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ORIGINAL ARTICLE

Hepatoprotective effects of soy protein isolate against dimethylnitrosamine-induced acute liver injury in Sprague Dawley rat

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Abstract Diseases and injuries impose oxidative stress on different organs and systems of the body. This study was conducted to investigate the hepatoprotective effects of soy protein isolate against acute liver toxicity induced by dimethylnitrosamine (DMNA). Forty-eight Sprague Dawley rats were randomly divided into three groups. Groups A and B consumed a casein containing diet and the Group C received a soy protein isolate (SPI) containing diet for 18 days. Group A was then given an intra-peritoneal saline injection and continued on a casein diet for another 4 days before being killed. Each animal from groups B and C was given a single intra-peritoneal injection of DMNA (30 mg/kg) on the 18th day of the study. All groups continued their diets for 4 days before their sacrifice. The serum ALT decreased and albumin increased significantly in rats fed 20 % SPI containing diet ($P < 0.05$). Histological results showed that SPI improved DMNA-induced alteration in the liver structure. Morphological and biochemical data suggest that soy protein isolate containing diet decreased DMNA-induced liver damage.

Keywords Hepatic failure · Biochemistry · Histopathology · Soy protein isolate

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Introduction

Acute liver failure (ALF) is a relatively uncommon clinical syndrome caused by severe hepatocyte dysfunction in the absence of pre-existing liver diseases and it occurs when the rate and extent of liver cell death are not adequately balanced by regenerative activity [1, 2].

Oxidative metabolism is essential for survival of the cells, whereas excessive formation of free radicals due to diseases such as acute liver failure induce destructive and lethal cellular effects such as apoptosis by oxidizing membrane lipids, cellular proteins, DNA and enzymes, thus shutting down cellular respiration [3].

Cells are naturally protected against oxidative insults by natural antioxidant products, notably glutathione, and by diverse antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase. Naturally, there is a balance between oxidants produced in the body and the level of antioxidant products. Oxidative stress develops when this balance is disturbed and reactive oxygen species (ROS) are generated in excess, combined with depleted antioxidant defenses in the organ [4–6].

Foods containing antioxidants may be able to recover this imbalance and retrieve the anatomy and physiology of the injured organ almost to its normal state [6]. Many plant and animal proteins such as whey and soy are known to possess significant antioxidant properties [7].

Accumulated data from epidemiologic as well as nutritional intervention studies in humans and animals suggest that consumption of soy and soy-based products has protective effects on a variety of diseases [8, 9]. In a study

using male Wistar rats, it was found that the intake of either soy protein isolate or soy peptide had the effect of reducing paraquat-induced oxidative stress. In this experiment, both soy protein isolate and soy peptide prevented the elevation of the serum thiobarbituric acid-reactive substance concentrations [10].

Since soy proteins have high biological value, possess antioxidant properties and thus have beneficial effects for patients with hepatic failure, therefore, the present study was conducted to determine the possible preventive effect of diet containing soy protein isolate on DMNA-induced acute liver failure in rats.

Materials and methods

Animal experiments

Forty-eight mature Male Sprague Dawley (SD) rats weighting 200 ± 10 g were obtained from the Experimental Animal Center, Razi Institute (Tehran, Iran). They were kept according to experimental design in individual steel cages of 22 × 30 × 25 cm dimension, at 22 ± 2 °C temperature and 50 ± 10 % humidity, with (12_h–12_h) light–dark cycle and food and water ad libitum. All experimental animal protocols were evaluated by the Ethics Committee of the Research Council of the Dean of Research Affairs of the Shiraz University of Medical Sciences.

The rats were randomly divided into 3 equal groups each having 16 animals. Prior to the experiment, the rats were allowed to adapt to the laboratory environment for 3 days. Groups A and B received a diet containing casein protein, in addition to all other components. Whereas, the rats of group C received the same diet as did the other two groups except for the protein content which was soy protein isolate (SPI). The protein content (on dry basis) and nitrogen soluble index (NSI) were 90 % minimum and 88 % minimum, respectively. Its urease activity was negative. The composition of the diets that was used during this experiment is shown in Table 1.

