Antioxidant characterization of soy derived products in vitro and the effect of a soy diet on peripheral markers of oxidative stress in a heart disease model

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Abstract: This study analyzed and compared the content of isoflavones in 2 soy products, the effectiveness of isoflavones as antioxidants, in vitro, and demonstrated the antioxidant effect of a soy diet in rats with myocardial infarction (MI). Isoflavone content was analyzed in soybean hypocotyl (SH) and isolated soy protein (ISP). The quality (TAR) and quantity (TRAP) of antioxidants present in the samples was quantified. The amount of daidzin was higher in SH (9 times) and genistein in ISP (5 times). SH presented a 3-fold increase in TAR, while both products exhibited same TRAP. The rats were fed an ISP diet for 9 weeks. Animals were distributed among 6 treatment groups: (*i*) Sham Casein; (*ii*) Infarct Casein < 25%; (*iii*) Infarct Casein > 25%; (*iv*) Sham Soy; (*v*) Infarct Soy < 25%; and (*vi*) Infarct Soy > 25%. MI was induced 5 weeks after the commencement of the diets. Lipid peroxidation (LPO), antioxidant enzyme activity, and levels of nitrites/nitrates were determined in blood. Rats receiving the ISP diet demonstrated increased activity of antioxidant enzyme activity and nitrite/nitrate content. In addition, the increase in LPO seen in rats subjected to MI was significantly mitigated when the ISP diet was given. These findings suggest a nutritional approach of using a soy-based diet for the prevention of oxidative-stress-related diseases such as heart failure.

Key words: isolated soy protein, soybean hypocotyl, lipid peroxidation, antioxidant potential, myocardial infarction, antioxidant enzymes, nitric oxide.

Résumé : Cette étude visait à analyser et à comparer le contenu en isoflavones de deux produits du soja, leur efficacité en tant qu'antioxydants in vitro et à démontrer un effet antioxydant d'une diète de soja chez les rats souffrant d'un infarctus du myocarde (IM). Le contenu en isoflavones de l'hypocotyle de la fève de soja (HS) et de la protéine de soja isolée (PSI) a été analysé. La qualité (TAR) et la quantité (TRAP) des antioxydants présents dans les échantillons ont été évaluées. La quantité de daidzéine était 9 fois plus élevée dans l'HS alors que la génistéine était 5 fois plus élevée dans la PSI. La TAR de l'HS était 3 fois plus élevée, alors que la TRAP était la même chez les deux produits. Des rats ont été soumis à une diète de PSI pendant 9 semaines. Les animaux ont été divisés en 6 groupes : (*i*) faux traitement/caséine; (*ii*) infarctus/caséine < 25 %; (*iii*) infarctus/caséine > 25 %; (*iv*) faux traitement/soja; (*v*) infarctus/soja < 25 %; (*vi*) et infarctus/soja > 25 %. L'IM a été induit après 5 semaines de diète. La peroxydation des lipides (LPO), l'activité des enzymes antioxydantes et le contenu en nitrites/nitrates ont été déterminés dans le sang. L'activité des enzymes antioxydantes et le contenu en nitrites/nitrates étaient accrus chez les rats soumis à la diète de PSI. De plus, l'augmentation de LPO observée chez les rats souffrant d'un IM était significativement atténuée par la diète à la PSI. Ces résultats suggèrent d'utiliser une approche nutritionnelle à base de soja pour prévenir les maladies reliées au stress oxydant comme l'insuffisance cardiaque.

Received 10 November 2011. Accepted 20 January 2012. Published at www.nrcresearchpress.com/cjpp on 18 July 2012.

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This article is one of a number of papers published in the Special Issue entitled "Heart Health and Care," which focuses on new knowledge of the physiology of cardiovascular functions in health, and pathophysiology of cardiovascular dysfunctions.

Mots-clés : protéine de soja isolée, hypocotyle de soja, peroxydation des lipides, potentiel antioxydant, infarctus du myocarde, enzymes antioxydantes, oxyde nitrique.

[Traduit par la Rédaction]

Introduction

Soy is known for having high concentrations of several physiologically active phytochemicals, including isoflavones such as genistein and daidzein and their respective β-glycosides, genistin and daidzin. Soybean and soy products are a particularly abundant source of isoflavones (Havsteen 2002). Isoflavones are a subclass of a large and ubiquitous group of nutraceuticals called flavonoids. One of the most useful properties of many flavonoids is their ability to scavenge free radicals (Larkin et al. 2008). Previous studies have indicated that isoflavones exhibit free-radical-scavenging action, and may increase the antioxidant defense system (Guo et al. 2002; Kawakami et al. 2004; Hagen et al. 2009).

