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Effects of Soybean Embryo on Liver Protection and Lipid Metabolism of Alcohol-Fed Rats

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Abstract In this study, the ameliorating effect of soybean embryos on the impact of alcohol consumption was investigated on rat hepatocytes and in reducing total serum cholesterol levels and total serum lipid levels. Liver histology and two clinically important enzyme markers, aspartate aminotransferase (AST) and alanine aminotransferase (ALT), of rats administered with both alcohol and soybean embryo were compared with a control group. The treatment regimen of soybean embryo significantly reduced the serum ALT and AST levels of the subjects, demonstrating the hepato-protective effects of soybean embryo. Electron microscopy indicated that the administration of soybean embryo preserved the important hepatocyte structures and prevented the presence of lipid droplets and secondary lysosomes. Furthermore, total cholesterol and total lipid levels were significantly reduced. These results indicate that treatment with soybean embryo can positively mediate the effects of alcohol on hepatocytes and general liver functions.

Keywords: soybean embryo, soybean, rat hepatocytes, alcohol

Introduction

Alcoholism is a global social and economic problem. 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 (DALYS) (1), even costlier than tobacco use.

Alcohol is rapidly absorbed in the upper portion of the small intestine. The alcohol-laden blood then travels to the liver via the veins and capillaries of the digestive tract, which affects nearly every liver cell. The impairment of bodily functions and the damage caused by the consumption of alcohol work mainly in two ways: (i) indirectly, by interfering with the body's normal processing of food, thereby causing malnutrition, and (ii) directly, through direct toxic effects causing organ pathology, the effects of which center particularly upon the liver (2). 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 itself and play a critical role in the body's metabolic processes (3).

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 pathology (4-6). However, these chemically derived compounds can have harmful and unforeseen side effects. Therefore, there has been a focus on natural or herbal treatments to alcohol-induced diseases.

A number of studies have researched the nutritional and physiological benefits of the soybean. Soybeans are a good source of carbohydrates, fat, protein, fiber, and calcium, making them a nutritionally valuable food source (7). Recently, the FDA has accepted the health claim that soy protein helps to reduce the blood cholesterol level, further highlighting the importance of the soybean. The nutritional, economic, and health benefits of this legume make it an important focus of future research. Aside from general positive nutritional factors, the beneficial effects of various compounds of the soybean are being researched. The positive effects most relevant to this study are the reduction of serum cholesterol and lipid levels, the protection of hepatocyte membranes and structures, the amelioration of nutritional deficiencies, and the prevention and/or breakdown of hepatic fibrosis.

Studies with soy protein in hypercholesterolaemia have hypothesized that the mechanism of the cholesterol reduction is most likely ascribable to an activation of the low-density lipoprotein (LDL) receptor activity (4, 8). In the presence of soy protein or soy peptides, cellular LDL receptors, depressed by hypercholesterolaemia, return to normal function, thus allowing cells to more efficiently dispose of cholesterol (4, 9, 10). This research also suggests that soy proteins may be effective in protecting the liver from alcohol-induced damage and detrimental conditions such as fatty liver and the development of fibrosis.

Dietary supplementation with soy phospholipids may also help patients with liver disease, alcoholism, or chronic parenteral nutrition reduce the risk of linoleic acid (LA) deficiency. In addition, soy phospholipid is reported to reduce the symptoms of liver disease, chronic hepatitis, or liver dysfunction due to malnutrition, such as loss of appetite and abdominal pain (9-11).

As mentioned above, soy-based compounds can potentially confer many beneficial health effects and even attenuate the damage induced by long-term alcohol consumption. This study investigated the potential positive effects of the soybean embryo which is a seldom used portion of the soybean in commercial contexts.

Materials and Methods

Preparation of soybean embryo Soybean embryos, which were segregated from the whole soybean by Dr. Chung's

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Food Co., Ltd., were used in this experiment. The dried soybean embryos were purchased from Dr. Chung's Food Co., Ltd. in February 2003. The dried soybean embryo solution was prepared in two steps: (1) boiling 100 g of the dried soybean embryo power and 1000 mL of distilled water for 10 min at 100°C while continuously stirring the solution, and (2) adjusting the final volume to 300 mL in an evaporator (Rotavapor R-200, BUCHI, Switzerland). The solution was cooled at room-temperature for 1 hr. This solution was prepared daily to prevent any loss of nutritional value.

