Ameliorative Effects of Soy 11S Protein on Liver Damage and Hyperlipidemia in Alcohol-Fed Rats

Kap Joo Park, a Hye Yun Kim, a Byung Joon Chang, b and Hyung Hoan Lee* a

a Department of Biological Sciences, Konkuk University; and b Department of Veterinary Medicine, Konkuk University; Seoul 143–701, Korea. Received March 10, 2004; accepted June 28, 2004

This study sought to investigate the ameliorating effects of soy 11S protein on the impacts of alcohol consumption in rat hepatocytes and in reducing total cholesterol levels and total lipid levels in the serum. Liver histology and the clinically important enzyme markers (Aspartate Aminotransferase: AST and Alanine Aminotransferase: ALT) of rats, administered with both alcohol and soy 11S protein treatments, were compared with those in the control group. The treatment regimen (11S soy protein extract) significantly reduced serum ALT and AST levels, indicating the hepato-protective effects of soy 11S protein. Furthermore, total cholesterol and total lipid levels were significantly reduced. In addition to preventing the presence of lipid droplets and secondary lysosomes, electron microscopy indicated that the administration of the soy 11S protein treatment preserved important hepatocyte structures. These results indicate that soy 11S protein can positively mediate the effects of alcohol on hepatocytes and general liver functions.

Key words 11S protein; soybean; rat hepatocytes; alcohol

Alcoholism is a social and economic problem that is global in scope. Alcohol is the most frequently abused drug throughout the world and has a long history of use. Alcoholism can also be considered one of the costliest diseases of the modern era in terms of life years lost,1) even costlier than tobacco use. Alcohol-laden blood then travels to the liver via the veins and capillaries of the digestive tract, which affects nearly every liver cell. The liver cells are the only cells in the body that can produce sufficient amounts of the enzyme alcohol dehydrogenase to oxidize alcohol at an appreciable rate.2) The impairment of bodily functions and the damage caused by the consumption of alcohol works mainly in two ways: 1) indirectly, by interfering with the body’s normal processing of food, thereby causing malnutrition; and 2) through direct toxic effects causing organ pathology, the effects of which focus particularly upon the liver.3) The liver is the body’s largest internal organ. The functions of the liver (filtration of circulating blood, removal and breakdown of toxic substances) are essential to life and play a critical role in the body’s metabolic processes.4) Ameliorating the effects of long-term alcohol consumption has long been a focus for many researchers and clinicians. There have been various efforts to develop compounds to ameliorate or treat alcohol-related pathology5–7) however, these chemically derived compounds can have harmful and unforeseen side effects. Therefore there has been a focus on natural or herbal treatments for alcohol-induced diseases. There are a number of studies that have researched the nutritional and physiologic benefits of the soybean. Soybeans are a good source of carbohydrates, fat, protein, fiber, and calcium, making them a nutritionally valuable food source. Recently, the FDA has accepted the health claim that soy protein helps to reduce blood cholesterol levels, further highlighting the importance of the soybean. The nutritional, economic, and health benefits of this legume make it an important focus for future research. Besides the general positive nutritional factors, various compounds of the soybean are being researched for their beneficial effects. Those most relevant to this study are the lowering of serum cholesterol levels, protection of hepatocyte membranes and structures, amelioration of nutritional deficiencies (focused in the liver), and the prevention and/or breakdown of hepatic fibrosis. A meta-analysis of 38 studies on human subjects confirmed this line of thought. Lovati found that the ingestion of diets containing the soy protein was associated with significant reductions in serum concentrations of total cholesterol, low-density lipoprotein (LDL), and triglycerides, accompanied by a non-significant increase in serum high-density lipoprotein (HDL) concentrations.8) Dietary supplementation with soy phospholipids may also help patients with liver disease, alcoholism, or chronic enteral nutrition to reduce their risk of linoleic acid (LA) deficiency. Phospholipids mostly contain LA, a fatty acid essential in cell membrane formation.9) When liver function is compromised, LA is deficient. Soy phospholipid 73.9% (3-sn-phosphatidyl) choline products in addition are reported to reduce symptoms of liver disease, chronic hepatitis, or liver dysfunction due to malnutrition, such as the loss of appetite and abdominal pain.10) Soy-based compounds can thus potentially confer many beneficial health effects and even attenuate the damage induced by long-term alcohol consumption. Now we are planing to develope a new functional 11S protein tofu, which was made using the 11S protein of Korean soybean and a new tofu congelator. We clearly need to identify the ameliorating effects of Korean soybean 11S protein on cholesterolomla. This study seeks to investigate the potential positive effects of the 11S storage protein (a seldom used portion of the soybean in commercial contexts).

