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ORIGINAL CONTRIBUTION

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# Soy isoflavones reduce heat shock proteins in experimental atherosclerosis

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**Summary** *Background* Soy isoflavones may affect several biochemical pathways like the synthesis of nitric oxide (•NO) and heat shock proteins (HSP) that are important factors for atherosclerosis development. *Aim of the study* The purpose of this study was to investigate the influence of soy isoflavones on the production of •NO and HSP60, HSP70 and HSC70 in experimental atherosclerosis. *Methods* One group of rabbits (New Zealand) was fed an atherogenic diet containing 27 % casein (CAS) and another group was fed the same diet supplemented with soy isoflavones (5 mg/kg/day) (ISO). Blood samples were obtained monthly and after six months of feeding, the rabbits were sacrificed and the aortas were removed. *Results* The ISO group showed a significant reduction of cholesterol in LDL (36.2 %) and in aorta (36 %), as well as, an increase of HDL-cholesterol (2.1 times) in relation to the CAS group. The concentration of •NO metabolites (NOx) in blood plasma and the levels of reactive antibodies to HSC70 in blood plasma and to HSC70 and HSP70 in aortic tissue were significantly decreased in the ISO group. Isoflavones promoted a reduction of content of HSP60, HSP70 and HSC70 in aortic arch analyzed by immunohistochemistry. The isoflavone supplementation promoted a reduction of cholesterol content in aorta (62.2 %) ($p < 0.05$). *Conclusions* Soy isoflavones reduced hypercholesterolemia, the production of HSP60, HSC70 and HSP70 and reactive antibodies to HSC70 in serum and to HSC70 and HSP70 in aorta, as well as, the cholesterol content in atherosclerotic lesions in rabbits fed a casein-based atherogenic diet.

**Key words** soy isoflavones – genistein – atherosclerosis – heat shock protein – nitric oxide

## Introduction

Atherosclerosis is a main cause of morbidity and mortality worldwide. Atherogenesis is a complex process, with multiple mechanisms contributing to its initiation and progression: infections, inflammation and autoimmunity have been associated with pathogenesis of the disease [1]. Autoimmunity is one of the more recently identified potential mechanisms, with heat shock proteins (HSP) identified as possible autoantigenic determinants acting as immune targets in the atherosclerotic process [2].

HSP60, HSP70 and HSC70 are present in human atherosclerotic lesions [3, 4]. Immunization with recombinant mHSP65 induces atherosclerotic lesions in normocholesterolemic rabbits [5]. Moreover, elevated levels of mHSP65 antibodies are significantly associated with

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carotid atherosclerosis and mHSP65 antibodies derived from human serum cross-react with human HSP60 [6]. The increase of •NO production may enhance HSP expression in some types of cells [7]. Furthermore, inducible nitric oxide synthase (iNOS) is also found in atherosclerotic lesions [8].

Isoflavones have important actions on inhibition of iNOS activity and expression [9]. It has been reported that genistein exhibits mild antiinflammatory properties that may, in part, involve the attenuation of nitric oxide release via iNOS and the formation of peroxynitrite, which involves a tyrosine kinase-dependent mechanism [10]. Genistein is a potent inhibitor of protein tyrosine kinase [11], inhibiting HSP expression in cells [12].

Considering that 1) the elevated •NO production by iNOS stimulates HSP expression, 2) in the initial stage of atherosclerosis there is an autoimmune reaction against these proteins and 3) HSPs and iNOS expression may be reduced by genistein in *in vitro* studies, we evaluated the effect of soy isoflavones on the formation of •NO, nitrotyrosine and anti-HSPs antibodies, as well as, on the iNOS and HSPs content in aorta of rabbits fed an atherogenic diet.

## Methods and materials

### Animals and diet

The experimental protocol conforms with the "Principles of laboratory animal care" (NIH Publication No. 85–23, revised 1985). Male New Zealand White rabbits (Procria S/A., Suzano, Sao Paulo, Brazil), 45 days old, and initial body weight 1.0–1.2 kg were used. Rabbits were maintained in individual metal cages with free access to food and water, at ~22 °C. The animals were divided randomly into two groups. One group of 5 rabbits was fed a purified atherogenic both cholesterol- and nitrate-free casein-based diet (casein 27 %) according to Roberts et al. (1981) [13] supplemented with isoflavones extracted from soy molasses (ISO) and compared to another group (5 rabbits) with the same diet (CAS) without isoflavone supplementation. From birth to the beginning of the experiments, the animals were fed with commercial rabbit chow (Nuvital Nutrientes Ltda, Colombo, Paraná, Brazil) and subsequently they were gradually transferred to the purified diet over 1 week and finally, the animals were fed with 100 % experimental chow during the following 6 months.

