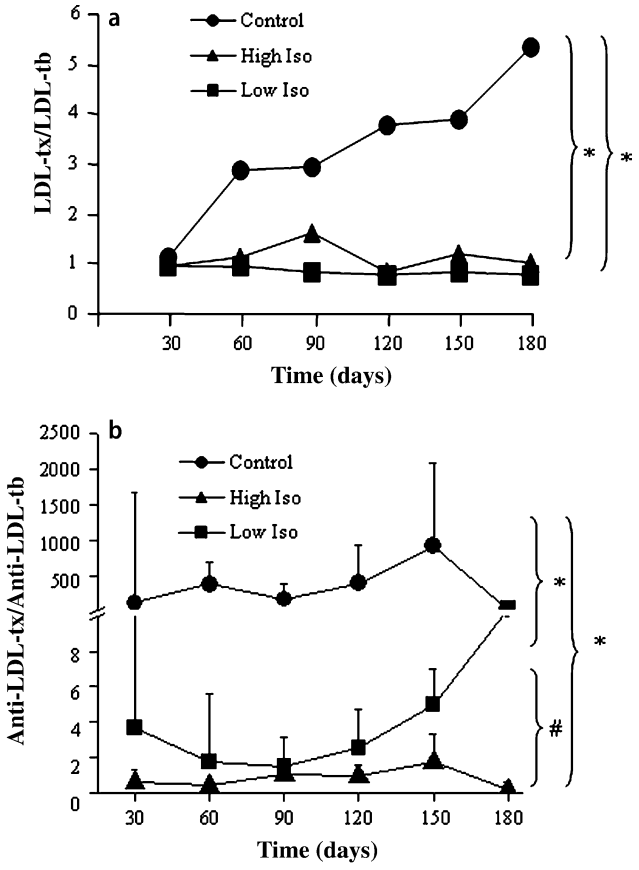

Fig. 1 (**A**) Detection of LDL$^-$ in plasma of groups Control ($n = 5$), High Iso ($n = 5$) and Low Iso ($n = 5$) using the anti-LDL$^-$ monoclonal antibody. (**B**) Detection of antibodies anti-LDL$^-$ in plasma of groups Control ($n = 5$), High Iso ($n = 5$) and Low Iso ($n = 5$) using the anti-LDL$^-$ monoclonal antibody. Significant differences of area under the curve were defined by Tukey's test, $P < 0.05$ using data obtained from zero to 180 days during experimental time for each animal

terol/HDL-cholesterol ratios, as well as, the percent of cholesterol in relation to the total lipids confirmed the hypocholesterolemic effect of soy isoflavones.

Detection of LDL$^-$ in plasma

The concentrations of LDL$^-$ (LDL^-_{tx}/LDL^-_{tb}) increased along atherosclerosis development in the Control group. In contrast, both groups supplemented with isoflavones showed lower LDL$^-$ concentrations in comparison with the Control group (Fig. 1A).

Detection of autoantibodies reactive to LDL$^-$ in blood plasma

The IgG equivalents reactive to LDL$^-$ were higher in Control group than in the High Iso and Low Iso groups (Fig. 1B). Interestingly, in the Low Iso group the IgG reactive to LDL$^-$ increased continously until 180 days of isoflavone supplementation reaching values similar to those of the Control group.

Detection of LDL$^-$ in atherosclerotic lesion

Electronegative low density lipoprotein immunostaining was predominant in intima and subintimal regions of aorta with higher intensity in the Control group (Fig. 2A-I) than in High Iso (Fig. 2A-II) and Low Iso (Fig. 2A-III) groups. Negative controls indicated absence of artifactual staining (Fig. 2A-IV).

Detection of autoantibodies reactive to LDL$^-$ in aorta

The arteries from animals that consumed isoflavones had higher levels of IgG reactive to LDL$^-$ in comparison with Control group (Fig. 2B).

Discussion

In the present study rabbits fed an atherogenic casein-based diet enriched with isoflavones had decreased LDL-cholesterol and increased HDL-cholesterol in comparison with rabbits fed the same diet without isoflavones. Our data are in agreement with findings observed in rats [30], hamster [31], monkeys [32], and humans [33] reinforcing that isoflavones can contribute to the hypocholesterolemic effect previously described for soy protein [34]. The isoflavone metabolite, equol, modulates the expression of hepatic cholesterol 7α-hydroxylase which results in increase of the fecal excretion of acidic steroids and decrease of cholesterol in blood plasma [35]. Moreover, equol also has an action on hepatic lipase activity and adipose tissue [36], as well as, on up-regulation of LDL receptors [37]. In our study, equol was present in plasma and urine of rabbits fed either the High Iso or the Low Iso diets and the observed hypocholesterolemic action of isoflavones may be related to its action on lipid metabolism.