MATERIALS AND METHODS

Preparation of Soy 11S Protein 0.01 M Tris–HCl buffer (pH 8.0) and soybeans (Dr. Chung’s Food. Co. Ltd., Korea) were mixed at a 10 : 1 ratio. The 10 : 1 ratio soybean mixture was precipitated, keeping the solution at a low temperature (4 °C). When the mixture precipitated, the sediment was washed with a magnetic bar for 1 h and centrifuged at 6000 rpm for 30 min. After centrifugation, the sediment was discarded and the supernatant was adjusted to pH 6.4 and kept at 0–6 °C for a period longer than 3 h and less than 24 h. Then the supernatant was centrifuged at 10000 g for...
30 min at 6°C. The supernatant was discarded, and dialysis was performed, adjusting the pH between 7.5 and 8 by adding distilled water to the sediment. After dialysis, the solution was quantitatively analyzed using the Biuret method. Segregated samples were used after storing at 4 °C.9)

Young adult male Sprague-Dawley rats weighing 200 ± 10 g were obtained from Daehan Biolink Co., Ltd. (Seoul, Korea). All rats were kept under constant laboratory conditions (temperature, 22 ± 2 °C; relative humidity, 45 ± 5%; 12-h light/dark cycle). The subjects were fed a diet (Samyang Assorted Diet produced by Samyang Co., Ltd., Korea) and were allowed free access to drinking water (distilled).

After a 4-week adaptation period, 24 rats (2 per cage) were then randomly assigned to four groups (n=6) as follows: normal controls were given water; negative controls were given ethanol/water; positive controls were given ethanol/water and a solution of liver disease cure solution (LCS) (Alcodex: Guju Pharmaceutical Co., Ltd., Seoul) and blood circulation promotion solution (BPS) (Vasoclean: Cho-A Pharmaceutical Co., Ltd., Seoul), both commercially available; and rats in the experimental treatment group were given ethanol/water and 11S protein solution. All groups of rats were treated with these various regimens for 6 weeks (Table 1).

Ethanol/water intake of 5 g/kg/d was achieved. After 4 weeks, the rats weighed an average of 330 g. Either a treatment solution or a placebo (distilled water for the normal control group, alcohol and distilled water for the negative control group) were orally administered (syringe-fed) to all rats daily for 6 weeks.

The syringe feedings were performed at about the same time everyday. Food consumption and water (or alcohol and water solution) measurements were taken daily. Body weight measurements were made weekly.

**Diet and Treatment Composition.**

(a) **Positive Control Group**
The positive control group solution was a mixture of a commercially available solution from Guju Pharmaceutical Co., Ltd., Seoul (Alcodex), referred to as LCS for the purposes of this experiment, and Vasoclean another commercially available solution from Cho-A Pharmaceutical Co., Ltd., Seoul, referred to as BPS. The ingredients of LCS (per 30 ml) as on the label are 2.193 g of l-arginine (U.S.P), 0.882 g of citric acid, betaine HCl, and betaine. The ingredients (per 60 ml) of BPS on the label are 120 mg of ginkgo biloba extract and 60 mg of sodium benzoate. The dosage administered was derived from the amount that is commercially recommended for humans (30 ml/60 kg of LCS + 60 ml/60 kg of BPS), and a ratio was found from the mean rat weight to calculate the proportionate dosage.