Isoflavones were extracted from soy molasses in 90 % ethanol in the proportion 1:5, (v/v) under constant agitation for 2 h [14]. The purified extract was concentrated in a rotatory evaporator at 40 °C. An adequate amount of isoflavones was added to the chow to achieve a mean isoflavone ingestion corresponding to 5 mg/kg/day considering the daily average consumption of chow and the weight of the rabbits.

### Blood withdrawn and sacrifice of animals

Rabbits were deprived of food for 12 h and samples of serum and plasma were collected before starting the atherogenic diet (basal) and after each month during a 6-month period of feeding the atherogenic diet. Blood withdrawn for isoflavones analysis was performed without fasting at basal and after 1, 2, 3, 4 and 5 months of feeding the atherogenic diet. After an experimental time of 6 months, the animals were anesthetized with an intramuscular injection of 5 mg/kg body weight Rompun (Bayer S. A., São Paulo, Brazil) and 50 mg/kg body weight Ketalar (Parke-Davis, São Paulo, Brazil) and then sacrificed with endovenous injection of 25 mg/kg pentobarbital (Parke-Davis, São Paulo, Brazil) and the aortas were removed and opened lengthwise. The aortas were washed in physiologic solution and two fragments of the aortic arch were frozen in Tissue-Tec (Leica®) for histological and immunohistochemical analyses. The remaining aortic tissue was frozen in liquid nitrogen and further macerated for analysis of cholesterol and antibodies anti-HSP.

### Analysis of cholesterol levels

A homogenate was prepared with 100 mg of macerated aortic tissue in 1 mL of 25 mM Tris-HCl buffer pH 7.4 containing 1mM EDTA, 1 mM EGTA, 15 mM CHAPS (tensoative), 2 µg/mL aprotinin, 2 mM benzamidine, 1 mM PMSF and 20 mM BHT, in ice bath. The homogenate was centrifuged at 10,000 x g for 15 minutes. The supernatant was utilized for cholesterol determination. The lipoproteins (VLDL, LDL and HDL) were separated from plasma by sequential ultracentrifugation [15]. All samples (serum, plasma and lipoproteins) were maintained at –80 °C until analysis. The measurements of total plasma, VLDL, LDL, HDL and aorta cholesterol were performed with commercial enzymatic reagents (BioSytems®).

### Isoflavones analysis

Isoflavones were extracted from blood plasma, urine and chow and analyzed according to Franke et al. [16] by HPLC. Genistein, genistin, daidzein, daidzin and equol were acquired from Endofine Chemical Co. (USA) and used as external standards. The inter-assay precision was determined by analyzing four levels of quality control samples on four different runs. The criteria for acceptability of the data included accuracy within 15 % de-

viation from the nominal values and a precision of 15 % relative standard deviation [17].

### Nitrate + nitrite (NOx) concentration

NOx concentration in blood plasma was measured by chemiluminescence in the gaseous phase in accordance with Pereira et al. [18].

### Measurement of area and volume of atherosclerotic lesions

A fragment of aortic arch was dehydrated with increasing ethanol concentrations, cleared in propylene oxide and embedded in paraffin. Consecutive semi-thin sections (n = 10; 5 µm) were taken serially from each fragment, mounted on glass slides, stained with hematoxilin/eosin and examined under the light microscope at X 100 magnification. Quantitative measurement was performed in each section using the Optimas imaging analysis system (BioScan, Edmonds, WA). The area ($\mu m^2$) was calculated as the linear length of the lesions in the 10 sections multiplied by 5 µm (thickness of each section). The volume ($\mu m^3$) was calculated as the lesion area in the 10 sections multiplied by 5 µm (thickness of each section). Data are presented as the percentage of the lesions in relation to the total area and volume of the sections from each segment of analyzed aortic arch.

