Letter

Effects of source of protein and supplementary extracted isoflavones and anthocyanins on longevity of Stroke-prone Spontaneously Hypertensive (SHRSP) rats

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ABSTRACT — Amount of dietary protein is known to influence blood pressure in humans and animal models. However, contradictory reports are available on the influence of source of dietary protein and soy isoflavones on blood pressure. Information on potential effect of anthocyanins, potent flavonoid antioxidants widely distributed in fruits and vegetables, on hypertension is also limited. Therefore, this study was conducted to examine whether source of dietary protein (casein vs. soybean protein, washed by alcohol to remove most isoflavones), dietary extracted isoflavones and anthocyanins modulate the lifespan of Stroke-prone Spontaneously Hypertensive (SHRSP) rats, one of the most suitable models for hemorrhagic stroke. Body weight and systolic blood-pressure matched groups of 47 day-old SHRSP rats (n = 16) received semi-purified diets containing 200 g/kg protein (casein or soybean) supplemented with 0 or 500 mg/kg isoflavones (NOVASOY™, a commercial soy isoflavones supplement extracted from soybean), and 0 or 500 mg/kg anthocyanins (extracted from elderberry). The drinking water contained 10 g/l sodium chloride to induce early hypertension. Survival times and survival rates of rats were determined. The survival rates were determined for each group and expressed as a percentage of the original number of rats still alive on a given day. The survival times and survival rates of animals fed casein and soybean protein diets were not different (P > 0.05). However, there was a significant effect of supplementation with isoflavones or anthocyanins on survival times and survival rates. Death occurred significantly earlier (P < 0.05) in the isoflavones- or anthocyanins-supplemented groups.

Key words: Extracted isoflavones, Anthocyanins, Rats, Hemorrhagic stroke, Survival time or rate

INTRODUCTION

Elevated blood pressure (a risk factor for stroke) is a common clinical observation in the United States, Canada and Europe. According to a recent survey, 24% of the American population (approximately 43 million people) have hypertension and only 47% have optimal blood pressure (systolic blood pressure, < 120 mm Hg, diastolic blood pressure, < 80 mm Hg (Burt et al., 1995). Similarly, 22% of Canadians (or 4.1 million people) have high blood pressure (Joffres et al., 1997). Efforts to reduce the prevalence of hypertension, an important goal for public health, have focussed on non-pharmacologic approaches that could produce favorable effects on blood pressure (Appel et al., 1997).

Amount of dietary protein is known to influence blood pressure (Obarzanek et al., 1996) and hemorrhagic strokes (Iso et al., 2001) in humans and animal models. Meta-analysis of data from observational studies suggested that a 5% increase in energy from protein might be associated with up to 2 mm Hg lower blood pressure (Obarzanek et al., 1996). Moreover, mean survival times of Stroke-prone Spontaneously Hypertensive (SHRSP) rats, one of the most suitable models for hemorrhagic stroke in humans, fed 10, 20 and 40% casein were 77, 96 and 99 days, respectively (Sarwar et al., 1999).

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Contradictory reports are, however, available on the influence of protein source (animal vs. vegetable) on blood pressure. Dietary intake of animal protein (rich in methionine) was inversely associated with blood pressure in three Chinese population samples (Zhou et al., 1994) and risk of hemorrhagic strokes in Japanese men living in Honolulu (Takeya et al., 1984). Low intake of animal protein was also associated with an increased risk of intraparenchymal hemorrhage (Iso et al., 2001), which may explain the high rate of this stroke in Asian countries. Similarly, SHRSP rats, fed a diet high in methionine (an amino acid found in abundance in animal proteins) had a delayed onset of hypertension and fewer strokes (Horie et al., 1987). However, in some clinical trials, the consumption of soy protein, when compared to casein protein, was reported to improve blood pressure in normotensive men and postmenopausal women (Teede et al., 2001). The potential protective effect of soy protein compared to casein protein observed in some clinical trials could be due to the presence of estrogenic isoflavones, higher level of hypotensive amino acids such as arginine or the presence of angiotensin converting enzyme (ACE) inhibitory peptides in soy protein.

Soy isoflavones, a subclass of phytoestrogens, are diphenolic compounds with structural similarities to natural and synthetic estrogens and antiestrogens. Soy isoflavones have gained increasing interest in recent years because of their potential health benefits in cases of certain cancers, cardiovascular disease, and alleviation of symptoms of menopause and bone loss in postmenopausal women (Setchell, 1998; Setchell and Cassidy, 1999). However, the consumption of extracted soy isoflavone supplements, in a randomized, double, blind, placebo controlled clinical trials, had no effect on blood pressure (Hodgson et al., 1999).