(b) **Experimental Treatment Group**
The 11S protein solution was prepared as explained above. The dosage administered was derived from the recommended daily protein allowance for a 60-kg Korean adult male, which is 1 g/kg/d (a standard value set by the Korea Dietitian Association, 2000). Because all rats were weighed an average of 330 g after a 4-week adaptation period, this standard value was chosen for the protein treatment dose (0.33 g/d). An amount of the 11S protein solution proportionate to this ratio was orally administered daily at the same time. The above ratio was used along with the average rat weight (average of the group, measured from the previous week) to calculate the proportionate dosage.

**Measurements and Post experimental Analysis.**

(a) **Body Weight, Food, and Water (or Alcohol and Water Solution) Measurements**
The body weights of the rats were measured every week: at the beginning of the first week, at the beginning of the adaptation period, to week 11, and at the end of the experimental period. Food consumption was measured every day at the same time. Water (or alcohol and water, depending on the experimental group) intake was also measured daily at about the same time.

(b) **Blood Samples and Serum Preparation**
At the end of the last day of the experiment, the rats were fasted for 14 h (overnight). The rats were then lightly anesthetized with ethyl ether and were left at room temperature for 30 min. Then more than 3 ml of blood was extracted from the inferior vena cava of each of the anesthetized rats using a syringe and a test tube (not treated with heparin). The serum was separated from the extracted blood by centrifuging at 3000 rpm and at 4°C for 15 min. The AST, ALT, total cholesterol, and total lipid levels were determined in the serum.

(c) **Biochemical Tests (AST, ALT, Total Cholesterol, and Total Lipids)**
To obtain the activity value measurement of the serum, an AST kit (Boehringer Mannheim, Germany) was used. The extinction value was measured at a wavelength of 340 nm by an automatic biochemical analysis machine (Hitachi 747, Japan). To obtain the activity value measurement of the serum, an ALT kit (Boehringer Mannheim, Germany) was used. The extinction value was measured at a wavelength of 340 nm by an automatic biochemical analysis machine (Hitachi 747, Japan). Total cholesterol was measured using a colorimetric test. The total cholesterol value was measured using the R reagent (YD diagnostics Co., Ltd., Korea) and an automatic biochemical analysis machine (Hitachi 747, Japan). The measurement of the total lipid value was obtained using sulfo-phosphovanillin. Sulfuric acid was added to the serum, heated, reacted with a mixture of phosphoric acid and vanillin, which subsequently reacted to form a pink band. This band was measured at 540 nm and it’s concentration was determined using a total lipid reagent kit (Medicos, U.S.A.) on an automatic biochemical analytical machine (Hitachi 747, Japan).

(d) **Liver Weight Measurements and Histologic Examination**
After blood samples were obtained, we removed the rat livers and rinsed the organs in cold physiologic salt solution. Once all excess surface moisture was wiped away, the weight of the livers were measured. Liver tissue samples (1×1×1 cm) were removed and prefixed for 2 h at 4°C in 4% paraformaldehyde solution with 0.1 M phosphate buffer (pH 7.3) and 2.5% glutaraldehyde. The tissue samples were
Table 2. Mean Changes in Body Weight, Average Weekly Dietary Intake, and Liver Weight as a Percentage of Total Body Weight

<table>
<thead>
<tr>
<th>Group</th>
<th>Body weight (g)</th>
<th>Dietary intake in 7 weeks (g)</th>
<th>Liver (% of body weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>121.55±16.292*</td>
<td>44.09±2.140***</td>
<td>2.900±0.1244***</td>
</tr>
<tr>
<td>Negative control</td>
<td>108.60±9.334</td>
<td>37.71±0.639</td>
<td>3.105±0.0749</td>
</tr>
<tr>
<td>Positive control</td>
<td>115.23±7.866*</td>
<td>36.76±0.586***</td>
<td>2.921±0.2185*</td>
</tr>
<tr>
<td>Experimental treatment</td>
<td>115.12±12.905*</td>
<td>36.97±0.645**</td>
<td>2.970±0.2150*</td>
</tr>
</tbody>
</table>

* Significantly different from the negative control group (\(p<0.5\), **\(p<0.1\), ***\(p<0.05\)) by Student's \(t\)-test.11)

