Whole soybean flour with high concentration of isoflavone and vitamin E modulates cardiovascular risk factors in Wistar rats

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Abstract
Cardiovascular diseases (CVD) are the main cause of morbidity and mortality worldwide. The intake of soybean (Glycine max (L.) Merrill), a source of bioactive compounds, can reduce the risk of developing CVD. Therefore, the present work evaluated the effect of whole soybean flour (UFVTN 105AP) on cardiovascular risk factors in rats. Male Wistar rats were divided into three groups (n = 10): AIN-93M (control diet - casein standard); SP100 group (control diet with 100% soybean protein) and SP50 group (control diet with 50% soybean protein). After 56 d, triglycerides, cholesterol and serum lipid peroxidation levels decreased in the SP100 group. Furthermore, an increase in the excretion of faecal lipids and liver fat was observed. The SP50 group presented low lipid peroxidation in the serum and lung, and increased lipid peroxidation in the duodenum and ileum villi. Thus, UFVTN 105AP whole soybean could be a potential functional food, which has cardioprotective effects and reduces cardiovascular risk associated with oxidative stress.

Introduction
Cardiovascular diseases (CVD) are the leading cause of death in patients with chronic non-communicable diseases, accounting for over 17.3 million deaths per year, mainly from heart attack and stroke (7.3 and 6.2 million per year, respectively) (Sacks et al., 2017). These complications are associated with atherosclerosis, hypertension, hyperglycaemia and dyslipidaemia (WHO, 2012).

A high intake of saturated fat, trans fat, cholesterol and salt, and low consumption of fish and vegetables contribute to the development of atherosclerosis (WHO, 2012). Among plant-based foods, soybean (Glycine max (L.) Merrill) stands out because of its functional properties which are attributed to nutrient composition and bioactive compounds. Soybean is a good source of protein, essential fatty acids, dietary fibre and phenolic compounds such as isoflavones (Han et al., 2017).

Studies have shown that soybean intake modulates risks associated with CVD, mainly in the treatment of hypercholesterolemia (Ramos Fonseca et al., 2014). This effect has been attributed to isolated proteins and peptides in soybean (Wang et al., 2017) or association with isoflavones and other antioxidant compounds such as vitamin E (Han et al., 2017). Isoflavones are the main phenolic compounds present in soybean, known to exert antioxidant effects. These bioactive compounds contribute to the reduction of lipid peroxidation and activation of antioxidant mediators, thus reducing the occurrence of CVD.

Although the intake of these compounds alone is beneficial to health, the consumption of whole soybean may accentuate the positive physiological effects of these compounds through the synergy between nutrients and bioactive compounds (Khan and Kang, 2017). Despite this advantage, the consumption of whole soybean is limited in certain populations due to its distinctive flavour which reduces palatability.
This flavour is related to the oxidation of unsaturated fatty acids by lipoxygenase (Ciabotti et al., 2006). Through genetic manipulation, new soybean cultivars with improved sensory, nutritional, and organoleptic characteristics can be created. An example is the new UFVTN 105AP soybean cultivar, which is lipoxygenase-free and has a high concentration of protein and isoflavones (Esteves et al., 2010).

Previous studies have demonstrated the efficiency of whole soybean flour in the improvement of CVD markers in experimental groups placed on high fat or cafeteria diets (Martino et al., 2011; Andrade et al., 2013). However, the performance of UFVTN 105AP whole soybean flour combined with a normal diet is unknown. Therefore, the present work evaluated the concentration of isoflavones and vitamin E in UFVTN 105AP whole soybean flour, and the effect of its intake on cardiovascular risk factors and histomorphometric properties of the liver and intestine in adult Wistar rats.