Considerable epidemiological evidence suggests a link between the consumption of diets rich in fruits and vegetables and a decreased risk of cardiovascular disease and cancers (Milbury et al., 2002). Anthocyanins, potent flavonoid antioxidants widely distributed in fruits and vegetables, have received attention as important dietary constituents that may provide potential health benefits such as antioxidant, anticarcinogenic, and antiinflammatory effects (Kay et al., 2004). A recent clinical study with 22 healthy volunteers and 25 patients with metabolic syndrome (MS) on the influence of anthocyanins from chokeberry on blood pressure, concentration of endothelin-1, serum lipids, fasting glucose, uric acid and membrane cholesterol in erythrocytes of patients with MS, concluded that anthocyanins may be of benefit to patients with MS as far as atherosclerosis prevention is concerned. The beneficial health effect appears to result from anthocyanins' influence on blood pressure; endothelin-1 level and serum lipid (Broncel et al., 2007). Further research using animal models, in which experimental conditions are highly controlled, is required to assess the potential influence of the source of protein (animal vs. vegetable) and associated isoflavones and anthocyanins on hypertension. Therefore, the effects of two protein sources (casein and alcohol-washed soy protein, devoid of isoflavones), supplementary extracted soy isoflavones and elderberry anthocyanins, on the lifespan of SHRSP rats were investigated in this study.

The SHRSP rats that exhibit nearly a 100% incidence of stroke, are a unique model in which environmental effects on stroke can be studied experimentally. This model was originally developed in Japan and the primary characteristics of SHRSP rats are rapid increase in blood pressure in the early stage followed by spontaneous development of both hemorrhagic and thrombotic strokes, mostly before the age of 10 months (Okamoto et al., 1974; Yamori, 1989). In this model, salt loading accelerates the development of stroke, whereas increases in the intakes of potassium, fiber, cholesterol and some fatty acids attenuate the deleterious effects of salt (Yamori, 1989; Yamori et al., 1984).

**MATERIALS AND METHODS**

**Diet ingredients and composition**

Alcohol-washed soy protein isolate (SPI, Pro fam 930, 90% protein) and NOVASOY™ soy isoflavone concentrate (source of supplementary isoflavones) were purchased from Archer Daniel Midland Company (Decatur, IL), while casein (90% protein) was purchased from ICN (Cleveland, OH, USA). The elderberry (Sambucus nigra) extract (no. 7412000, lot L98059) was purchased from Artemis International (Fort Wayne, IN, USA), and contained 4 major anthocyanins, cyanidin-3-glucoside, cyanidin 3-sambubioside, cyanidin 3-sambubioside-5-glucoside and cyanidin-3, 5-diglucoside.

The compositions of the six experimental diets (casein; casein + isoflavones, 500 mg/kg; casein + anthocyanins, 500 mg/kg; SPI; SPI + isoflavones, 500 mg/kg; and SPI + anthocyanins, 500 mg/kg) are shown in Table 1. All six experimental diets contained 20% protein (N X 6.25) either from casein or SPI; 10% soybean oil and required levels of minerals and vitamins for rat growth. The six experimental diets were isoenergetic, and the metabolizable energy per kg diet of the six diets was 16.48 MJ. Metabolizable energy was calculated using the Atwater factors of 17, 37 and 17 kJ/g for protein, fat and available carbohydrates, respectively.

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Table 1. Composition (g/kg) of six experimental diets fed to SHRSP rats

<table>
<thead>
<tr>
<th>Diet</th>
<th>Casein</th>
<th>SPI</th>
<th>NOVASOY&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Anthocyanins&lt;sup&gt;3&lt;/sup&gt;</th>
<th>Cornstarch</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>222.2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>477.3</td>
</tr>
<tr>
<td>Casein + isoflavones</td>
<td>222.2</td>
<td>-</td>
<td>1.7</td>
<td>-</td>
<td>475.6</td>
</tr>
<tr>
<td>Casein + anthocyanins</td>
<td>222.2</td>
<td>-</td>
<td>-</td>
<td>3.8</td>
<td>473.5</td>
</tr>
<tr>
<td>Soy protein isolate, SPI</td>
<td>-</td>
<td>222.2</td>
<td>-</td>
<td>-</td>
<td>477.3</td>
</tr>
<tr>
<td>SPI + isoflavones</td>
<td>-</td>
<td>222.2</td>
<td>1.7</td>
<td>-</td>
<td>475.6</td>
</tr>
<tr>
<td>SPI + anthocyanins</td>
<td>-</td>
<td>222.2</td>
<td>-</td>
<td>3.8</td>
<td>473.5</td>
</tr>
</tbody>
</table>

<sup>1</sup> Each diet also contained (g/kg): L-methionine, 3; sucrose, 100; AIN-93-G mineral mixture (Reeves et al., 1993), 35; AIN-93G vitamin mixture (Reeves et al., 1993), 10; choline bitartrate, (Sigma Chemical, St. Louis, MO, USA), 2; soybean oil, 100; and cellulose (Alfa-floc, Teklad Diet, Madison, WI, USA), 50.