Table 3. Mean Serum Levels of AST, ALT, Total Cholesterol, and Total Lipids in Alcohol-fed Rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>AST (U/l)</th>
<th>ALT (U/l)</th>
<th>Total cholesterol (mg/l)</th>
<th>Total lipids (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>71.67±6.593***</td>
<td>45.50±5.206*</td>
<td>88.00±11.331**</td>
<td>331.33±7.873***</td>
</tr>
<tr>
<td>Negative control</td>
<td>87.83±10.741</td>
<td>94.83±13.197</td>
<td>114.67±21.210</td>
<td>392.67±23.534</td>
</tr>
<tr>
<td>Positive control</td>
<td>71.00±4.940***</td>
<td>43.67±7.711*</td>
<td>92.17±9.432***</td>
<td>347.17±39.398***</td>
</tr>
<tr>
<td>Experimental treatment</td>
<td>68.67±10.930***</td>
<td>42.67±9.522*</td>
<td>94.83±9.368**</td>
<td>342.83±43.605***</td>
</tr>
</tbody>
</table>

*,# Significantly different from the negative control group (\(p<0.5\), **\(p<0.1\), ***\(p<0.05\), #\(p<0.001\)) by Student's \(t\)-test.11)

RESULTS

Changes in Body Weight Table 2 shows changes in rat weights after the administration of alcohol for 6 weeks. Increases in body weight were seen over the total course of the experiment. Alcohol consumption for a prolonged period (6 weeks) stunted the normal growth of the rats, as evidenced by a minimal gain in body weight of the negative control group in comparison with the other experimental groups. The final change in body weight of the normal control group was 121.55±16.292 g and of the negative control group was 108.60±9.334 g. 115.25±7.866 g weight gain was observed in the positive control group, while the experimental treatment group was seen to have gained 115.12±12.905 g. These results are strengthened by the fact that all experimental groups, with the exception of the normal control group, consumed roughly the same amount of food, as shown in Table 2 (average weekly dietary intake). The negative control group, on average, consumed 37.71±0.639 g while the positive control group consumed an average of 36.76±0.586 g of food per week. The experimental treatment group consumed 36.97±0.645 g of food respectively. The only exception was the normal control group, whose average weekly dietary intake was 44.09±2.140 g (this may be evidence of the addictive properties of alcohol, since alcohol replaced food in the diet). These results show that weight gain, or the lack of it in the case of negative controls, cannot be attributed to the differences in the amount of food consumed.

Weight Ratio of the Liver Table 2 shows liver weights as a percentage of the rat's total body weight. The liver weight percentage in the experimental treatment group was 2.970±0.2150%. This result seemed to be significantly \((p<0.5)\) close to the results \((2.900±0.1244\%)\) in the normal control group. The results for the negative control group were found to be higher than the others.

Serum Levels of AST and ALT. (a) Serum Level of AST Table 3 shows the AST levels in the serum. As evidenced by the data, AST levels in the serum of the normal control group was 71.67±6.593 U/l, significantly lower \((p<0.05)\) than the negative control group’s serum level of 87.83±10.741 U/l. This elevated AST level can be attributed to the liver damage induced by long-term alcohol consumption. The AST levels of experimental group was found to be 68.67±10.930 U/l, also significantly lower in comparison to the negative control group \((p<0.05)\) and similar to the normal control group.

(b) Serum Level of ALT Table 3 shows the ALT levels in the serum. ALT levels in the serum of the normal control group was 45.50±5.206 U/l, significantly lower \((p<0.0001)\) than the negative control group’s level of 94.83±13.197 U/l. These results confirm the extent of hepatic damage the negative control group sustained due to long-term alcohol consumption. ALT levels of experimental treatment group \((42.67±9.522 U/l)\) was lower than the negative control group \((p<0.0001)\) and was similar in comparison to the normal control group.