Preparation of animal models 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 of 22±2°C temperature, and 45±5% relative humidity, in a 12 hr 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 water).

After a 4-week adaptation period, 24 rats (2 per cage) were then randomly assigned to 4 groups (n=6) for the following 6-week regimens: (i) normal controls were given water, (ii) negative controls were given an ethanol/water mixture, (iii) positive controls were given ethanol/water and a mixture 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.), and (iv) rats in the experimental treatment group were given ethanol/water and soybean embryo solution (Table 1).

An ethanol/water intake of 5 g/kg/day was achieved. At the end of a 5-week period consisting of 4 weeks adaptation and 1 week of alcohol, the rats weighed an average of 330 g. Either a treatment solution or a placebo (distilled water for the normal control group, and 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 the same time everyday. Food consumption and water (or alcohol and water solution) measurements were taken daily. Body weight measurements were made weekly.

Positive control group The solution for the positive control group was a mixture of Alcodex, referred to as alcoholic liver disease cure solution (LCS) for the purposes of this experiment, and Vasoclean, referred to as blood circulation promotion solution (BPS). The ingredients of LCS (per 30 mL) as described 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 as described on the label are 120 mg of ginkgo biloba extract and 60 mg of sodium benzoate. The administered rat dosage based on the average rat weight was derived from the amount that is commercially recommended for humans (30 mL /60 kg of LCS+60 mL/60 kg of BPS).

Table 1. Experimental designs

Groups	No. of rats treated	Treatments[1)]
Normal control	6	H_2O
Negative control	6	AH_2O
Positive control	6	AH_2O+LCS+BPS
Experimental treatment	6	AH_2O+Soybean embryos

[1)]H_2O, distilled water; AH_2O, alcohol and distilled water (5 g/kg/day: calculation of alcohol concentration explained in the text); LCS, alcoholic liver disease cure solution (*Alcodex*, Guju Pharmaceutical Co., Ltd., Seoul); and BPS, blood circulation promotion solution (*Vasoclean*, Cho-A Pharmaceutical Co., Ltd., Seoul).

Experimental treatment group The soybean embryo solution was prepared as explained above. The dosage administered was derived from the recommended daily protein allowance for a 70 kg Korean adult male of 1 g/kg/day (a standard value set by the KDA: Korea Dietitian Association, 2000). Ten percent of this standard value was chosen for the protein treatment dose of 0.1 g/kg/day. A proportional amount of the soybean embryo solution, based on the average rat weight (average of the group, measured from the previous week), was orally administered daily at the same time.

Diet The rats were fed a commercially available diet from Samyang Co., Ltd., Korea (Samyang Assorted Diet, commercial name). The diet was composed of more than 22.1% crude protein, more than 3.5% crude fat, less than 5.0% crude fiber, less than 8.0% crude ash, more than 0.4% total phosphorus and more than 0.6% calcium.

Body weight, food, and water (or alcohol and water solution) measurements The body weights of the rats were measured every week from the beginning of the 1st week, at the beginning of the adaptation period, to week 11, and at the end of the experimental period. Food consumption was measured everyday at the same time. Water (or alcohol and water, depending on the experimental group) intake was also measured daily at about the same time.

Blood sample and serum preparation At the end of the last day of the experiment, the rats were not fed for 14 hr (overnight). They were then lightly anesthetized with ethyl ether and left at room temperature for 30 min, after which more than 3 mL of blood was extracted from the inferior vena cava of each anesthetized rat using a syringe and a test tube (not treated with heparin). The serum was separated from the extracted blood by centrifuging at 3,000 rpm for 15 min at 4°C. The aspartate aminotransferase (AST, serum SGOT), alanine aminotransferase, (ALT, serum SGPT), total cholesterol, and total lipid levels were determined in the serum.

Liver weight measurements and histological examination After the blood samples were obtained, the rat liver was removed and rinsed in a cold physiological salt solution. Once all excess surface moisture was wiped away, the livers were weighed. Liver tissue samples (1×1×1 cm) were removed and pre-fixed for 2 hr at 4°C in 4% paraformaldehyde solution with 0.1 M phosphate buffer (pH 7.3) and 2.5% glutaraldehyde. The tissue samples were then dehydrated in ethanol, embedded in Epon 812, and polymerized. The tissue samples were cut using an LKB 2088 ultramicrotome, stained with 1% uranyl acetate and lead citrate, and examined with a transmission electron microscope (JEM-2000 EXII, 80 KV, Japan).