<sup>2</sup> Addition of 1.7 g of NOVASOY™ (a commercial isoflavones supplement extracted from soybeans) provided 500 mg of isoflavones/kg diet. Total isoflavones in NOVASOY™ were determined in our laboratory (Wang and Murphy, 1994), and the analyzed value was used in formulating the NOVASOY™-supplemented diets.

<sup>3</sup> Addition of 3.8 g of anthocyanins (extracted from alderberry) provided 500 mg of anthocyanins/kg diet.

Animals

Young male SHRSP rats (age 29 d) obtained from the breeding colony of Health Canada, Ottawa were used. The animals, with initial body weight of 98 ± 5 g, were placed into six dietary groups of sixteen rats per group in a completely randomized design. The animals were housed individually in metal cages in a climate-controlled facility with a 12 hr d/12 hr night cycle (Gilani et al., 2006). The drinking water contained 10 g/l NaCl to induce early hypertension. Records of weekly body weight and food intake and lifespan were kept. To include body weights and food consumption data for all animals, the data were recorded after 31 days of feeding or at the age of 60 days, a time period was set well in advance of the start of death of animals on any treatment group that was based on our previous experience with SHRSP rats.

Rats were identified for potential termination if they showed clinical signs caused from severe stroke such as paralysis, tremor and spasms. Animals were slaughtered (deep inhalation anesthesia and bleeding) if there was no evidence for recovery or if they were in pain. Stroke was either the cause of death or the major contributory factor for euthanasia as confirmed by necropsy. The skull of SHRSP rats skull was carefully opened and stroke was diagnosed if intracranial haemorrhagic was present in the form of meningeal or intraventricular bleeding or blood clots. Canadian Council on Animal Care guidelines for care and use of animals were followed, and the experimental protocol was approved by the Animal Care Committee of Health Canada.

Statistical analysis

Effects of experimental treatments on food consumption, weight gain and survival time were investigated using a one-way ANOVA with the Statistical Systems for Personal Computers (SAS Institute, Cary, NC, USA). To compare survival curves for the effects of dietary treatments, analysis of survival rates was performed by using Wilcoxon's nonparametric test (Lawless, 2003). Post hoc comparisons of means were performed using Tukey's honest significant difference test (Steele and Torrie, 1980). Statistical significance was established at $P < 0.05$.

RESULTS

The data on weight gain and food consumed after 31 d of feeding, and mean survival time, are shown in Table 2. The experimental treatments had no significant effects on weight gain and food consumed but had significant effect on mean survival time. The mean survival times for SHRSP rats fed unsupplemented casein and SPI diets were not different, but the supplementation with isoflavones or anthocyanins of the casein or SPI diet resulted in significantly lower survival time of SHRSP rats.

The data on survival rates of rats fed the casein and SPI diets are shown in Figs. 1 and 2, respectively. Survival rate was determined for a group and expressed as a percentage of the number of animals still alive on a certain day to their original number. There was a significant effect of supplementary isoflavones or anthocyanins on survival rates of SHRSP rats. The death rates of rats fed casein and SPI diets were not different ($P > 0.05$). However, death occurred significantly earlier ($P < 0.05$) in the isoflavones- or anthocyanins- supplemented groups.
Table 2. Growth and mean survival time of SHRSP rats fed six experimental diets

<table>
<thead>
<tr>
<th>Diet</th>
<th>Weight gain (g/31 d)</th>
<th>Food consumed (g/31 d)</th>
<th>Mean survival time (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>141 ± 12</td>
<td>450 ± 30</td>
<td>97.4 ± 16.0a</td>
</tr>
<tr>
<td>Casein + isoflavones</td>
<td>131 ± 11</td>
<td>424 ± 24</td>
<td>85.9 ± 10.2c</td>
</tr>
<tr>
<td>Casein + anthocyanins</td>
<td>138 ± 12</td>
<td>430 ± 33</td>
<td>88.7 ± 13.2c</td>
</tr>
<tr>
<td>Soy protein, SPI</td>
<td>134 ± 16</td>
<td>424 ± 39</td>
<td>98.8 ± 18.2c</td>
</tr>
<tr>
<td>SPI + isoflavones</td>
<td>132 ± 10</td>
<td>418 ± 25</td>
<td>90.2 ± 13.9b</td>
</tr>
<tr>
<td>SPI + anthocyanins</td>
<td>135 ± 14</td>
<td>420 ± 36</td>
<td>90.4 ± 16.8b</td>
</tr>
</tbody>
</table>

* Data are means ± S.D. (n = 16). Values within a column with unlike superscript letters (a, b, c) were significantly different: p < 0.05.