(c) Serum Levels of Total Cholesterol and Total Lipids Table 3 shows levels of total cholesterol found in the serum. The treatment groups had levels similar to the normal controls. In comparing the normal control \((88.00±11.331 mg/dl)\) and experimental treatment group \((94.83±9.368 mg/dl)\) to the negative control group \((114.67±21.210 mg/dl)\), significant differences \((p<0.05)\) were found in the levels of serum cholesterol, indicating that the treatments were effective in ameliorating the effects of alcohol consumption. This finding parallels the findings of other studies in which soy proteins lowered serum cholesterol levels.11,12,15

Table 3 shows the levels of total lipids found in different experimental groups. The normal controls had a mean total lipid level of 331.33±47.873 mg/dl, while the negative controls were measured at 392.67±23.534 mg/dl. The experimental treatment group \((342.83±43.605 mg/dl)\) had a signifi-
cantly lower level than the negative controls ($p < 0.05$) in total lipid levels.

**Histologic Studies.**

**(a) Light Micrographs of Hepatic Tissue**

Figure 1 shows the light micrographs of hepatocytes from the experimental groups. Columns A, B1, C, and D in Fig. 1 show the central and portal areas of normal control group, negative control group, positive control group and experimental treatment group (A, B1, C and D, ×100), and a high magnification of the central vein area of negative control group (B2—B3, ×400) respectively.

Figure 1A shows the normal hepatic tissue from the normal control group (cv, central vein; pv, portal vein). Figure 1B (B1—B3) shows the hepatic tissues from the negative control group with evidence of liver damage from long-term alcohol consumption and degeneration of hepatocytes (B2 and B3, open arrow), inflammatory cell (B2, arrowhead), mallory body (B3, closed arrow), and the dilatation of the sinusoids (B2 and B3, asterisks). A few eosinophilic Mallory bodies (B3, closed arrows) and accumulation of small lipid droplets can be seen. Figure 1C shows hepatic tissues of the positive control group which has morphology similar to that of the normal control group except for the infiltration of inflammatory cells (arrowhead) near the portal area. Figure 1D shows hepatic tissues of the experimental treatment group which has morphology similar to that of the normal control group.

**(b) Electron Micrographs of Hepatocytes**

Figure 2 shows the electron micrographs ($×15000$) of hepatocytes from the experimental groups (N, nucleus of hepatocyte; M, mitochondria; R, rough endoplasmic reticulum; L, lipid droplet; BC, bile canaliculus). The normal control group, Fig. 2A, exhibits normal hepatocyte morphology. Figures 2B, 2C and 2D are electron micrographs of the negative control group. Hepatocytes in Fig. 2B show degenerative signs (asterisks). The hepatocytes also exhibit a diffuse accumulation of lipid droplets (L) in the cytoplasm. Figure 2D shows the increase of collagen in the hepatic lobule. Figure 2E is electron micrograph of the positive control group, which exhibit normal hepatocyte structures. Figure 2F is the electron micrograph of the experimental treatment group. It shows the normal morphology of hepatocytes (as in the normal control group) with none of the degenerative signs exhibited in the negative control group.

**DISCUSSION**

As seen in the data, alcohol consumption for a prolonged period (6 weeks), stunted the normal growth of the subjects, as evidenced by the minimal gain in body weight of the negative control group in comparison with the other experimental groups. The experimental treatment group had statistically significant ($p < 0.05$) gains in weight (in comparison to the negative controls). Furthermore, these gains were higher than the other alcohol-treated groups. These results are significant by the fact that all experimental groups, with the exception of the normal control group, consumed roughly the same amount of food, as shown in Table 2 (average weekly dietary intake). These results show that the weight gain or lack of it in the case of the negative controls cannot be attributed to the differences in the amount of food consumed.