Biochemical tests After the EDTA-free tube was filled with more than 3 mL blood, the sample was allowed to sit for 30 min at room temperature and was then centrifuged at 3,000 rpm for 15 min at 4°C. This serum was used to measure the AST, ALT, total cholesterol, and total lipid levels.

AST and ALT To obtain the activity value measurements of the serum, an AST kit and an ALT kit (both, Boehringer Mannheim, Germany) were used. The extinction values were measured at 340 nm wavelength by an automatic biochemical analysis machine (Hitachi 747, Japan).

Total cholesterol The 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).

Total lipids The total lipid level was measured using sulfo-phospho-vanillin. Sulfuric acid was added to the blood serum, heated and 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 the concentration was determined using a total lipid reagent kit (Medicos, USA) on an automatic biochemical analytical machine (Hitachi 747, Japan).

Statistical analysis All the results are shown as mean± standard deviation. Statistical evaluation of the data was performed using student's t-test (12) to make comparisons among groups.

Results and Discussion

Changes in body weight Table 2 shows the changes of rat weights after the administration of alcohol for seven weeks (1 week of acclimation plus 6 weeks of treatment). The increased body weight over the total course of the experiment was attributed to normal growth. Alcohol consumption for a prolonged period of 7 weeks stunted the normal growth of the rats, as evidenced by the 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 that of the negative control group was 108.60± 9.334 g. A weight gain of 115.25±7.866 g was observed in the positive control group, and 116.88±10.398 g in the experimental treatment group. These results are strengthened by the fact that all experimental groups, with the exception of the positive control group, consumed roughly the same amount of food, as shown in Table 3 (average weekly dietary intake). The negative and positive control groups consumed an average of 37.71±0.639 g and 36.76±0.586 g of food per week, respectively. The experimental treatment group consumed 36.78±1.737 g of food. The only exception was the normal control group, whose average weekly dietary intake was 44.09±2.140 g. This maybe evidence of the addictive properties of alcohol, since alcohol replaced food in the diet. These results show that the weight gain, or the lack of it in the case of the negative control, cannot be attributed to the differences in the amount of food consumed.

Table 2. Changes in rat body weights over time

Groups	Changes in the body weight (g) for 6 weeks Mean±SD
Normal control	121.55±16.292*
Negative control	108.60±9.334
Positive control	115.25±7.866*
Experimental treatment	116.88±10.398*

*significantly different from the negative control group at $p<0.05$ by Student's t-test.

Table 3. Average weekly dietary intake

Groups	Average dietary intake in 7 weeks (g) Mean±SD
Normal control	44.09±2.140***
Negative control	37.71±0.639
Positive control	36.76±0.586***
Experimental treatment	36.78±1.737*

*,***significantly different from the negative group at $p<0.005$ and $p<0.005$ by Student's t-test, respectively.

Liver weight Table 4 shows the liver weight relative to the rat's total body weight. The liver weight ratio in the experimental treatment group was 2.962±0.1996%, which was close to the ratio of 2.900±0.1244% in the normal control group. The ratio for the negative control group was higher than the others at 3.105±0.0749%.

Serum level of AST Table 5 shows the AST levels in the serum. The AST level in the serum of the negative control group, 87.83±10.741 U/L, was significantly higher than that of the normal control group, 71.67±6.593 U/L ($p<0.05$). This elevated AST level can be attributed to the liver damage induced by long-term alcohol consumption. The AST level of the experimental group was 72.67±2.805 U/L, which was similar to the normal control group and also significantly lower than the negative control group ($p<0.05$).

Table 4. Mean liver weight as a percentage of total rat body weight

Groups	Liver (% of body weight) Mean±SD
Normal control	2.900±0.1244***
Negative control	3.105±0.0749
Positive control	2.921±0.2185*
Experimental treatment	2.962±0.1996*

*,***significantly different from the negative control group at $p<0.05$ and $p<0.005$ by Student's t-test.