Table 1 Composition of the diets

Ingredients	Contents (gm kg^{-1})
Casein and SPI[a]	200
Corn starch	560.7
Sucrose	100
Corn oil	40
Cellulose	50
AIN-93M mineral mixture[b]	35
AIN-93M vitamin mixture[b]	10

[a] Dor Shimi Marjan, Tehran, Iran; Behin Azma, Shiraz, Iran

[b] (Razi and Osveh Companies, Tehran, Iran)

On day 18th of the study, the animals of Groups B and C were treated with 30 mg/kg dimethylnitrosamine (Sigma Chemical, St. Louis, MO, USA) and the rats of Group A received saline injection.

The animals were weighed at the beginning of the study and every 4 days thereafter for the entire experimental period. Food intake was also determined every 4 days.

Food efficiency ratio (FER) of the different diets was calculated as the gain in body weight (g) per food intake (g) [11], and protein efficiency ratio (PER) was calculated as weight gained (g) per protein intake (g) in the time period of the experiment [12].

All animals were euthanized under anesthesia with diethyl ether 4 days after DMNA or saline injection. Blood samples were collected from the heart and the sera and were separated for different assays. Liver was removed, and the liver sections of approximately 1 cm^3 in thickness were isolated immediately and rinsed in cold saline and were then fixed in 10 % neutral buffered formalin.

Serum analysis and histopathological studies

After blood collection, serum was separated by centrifugation at 2,500 rpm and 4 °C for 20 min. The serum aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), total protein, albumin and total bilirubin values were measured using commercially available kits (Zist Chemi and Pars Azemoon, Tehran, Iran). ALT and AST were estimated by colorimetric methods according to Reitman and Frankel [13], and ALP according to Belfield and Goldberg [14]. The level of lipid peroxides, expressed as nanomoles of malondialdehyde (MDA) per milliliters of serum was assayed as thiobarbituric acid reactive substances (TBARS) using a colorimetric method. Briefly, 0.5 ml serum was added to 2 ml thiobarbituric acid (TBA) reagent containing 0.375 % TBA (Sigma Chemical, St. Louis, MO, USA), 15 % trichloroacetic acid and 0.25 mol/l HCl. The mixture was boiled for 15 min, cooled at 4 °C and centrifuged at 1,700×g for 15 min. The absorbance of the supernatant was measured at 532 nm. The TBARS concentration was calculated using 1, 1, 3, 3-tetraethoxy propane (TEP) (Sigma Chemical, St. Louis, Mo, USA) as a standard [15].

After euthanizing, the abdomen was opened immediately by a midline incision and the liver was removed. The whole liver was weighed. A small portion of the liver of approximately 1 cm^3 in dimensions was isolated immediately and rinsed in cold saline before being placed in 10 % neutral buffered formalin. Liver samples were then dehydrated with graded ethanol solutions, embedded in paraffin wax and sections of 5 μm in thickness were stained with

haematoxylin–eosin and studied by a routine light microscope.

Statistical analysis

Results were expressed as mean ± SD, and data were analyzed using one-way ANOVA followed by Scheffe or Tamhane's post hoc comparison test for significant difference. Statistical significance was defined as $P < 0.05$. All statistical procedures were performed with SPSS software (version 13: SPSS Chicago, IL, USA).

Results

The effect of soy protein isolate in comparison with the casein diet is shown in Table 2. Although food and macronutrient intake (protein, fat, and carbohydrate) of rats in the CAS + DMNA and SPI + DMNA were significantly lower than the control group ($P < 0.05$), weight gain, final weight, food efficiency ratio (FER) and protein efficiency ratio (PER) were lower in the SPI + DMNA group. There were no significant differences between hepatic and relative hepatic weight between the groups.

Serum total protein did not differ between the groups. A significant elevation in serum AST, ALP, and total bilirubin, and a decrease in albumin level were recorded in the groups treated with DMNA in comparison with the control group. As shown in Table 3, soy protein isolate containing diet ameliorated the increase of ALT ($P < 0.05$), and total bilirubin. In addition, the soy protein isolate containing diet elevated the decrease in serum albumin level caused by DMNA ($P < 0.05$). Levels of AST in the sera were not significantly decreased by soy protein isolate containing

Table 2 Food and macronutrient intake, final weight, weight gain, food efficiency ratio (FER), protein efficiency ratio (PER), hepatic weight, relative hepatic weight, of the control group and liver injured rats fed casein or SPI