**Materials and methods**

**Experimental design**

A total of 30 adult male rats (*Ratus norvegicus albinus* Wistar) were used in the present work, 75 d of age with weight between 250 and 350 g. The rats were individually housed in temperature-controlled (22 ± 2°C) cages, with a 12-h photoperiod and access to food and distilled water ad libitum. Three groups of ten rats each were formed, and each group was given a specific pelleted diet as follows: (i) AIN-93M (control diet - casein standard) (Reeves et al., 1993); (ii) SP50: control diet with 50% replacement of casein with whole soybean protein flour; (iii) SP100: control diet with 100% replacement of casein with whole soybean protein flour. Dietary intake was assessed through daily records of diet intake in grams. Weight gain was calculated considering the difference between the final and initial weight in grams. Weight gain was calculated considering the diet consumed.

On the 56th day of the protocol (Andrade et al., 2013), after a 12-h fast, the animals were anesthetised with Isoflurane® and sacrificed by exsanguination. Blood samples were collected and centrifuged at 1,000 g for 15 min to obtain serum samples, which were stored at -80°C. The liver, lung and small intestine were maintained in liquid nitrogen and subsequently lyophilised. Faecal samples were collected and stored at -24°C.

**Soybean samples**

The lipoxygenase-free UFVTN 105AP cultivar (LOX 1, LOX 2 and LOX 3) with high protein content was developed by BIOAGRO (Institute of Biotechnology Applied to Agriculture, Universidade Federal de Viçosa), using backcrossing as described by Martino et al. (2011). Briefly, we obtained lipoxygenase-free backcross isolines with high protein content through a recurrent parent (cultivar Monarch), lipoxygenase-free donors (Japanese cultivars) and a donor of genes for high protein content (American variety BARC-8).

**Whole soybean flour**

The soybean samples were washed with water and dried at ambient temperature. The grains were heated at 150°C for 30 min in an oven with air circulation (New Ethics® model 400/6ND, Vargem Grande Paulista, Brazil) to inactivate antinutritional factors and improve protein quality (Martino et al., 2011). Subsequently, the grains were cooled and stored at -20°C in polyethylene packaging bags. The grains were shelled and ground in a knife mill (Brabender® model Rotary Mill, Duisburg, Germany) and then passed through a 60 mesh sieve (0.25 mm) to obtain the flour.

The chemical composition of the UFVTN 105 whole soybean flour was previously determined by Martino et al. (2011). Vitamin E and its isomers were analysed by High-Performance Liquid Chromatography (HPLC), using the chromatographic conditions and procedures proposed by Guinazé et al. (2009). The total content of vitamin E was calculated as the sum of the isomers.

The total phenols present in the alcoholic extract of the soybean flour samples were determined using the Folin-Ciocalteu method (Singleton et al., 1999), by interpolating the absorbance of the same from a standard curve of gallic acid (equation: \( y = 24.888x + 0.0246; R^2 = 0.99 \)). The contents of isoflavone and isomers were determined by HPLC following the method described by Murphy et al. (1999).

**Experimental diets**

The composition of the SP100 and SP50 diets was based on the AIN-93M diet (Reeves et al., 1993). Casein in the control diet was replaced by soybean protein flour, with adjustments in macronutrients and dietary fibre. Accordingly, the diets were isocaloric and contained 50% (SP50 diet) or 100% (SP100 diet) protein content from UFVTN 105 whole soybean flour (Table 1).

The diet ingredients were weighed and manually mixed, and homogenised in a semi-industrial mixer.
(Lieme®) for 15 min. The diets were moulded into pellets, wrapped in plastic bags and stored under refrigeration.

The chemical composition of the diets was determined according to the Association of Official Analytical Chemists (AOAC, 2002) (Table 1). The energy in the foods was calculated using the conversion factors of 4, 4 and 9 kcal/g for carbohydrates, proteins, and lipids, respectively.