### Detection of iNOS, nitrotyrosine and heat shock proteins in aorta

A fragment of the aortic arch from the animals was embedded in Tissue-Tec (Leica®), frozen in isopentane and later stored in liquid nitrogen. Sections of 5 µm were mounted on microscope slides and fixed in acetone. After that, the sections were washed with PBS, for 5 minutes. Endogenous peroxidase activity was blocked by incubating the sections in $H_2O_2$ solution (100 ml of PBS + 1 ml of $H_2O_2$ 30 %) for 5 minutes, and then, the sections were washed 3 times with PBS. The sections were covered with the respective primary antibody (monoclonal antibodies anti-HSP60, -HSP70, -HSC70, 1:200, StressGen Biotechnologies, Victoria, BC; anti-iNOS, Transduction Laboratories or anti-nitrotyrosine, Upstate Biotechnology 1:100) and incubated overnight at 37 °C in a humid chamber. The slides were washed 3 times with PBS and the secondary biotinylated antibody 1:500 (anti-Mouse IgG, StressGen Biotechnologies, Victoria, BC) was added and incubated for 30 minutes at 37 °C, in a humid chamber. After washing 3 times with PBS, the tertiary reagent was added (Streptavidin peroxidase conjugate, 1:500, StressGen) and incubed for 1 hour at 37 °C, in a humid chamber. Then, the sections were washed 3 times in PBS and 0.4 mg/mL DAB (diaminobenzidina, Sigma) and hydrogen peroxide (Sinth, 6 µL/100 mL) were added. Further, the sections were counterstained with eosin. A brown-to-black precipitate was indicative of the presence of HSP, nitrotyrosine or iNOS. Negative controls were performed in the absence of both primary and secondary antibodies as well as the peroxidase substrate. HSP cross sections were analyzed with an Axioplan microscope connected to a video camera (JVC TK128 OV), a computer, an image digitizing board (Coreco OCTX) and a video monitor (Sony Trinitron) by using the software Bioscan Optimas 4.10, Bioscan (Edmonds, WA). To determine the staining density corresponding to immunoreactivity to HSP60, HSP70, HSC70, iNOS and nitrotyrosine, five readings of each cross section (9 sections for each animal) were made. The average of the measurements in the gray scale (indicating the immunoreactivity staining density) was expressed on a linear scale from 0 (white) to 255 (black) (in accordance with Johnson et al. [19]). The stained areas of each analyzed image were measured and related to the total area of the image and expressed as percentage of area (%).

### ELISA for determination of reactive antibodies to HSP60, HSP70 and HSC70 in serum and aorta

Autoantibodies anti-HSPs in serum were determined as described by Xu et al. [20] with the following modifications: (i) serum was diluted 1:10 to 1:10,000 in PBS with 0.1 % defatted milk; (ii) results were expressed as the last reactive titer (minimum absorbance 0.2 above that of the blank) multiplied by the respective absorbance value. For determination of IgG reactive to HSPs in aorta tissue, proteins were extracted from macerated aorta by homogenization with 2 mM Tris-HCl buffer, pH 7.4 containing 0.32 M saccharose (1:10, m/v), followed by centrifugation at 1000 x g for 10 min at 4 °C. The supernatant was conserved in ice and the sediment was newly homogenized in the same extraction buffer (1:5, m/v) and centrifuged. The supernatants were joined and purified in a desalinization column (Econo-PAC 10 DG, Bio-Rad). Fractions of 1 mL were collected and the absorbance was measured at 280 nm to determine the protein peak. The content of the tubes corresponding to the protein peak was applied on a column for IgG purification (Econo-PAC, Bio-Rad). The eluate was monitored at 280 nm and the fractions (1 mL) corresponding to the peak of absorption of the IgG fraction were concentrated in a Speed Vac (SC 110, Savant) to the volume of 1 mL. This concentrated fraction was used to determine the levels of antibodies reactive to HSP60, HSP70 and HSC70 by ELISA and the total protein content [21]. The positive reactivity to HSPs of IgG purified from aortas

was evaluated by chemiluminescence after adding 100 µL of 2.3 mM isoluminol, 0.9 mM p-iodofenol and 25 µL of 0.6 % hydrogen peroxide to each well of the 96-well white plates (Nunc-Immuno™ plates, MaxiSorp™ surface). The luminescence was immediately detected in a plate luminometer (LumiCount™, Packard).