Free radicals have been implicated in the pathophysiology of a number of disorders such as cardiovascular, neurodegenerative diseases, and cancer. Their detrimental role has been attributed to their high reactivity and deleterious effects on cell structures, interacting with biomolecules, and inducing oxidative injury (Rocha et al. 2009).

Heart failure is the major cause of mortality in Western countries, and the incidence and prevalence of coronary heart disease continues to rise (WHO 2004). For this reason, cardioprotective strategies including the use of antioxidants have gained interest in the experimental and clinical settings (Monnet and Chachques 2005; Xiao 2008; Hagen et al. 2009).

Flavonoids, such as isoflavones, are excellent agents for keeping oxidative stress under control, but their uptake should be regular and in considerable amounts, owing to their short half-life in the body (1–2 h) (Vinson 1998; Xiao 2008). The isoflavones genistein and daidzein, at physiologically achievable levels, were recently shown to significantly elevate the concentration of reduced glutathione; the most prevalent antioxidant in human endothelial cells (Guo et al. 2002). Furthermore, it has been demonstrated that genistein has an inhibitory effect on the production of hydrogen peroxide, decreases lipid peroxidation (LPO), and increases the activity of antioxidant enzymes such as catalase, superoxide dismutase, glutathione peroxidase, and glutathione reductase.

As different soy products are widely used in food industry and research studies (Cassidy et al. 2006), the characterization of such products is needed to define the optimal effect of their various components on human health. Isolated soy protein is the soy-derived product most used in food industry, for example, in sausages and other processed meat products. The main reason for this comes from the functionality of isolated soy proteins, for example, it has gel formation properties, water retention properties, and emulsifying properties. Isolated soy is 92.2% protein, while the soybean hypocotyl is 42.6% protein, 43.4% carbohydrates, and 11.4% fat (Stauffer 2005). The fat content means that it cannot be stored for long periods. However, the cost for the industrialization of food products using this ingredient would be cheaper. This fact has attracted the attention of the food industry.

For this reason, the aim of this study was to analyze and compare the isoflavone content, as well as antioxidant potential and reactivity, in 2 soy products, and the effectiveness of their peripheral antioxidant protection in an in-vivo model of myocardial infarction.

Materials and methods

Chemicals and components of the diets

Casein was purchased from Farmaquímica, Porto Alegre, Brazil. Isolated soy protein (Samprosoy 90LH) and soybean oil were a gift from Solae, LLC (Brazil). A mineral mixture and a vitamin mixture were obtained from Roche Ltd., fiber from Colorcon do Brazil, and choline bitartrate from Valdequímica Produtos Químicos Ltda, Porto Alegre, Brazil. L-Methionine was purchased from Merck, Rio de Janeiro, Brazil. *tert*-Butylhydroquinone (TBHQ) was purchased from Aldrich Chemical Co. (Milwaukee, Wisconsin, USA). All other compounds were purchased from Sigma (St. Louis, Missouri, USA).

Experimental procedures

Initially, we tested 2 soy-derived products in vitro: (*i*) soybean hypocotyl (SH), and (*ii*) isolated soy protein (ISP). Total antioxidant reactivity (TAR) and total radical-trapping antioxidant potential (TRAP) of these 2 products were determined, as well as their isoflavone content.

Additionally, an ISP-based diet was offered to rats submitted to myocardial infarction surgery, and the parameters of oxidative damage, antioxidant enzymatic defenses, and nitric-oxide metabolites were analyzed in the peripheral blood.

Isoflavone analysis

Isoflavone analysis was performed at The Physical Chemistry Laboratory of Embrapa Soybean, located at Londrina, Brazil. Isoflavone quantitative analysis was carried out by high pressure liquid chromatography (HPLC) according to Berhow (2002). The isoflavones were extracted according to Carrão-Panizzi et al. (2002). Approximately 100 mg of defatted milled roasted soybean hypocotyl (SH) and isolated soy protein (ISP) samples were extracted in test tubes with 4.0 mL of ethanol 70% containing 0.1% of acetic acid for 1 h at room temperature. They were then transferred to Eppendorf tubes and centrifuged. The supernatants were filtered in 0.45 μm filters (Millipore) and 20 μL was used to separate and quantify the isoflavones in a chromatographer (Waters 2690) equipped with a photodiode array detector (Waters 996) with the temperature setting at 20–22 °C. The isoflavones were eluted in a reverse phase column (YMC Pack ODS-AM C18; 4.6 mm × 250 mm, 5 μm particle size) by the linear gradient system. The initial gradient system condition consisted of 20% methanol acidified with 0.025%

trifluoracetic acid (pH 3.0), and 80% H_2O acidified with 0.025% trifluoracetic acid (pH 3.0). The proportion of 90% of methanol and 10% of H_2O was reached in 35 min of elution, and the isoflavones were separated and detected at 254 nm. The gradient proportion of 100% methanol and was reached at 40 min, remaining in this condition for 5 min for column cleaning, and then returned to the initial condition of 20% methanol and 80% H_2O in 20 min. The final elution time was 60 min. The solvent flow rate was 1 $mL \cdot min^{-1}$.