### Table 1. Ingredients and nutrient composition of experimental diets.

<table>
<thead>
<tr>
<th>Ingredient (g/100 g)</th>
<th>AIN-93M</th>
<th>SP50</th>
<th>SP100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein (% protein)</td>
<td>15.40</td>
<td>7.70</td>
<td>-</td>
</tr>
<tr>
<td>Sucrose</td>
<td>10.00</td>
<td>10.00</td>
<td>10.00</td>
</tr>
<tr>
<td>Corn starch</td>
<td>43.92</td>
<td>42.34</td>
<td>40.77</td>
</tr>
<tr>
<td>Dextrinised starch</td>
<td>15.50</td>
<td>15.50</td>
<td>15.50</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>5.25</td>
<td>2.63</td>
<td>0.00</td>
</tr>
<tr>
<td>Microcrystalline cellulose</td>
<td>5.00</td>
<td>2.50</td>
<td>2.50</td>
</tr>
<tr>
<td>Mineral Mix AIN93M</td>
<td>3.50</td>
<td>3.50</td>
<td>3.50</td>
</tr>
<tr>
<td>Vitamin Mix AIN93M</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>L-cystine</td>
<td>0.18</td>
<td>0.18</td>
<td>0.18</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>Integral soy flour</td>
<td>0</td>
<td>13.83</td>
<td>27.67</td>
</tr>
</tbody>
</table>

### Nutritional composition

**Macronutrients (g/100 g)**

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>AIN-93M</th>
<th>SP50</th>
<th>SP100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>9.52 ± 0.52</td>
<td>9.47 ± 0.16</td>
<td>9.20 ± 1.07</td>
</tr>
<tr>
<td>Lipid</td>
<td>2.25 ± 0.65</td>
<td>1.80 ± 0.05</td>
<td>2.10 ± 0.02</td>
</tr>
<tr>
<td>Ash</td>
<td>1.87 ± 0.02</td>
<td>2.30 ± 0.02</td>
<td>2.67 ± 0.01</td>
</tr>
<tr>
<td>Moisture</td>
<td>32.20 ± 0.08</td>
<td>32.99 ± 0.26</td>
<td>32.58 ± 0.24</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>49.16 ± 1.16</td>
<td>51.17 ± 0.11</td>
<td>50.09 ± 0.89</td>
</tr>
<tr>
<td>Dietary fibre</td>
<td>5.00 ± 0.13</td>
<td>3.86 ± 0.02</td>
<td>5.25 ± 0.04</td>
</tr>
</tbody>
</table>

**Caloric density and bioactive compounds**

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>AIN-93M</th>
<th>SP50</th>
<th>SP100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caloric density (Kcal/g)</td>
<td>2.55 ± 0.03</td>
<td>2.44 ± 0.01</td>
<td>2.39 ± 0.01</td>
</tr>
<tr>
<td>Vitamin E (mg/100 g)</td>
<td>-</td>
<td>0.53</td>
<td>1.06</td>
</tr>
<tr>
<td>Mioinositois phosphates (g/100 g)</td>
<td>-</td>
<td>0.15</td>
<td>0.31</td>
</tr>
<tr>
<td>Total phenols (mg de EAG/100 g)</td>
<td>-</td>
<td>8.30</td>
<td>16.60</td>
</tr>
<tr>
<td>Isoflavones (mg/100 g)</td>
<td>-</td>
<td>362.47</td>
<td>724.95</td>
</tr>
</tbody>
</table>

AIN-93M: control diet; SP50: group with a replacement of 50% of casein by whole soybean flour protein; SP100: group with total replacement of casein by whole soybean flour protein; Estimated content based on nutritional composition of whole soybean flour added on experimental diets.

Biochemical analysis

Total cholesterol, HDL-C, and triglycerides were determined by the enzymatic colorimetric method using commercial kits (Human in Brazil). Following the reaction, absorbance was measured in a spectrophotometer (Shimadzu model UV 1601) at 415 nm. HbA1c concentration was expressed as % of total haemoglobin. The activities of aminotransferases, alanine transaminase (ALT) and aspartate aminotransferase (AST) were measured by the UV kinetic method, using commercial kits (Human in Brazil).

Lipid peroxidation

Lipid peroxidation in the serum, liver and lung was assessed by Thiobarbituric Acid Reactive Substances Test (TBARS), following the method described by Buege and Aust (1978). The lyophilised tissue was re-suspended in 0.1 M phosphate buffer, pH 7.4, 1:10 (m/v) to obtain a homogenate for analysis.