	Control ($n = 16$)	CAS + DMNA ($n = 13$)	SPI + DMNA ($n = 14$)
Food intake (gm/day)	13.02 ± 1.20	10.33 ± 1.52[a]	10.54 ± 0.94[a]
Protein intake (gm/day)	2.60 ± 0.24	2.07 ± 0.30[a]	2.11 ± 0.19[a]
Fat intake (gm/day)	0.52 ± 0.05	0.41 ± 0.06[a]	0.42 ± 0.04[a]
Carbohydrate intake (gm/day)	9.25 ± 0.86	7.34 ± 1.08[a]	7.49 ± 0.67[a]
Final weight (gm)	243.56 ± 18.69[b]	233.92 ± 12.38[b]	213.21 ± 17.20
Weight gain (gm)	43.50 ± 14.09[b]	31.77 ± 13.64[b]	13.64 ± 14.18
Food efficiency ratio	0.15 ± 0.04[b]	0.13 ± 0.05[b]	0.05 ± 0.06
Protein efficiency ratio	0.75 ± 0.19[b]	0.65 ± 0.27[b]	0.28 ± 0.31
Hepatic weight (gm)	11.49 ± 1.58	12.10 ± 1.30	10.74 ± 1.58
Relative hepatic weight	4.752 ± .64	5.18 ± 0.60	5.03 ± 0.52

Numbers in parenthesis indicate # of rats at the end of the study. Data are expressed as the mean ± SD

[a] $P < 0.05$. Significantly different with control group; one-way ANOVA test

[b] $P < 0.05$. Significantly different with SPI group; one-way ANOVA test

Table 3 Biochemical serum parameters of the control group and liver injured rats fed casein or SPI

	Control ($n = 16$)	CAS + DMNA ($n = 13$)	SPI + DMNA ($n = 14$)
AST (IU/l)	134.82 ± 73.01	469.99 ± 343.33[a]	461.43 ± 342.51[a]
ALT (IU/l)	194.82 ± 52.56[b]	826.70 ± 766.22	270.23 ± 493.57[b]
ALP (IU/l)	159.12 ± 71.12	339.08 ± 103.88[a]	340.71 ± 89.51[a]
T-Protein (gm/dl)	6.79 ± 0.72	7.34 ± 0.63	6.80 ± 0.59
Albumin (gm/dl)	5.16 ± 1.05[c]	3.37 ± 0.75	4.34 ± 0.66[d]
T-bilirubin (mg/dl)	1.65 ± 0.62	3.51 ± 1.58[a]	2.86 ± 1.30[a]

Numbers in parenthesis indicate # of rats at the end of the study. Data are expressed as mean ± SD

AST aspartate amino transferas, *ALT* alanine amino transfrase, *ALP* alkaline phosphatase

[a] $P < 0.05$. Significantly different with control group; one-way ANOVA test

[b] $P < 0.05$. Significantly different with CAS group; one-way ANOVA test

[c] $P < 0.05$. Significantly different with CAS and SPI group; one-way ANOVA test

[d] $P < 0.05$. Significantly different with CAS and control group; one-way ANOVA test

diet. Surprisingly, ALP was increased in rats receiving the soy protein isolate diet, however, the increase was not significant. Compared to the other two groups, MDA was significantly elevated in SPI + DMNA group.

Histopathological findings

Livers of the rats of the control group were grossly and histopathologically normal. The liver of all the 13 rats of the CAS + DMNA group showed different ranges of cell necrosis, congestion, intravascular coagulation of the central and/or portal veins and hemorrhages. Nine rats of this group showed severe necrosis of the hepatocytes in central, midzonal and periportal areas with massive hemorrhages and disruption of tissue architecture (Fig. 1), while the liver of the rest of the animals of this group was moderately to mildly necrotic and the hepatocytes close to the central veins and/or midzonal regions (Figs. 2 and 3) showed necrotic changes. Congestion and hemorrhages were commonly seen in the areas of most intense necrosis especially around the central veins and in midzonal areas. Most of the hepatocytes in the centrilobular region were lysed and disappeared and the nuclei of the remaining cells in this area and to a lesser extent midzonal region were either piknotic, fragmented or karyolysed. Lymphocytes, plasma cells and macrophages were moderately to mildly infiltrated in the portal areas of the liver of some of the rats of this group. The remaining hepatocytes in the midzonal and periportal areas of the liver of seven rats of this group were swallowed and showed severe fatty degeneration and ballooning or eosinophilic changes of the cytoplasm and exhibited coarse chromatin granules. Disseminated intravascular coagulation (DIC) was seen in the liver of seven and telangiectasis was evident in the liver section of four rats of this group. No mitotic figures and regeneration of the hepatocytes was evident in the liver of the animals of this group.