Isoflavones were identified and quantified by comparing the standard curves for genistin, daidzin, and glycitin (Sigma). The malonyl-glycosides and aglycone concentration were calculated from the standard curves of their corresponding β-glycosides, using the similarity of the molar extinction coefficients of malonyl-isoflavones and their β-glycosides (Coward et al. 1998). The isoflavone contents were expressed in $mg \cdot (100\ g)^{-1}$ of defatted soybean flour on a dry mass basis.

Total antioxidant reactivity assay

The TAR assay is based on luminol-enhanced chemiluminescence (CL) induced by an initiator 2.2′azo-bis (2-amidinopropane) dihydrochloride (ABAP). CL was measured in a liquid scintillation counter in the out-of-coincidence mode (LKB Rack Beta Liquid Scintillation Spectrometer 1215; LKB, Produkter AB, Sweden). The assay conditions are described elsewhere (Siqueira et al. 2005). TAR values were expressed as equivalents of Trolox concentration per milligram of protein.

Total radical-trapping antioxidant potential

TRAP represents the total antioxidant capacity of the soy product, and was determined by measuring the luminol CL intensity induced by ABAP (Siqueira et al. 2005). The results were represented as $nmol \cdot (L\ Trolox)^{-1} \cdot (mg\ protein)^{-1}$.

Animals and experimental groups

The experimental protocol used in this study was reviewed and approved by the Ethical Committee of Federal University of Rio Grande do Sul, number 2002-62, and it was in accordance with the principles and guidelines of the Canadian Council on Animal Care. Male Wistar rats were obtained from the Central Animal House of the Federal University of Rio Grande do Sul.

Twenty-one-day-old male Wistar rats were obtained from the Central Animal House of the Federal University of Rio Grande do Sul. Animals were housed in metabolic cages (one animal each), received water and food ad libitum and were maintained under standard laboratory conditions (controlled temperature of 21 °C, 12 h (light) –12 h (dark) cycle). All animals were fed with the experimental diets for 9 weeks and were weighed weekly throughout the study. Food intake was measured 3 times a week.

Animals were assigned into 6 groups: (*i*) Sham Casein (SC), sham-operated, fed with casein; (*ii*) Infarct Casein < 25% (IC < 25%), infarct size less than 25%, fed with casein; (*iii*) Infarct Casein > 25% (IC > 25%), infarct size greater than 25%, fed with casein; (*iv*) Sham Soy (SS), sham-operated, fed with ISP; (*v*) Infarct Soy < 25% (IS < 25%), infarct size less than 25%, fed with ISP; and (*vi*) Infarct Soy > 25% (IS > 25%), infarct size greater than 25%, fed with ISP.

Diet

Diet composition was formulated according to the specifications of the AIN-93G (Reeves et al. 1993) and contained similar amounts of protein (21%), fat (7%), carbohydrates (62%), minerals and vitamins, except for the protein source (casein, 86.7%) or ISP (a concentrated product containing 92.2% protein). The content of the diets offered to the animals was described previously by our research group (Hagen et al. 2009).

Surgical induction of myocardial infarction

Five weeks after the beginning of the diet treatments, myocardial infarction (MI) was produced by occlusion of the left coronary artery according to a technique described previously (Pfeffer et al. 1979). The animals were submitted to surgical procedure of ligature of the descending anterior branches of the left coronary artery or to a sham-operation as described in a previous study from our group (Hagen et al. 2009).

Blood samples preparation

Immediately before the sacrifice, blood samples were collected by cardiac punction (2 mL in heparinized tubes). The samples were centrifuged for 10 min at 1000*g* (Sorval RC 5b-rotor SM 24, Du Pont Instruments, USA), and the plasma was separated for further assays of nitrites and nitrates. Afterwards, the erythrocytes were washed out with a saline solution (NaCl 0.9%), and used for further assays of oxidative stress. After blood collection, animals were killed by decapitation.

Determination of infarct size

The ventricular chambers were dissected, blotted, and weighed. The presence of infarction was easily confirmed by gross visualization of the fibrous scar on the anterolateral region of the left ventricle. The scar tissue was separated from the remaining left ventricular myocardium in the infarcted hearts under a microscope. Both fragments were blotted and weighed, and their outlines were drawn on graph paper to estimate the respective areas. Infarct size was calculated and reported as percent of the ventricular endocardial surface covered with scar tissue (Mill et al. 1990). The infarct size median was 25% of the left ventricle area and it was used to stratify the groups.