### Statistical analysis

Data are represented as mean ± standard deviation. CAS and ISO groups were compared by multivaried variance analysis of repeated measurements to determine the effect of isoflavones on the analyzed variables during the experimental time course (SPSS/PC+ software, Chicago, IL). The isolated values of two groups at the same time and the area under the curve values between the groups were compared through a one-way analysis of variance using ANOVA (ORIGIN 5.0 software). Correlation analysis was done by Pearson Product Moment method with SigmaStat 1.0 software. The $p < 0.05$ value was used as the level of significance in all the statistical analyses.

## Results

A similar average weight gain was seen for rabbits of both groups during the experimental time course. The isoflavone composition of the extract was genistin – 57 %, daidzin – 33 %, genistein – 5 % and daidzein – 5 %. The consumption and concentrations of isoflavones in plasma and urine are shown in Table 1. Isoflavones were not detected in plasma and urine of the CAS group.

### Lipid profile

Hypercholesterolemia was observed in rabbits 1 month after feeding the atherogenic diet. The increment of cholesterol concentration in blood plasma was associated with the increase of LDL-cholesterol and VLDL-cholesterol (Fig. 1). The peak of cholesterol concentration in blood plasma was found after feeding the atherogenic diet for two months. The ISO group also developed hypercholesterolemia, although less intense than in the CAS group. LDL-cholesterol was 26 % lower (area under curve) in the ISO group in relation to the CAS group. HDL-cholesterol was significantly reduced in the CAS group after feeding the atherogenic diet for one to two months, while HDL-cholesterol of the ISO group was increased by 28 % (area under curve). The concentrations of genistein, daidzein and equol in blood plasma were negatively correlated (r/p, correlation coefficient/significance level) with VLDL (–0.31/0.017, –0.28/0.031, –0.29/0.027) and LDL-cholesterol (–0.29/0.025, –0.26/0.048, –0.31/0.016), respectively; equol concentrations were correlated positively with HDL-cholesterol (0.29/0.0239).

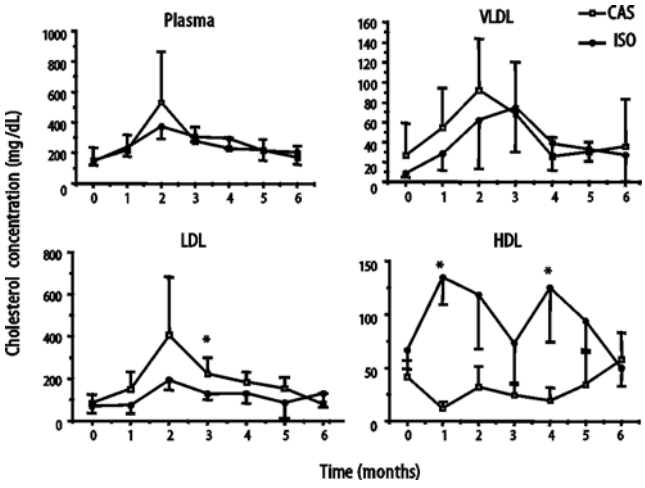


**Fig. 1** Lipid profile of animals from the CAS and ISO groups during the experimental time course. The values are presented as mean ± standard deviation
* Significant difference between groups. * LDL ($p = 0.032$), area under the curve ($p < 0.042$). * HDL ($p = 0.021$), area under the curve ($p < 0.001$)

**Table 1** Isoflavone consumption, plasma concentration and urinary excretion (24 h) in animals of group ISO (n = 5)

| | 1st month | 2nd month | 3rd month | 4th month | 5th month |
|---|---|---|---|---|---|
| Consumption (mg/kg/day) | | | | | |
| Total isoflavone | 3.0±0.3 | 4.1±0.2 | 5.1±0.4 | 5.6±0.7 | 5.9±0.5 |
| Plasma µM | | | | | |
| Daidzein | 0.70±0.10 | 0.33±0.06 | 0.30±0.05 | 0.27±0.06 | 0.3±0.03 |
| Genistein | 0.20±0.03 | 0.14±0.04 | 0.11±0.06 | 0.09±0.03 | 0.17±0.09 |
| Equol | 1.71±0.08 | 1.03±0.15 | 1.32±2.8 | 1.33±0.3 | 1.01±0.18 |
| Urine µmol/24 h | | | | | |
| Daidzeine | 1.9±0.6 | 2.3±1.2 | 4.3±1.3 | 3.5±1.1 | 4.6±1.0 |
| Genistein | 1.4±0.5 | 1.6±0.9 | 4.0±1.2 | 2.9±0.9 | 3.9±0.9 |
| Equol | 12.2±7.7 | 10.9±9.1 | 27.7±6.8 | 21.8±7.2 | 25.2±5.9 |