Excretion of lipids

Faecal excretion of lipids was determined by the content of fat in faeces. For this purpose, the
faeces were dried and crushed, and total lipids were extracted in a Soxhlet apparatus, using ethyl ether for 8 h under reflux, following the AOAC Method Ba 3-38 (AOAC, 2002).

**Histomorphometric analyses of the liver and intestine**

Histological analyses were performed using fragments of the liver and proximal (duodenum) and distal (ileum) small intestines. The intestine and liver portions were fixed in Bouin’s fluid for a minimum of 24 h and preserved in 70% alcohol. Nine semi-serial transverse sections of the duodenum, ileum and liver, 3 μm in thickness, were stained with hematoxylin/eosin. The slides were examined under an Olympus CX31 light microscope, and images were obtained using a SC 020 digital camera via Analysis GET IT Olympus software. Images of the histological sections were captured with a 10× objective for the visualisation and quantification of fat deposits in the liver tissue and muscle layers of the intestine. A 4× objective was utilised for the morphometric evaluation of the villi and crypts of the intestine (Andrade et al., 2013).

Fat accumulation in the liver was determined using computational quantification of fat droplets and the values were obtained through an algorithm developed using software Scilab 4.1 (developed by INRIA and ENPC, version released in 2006) programming language based on the thresholding method (Sabarense et al., 2012).

In both duodenum and ileum, the following measurements were taken with the aid of Image-Pro® Plus version 4.5 (Media Cybernetics): eight random fields were selected per animal, totalling 40 villi per experimental group. Only villi with defined and visible conjunctival epithelium were utilised.

The hepatosomatic index (HSI) was calculated by the formula: (liver weight/final animal weight) × 100.

**Statistical analysis**

The experiment was conducted using a randomised block design with three replicates and ten treatments. Four replicates were used for the histological analysis of the intestine. The data were analysed using ANOVA (analysis of variance), and the results were expressed as mean ± standard deviation. Significant differences between groups were detected by the Duncan test, using SAEG-UHV 9.1 software for parametric statistics. Liver fat deposition was analysed by the Kruskal-Wallis test using Sigma Stat Software version 2.03. The level of significance was set at 5%. The chemical composition of the soybean flour diets and replicates were analysed and presented as mean values.

**Ethical aspects**

The project was approved by the Ethics Committee on Animal Research of the Federal University of Minas Gerais (UFMG-CETEA) (protocol number 212/2009) and was conducted in accordance with the Ethical Principles of Animal Experimentation.

**Results**

**Chemical composition of whole soybean flour**

The soybean flour contained higher amounts of vitamin E and its isomers, with tocopherol present in larger quantities. The amount of vitamin E per 100 g of soybean flour corresponded to 25.6% according to Dietary Reference Intake (IOM, 2005). In addition, the soybean meal presented high amounts of total phenolics, among them isoflavones, mainly genistein (Table 2).

<table>
<thead>
<tr>
<th>Phytochemical</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin E (mg/100 g)</td>
<td>3.84</td>
</tr>
<tr>
<td>α-tocopherol</td>
<td>0.42</td>
</tr>
<tr>
<td>γ-tocopherol</td>
<td>2.53</td>
</tr>
<tr>
<td>δ-tocopherol</td>
<td>0.89</td>
</tr>
<tr>
<td>Total phenols (mg de EAG/100 g)</td>
<td>60</td>
</tr>
<tr>
<td>Genistearin</td>
<td>753.34</td>
</tr>
<tr>
<td>Daidzein</td>
<td>658.23</td>
</tr>
<tr>
<td>Glicitein</td>
<td>155.25</td>
</tr>
</tbody>
</table>

