Oral toxicological studies of black soybean (Glycine max) hull extract: Acute studies in rats and mice, and chronic studies in mice

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Abstract

Black soybean (Glycine max) has been used for traditional medicine and food in Asian countries, but safety of its hull has not been studied. We conducted acute and chronic oral toxicity studies. For the acute study, an extract of black soybean hull (BE; 2.5 g/kg body weight) was administered singly by intragastric intubation to Sprague–Dawley rats and C57BL/6 mice. There was no death or significant decrease in body weight in rats and mice, and the oral LD₅₀ of BE was >2.5 g/kg body weight. In the chronic study, BE was administered at dietary levels of 0% (control), 2.0%, and 5.0% to male and female C57BL/6 mice for 26 weeks. No mortality or toxicologically significant clinical changes were observed through the experimental period. Although body weights, as well as abdominal fat, blood levels of triglyceride and total cholesterol in 5.0% males were significantly lower than that in control and 2.0% groups, these changes were considered not to be adverse. Hematological and histopathological observation revealed no toxicologically significant changes. The no-observed adverse-effect-level of BE was estimated to be 5.0% in the diet (5074.1 mg/kg body weight/day for males and 7617.9 mg/kg body weight/day for females).

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1. Introduction

Soybean (Glycine max) has been widely used as a traditional medicine and food in Asian countries. It has been reported that Asians consume 20–80 g of soy foods every day (Omoni and Aluko, 2005). Black soybean has been used as an herbal medicine to treat jaundice and edema; it has also been used to treat enuresis by affecting the functions of the kidney and spleen. The hull of the black soybean has been used for the treatment of vertigo and headache, as well as for detoxification and diuresis. Recently, the extract of the black soybean hull (BE) has been used as a dietary ingredient including nutraceuticals and pigments.

Abbreviations: ALB, albumin; ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate transaminase; BE, extract of black soybean hull; BW, body weight; BUN, blood urea nitrogen; CRE, creatinine; GLU, glucose; TG, triglycerides; CHL, total cholesterol; WBC, white blood cells; RBC, red blood cells; HGB, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; FLT, platelets; NEUT, neutrophils; LYMPH, lymphocytes; MONO, monocytes; EO, eosinophils; BASO, basocytes; NOAEL, no-observed adverse-effect-level.

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Soy foods contain various biologically active compounds. These include saponins, phytoestrogens, protease inhibitors, phenolic acids, lecithin, phytosterols, isoflavones, and omega-3 fatty acids (Omoni and Aluko, 2005). In addition to these compounds, black soybean has unique properties owing to its black hull (which is different from yellow soybean and green soybean). The black hull contains various polyphenols, such as anthocyanins, procyanidins, and catechins (Kanamoto et al., 2011). It was reported that dietary cyanidin 3-glucoside-rich purple corn color prevents high-fat diet-induced obesity in mice (Tsuda et al., 2003). It was also reported that propanidin from the chardonnay grape seed extract prevents high-fat diet-induced obesity in hamsters (Décordé et al., 2009). These reports suggest that BE rich in anthocyanins and procyanidins shows similar biological activities.

The toxicological effects of anthocyanins (Bentivegna and Whitney, 2002; Soulimani et al., 2001), procyanidins (Bentivegna and Whitney, 2002; Hanamura and Aoki, 2008; Ray et al., 2001a,b; Yamakoshi et al., 2002), and catechins (Chengelis et al., 2008; Fuji et al., 2007; Isbrucker et al., 2006a,b; Lambert et al., 2010) have been studied. However, the oral toxicological effects of BE have not been studied.

In the present study, acute and chronic oral toxicity studies of BE were conducted in rats and mice. The results of this study will
provide an important reference of BE for usage as a food supplement for humans.

2. Materials and methods

2.1. Test substance

BE (Choronos Care™) is manufactured commercially by Fujico Company, Limited (Kobe, Japan). It is produced as a dark red-purple powder. Briefly, black soybean hull was extracted with acidic water and ethanol, purified using absorbent resin, and powdered by spray drying. The total amount of polyphenols contained in BE was 67.0% (w/w). BE contained epicatechin (6.2%), cyanidin 3-glucoside (9.2%), and procyanidin (39.7%).