Due to the accumulation of lipids with long-term alcohol consumption, hypertrophy of the liver can be seen as an indicator of fatty liver and liver fibrosis.15) The results of our study are inclined to agree with this finding. Lee16) showed that liver’s weight as a percentage of the total body weight in subjects with alcohol-induced liver damage, were higher than in subjects that obtained from a normal or a treatment. The group that consumed a treatment solution had a lower liver weight to body weight ratio in comparison with the alcohol-only group: this finding was also paralleled in Lee’s research.16) Fatty liver is the first stage of liver deterioration in heavy drinkers and interferes with the distribution of oxygen and nutrients to the liver cells.17) Alcohol also directly affects lipocytes in the liver, causing the deposition of collagen, a characteristic protein of the fibrous tissues. Long-term alcohol consumption transforms lipocytes into collagen-pro-
ducing myofibroblast-like cells.\textsuperscript{18,19} The light micrographs of the negative controls show the degeneration of hepatocytes, as evidenced by the dilatation of the sinusoids, presence of eosinophilic Mallory bodies, accumulation of inflammatory cells, and accumulation of small lipid droplets. Mallory bodies are characteristic cytoplasmic hyaline inclusions in hepatocytes reflecting a morphologic manifestation of chronic liver cell injury.\textsuperscript{20} These findings are typical of alcohol-induced liver damage. On the other hand, the hepatic tissue of the experimental treatment group exhibited morphology very similar to that of the normal control group, which is physical evidence of the hepatoprotective effects of soybean 11S protein solution. Further physical evidence can be found in the electron micrographs of the negative control group, which show degenerative signs, such as, clear areas and dilated biliary canaliculi without the microvilli. In addition, the hepatocytes exhibit a diffuse accumulation of lipid droplets in the cytoplasm and the electron micrographs show an increase in collagen in the hepatic lobule. The experimental treatment group showed the normal morphology of hepatocytes (as in the normal control group) with none of the degenerative signs exhibited in the negative control group. The histologic studies showed that the 11S protein solution exhibits hepatoprotective effects, ameliorating the impact of long-term alcohol consumption. The degradation of normal liver functions is indicated by the increased levels of serum AST and ALT enzymes that are normally concentrated in the hepatic tissue. Increased levels of AST and ALT enzymes in the serum can be caused by fatty liver or exposure to by-products, resulting in metabolic problems of the liver and hepatocyte death. The elevated AST level in the negative control group can be attributed to the liver damage induced by long-term alcohol consumption, confirming previous research.\textsuperscript{16} The serum AST levels of the experimental group were significantly lower in comparison with those in the negative control group and similar to those in the normal control group, indicating the hepatoprotective effects of soy 11S protein. Serum ALT levels of the experimental treatment group were significantly lower than those in the negative control group and were similar to those in the normal control group. These results may also confirm the extent of hepatic damage the negative control group sustained due to long-term alcohol consumption.

Fig. 2. Electron Micrographs of Hepatocytes
(A) Hepatocytes from the normal control group. (B, C, D) Hepatocytes from the negative control group (fed alcohol and water only). B. Hepatocytes show degenerative signs (asterisks) and show increased collagen fibrils in the hepatic lobule. C. Hepatocytes exhibit diffuse accumulation of lipid droplets in the cytoplasm. D. Bile canaliculi (BS) show dilated morphology with short microvilli. (E) Hepatocytes from the positive control group, which was treated with alcohol, LCS, and BPS. Hepatocytes show normal morphology. (F) Hepatocytes from the experimental treatment group. Hepatocytes show normal morphology. M, mitochondria; N, nucleus of the hepatocyte; R, rough endoplasmic reticulum; L, lipid droplet; BC, bile canaliculus; asterisk, hydropic degenerative sign of hepatocyte. Scale bar = 1 µm; magnification ×15000.
With regard to the serum total cholesterol level, comparing the normal control group and to the treatment and negative control groups, significant differences were found in levels of serum cholesterol, indicating that the treatment was effective in ameliorating the effects of alcohol consumption. This finding parallels the findings of other studies in which it was found that soy proteins lowered the serum cholesterol levels of the subjects.\textsuperscript{12,13,21,22} With regards to the total lipid level, the experimental treatment group was found to be significantly different from the negative controls. This finding also confirms the results of Potter,\textsuperscript{5} Lovati \textit{et al.},\textsuperscript{3} and Baum\textsuperscript{23} and this result is significant since high levels of cholesterol are major causal factors in the development of atherosclerosis and subsequent cardiovascular disease.

This research highlights the various potential benefits (protective effect against hypercholesterolemia with long-term alcohol consumption) of 11S protein in soybean, suggesting future areas for research.

**Acknowledgment** This study was supported by a grant from the Korea Ministry of Agriculture and Forest.

**REFERENCES**