Results are presented as mean ± standard deviation

## NOx, iNOS and nitrotyrosine

Fig. 2 shows the NOx concentrations in blood plasma. There was a peak nitrate concentration in both groups after 1 month of feeding the atherogenic diet. Comparing the area under the curve, the ISO group shows a lower NOx concentration (10.4 %). iNOS and nitrotyrosine were detected in rabbit aortas; however, no significant differences between CAS and ISO groups (Table 2) were observed. iNOS and nitrotyrosine were colocalized in the subendothelial region of aorta (data not shown).

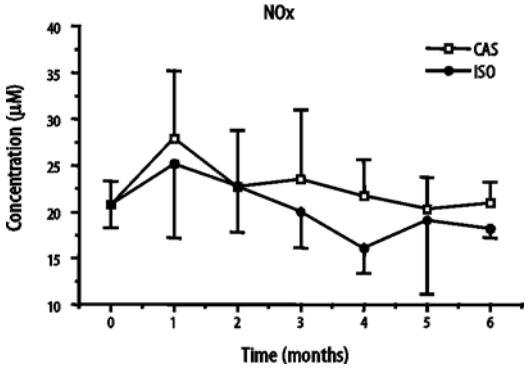


**Fig. 2** NOx (nitrite + nitrate) concentrations of animals from the CAS and ISO groups during the experimental time course. The values are presented as mean ± standard deviation. *Significant difference between groups (area under the curve, p = 0.047)

## HSPs and anti-HSPs antibodies

HSC70 was the heat shock protein that showed the most intense stained area in the atherosclerotic lesions, followed by HSP60 and HSP70 (Table 2). The consumption of isoflavones reduced the expression of all evaluated HSP (Table 2). Both groups developed antibodies against HSP60, HSP70 and HSC70; however, the ISO group showed lower titers of reactive antibodies to HSP as compared to the CAS group (Fig. 3), but there was a significant difference only in reactive antibodies to HSC70. We found negative correlations (r/p) between the concentrations of plasma isoflavones (daidzein, genistein and equol, respectively) and the levels of anti-HSP60 (–0.28/0.033; –0.27/0.039; –0.31/0.016) and anti-HSC70 (–0.30/0.021; –0.28/0.031; –0.34/0.007) antibodies. Fig. 4A shows the presence of anti-HSPs antibodies in rabbit aortas. A significant reduction of antibodies reactive to HSP70 and HSC70 was found in the aortas of the ISO group in comparison to the CAS group. No significant differences were found for reactive antibodies to-HSP60 and total IgG (Figs. 4A and 4B).

## Distribution, area and volume of atherosclerotic lesions

Macroscopically visible lesions were detected in the aortic arch of 4 of 5 rabbits (80 %) and in the thoraxic aorta of 3 of 5 rabbits (60 %) of the CAS group. In the ISO group a lower number of lesions was observed (data not shown). From the 5 rabbits of the ISO group, only 3 had

**Table 2** iNOS, nitrotyrosine, HSP60, HSP70 and HSC70 in aortic arch from rabbits of CAS (n = 5) and ISO (n = 5) groups

| | iNOS | | Nitrotyrosine | | HSP60 | | HSP70 | | HSC70 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | CAS | ISO | CAS | ISO | CAS | ISO | CAS | ISO | CAS | ISO |
| Density | 140.0±5.8 | 122.5±1.3 | 136.8±3.8 | 127.4±2.98 | 88.9±1.9 | 74.3±1.7** | 86.1±2.0 | 74.6±3.5** | 84.8±1.0 | 72.4±2.2** |
| Area (%) | 45.09±20.1 | 38.6±10.5 | 45.2±4.4 | 41.6±2.7 | 21.4±5.4 | 6.2±3.5* | 15.7±5.5 | 5.0±3.7* | 22.3±6.7 | 11.8±5.3* |