After infarct size analysis, the animals were assigned into 6 groups: (*i*) Sham Casein (SC), sham-operated, fed with casein; (*ii*) Infarct Casein < 25% (IC < 25%), infarct size less than 25%, fed with casein; (*iii*) Infarct Casein > 25% (IC > 25%), infarct size larger than 25%, fed with casein; (*iv*) Sham Soy (SS), sham-operated, fed with ISP; (*v*) Infarct Soy < 25% (IS < 25%), infarct size less than 25%, fed with ISP; and (*vi*) Infarct Soy > 25% (IS > 25%), infarct size larger than 25%, fed with ISP.

Peripheral markers of oxidative stress

To evaluate LPO, *tert*-butyl hydroperoxide (tBOOH)-initiated CL technique was analyzed in the red blood cells. CL was measured in a liquid scintillation counter in the out-of-coincidence mode (LKB Rack Beta Liquid Scintillation Spectrometer 1215; LKB, Produkter AB). Erythrocyte samples were placed in low-potassium vials at a protein concentration of 0.5–1.0 $mg \cdot mL^{-1}$ in a reaction medium consisting of

120 mmol·L^{-1} KCl and 30 mmol·L^{-1} phosphate buffer (pH = 7.4). Measurements were started by the addition of 3 mmol·L^{-1} tBOOH, and the data are expressed as counts per second per milligram of the blood sample hemoglobin (Hb) (cps·(mg Hb)$^{-1}$) (Gonzalez Flecha et al. 1991). Antioxidant enzyme activities were also determined. Superoxide dismutase (SOD) activity, expressed as units per milligram of protein, was based on the inhibition of the superoxide radical reaction with pyrogallol. The reaction medium contained Tris buffer (50 mmol·L^{-1}, pH = 8.2, pyrogallol (24 mmol·L^{-1}), and catalase (30 mmol·L^{-1}). Absorbance changes were measured at 420 nm for 2 min (Marklund 1985). Catalase (CAT) activity was determined by following the decrease in 240-nm absorption in a reaction medium containing 50 mmol·L^{-1} phosphate buffer (pH = 7. 2) and 10 mmol·L^{-1} hydrogen peroxide (H_2O_2), and expressed as picomol of H_2O_2 reduced per minute per milligram of protein (Aebi 1984). Glutathione peroxidase (GPx) activity, expressed as nanomols of peroxide/hydroperoxide reduced per minute per milligram of protein, was measured by following NADPH oxidation at 340 nm in a reaction medium containing 0.17 mmol·L^{-1} reduced glutathione, 0.2 U·mL^{-1} glutathione reductase, 0.5 mmol·L^{-1} *tert*-butyl hydroperoxide, as described by Flohé and Günzler (1984).

Nitrites and nitrates

Total plasma nitrite and nitrate concentrations were determined according to the method of Granger et al. (1999). The plasma samples were incubated with enzymatic cofactors and nitrate reductase (1.75 U·mL^{-1}) for 30 min at room temperature, to convert nitrite to nitrate. Adding Griess reagent (1 g sulfanilamine, 0.1 g naphthalene diamine, 2.3 mL orthophosphoric acid 85%, and 97.7 mL water) to the reaction medium evoked the reaction. The reaction medium cosisted of the following: Tris 1 mol·L^{-1}, pH 7.5; NADPH 0.02 mmol·L^{-1}; glucose-6-phosphate 5 mmol·L^{-1}; glucose-6-phosphate-dehidrogenase 10 U·mL^{-1},· and nitrate reductase 0.1 UmL^{-1}. Absorbancy was read at 540 nm in a spectrophotometer and the results were expressed in μmol·L^{-1}.

Protein determination

For the soybean products, the protein content was determined by the Kjeldahl method according to the methodology preconized by AOAC (2009).

The rat erythrocyte protein was measured by the method of Lowry et al. (1951), using bovine serum albumin as the standard.

Hemoglobin determination

Hemoglobin content was assessed by the conversion of hemoglobin to cyanomethaemoglobin by Drabkin reagent measured against a standard curve (Drabkin and Austin 1935).

Statistical analysis

In-vitro data

Data are expressed as the mean ± SD. Student's *t* test was used to compare data from 2 groups. Graphpad Instat 3.0 software (San Diego, California, USA) was used. Values of $P < 0.05$ are considered statistically significant.

In-vivo data

Data were compared by one-way analysis of variance and are expressed as the mean ± SD. The Student–Newman–Keuls post-hoc test was used to determine significant differences among individual groups. Graphpad Instat 3.0 software was used. Values of $P < 0.05$ are considered statistically significant.