**DISCUSSION**

All the casein and SPI diets were supplemented with the limiting amino acid (L-methionine), and the methionine-supplemented diets met or exceeded the indispensable amino acid requirements of rat growth (NRC, 1995) as reported previously (Gilani et al., 2006). Therefore, the source of protein (casein or SPI) had no effects on rat growth, and all the experimental diets supported adequate growth.

Dietary proteins (casein or SPI) tested in this study had no effect on survival times of SHRSP rats. The effects of the amount and source of dietary protein on blood pressure have been recently reviewed (Hodgson, 2007). According to this review, most studies consistently found that animal (including casein) and vegetable (including soy protein) proteins did not differ with respect to effects on blood pressure.

The addition of isoflavones or anthocyanins to the casein or SPI diets resulted in significantly shorter survival times in this study. Blood pressure was not measured in this study. However, based on our previous studies with SHRSP rats (Ratnayake et al., 2000a, 2000b), it could be suggested that isoflavones or anthocyanins may be shortening the lifespan of SHRSP rats without altering blood pressure and renal function. For example, vegeta-
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![Graph showing survival rate against age (Days)]

**Fig. 2.** Effect of soy protein isolate (SPI) diets on survival of SHRSP rats (each curve represents the proportion surviving to the indicated age in days; each step indicates the death of one or more rat).

Vegetable oils high in phytosterols resulted in lower cholesterol concentrations and made the cell membrane more rigid, which might be responsible for shortened life span of SHRSP rats fed diets containing these oils (Ratnayake et al., 2000b).

Like SHRSP rats, humans with a history of hemorrhagic stroke also have cell membrane abnormalities due to lower cholesterol concentrations in the cell membrane (Tsuda et al., 1992). Moreover, in prospective studies, hemorrhagic stroke has been found to occur at higher rates in persons with lower concentrations of blood cholesterol compared to those with higher blood cholesterol concentrations. This unique lipid-stroke relationship has been reported in Japanese populations (Ueda et al., 1998), in Japanese men living in Hawaii (Iribarren et al., 1996) and in Caucasian men and women in USA (Iribarren et al., 1995). The reasons behind these associations are not known. However, it is quite plausible that, as in SHRSP rats, the weakening of cell membrane due to low circulating cholesterol may influence arterionecrosis.

Blood cholesterol concentrations were not determined in the present study. However, a meta-analysis of eight randomized controlled trials revealed that with identical soy protein intake, high isoflavone intake led to significantly greater decreases in serum low density lipoprotein (LDL) cholesterol than low isoflavone intake; demonstrating that isoflavones have LDL cholesterol-lowering effects independent of soy protein (Zhuo et al., 2004). Therefore, it is quite possible that feeding isoflavone-supplemented diets in the current study may have resulted in lower blood cholesterol which could have an adverse effect on the longevity of SHRSP rats. Reports on the effects of anthocyanins (extracted from elderberry) on blood cholesterol are not available. However, like isoflavones, supplementary anthocyanins may have caused reduction in blood cholesterol and ensuing in a reduction of the lifespan of SHRSP rats.

In this study, the supplementary isoflavones and anthocyanins were added at the level of 500 mg/kg diet. That would translate to about 28 mg of isoflavones or anthocyanins per kg body weight of rats (assuming about 14 g of food intake per day in rats weighing about 250 g after 31 days). Although it would be rather difficult to obtain this level through consumption of conventional foods, it could be very easily obtained through the consumption of extracted supplements. Soy isoflavones are now marketed as powders, and tablets may soon become available over the counter. Although there is a long history of safe use of soy foods containing isoflavones and of fruits and vegetables containing anthocyanins, this conclusion may not apply to the use of supplements, especially in humans with a history of hemorrhagic strokes.
Therefore, further studies are required to determine the potential risk associated with consumption of soy isoflavones and anthocyanins concentrates.

ACKNOWLEDGMENTS

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