Intensity of immune staining is expressed as relative density and percentage of the stained area (%). Immune reactivity is showed as the mean optical density on a scale of 0 (white) to 255 (black). Values are mean ± standard deviation. * $p < 0.05$; ** $p < 0.001$

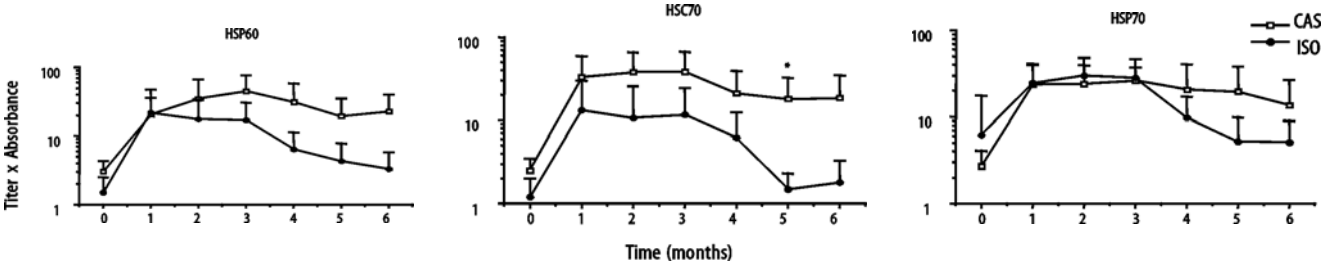


**Fig. 3** Titer of antibodies reactive to HSP60, HSP70 and HSC70 of animals from the CAS and ISO groups during the experimental time course. The values are presented as mean ± standard deviation. * Significant difference (p = 0.049) between groups; different area under curve for anti-HSC70 ($p < 0.024$). The results are presented on a logarithmic scale

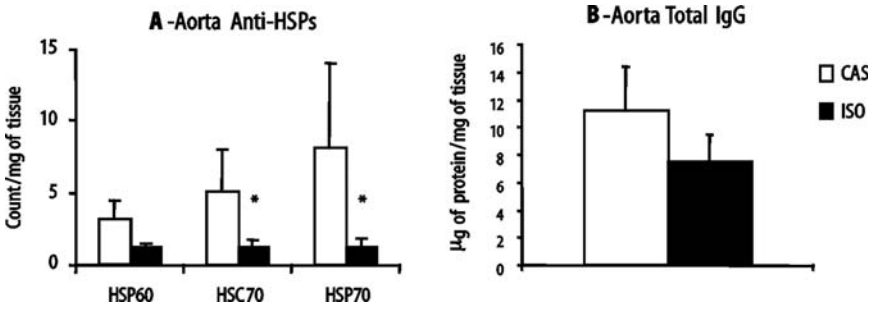


**Fig. 4** **A** Reactivity of antibodies anti-HSP60, -HSP70 and -HSC70 in purified IgG fraction of the CAS and ISO groups determined by ELISA. * Significant difference between groups, HSC70 ($p = 0.034$) and HSP70 ($p = 0.033$). **B** Protein values (mg of protein/mg of tissue) of purified IgG fraction of CAS and ISO groups. The values are presented as mean ± standard deviation

macroscopically visible lesions in the aorta with a smaller area occupied by lesions than in the CAS group, while 2 rabbits had lesions only in the aortic arch and 1 rabbit only in the thoraxic aorta. One rabbit of the ISO group did not show macro- or microscopically detectable lesions. The area and volume of atherosclerotic lesions from the aortic arch were 52 % and 48 % lower, respectively, in the ISO group than in the CAS group, but no significant difference was observed between groups (Fig. 5A). Moreover, the cholesterol content was reduced in the aorta of the ISO group as compared to the CAS group (Fig. 5B).

## Discussion

This study showed that soy isoflavones improved the lipid profile and reduced the nitric oxide metabolites, the heat shock proteins (HSP60, HSP70 and HSC70), the reactive antibodies to HSC70 in serum and antibodies to HSC70 and HSP70 in aorta as well as the aorta cholesterol content of rabbits fed a casein-based diet.