Results

Isoflavone content

Table 1 shows the content of isoflavones in SH and ISP analyzed by HPLC, as well as its aglycone equivalents. The samples were not analyzed for the content of glycitein or glucosides forms because the HPLC column utilized was not able to separate these forms.

The SH showed 9-fold higher levels of total isoflavones and higher levels of daidzin, genistin, and mal-daidzin than ISP. However, ISP demonstrated a 4.5 fold higher levels of genistein and Mal-genistin when compaired with SH.

Antioxidant potential

To determine the final concentration of SH and ISP to be used in the in-vitro studies, different concentrations were tested for TAR and TRAP (data not shown). The final concentration (2.0% for both products) showed the most optimal sensitivity and specificity for the luminescent analysis and was chosen as the working concentration for the following results. Concentrations greater or less than 2% were outside the optimum range of reading of the instruments.

Figures 1A and 1B shows the comparison of the 2 products at the final concentration of 2%, in terms of TAR and TRAP. SH had about 3 times more TAR than ISP. However, both soybean products presented the same TRAP, which means that although they have different quality of antioxidants the total amount of antioxidant is similar in both products.

In-vivo study

SH is 42.6% protein, while the ISP is a concentrated product that is 92.2% protein. Thus, the ISP diet composition is similar to casein (86.7% protein), which is usually utilized as a control diet. Therefore, we offered an ISP diet to rats submitted to myocardial infarction to study the peripheral markers of oxidative stress, 4 weeks after surgery. The mean daily food intake per rat was 18 g·day^{-1}. Since the isoflavone content in ISP was reported to be 189 mg·(100 g ISP)$^{-1}$, the animals consumed around 7.0 mg of total isoflavones per day. This amount represents 1.6 g of total isoflavones or 1.10 g of aglicone equivalents per day for a human adult with body mass of 70 kg.

The infarct size areas were measured and were in average 36% (data not shown); ISP administration did not significantly change this parameter.

The systemic profile of antioxidant enzymes in rats treated with ISP or casein diet is presented in Table 2. SOD, CAT, and GPx activities were determined in erythrocytes. In the ISP-fed rats, the antioxidant enzymes were found to be increased (49% and 142%, for SOD and CAT, respectively, compared to their respective control groups). MI induced a decrease in the activities of SOD and CAT in both ISP and

Table 1. Content of isoflavones (in milligrams per 100 g of the product) in soybean hypocotyl and isolated soy protein.

	Soybean hypocotyl		Isolated soy protein	
Isoflavone profile	Aglycones and conjugated	Aglycone equivalents	Aglycones and conjugated	Aglycone equivalents
Daidzein	21.8	21.8	24.4	24.4
Genistein	5.2	5.2	23.2	23.2
Daidzin	186.5	113.9	19.7	12.0
Genistin	63.2	39.5	34.1	21.3
Mal-daidzin	52.7	26.7	21.4	10.8
Mal-genistin	14.6	7.6	66.2	34.5
TOTAL	344	214.7	189	126.2

Note: Content analysis was performed at The Physical Chemistry Laboratory of the Embrapa Soybean, Londrina, Brazil. Aglycone forms: daidzein, genistein. Conjugated forms: daidzin, genistin, Mal-daidzin, Mal-genistin.

Table 2. Antioxidant enzyme activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) in erythrocytes after 9 weeks of diet treatment.

Parameter	SC	CI < 25%	CI > 25%	SS	SI < 25%	SI > 25%
SOD (U·(mg prot.)$^{-1}$)	7.8±1.1	5.9±0.8[a]	5.1±0.9[b]	11.6±1.3[a]	9.7±0.8[a,c,d,e]	8.6±0.8[d,f]
CAT (pmol·(mg prot.)$^{-1}$)	2.4±0.3	1.1±0.2[g]	0.6±0.1[g,h]	5.8±0.5[g]	3.8±0.3[c,d,f,g]	1.9±0.3[a]
GPx (nmol·(min)$^{-1}$·(mg prot.)$^{-1}$)	7.2±0.6	6.3±0.3	5.6±0.6	7.8±0.5	6.9±0.5	6.4±0.6

Note: Values are expressed as the mean ± SE of 6–8 animals per group. SC, sham casein; CI < 25%, casein infarct < 25%; CI > 25%, casein infarct > 25%; SS, sham soy protein isolate; SI < 25% soy protein isolate infarct < 25% and SI > 25% soy protein isolate infarct > 25%.