Isoflavones have antioxidant action and protect LDL from oxidation besides increasing the total antioxidant capacity of blood plasma [38]. In fact, we observed that isoflavones decreased the amount of electronegative low-density lipoprotein (LDL$^-$) in plasma and aorta. This effect occurred for both High Iso and Low Iso groups. It was previously reported that the soy protein containing isoflavones is more effective for inhibiting LDL oxidation than the isoflavone depleted soy protein [39]. In contrast to our results, other studies [40] did not find a protective

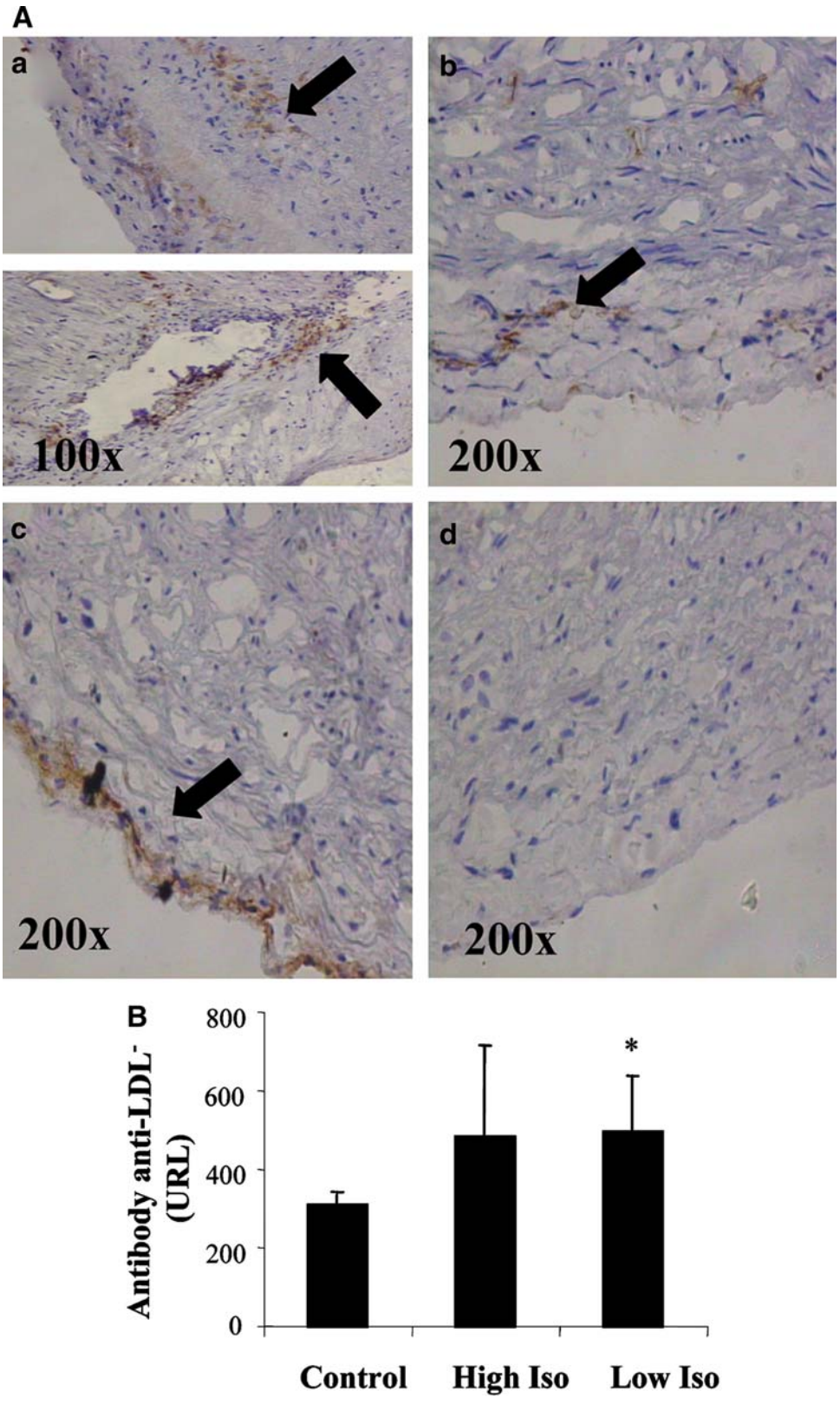

Fig. 2 (**A**) Photomicrography of atherosclerotic lesions of aortic arch showing the detection of LDL⁻ by immunohistochemistry with the anti-LDL⁻ monoclonal antibody. I, Control group; II, High Iso group; III, Low Iso group; IV, normal rabbit aortic arch. (**B**) Detection of antibodies anti-LDL⁻ in atherosclerotic lesions of groups Control ($n = 5$), High Iso ($n = 5$) and Low Iso ($n = 5$). *Significant differences between groups were defined by Tukey's test, $P < 0.05$