Hypercholesterolemia promoted by the casein-based diet is transient (Fig. 1). Casein increases intestinal cholesterol absorption, reduces cholesterol biosynthesis and the expression of LDL receptors, which causes a reduction of LDL uptake and induces hypercholesterolemia [22]. After the third month of a casein-based diet, the plasma cholesterol concentration was reduced as previously reported [23].

Our results showed that an extract of isoflavones added to a diet containing casein was able to reduce LDL-cholesterol and increase HDL-cholesterol (Fig. 1). Moreover, isoflavones of soy molasses extract were bioavailable, as isoflavones were detected in blood plasma and urine of animals (Table 1). Importantly, the concentrations of genistein, daidzein and equol in blood plasma were negatively correlated with VLDL and LDL-cholesterol and equol concentrations were correlated positively with HDL-cholesterol, reinforcing the positive effect of isoflavones on lipid profile. Demonty et al. [24] reported that available data more strongly support the link between soy isoflavones and both cholesterol and triglyceride metabolism in rabbits. In primates and humans, however, there is less evidence that soy isoflavones may affect circulating lipid levels [25]. A recent meta-analysis [26] concluded that isolated isoflavones seem to have no clinical benefits on serum lipids. In accordance to our data, Crouse et al. [27] showed a parallel between a reduction in the LDL cholesterol concentrations and an increase in the isoflavone content (from 3 to 62 mg) from a 25 g/day soy protein diet. Moreover, equol, an intestinal metabolite of daidzein, has potent estrogenic and antioxidant activities [28] and affects HDL metabolism [29]. However, because of these contradictory results the FDA has stated that confirmation of a hypocholesterolemic role of dietary isoflavones cannot be made at this time (Federal Register 57699–577733). Neverthless, consistent and beneficial effects of isoflavones like antioxidant [30] and on vascular reactivity, especially after 6 months of intervention [31] have been shown.

Hypercholesterolemia is associated with increased

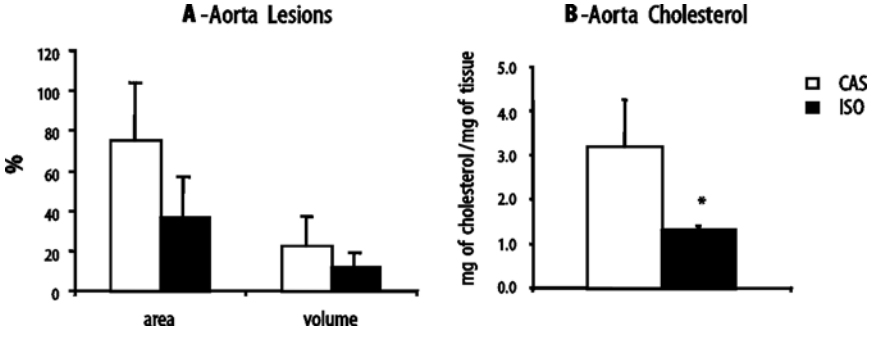


**Fig. 5** **A** Area and volume of atherosclerotic lesions in the aortic arch from rabbits of the CAS and ISO groups. Values are expressed as mean of % values obtained from 10 specimens of aortic arch. **B** Cholesterol content of the aortic arch from rabbits of the CAS and ISO groups. * Significant difference between the groups ($p = 0.014$)

production of •NO and the superoxide radical ($O_2^{\bullet -}$). Moreover, the subsequent interaction of •NO with superoxide anion generates peroxynitrite that can attack tyrosine from proteins, producing nitrotyrosine [18]. In this study, a transient increase of plasma •NO derivatives (NOx, nitrite + nitrate) was observed in both groups studied (Fig. 2). Similar results were found by Freyschuss et al. [32] who showed an increment of plasma nitrate in rabbits fed a diet containing 1 % cholesterol, between 3 and 6 weeks of feeding, with further decline to the basal levels after the ninth week. Interestingly, the peak of NOx was timely coincident with an increase of plasma cholesterol.