[a]Compared with SC ($P < 0.01$).
[b]Compared with SC ($P < 0.001$).
[c]Compared with CI < 25% ($P < 0.001$).
[d]Compared with CI > 25% ($P < 0.001$).
[e]Compared with SS ($P < 0.01$).
[f]Compared with SS ($P < 0.001$);
[g]Compared with SC ($P < 0.001$).
[h]Compared with CI < 25% ($P < 0.05$).

casein-treated groups. However, in the infarcted animals treated with ISP, antioxidant enzyme activities were significantly higher than those treated with casein. It is important to highlight that CAT activity was about 3 times higher in ISP-treated rats, not only in the control but also in the 2 infarcted groups (SI < 25% and SI > 25%). GPx activity did not change in any group.

LPO was evaluated in erythrocytes by CL and did not change in the SS group when compared with SC (Fig. 2A). In the infarcted groups, LPO was higher than the respective controls; however, LPO was significantly less in ISP-fed rats when compared with the casein-treated animals.

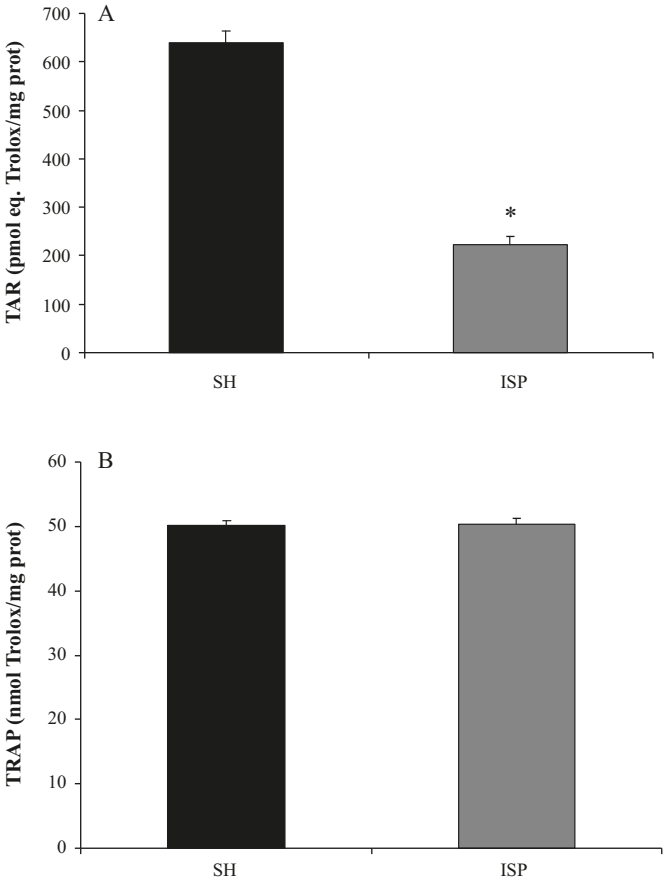
Plasma levels of nitrites and nitrates are represented in Figs. 2B and 2C. There was a significant increase in nitrite/nitrate levels (78%, and 50%, respectively) in SS as compared with SC. MI produced a decrease in this parameter; however, in ISP-fed rats, this decrease was attenuated compared with the casein-treated groups. Nitrites were preserved by about 140% and nitrates by 100% in the IS > 25% group when compared with the IC > 25% group.

Discussion

Other studies have demonstrated the potential benefit of soy-derived products consumed in the diet, associating their effects with the presence of antioxidants (Yousef et al. 2004; Engelman et al. 2005; Liu et al. 2005). In this regard, we have previously reported that in an animal model of myocardial infarction, the characteristic changes of congestive heart failure were attenuated by the administration of a diet with ISP. The efficacy of the diet with ISP in reducing myocardial dysfunction after infarction was attributed to the antioxidant status improvement seen in the studied animals (Hagen et al. 2009). Our intention, in the present study, was to compare 2 soy-derived products in terms of their antioxidant capacity in vitro, and to test the antioxidant potential, in vivo, through peripheral markers of oxidative stress, which are considered minimally invasive methods with clinical relevance (Repetto et al. 1996).

Our data demonstrated that the 2 soy-derived products tested, SH and ISP, have similar antioxidant potential (evaluated by TRAP). We also observed that SH has greater TAR values than ISP. The antioxidant properties may be associated to their isoflavone content. In this regard, the chemical analysis of the products was performed showing that they present the same flavonoids but in different concentrations. SH has greater amounts of the glycoside isoflavones (especially daidzin), whereas in ISP, daidzein and genistein are the main aglycone isoflavones, as also reported by others (Kroon et al. 2004; Kruk et al. 2005). These differences can justify the distinct antioxidant reactivity of the 2 compounds. TRAP evaluation of these compounds revealed that both products