2.2. Animals

Animal experiments in the present study were approved by the Animal Care and Use Committee of Kobe University (Kobe, Japan). The permission numbers were 19-5-06 for rats and 19-5-32 for mice, and the experiments were carried out according to the Animal Experimentation Regulations of Kobe University. Nine male and nine female Sprague–Dawley (SD) rats (age, 6 weeks) as well as 42 male and 44 female C57BL/6 mice (age, 6 weeks) were purchased from Japan SLC (Shizuoka, Japan). They were acclimatized for 1 week before the start of experimentation. Animals were maintained at 22 ± 3°C on a 12-h light/dark cycle and allowed free access to purified water and a standard diet.

2.3. Acute studies

In acute studies, male and female SD rats and C57BL/6 mice were fasted for 12 h. They were administered a single oral dose of BE (2.5 g/kg body weight; n = 6) or purified water (5 mL/kg body weight, n = 3) by intragastric intubation. Rats and mice were observed for 14 and 15 days, respectively, for signs of morbidity or mortality, and body weights measured every day. On day 15 for rats and day 16 for mice, animals were killed by heart exsanguination under anesthesia, and green pathological examination undertaken. Blood samples were collected in heparinized tubes and plasma obtained by centrifugation at 860 × g for 10 min at 4°C. Plasma was subjected to clinical chemistry determinations: aspartate transaminase (AST), alanine transaminase (ALT), triglyceride (TG), glucose (GLU), total cholesterol (CHL), creatinine (CRE), albumin (ALB), and alkaline phosphatase (ALP). Livers, spleens, hearts, thymus glands, and kidneys were weighed.

2.4. Chronic studies

In chronic studies, 33 male and 35 female C57BL/6 mice were randomly divided into three groups and fed anAIN-93M-based diet for 26 weeks. BE was added to the diet at concentrations of 0% (control; n = 11 for males and females), 2.0% (n = 11 for males, n = 12 for females), or 5.0% (n = 11 for males, n = 12 for females). Mice were observed for signs of morbidity or mortality at least once a day during the experimental period, and individual body weights measured once a week. Replacement of diets and measurement of the consumption of food and water per cage were carried out three times a week. At week 26, abdominal fat was measured under anesthesia using an X-ray CT (LaiGer™, Hitachi-Aloca, Tokyo, Japan) according to the manufacturer’s protocol.

At the end of week 26, mice were killed by heart exsanguination under anesthesia, and gross pathological examination conducted. Blood samples were collected in ethylenediamine tetra-acetic acid (EDTA)-2K-treated and heparinized tubes, and measured according to the manufacturer’s instructions (Kainos Laboratories, Incorporated, Tokyo, Japan). Histopathological examinations were undertaken at the DIMS Institute of Medical Science. Histopathological examinations of the duodenum and liver in the 2.0% group of both sexes were also done because the changes caused by the test substance were observed in these organs in the 5.0% group of both sexes.

2.5. Experimental procedure

Levels of GLU, TG, CHL, ALB, BUN, CRE, ALT, and AST were measured using commercial assay kits according to manufacturers’ instructions (Glucose Clinitest™, Tri-glyceride E-test, Cholesterol E-test, Albumin B-test, Urea nitrogen B-test, Creatinine test, and Transaminase Clit-test; Wako Pure Chemicals, Osaka, Japan). ALP was measured using a commercial assay kit according to manufacturers’ instructions (Kainos Laboratories, Incorporated, Tokyo, Japan).

2.6. Statistical analyses

For data relating to body weight, organ weight, hematology, and clinical chemistry, values are the mean with the standard error of the mean (SE); statistical significance was determined using the Scheffe multiple comparison test with p < 0.05 considered significant. For the prevalence of histopathological lesions, statistically significant differences were determined by the one-sided Fisher’s exact test. For comparison of the degree of change, the two-sided Wilcoxon’s rank sum test was employed. The levels of significance were set at p < 0.05 and p < 0.01.