**Effect on biochemical parameters, body weight and food intake**

The body weight gain (g/day) was similar among the experimental groups (2.43, 2.99 and 2.60 for AIN-93M, SP50 and SP100, respectively). In addition, food intake was also similar among the experimental groups (31.90, 32.18 and 31.57 g/day for AIN-93M, SP50 and SP100, respectively). Likewise, the feed intake coefficient was the same among the experimental groups, AIN-93M, SP50 and SP100 (0.08, 0.09 and 0.07, respectively). The serum levels of HDL-C, HDL-C/TC ratio, glycated haemoglobin, alanine transaminase and aspartate aminotransferase did not differ among the experimental groups (Figure 1). However, the SP100 group showed lower values of total cholesterol (Figure 2A) and triacylglycerols (Figure 2B). The SP50 group did not differ from the control group in any of these markers.
Figure 1. Effect of experimental diets on biochemical and metabolic parameters. Data are means ± SE. Different letters indicate significant difference ($p < 0.05$) by ANOVA and post hoc Duncan test. AIN-93M: control group ($n = 10$); SP50: group with a replacement of 50% of casein by whole soybean flour protein ($n = 10$); SP100: group with total replacement of casein by whole soybean flour protein ($n = 10$); A: AST (aspartate aminotransferase); B: ALT (alanine transaminase); C: HDL-c (high density lipoprotein cholesterol); D: HDL-c/CT rate; E: GHb (glycated hemoglobin); F: hepatic weight (mg); G: hepatosomatic index; H: faecal moisture (%).
Effect on lipid peroxidation

The concentration of serum TBARS was low and high in the SP100 group and control group, respectively. The SP50 group showed lower values of TBARS in the liver, as compared to the other experimental groups. For TBARS in the lung, the SP100 group had the highest values (Figure 3).

Excretion of faecal lipids

The water content of the faeces did not differ among the experimental groups (Figure 1H) but faecal excretion of lipids was higher in the groups that received soybean flour, and this property improved with increasing proportion of soybean as observed in the group that received the highest content (group SP100, $p < 0.05$) (Figure 2D).

Effect on hepatic parameters

Liver weight and hepatosomatic index (HSI) were similar among the experimental groups (Figure 1G). Therefore, the consumption of whole soybean flour did not alter liver weight; however, there was a higher percentage of hepatic fat in the SP100 group animals (Figure 2C).
Effect on intestinal parameters

Histomorphometric analysis of the intestine showed that whole soybean flour consumption lowers duodenal villus height, mainly in the group with total protein substitution (group SP100) (Figure 2E). However, an increase in ileum villus height was observed in the group that received only 50% soybean protein flour (group SP50), whereas the SP100 group had the lowest value (Figure 2F).

Discussion

Soybean and its products have a high economic value because of their high nutrient and phytochemical content (Al Loman and Ju, 2017). Studies have shown that UFVTN 105AP soybean variety has a high content of indispensable amino acids, minerals, linoleic acid, isoflavones, and phytate as compared to conventional cultivars (Esteves et al., 2010; Martino et al., 2011; Carvalho et al., 2013). In the present work, we observed a high concentration of vitamin E and total phenolic compounds, among them isoflavone, as compared to other studies that evaluated other soybean cultivars (Esteves et al., 2010; Ebert et al., 2017).

The high concentration of these bioactive compounds in UFVTN 105 soybean provided the animals which consumed only 50% of soybean protein with 0.35 mg/kg of vitamin E, 5.51 mg/kg of total phenolics and 240.74 mg/kg of isoflavones daily. Since these compounds are antioxidants (Ramos Fonseca et al., 2014), they may have exerted a synergistic effect responsible for the lower lipid peroxidation in the serum and liver of the animals that received soybean protein. However, in the lung, the animals fed with 100% soybean protein showed the highest lipid peroxidation. A possible explanation is that soybean flour has the highest amount of γ-tocopherol, vitamin E isoform, which is inversely related with lung function, and thus the consumption of soybean flour at high concentrations had a damaging effect on the lung. A possible mechanism is
that γ-tocopherol may elevate inflammation through endothelial cell signal regulation during leukocyte recruitment. On the other hand, vitamin E and α-tocopherol are related with antioxidant and anti-inflammatory effects and lung health (Hanson et al., 2016).

Isoflavones, the bioactive compound present in the highest concentration, may reduce lipid peroxidation in the serum and tissues and increase nuclear factor (erythroid-derived 2)-like 2 (Nrf2) activity, a transcription factor that protects vascular aging by upregulating antioxidant enzyme gene transcription.