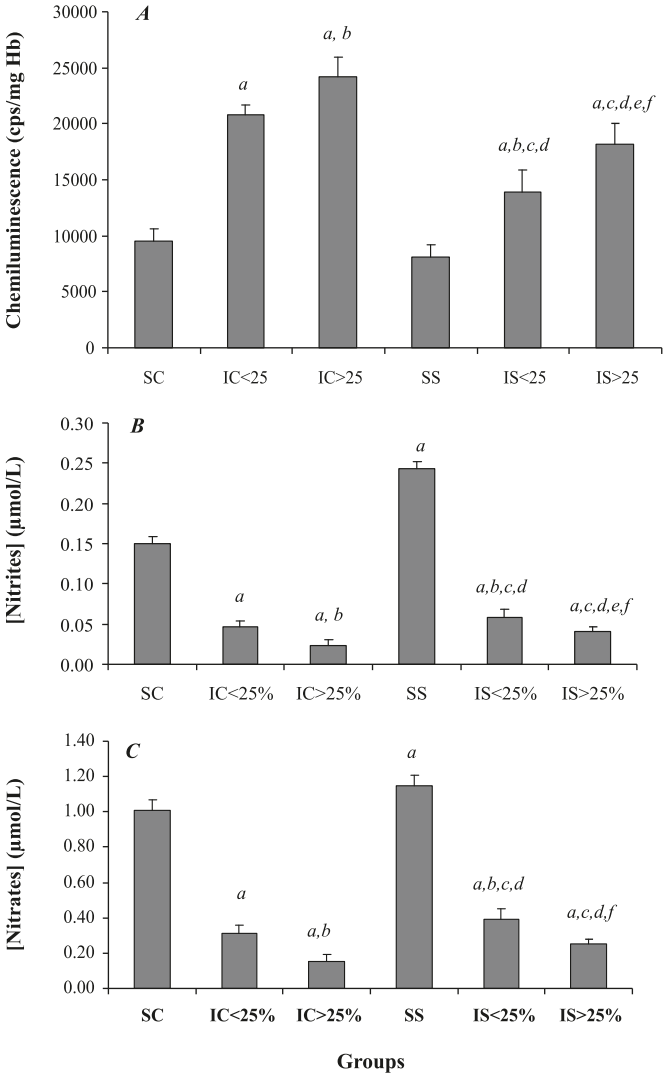
Fig. 1. (A) Total antioxidant reactivity (TAR) levels of soybean hypocotyl (SH) and isolated soy protein (ISP). Results are the mean ± SD of experiments performed in triplicate. TAR is calculated as picomole equivalent of Trolox per milligram of protein. *, $P < 0.01$ compared with SH. (B) Total radical-trapping antioxidant potential (TRAP). Results are the mean ± SD of experiments performed in triplicate. TRAP is calculated as nanomole of Trolox per milligram of protein.



have similar levels, which means they may be able to scavenge the same quantity of free radicals.

Moreover, although the ISP showed lower antioxidant reactivity than the SH in our study, the bioavailability of isoflavones appears to be greater in the ISP, owing to the presence of larger amounts of aglycones. Previous studies have demonstrated that the isoflavone glycosides, more abundant in SH, are not absorbed directly, but are hydrolyzed in the intestine by intestinal enzymes (β-glucosidase) (Setchell et al. 2002; Németh et al. 2003). According to Izumi et al. (2000), the aglycones are absorbed faster and in greater quantities than the glycosylated forms. Another study demonstrated that genistein has better antioxidant activity than the genistein glycosides in human LDL-cholesterol (Lee et al. 2005). In addition to its antioxidant capacity, the ISP has an amino-acid pattern similar to the reference standard for essential amino acids recommended by FAO/WHO (1991), which gives the ISP a nutritional quality. In this regard, our in-vivo studies were conducted with the ISP diet. We believe that ISP could be a better candidate than SH in human dietetic practice, owing to the high quality of amino acids as well as the feasibility for being included in food processing.

Fig. 2. Effects of 9 weeks of the diet treatment on (A) lipid *tert*-butyl hydroperoxide induced chemiluminescence (CL, in cps·(mg Hb)$^{-1}$) in erythrocytes, (B) the concentration of nitrites (μmol·L^{-1}) in plasma, and (C) the concentration of nitrates (μmol·L^{-1}) in plasma. Values are expressed as the mean ± SD of 6–8 animals per group. SC, sham casein (sham-operated, fed with casein); IC<25, infarct casein < 25% (infarct size less than 25%, fed with casein); IC>25, infarct casein > 25% (infarct size greater than 25%, fed with casein); SS, sham soy (sham-operated, fed with isolated soy protein (ISP)); IS<25, infarct soy < 25% (infarct size less than 25%, fed with ISP); and IS>25, infarct soy > 25% (infarct size greater than 25%, fed with ISP). [a]Compared with SC ($P < 0.001$); [b]compared with CI < 25% ($P < 0.001$); [c]compared with SS ($P < 0.001$); [d]compared with CI > 25% ($P < 0.001$); [e]compared with CI < 25% ($P < 0.01$); and [f]compared with SI < 25% ($P < 0.001$).