In our study, isoflavones reduced NOx concentration in blood plasma. This finding is in accordance with that of Yen and Lai [33] showing that oral administration of isoflavones and soy-based products significantly decreased serum nitrite and nitrate, and nitrotyrosine levels in LPS treated rats. Genistein is a potent inhibitor of protein tyrosine kinase, inhibiting •NO production in cell culture [11] and the induction of iNOS by LPS [34]. Thus, it is possible that the reduction of NOx showed by our data could be caused by the decrease of •NO synthesis by inhibiting iNOS activity or expression, without modification of eNOS. This hypothesis was tested by measuring iNOS and nitrotyrosine in the aortic arch of animals as in this region the shear stress is disturbed, which would lead to a higher expression of the iNOS and, consequently, greater nitrotyrosine formation [35]. However, animals of the ISO group did not show significant differences in iNOS and nitrotyrosine in the aortic arch compared to controls. As the immunohistochemical analysis was performed only in this region of aorta, differences of iNOS and nitrotyrosine either in other aorta segments or arteries can not be excluded. In addition, as nitrotyrosine may be generated by multiple pathways it is not a specific "footprint" for $ONOO^-$ formation *in vivo* [36]. Furthermore, the small number of animals utilized in this study (5 per group) may have restricted the statistical significance of the data.

In this study, atherosclerosis induced by the atherogenic casein-based diet resulted in high concentrations of HSP in aortas of rabbits. This finding was expected as HSP60, HSP70 and HSC70 are increased in atherosclerotic lesions [3, 4]. However, low serum levels of HSP70 have been associated with atherosclerosis in subjects with established hypertension [37] and coronary artery disease [38]. HSP 70 has recently been found to be markedly low in the skeletal muscle of individuals with type 2 diabetes [39]. Such results suggest that reduced HSP 70 may be a primary event in the development of atherosclerosis. A mechanism by which HSP70 might modify the establishment and/or progression of atherosclerosis is via an antiinflammatory effect. Intracellular HSP70 has been shown to attenuate inflammatory responses, as increasing intracellular levels of HSP70 in the vasculature reduces leukocyte adhesion at inflammatory sites [40]. In contrast, serum HSP60 levels are associated with early atherosclerosis in clinically normal individuals [41] and HSP70 levels are elevated in peripheral vascular disease [42]. Antibody titers to HSP60, 65 and 70 have been positively associated with coronary risk factors, increased risk for and severity of cardiovascular disease (CVD) and vascular endpoints in patients with established disease [43]. These differences in levels of circulating HSP or HSP antibodies in non-CVD and CVD patients could be a consequence of immune complex formation. In addition, recent data [44] showed that HSP70 is released from macrophages in response to oxLDL treatment and that HSP70 may be a major paracrine inducer of cytokine expression and release in human macrophages, confirming its participation in the atherosclerotic process. It is worthy to note that isoflavones can provide protection against LDL oxidation induced by peroxynitrite [45] which is in line with data showed here.

Isoflavones ingestion reduced HSPs in atherosclerotic lesions (Table 2). It is reported that genistein reduces HSPs expression in cell culture [12]; however, it is not elucidated yet if isoflavones can modulate *in vivo* HSP expression. Isoflavones could reduce HSP expression by: 1) protein tyrosine kinase inhibition [11], 2) reduction of iNOS expression [10], 3) decrease of cholesterolemia [27] and 4) inhibition of LDL oxidation [30]. Thus, the antioxidant activity of isoflavones may also contribute to the reduction of HSP found in this study. Probably by reducing HSP expression, isoflavones would reduce the intensity of autoimmune response mediated by these proteins and the formation of immune complexes in the aorta (Fig. 4A).

Isoflavones reduced the cholesterol content of aorta and promoted a nonstatistically significant decrease of area and volume of lesions. This could be attributed to soy isoflavones antiproliferative properties already described *in vitro* [46]. In fact, isoflavone-rich extract, free of soy protein, attenuated atherosclerosis development in cholesterol-fed rabbits [47]. In conclusion, this study showed that soy isoflavones had a beneficial effect on plasma lipids by reducing LDL-cholesterol and increasing HDL-cholesterol, diminishing NOx concentration in blood plasma, decreasing the content of cholesterol, HSP60, HSC70 and HSP70 in atherosclerotic lesions, as well as by reducing the antibodies reactive to HSC70 in serum and the antibodies anti-HSP70 and anti-HSC70 in aorta of rabbits fed an atherogenic diet. Thus, these results indicate that soy isoflavones may modulate some pathways of the immune-inflammatory process in atherosclerosis.

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