3. Results

3.1. Acute studies

3.1.1. Necropsy, body weights, and organ weights

No death or significant decrease in body weight was observed during the experimental period in rats and mice (data not shown). The oral median lethal dose (LD50) of BE in SD rats and C57BL/6 mice was >2.5 g/kg body weight. Black feces due to BE pigments were observed in rats and mice 2 days after oral administration of BE (data not shown). Necropsy and organ weights at the end of the experiment did not reveal gross pathological abnormalities in rats and mice (data not shown).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>BE</td>
<td>Control</td>
</tr>
<tr>
<td>AST (IU/L)</td>
<td>65 ± 7.4</td>
<td>47 ± 4.7h</td>
</tr>
<tr>
<td>ALT (IU/L)</td>
<td>16 ± 0.9</td>
<td>16 ± 0.9</td>
</tr>
<tr>
<td>TG (mg/dL)</td>
<td>178 ± 12</td>
<td>159 ± 13</td>
</tr>
<tr>
<td>GLU (mg/dL)</td>
<td>216 ± 80</td>
<td>200 ± 3.4</td>
</tr>
<tr>
<td>CHL (mg/dL)</td>
<td>48 ± 0.8</td>
<td>49 ± 3.9</td>
</tr>
<tr>
<td>CRE (mg/dL)</td>
<td>0.68 ± 0.02</td>
<td>0.65 ± 0.02</td>
</tr>
<tr>
<td>ALB (g/dL)</td>
<td>6.8 ± 0.0</td>
<td>6.9 ± 0.1</td>
</tr>
<tr>
<td>ALP (IU/L)</td>
<td>214 ± 33</td>
<td>392 ± 80</td>
</tr>
</tbody>
</table>

Values are mean ± SE. Values with different superscripts in each sex indicate a significant difference (p < 0.05).

3.2. Chronic studies

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>BE</td>
<td>Control</td>
</tr>
<tr>
<td>AST (IU/L)</td>
<td>48 ± 7.3</td>
<td>39 ± 3.3</td>
</tr>
<tr>
<td>ALT (IU/L)</td>
<td>18 ± 0.2</td>
<td>19 ± 1.7</td>
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<tr>
<td>TG (mg/dL)</td>
<td>83 ± 17h</td>
<td>141 ± 13h</td>
</tr>
<tr>
<td>GLU (mg/dL)</td>
<td>268 ± 22</td>
<td>232 ± 7.1</td>
</tr>
<tr>
<td>CHL (mg/dL)</td>
<td>71 ± 5.7</td>
<td>70 ± 3.7</td>
</tr>
<tr>
<td>CRE (mg/dL)</td>
<td>0.58 ± 0.05</td>
<td>0.55 ± 0.06</td>
</tr>
<tr>
<td>ALB (g/dL)</td>
<td>6.4 ± 0.23</td>
<td>6.0 ± 0.14</td>
</tr>
<tr>
<td>ALP (IU/L)</td>
<td>479 ± 8.6</td>
<td>497 ± 20</td>
</tr>
</tbody>
</table>

Values are mean ± SE. Values with different superscripts in each sex indicate a significant difference (p < 0.05).
3.1.2. Clinical chemistry

Significant decreases in the levels of AST in BE male rats (Table 1) and increase in TG levels in BE male mice (Table 2) were observed. However, these changes were considered not to be treatment-related significant adverse effects. No statistically significant changes were observed in the levels of ALT, ALP, TG, ALB, GLU, and CRE.

3.2. Chronic studies

3.2.1. Clinical observations and body weights

Death or abnormal physiological signs were not observed during the experimental period in mice. However, a significant reduction in the body weights of 5.0% males from week 4 and week 16 compared with 2.0% males and control males, respectively, to the end of the experiment was observed (Fig. 1). The body weights of 5.0% females were also reduced, but these changes were not continuous. At week 26, abdominal fat was measured using an X-ray CT (Table 3): the abdominal fat of 5.0% males was 40% lower than that of controls. There was no change in the abdominal fat of females. These results indicated that reduction in the body weights of 5.0% males was not due to the adverse effects of BE, but that BE suppressed accumulation of abdominal fat. Black feces and red-purple urine due to BE pigments were observed in 5.0% males and 5.0% females.

3.2.2. Consumption of food and water

The consumption of food and water of mice fed a diet containing BE was similar to that of controls throughout the experimental period (Table 4). Despite the decrease in body weights, 5.0% males consumed a similar amount of food compared with the controls. Similarly, the food consumption of females was similar to that of males even though the body weights of females were lower than those of males. There was no treatment-related significant adverse effect of BE on the consumption of food and water. The mean intake of BE for males and females was 1468.9 and 2621.0 mg/kg body weight/day, respectively, in the 2.0% group, and 5074.1 and 7617.9 mg/kg body weight/day, respectively, in the 5.0% group.

3.2.3. Hematology and clinical chemistry

The hematological parameters for mice are summarized in Table 5. The WBC in 5.0% males and RBC, HGB, and the HCT in 5.0% females were significantly increased as compared with controls. The MCH was decreased in 5.0% females. With respect to blood chemistry, levels of TG and CHL in 5.0% males were significantly decreased (Table 6). In females, TG levels and BUN were decreased in the 5.0% group. No statistically significant change was observed in the other parameters examined.