Table 5. Serum levels of AST and ALT in alcohol-fed rats

Groups	AST (U/L) Mean±SD	ALT (U/L) Mean±SD
Normal control	71.67±6.593***	45.50±5.206#
Negative control	87.83±10.741	94.83±13.197#
Positive control	71.00±4.940***	43.67±7.711#
Experimental treatment	72.67±2.805***	46.17±5.981#

*significantly different from the negative control group
***,#at $p<0.005$ and $p<0.001$ by Student's t-test.

Table 6. Levels of total serum cholesterol and total serum lipids in alcohol-fed rats

Groups	Total cholesterol (mg/dL) Mean±SD	Total lipids (mg/dL) Mean±SD
Normal control	88.00±11.331**	331.33±47.873***
Negative control	114.67±21.210	392.67±23.534
Positive control	92.17±9.432***	347.17±39.398***
Experimental treatment	92.67±5.164**	372.17±16.461**

*,significantly different from the negative control group
,*at $p<0.01$ and $p<0.005$ by Student's t-test.

Serum level of ALT Table 5 shows the ALT levels in the serum. The ALT level in the serum of the negative control group was 94.83±13.197 U/L, which was significantly higher than the normal control group's level of 45.50±5.206 U/L ($p<0.001$). This increase confirms the extent of hepatic damage the negative control group sustained due to long-term alcohol consumption. The ALT level of the experimental treatment group (46.17±5.981 U/L) was similar to the normal control group and lower than the negative control group ($p<0.001$).

Serum levels of total cholesterol and total lipids The total cholesterol level is composed of free cholesterols and ester cholesterols. Table 6 shows the levels of total cholesterol found in the serum. The normal control (88.00 ±11.331 mg/dL) and experimental treatment group (92.67 ±5.164 mg/dL) had similar serum cholesterol levels, and both were significantly lowered compared to the serum cholesterol level of the negative control group (114.67± 21.210 mg/dL; $p<0.01$), indicating that the treatments were effective in ameliorating the effects of alcohol consumption. This finding parallels that of other studies in which soy proteins lowered serum cholesterol levels (7, 13-15).

The total lipids level is mainly composed of total cholesterol, phospholipids, triglycerides, and free fatty acids. Table 6 shows the levels of total lipids found in the different experimental groups. The normal control had a total lipids level of 331.33±47.873 mg/dL while the negative control was measured at 392.67±23.534 mg/dL. The experimental treatment group (372.17±16.461 mg/dL) was different from the negative control.

Light micrographs of hepatic tissue Fig. 1 shows the light micrographs of the hepatocytes from the experimental groups. Fig. 1A shows the normal hepatic tissue from the normal control group (CV, central vein; PV, portal vein). Fig. 1B (B1-B3) shows the hepatic tissues from the negative control group with the evidence of liver damage from long-term alcohol consumption and the degeneration of the hepatocytes (B2, arrowhead) and the dilatation of the sinusoids (B3, asterisks). A few eosinophilic Mallory bodies (B3, arrows) and accumulation of small lipid droplets can also be seen. Fig. 1C shows the hepatic tissue of the positive control group, which has a morphology similar to that of the normal control group except for the infiltration of inflammatory cells (arrow heads) near the portal area. Fig. 1D shows the hepatic tissues of the experimental treatment group, which has a morphology similar to that of the normal control group.

Electron micrographs of hepatocytes Fig. 2 shows the electron micrographs (×10,000) of hepatocytes from the experimental groups (N, nucleus of hepatocyte; M, mitochondria; R, rough endoplasmic reticulum; L, lipid droplet; and BC, bile canaliculus). The normal control group, Fig. 2A, exhibits normal hepatocyte morphology. Figs. 2B, 2C, and 2D are electron micrographs of the negative control group. Hepatocytes in Figs. 2B and 2C show degenerative signs (asterisks) such as clear areas and dilated biliary canaliculi (arrowhead) without microvilli. The hepatocytes also exhibit a diffuse accumulation of lipid droplets (L) in the