Besides antioxidant capacity, soybeans demonstrate hypolipidemic effects (Ramos Fonseca et al., 2014), as observed in the present work in which lower levels of triacylglycerols and cholesterol were observed in the animals that consumed 100% soybean protein. Such effects may be due to the bioactive peptides produced after gastrointestinal digestion of soybean protein (Wang et al., 2017).

Among these bioactive peptides, β-conglycinin activates β-oxidation (Moriyama et al., 2004), reducing the synthesis of fatty acids in the liver, increasing the activity of the LDL receptor (Mochizuki et al., 2009) and inhibiting HMG-CoA reductase (Pak et al., 2005). Lunasin, a bioactive peptide derived from soybean proteins, may control the migration and aggregation of cells, besides acting in the inflammatory process; thus acting directly in the pathogenesis of atherosclerosis (Cam and de Mejia, 2012).

Another bioactive peptide present in soybean is glycine. It has a homologous structure as enterotastin, which increases the excretion of bile acids in the faeces (Cam and de Mejia, 2012), as observed in the present work. The animals that consumed soybean flour showed greater faecal excretion of lipids as compared to controls. This result can be attributed to the presence of soluble fibres present in the soybean composition. This fibre can bind bile acids during the formation of intraluminal micelles that leads to increased bile acid synthesis, reduction in hepatic cholesterol content and up-regulation of LDL receptors (Shimada and Ebihara, 2017).

Despite the higher faecal lipid excretion in the groups that consumed soybean flour, the animals in the SP100 group had a higher percentage of fat in the liver. This can be explained by the reduction of intestinal absorption of cholesterol and bile acids. Thus, the low amount of cholesterol in the liver increases bile acid synthesis, which results in higher activity of the hepatic LDL cholesterol receptor, thus increasing the uptake and oxidation of cholesterol (Torres et al., 2006).

However, this result did not have a detrimental effect on the function of the liver, since the liver weight and hepatosomatic index were similar among the experimental groups, as well as serum levels of alanine aminotransferase and aspartate aminotransferase, markers of hepatic function (Kazemi-Bonchenari et al., 2016). Therefore, we observed that despite replacing 100% of casein (animal protein) with soybean protein (vegetable), hepatic function was not impaired.

In addition, the histomorphometric analysis of the intestine showed that the addition of soybean to diet resulted in a lower duodenal villus height (VH). Ileum VH was greater for the SP50 group. This is probably due to the glycinin and β-conglycinin proteins present in soybeans, which are involved in hypersensitivity reactions and promotion of intestinal villous atrophy by increasing the rate of mitosis and migration of enterocytes. Subsequently, there is a reduction in the number of mature villus enterocytes and thus, the digestive and absorptive capacity of the gut is reduced (Babot et al., 2016).

The lower ileum and duodenal VH of the SP100 group corroborate the results observed for some of the parameters of lipid metabolism, since a smaller absorptive capacity may have contributed to lower serum triglycerides and total cholesterol, and increased excretion of faecal lipids in this group.

Finally, we did not observe alteration in the biochemical markers, weight gain and food consumption. These results are justified since all groups received standard diets containing the same concentrations of carbohydrates, proteins, and lipids, unlike other studies in which hyperlipidic diets or soybean was offered in addition to diet (Andrade et al., 2013).

The positive effects found with the substitution of casein by whole soybean flour may be applicable for the modulation of CVD risk factors, since it would increase the number of compounds that may interfere with the morphology of the intestine and hepatic metabolism, with regards to oxidation of fatty acids and cholesterol clearance.

**Conclusion**

A 50% substitution of casein with soybean protein reduced lipid peroxidation and liver fat, and improved intestinal morphology, while a 100% substitution reduced cholesterol and triglyceride levels. Therefore, whole soybean, a source of vitamin E and isoflavone, is a functional food, which has cardioprotective effects and reduces cardiovascular disease risk associated with oxidative stress.
Acknowledgement

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