effect of isoflavones extract against LDL oxidation. These controversial findings may be related to the amount of supplemented isoflavones, the differences of isoflavones bioavailability among the species and the time of supplementation in the different studies. In our study we evaluated two doses of isoflavones (0.73 and 7.73 mg isoflavones per kg/body) and observed that both decreased the levels of LDL^- in blood plasma and aorta. The isoflavone dose administered to the Low Iso Group was calculated considering the mean protein consumption (30 g/day) of men with standard weight (70 kg) and diet. According to Eldrigde [41] and Wang & Murphy [42], 100 g of soy protein contain a mean isoflavone amount of approximately 122 mg. Thus, the mean isoflavone consumption of humans (men, 70 kg) ingesting soy protein would be approx. 36.6 mg/day. Taking the mean weight of rabbits (1.4 kg) at the beginning of the experiments and considering this mean daily soy consumption of humans we calculated that the amount of isoflavones to be consumed by each rabbit would be equal to 0.73 mg/kg body weight/day. According to this, the High Iso group had ten times higher isoflavone consumption than that provided by a mean daily soy protein consumption by humans. This finding suggests that even at the concentrations usually found in soy protein-based diets isoflavones can have protective effects on lipid metabolism and lipoprotein oxidation without need of further supplementation.

Another important point of our experimental design is the age of rabbits (15-days-old) at the beginning of the study. The responsiveness of rabbits even at the lower isoflavone dose may be related to the age of rabbits (15-days-old) at the beginning of the study. As reported by Subbiah et al. [43] rabbits in early life are more susceptible to diet-induced atherosclerosis. Thus, in these animals the atheroprotective effects of isoflavones could be related to the higher responsiveness of their arteries to the actions of these compounds on cells of the vessel wall. Several studies have found an important association between oxidized LDL and atherosclerosis [44]. Electronegative LDL has been found to be elevated in hypercholesterolemia and atherosclerosis in animals and humans [26] reinforcing its role in the pathogenesis of atherosclerosis. It has been demonstrated that neo epitopes presenting LDL^- and chemically modified forms of LDL (e.g., LDL-MDA, LDL-4HNE, LDL-$CuSO_4$) are recognized by the immune system as non-self antigens [45] contributing to an autoimmune response. Actually, atherosclerosis is considered as a chronic immune-inflammatory disease that involves cellular and humoral components [46, 47]. In fact, antibodies reactive to LDL^- were found in blood plasma of rabbits fed the atherogenic diet. Both, Low Iso and High Iso groups showed lower levels of anti-LDL^- antibodies in blood plasma than the Control group. In aorta, the isoflavone dose-response effect on autoantibodies formation is not conclusive due to the high variability observed in the High Iso group (Fig. 2B). Considering that the anti-LDL^- antibodies are generated in response to the presence of neo-epitopes in LDL^-, the inhibition of LDL oxidative modification by isoflavones could be related to a less intense stimulation of the humoral immune response. Another possible explanation is that high levels of LDL^- and anti-LDL^- antibodies stimulate IgG-LDL^- immunocomplexes generation and, consequently, the reduction of free antigen and antibodies, as previously suggested [48]. A different scenario was found in aorta where higher levels of antibodies reactive to LDL^- and less intense LDL^- immunostaining were found in rabbits fed isoflavones in comparison with the controls. In the latter group, the opposite was observed where high levels of LDL^- in plasma and aorta were associated with high levels of anti-LDL^- IgG in plasma and low amount in aortas. This suggests a direct relationship between the concentration of LDL^- in blood plasma and its uptake by macrophage scavenger receptors in the aorta wall and an inverse relation with the local levels of immunoglobulins reactive to LDL^-. This could be explained by the consumption of immunoglobulins of the artery wall to form oxLDL-IgG immunocomplexes that would be taken up by macrophages via Fcγ receptor [45], or, to decreased local immunoglobulin production induced by isoflavones. Future studies are needed to elucidate this point.

An interesting finding observed in our study was that even though rabbits from the Low and High Iso groups consumed less chow than the control non-supplemented animals, their weight increased along the experimental time. This effect may be due to the estrogenic effect of isoflavones as reported by Wagner et al. [49] and Jenkins et al. [50]. As previously described Wu et al. [51] and Manzoni et al. [52], isoflavone consumption by rats and mice has a direct relation with weight reduction and adiposity prevention. In addition, Hertrampf et al. [53] reported that rats fed isoflavone supplemented-diet did not have weight gain in comparison to controls (without isoflavone supplementation). Furthermore, it was observed that soy isoflavones increased preprandial peptide YY (PYY) but had no effects on ghrelin and body weight in healthy postmenopausal women [54]. Although isoflavones may affect the control of body weight, this effect can vary among different species and the increase of body weight observed in our study may be a specie-specific effect and warrants further investigation. In conclusion, soy isoflavones may have beneficial effects on lipid metabolism and atherosclerosis by decreasing LDL^- in blood plasma and aorta, diminishing LDL-cholesterol, as well as,

increasing HDL-cholesterol. These findings stimulate further investigations on the role of isoflavones isolated or associated to other soy derivatives on prevention of cardiovascular diseases.

Acknowledgments This study was supported by FAPESP (grant 97/05090 to D.S.P.A. and scholarship 97/09686-8 to N. R. T. D.) and PRONEX. The authors thank Cleiton Alves for technical assistance and Bunge Alimentos S/A for donation of soy isoflavones.

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