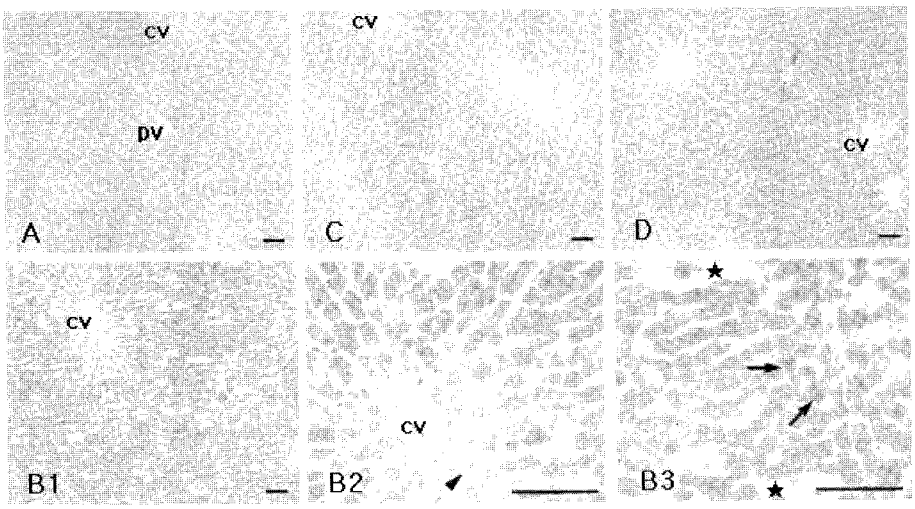

Fig. 1. Light micrographs of hepatic tissue in alcohol-fed rats. (A) Hepatic tissue from the normal control group. (B1, B2, B3) Hepatic tissue from the negative control group (fed alcohol and water only). (C) Hepatic tissue from the positive control group (treated with alcohol, alcoholic liver disease cure solution and blood circulation promotion solution). (D) Hepatic tissue from the experimental treatment group (treated with a soybean embryo solution). CV, central vein; PV, portal vein; BD, bile ductile; asterisk, dilatation of sinusoids; arrow, Mallory body; and arrowhead, inflammatory cell. Scale bar=40 μm. Cell staining was carried out by the H&E staining method. Magnification: A, B1, C, and D (×100), B2 and B3 (×400).

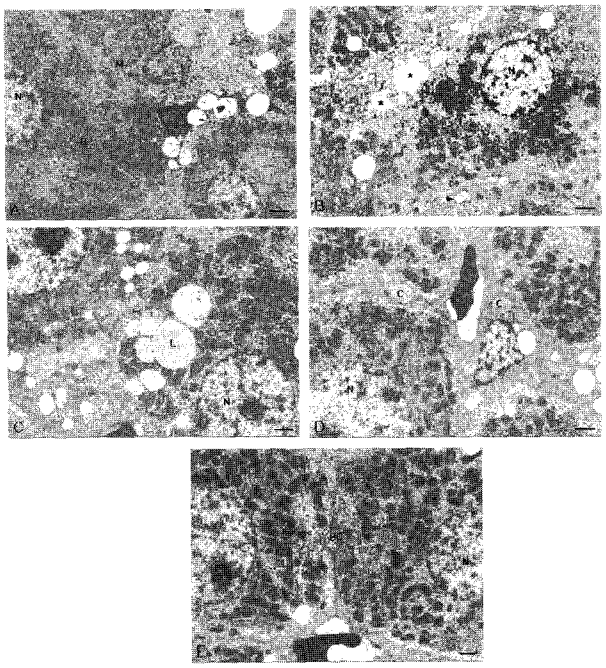

Fig 2. Electron micrographs of hepatocytes. (A) Hepatocytes from the normal control group. (B, C, D) Hepatocytes from the negative control group (fed alcohol and water only). (E) Hepatocytes from the positive control group, which was treated with alcohol, alcoholic liver disease cure solution and blood circulation promotion solution, showing normal morphology. (F) Hepatocytes from the experimental treatment group, showing normal morphology. M, mitochondria; N, nucleus of the hepatocyte; R, rough endoplasmic reticulum; L, lipid droplet; BC, bile canaliculus; asterisk, degenerative sign of hepatocyte; and arrowhead, dilated bile canaliculus. Scale bar=1 μm, magnification: ×10,000.

cytoplasm. Fig. 2D shows the increased collagen in the hepatic lobule. Fig. 2E is an electron micrograph of the positive control group, which exhibited normal hepatocyte structures. Fig. 2F is an electron micrograph of the experimental treatment group, which shows the normal morphology of hepatocytes (as in the normal control group) with none of the degenerative signs exhibited in the negative control group.