3.2.4. Organ weights

The final body weight of 5.0% males was significantly lower than that of controls and 2.0% males (Table 7). Relative weights...
of the spleen, brain, and kidney were increased in 5.0% males, whereas absolute weights of the liver were decreased in 5.0% males. In females, there was no significant change in final body weights, but relative weights of the spleen in the 5.0% group were increased. Absolute weights of heart, liver, and kidney were decreased, but that of spleen was increased in 5.0% females.

3.2.5. Necropsy/histopathology

Histopathological findings in each group are summarized in Table 8. In the duodenum, slight pigment accumulation in histiocytes of the lamina propria was found in all 2.0% and 5.0% males and females, and the change was statistically significant (Fig. 2 and Table 8). In livers, slight accumulation of pigment in Kupffer cells was found in all 5.0% males and females, and minimal accumulation of pigment in all 2.0% males and females was seen, and both were statistically significant (Fig. 3 and Table 8). Minimal or slight hepatocellular vacuolation was found in several 5.0% males, whereas minimal to marked hepatocellular vacuolation was found in control males, and the grade of lesion was significantly decreased by BE treatment. The prevalence of other
4. Discussion

BE has recently become commercially available as a dietary ingredient, but a safety assessment of BE has not been completed. In acute studies, there was no change in body weights, organ weights, and gross pathological abnormalities in both rats and mice. However, AST levels were lower in BE male rats, and TG was higher in BE male mice than that in the controls. There was no chemical parameter-related pathological change, so these changes were not considered to be toxicologically significant. From acute studies, the oral LD50 of BE in SD rats and C57BL/6 mice was >2.5 g/kg body weight.

In chronic studies, body weights in 5.0% males decreased significantly as compared with control and 2.0% groups. In addition to the decrease in body weights, abdominal fat, and blood levels of TG and CHL in 5.0% groups were significantly lower than those in the control and 2.0% groups. It was demonstrated that dietary cyanidin 3-glucoside-rich purple corn color prevents high-fat diet-induced obesity in mice (Tsuda et al., 2003). Procyanidin from the chardonnay grape seed extract was reported to prevent high-fat diet-induced obesity in hamsters (Décordé et al., 2009). BE used in the present study contained polyphenols, including cyanidin 3-glucoside and procyanidin. The above-mentioned reports suggest that the polyphenols contained in BE prevent spontaneous obesity, but do not show adverse effects (including growth arrest) in male mice. Indeed, the abdominal fat in 5.0% males was identical to that in females, which showed a moderate increase in body weights.

With respect to hematological parameters, RBC, HGB, and the HCT increased significantly in 5.0% females, but MCHC (which is commonly used for the diagnosis of erythron disease) was not altered by BE treatment. The WBC in 5.0% males was twice as high as that in controls, but this change was not considered to be abnormal. The relative weights of hearts, livers, and spleens in 5.0% males and the relative weights of hearts and spleens in 5.0% females were significantly altered as compared with control groups. However, histopathological findings in these organs did not change, and clinical parameters including AST and ALT, which related to liver injury, did not change.

We found slight accumulation of pigments in histiocytes of the lamina propria in the duodenum and minimal accumulation of pigments in Kupffer cells in livers in all 2.0% and 5.0% males and females. The contents of the duodenum were tinged with black in 2.0% and 5.0% groups: this change was considered to be a
treatment-related effect. It has been suggested that histiocytes “englobe” pigments as xenobiotics, and then the pigments accumulate in histiocytes. Such pigment accumulation in histiocytes was not observed in stomachs, esophagus’ or large intestines, indicating that BE pigments were absorbed mainly through the duodenum. Xenobiotics that contain pigments are metabolized in the liver. Similar to pigment accumulation in histiocytes, the pigments were englobed and accumulated in Kupffer cells in the liver. The prevalence of hepatic vacuolation in 5.0% males was significantly lower than that in controls, but the same phenomenon seen in males was not observed in females. This result indicated that the effects of BE on hepatic vacuolation differ in males and females. In the present study, we performed the chronic study using mice for 26 weeks, but rats are usually more sensitive than mice. Therefore, sub-chronic study using rats might be needed in the future.

In conclusion, the no-observed adverse-effect-level (NOAEL) of BE was found to be 5074.1 mg/kg body weight/day for males and 7617.9 mg/kg body weight/day for females in mice.

Conflict of Interest

The authors declare that there are no conflicts of interest.
Acknowledgment

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References