In this study, the systemic oxidative stress biomarkers analysis demonstrated that the ISP diet given to healthy rats has the ability to improve the majority of the parameters analyzed. It was interesting to note that the soy diet leads to a pronounced increase in the SOD and CAT antioxidant enzyme activities. This is in agreement with other reports in the literature showing that isolated isoflavones induce in-

creased expression of antioxidant enzymes (Röhrdanz et al. 2002). Nitric oxide (NO) metabolite (nitrite and nitrate) levels were also higher in the present study in ISP-fed rats when compared with the casein-fed controls. In fact, an increase in endothelial NO synthase expression had already been reported when genistein was included in the diet (Squadrito et al. 2000). Moreover, genistein has been reported to relax vascular smooth muscles (Li et al. 2008). These findings demonstrate that ISP diet might be able to improve the antioxidant reserve as well as NO parameters in healthy rats.

In our study, when rats were subjected to a coronary artery ligation producing myocardial infarction, the evident increase in lipid peroxidation estimated by CL demonstrated that infarcted animals were exposed to oxidative damage. It has been previously reported that MI produces a great elevation in reactive oxygen species (ROS) production (Ide et al. 2000; Singal et al. 2000). However, in the animals that received the ISP diet, CL was significantly lower than the respective controls, possibly due to the ROS scavenging action of the phenolic compounds present in diet.

Considering the fact that MI generates a great amount of superoxide anion ($O_2^{\bullet -}$) (Singal et al. 2000), our data showed that SOD, the enzyme responsible for $O_2^{\bullet -}$ detoxification, is more preserved in the ISP-fed rats, suggesting that part of the toxic effect of $O_2^{\bullet -}$ would be neutralized in these animals. In a subsequent reaction, H_2O_2 formed from the dismutation reaction of $O_2^{\bullet -}$ would be rapidly consumed by catalase, an enzyme that was found increased by about 140% in the healthy animals that consumed the ISP diet (SS). These findings suggest that when oxidative stress is caused by MI induction, animals receiving an ISP diet are better adapted than the control group (no ISP), since CAT activity was significantly enhanced in the SS group, eliminating the H_2O_2 formed in the oxidative process.

The third antioxidant enzyme studied (GPx) did not show the same profile previously described in the myocardium (Hagen et al. 2009). In fact, GPx is known to have a higher activity, and thus, to play a more important role in heart tissue than in erythrocytes, where CAT predominates in H_2O_2 detoxification.

In terms of plasmatic NO metabolites, our findings have shown that animals submitted to myocardial infarction and receiving a casein or ISP diet demonstrated a significant decrease in these parameters. However, this reduction was not so marked in the ISP-fed rats. As mentioned earlier, the increased ROS production in MI (Ide et al. 2000), added to elevated NO levels, would yield to the production of peroxynitrite ($ONOO^-$), which is a very strong oxidant (Ohshima et al. 1998). In the ISP treated animals, since SOD activity is higher than in controls, $O_2^{\bullet -}$ levels in erythrocytes would be less, generating less $ONOO^-$. This could promote a greater NO bioavailability, contributing to the vasodilatation that has been previously described when genistein was given in the diet (Smith et al. 2005). Beyond its antioxidant properties, isoflavone actions include hypocholesterolemic (Zhuo et al. 2004), blood-pressure-lowering (Welty et al. 2007) and estrogen-like effects (Lissin and Cooke 2000), which contribute to reduce cardiovascular risk factors. These combined effects may be relevant in the reduction of cardiovascular events.

Conclusion

Taken together, our data demonstrate that 2 different soy products with distinct isoflavone content exhibit properties associated with high levels of antioxidants.

Our results reveal the importance of soy-derived product consumption, not only in disease conditions, but also to healthy subjects since these products are able to increase the antioxidant reserve in blood. The in-vitro and in-vivo effects of the soy products observed in this study can be associated to their antioxidant ability, making them promising agents against lipid peroxidation and other free radical-mediated cell injuries.

Furthermore, our findings highlight a soy-based diet as an alternative solution for the prevention and management of oxidative stress-related diseases such as heart failure.

Acknowledgement

This work was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), the Fundação de Amparo à Pesquisa do Rio Grande do Sul (FAPERGS), the Secretaria de Ciência e Tecnologia do Estado do Rio Grande do Sul (SCT-RS) and The Solae, LLC (Brazil). Ana Ludke is supported by a studentship from Manitoba Health Research Council

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