Alcohol consumption for a prolonged period of 7 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. This data is in agreement with the fact that the consumption of alcohol can have malnutritive effects. For example, when alcohol is substituted for carbohydrates subjects tend to lose weight, indicating that they derive less energy from alcohol than from food (16). This result also implies that the subjects treated with soy solution were observed to absorb more nutrients (weight gain), even in the face of chronic alcohol consumption. The experimental treatment group gained weight compared to the negative control, and this gain was higher than that of the other alcohol-treated groups. 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 3 (average weekly dietary intake). These results show that the weight gain, or lack of it in the case of the negative control, cannot be attributed to differences in the amount of food consumed.

Hypertrophy of the liver can be regarded as an indicator of fatty liver and fibrosis of the liver caused by the accumulation of lipids due to long-term alcohol consumption (17). The results of our study agree with this finding. Lee (18) showed that the liver's weight, as a percentage of the total body weight, in subjects with alcohol-induced liver damage was higher than in normal subjects or those who had undergone treatment with experimental materials. The group that consumed a treatment solution of experimental materials had a lower liver weight to body weight ratio than the alcohol-only group, which was also in agreement with the finding of this study. Furthermore, 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 (19).

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-producing, myofibroblast-like cells (20, 21). The light micrographs of the negative control showed the degeneration of hepatocytes, as evidenced by the dilatation of the sinusoids, the presence of eosinophilic Mallory bodies, and the accumulation of inflammatory cells and small lipid droplets. Mallory bodies are characteristic cytoplasmic hyaline inclusions in hepatocytes, reflecting a morphologic manifestation of chronic liver cell injury (22). These findings are typical of alcohol-induced liver damage. On the other hand, the hepatic tissue of the experimental treatment group exhibited a morphology very similar to that of the normal control group, which is physical evidence of the hepato-protective effects of soybean embryo solution. Further physical evidence can be found in the electron micrographs of the negative control group, which showed degenerative signs, such as clear areas and dilated biliary canaliculi without the microvilli. In addition, the hepatocytes exhibited a diffuse accumulation of lipid droplets in the cytoplasm and the electron micrographs showed the increased collagen in the hepatic lobule. The experimental treatment group showed the same normal morphology of hepatocytes that was evident in the normal control group, with none of the degenerative signs exhibited in the negative control group. The histological studies showed that the soybean embryo solution exhibits hepato-protective effects and thereby ameliorates the impact of long-term alcohol consumption.

Finally, the results of serum ALT and AST levels were in agreement with the histological data. 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. Free radicals, byproducts of oxidation, attack the hepatocytes, causing the degradation of cell membranes (lipid peroxidation). This process may destroy the integrity of the membranes both within and surrounding the cell, seriously compromising cell function (23). The increased levels of serum AST and ALT enzymes can be caused by fatty liver, or exposure to by-products, resulting in metabolic problems of the liver and the death of hepatocytes. The elevated AST levels in the negative control group can be attributed to the liver damage induced by long-term alcohol consumption, confirming the findings of previous research. The serum AST level of the experimental treatment group was relatively low in comparison with that

in the negative control group ($p<0.05$) and was similar to that in the normal control group, indicating the hepato-protective effects of the treatments. The serum ALT level of the experimental treatment group was significantly lower than that of the negative control group ($p<0.001$) and was similar to that in the normal control group. These results also confirm the extent of hepatic damage sustained by the negative control group due to long-term alcohol consumption.

As for the serum total cholesterol level, the normal control and the treatment groups were significantly lower than the negative control group, indicating that the treatment was effective in ameliorating the effects of alcohol consumption. This finding is in agreement with the findings of other studies which found that soy proteins lowered the serum cholesterol levels of the subjects (24-27). With regards to the total lipids level, the experimental treatment group was significantly lower than the negative control ($p<0.05$), a finding which also supports the research of Lovati and Baum (4, 7, 8, 10, 15). These results are significant since high levels of cholesterol are major causal factors in the development of atherosclerosis and subsequent cardiovascular diseases.

The present study highlights the various potential benefits of soybean embryos such as a protective effect against hypercholesterolemic with long-term alcohol consumption, and suggests future research areas.

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