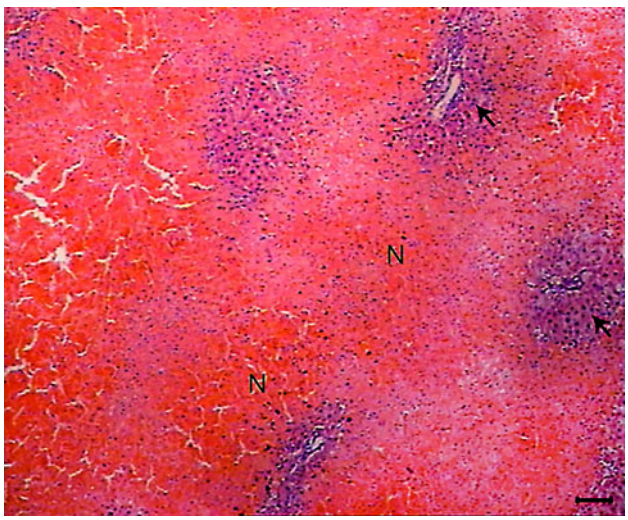

Fig. 1 Section from the liver of a DMNA + Casein treated rat shows massive necrosis (N) and hemorrhages. Only few rows of the hepatocytes surrounding the portal tracts (*arrows*) remained almost alive (H and E, *scale bar* = 210 μm)

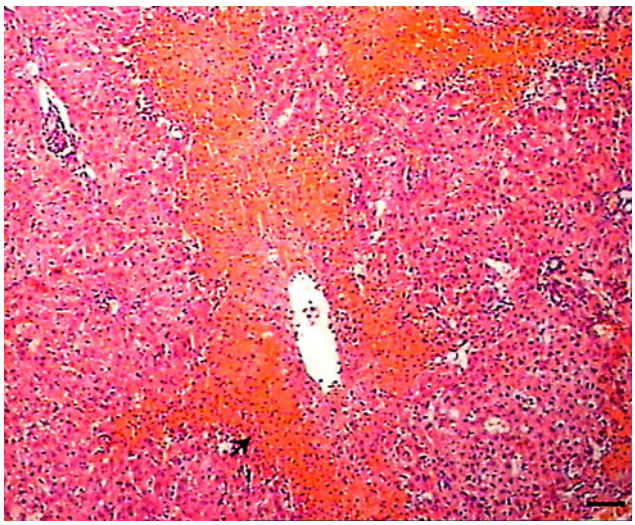

Fig. 2 Section from the liver of a DMNA + Casein treated rat with severe necrosis of the hepatocytes around a central vein (*arrow*) (H and E, *scale bar* = 210 μm)

Soy protein isolate ameliorated destruction of cells caused by DMNA and from the 14 remaining rats of this group at the end of the experiment; massive necrosis was present only in the liver of 3 rats. The liver of seven animals showed mild necrosis and only 1–3 rows of the hepatocytes around the central vein showed necrosis (Fig. 4) and some of the hepatocytes showed mild degrees of fatty changes. Some of the hepatocytes of the liver of these animals started to regenerate and few recently regenerated hepatocytes were present in the tissue section of the liver of these animals as a result of protective effect of this protein. The livers of the remaining four animals of this group looked like almost normal.

Discussion

Macronutrient intake in groups treated with DMNA was significantly lower than the control group. This is possibly due to the stress and liver damage [16] caused by DMNA which reduces appetite. In addition, effects of different peptides in causing satiety signals through intestinal opioid and cholecystokinin-A receptor, although to a lower extent, might be another reason for reduced food intake [17]. Specific fragments of soy peptides rich in arginine residues have strong food intake suppressor activity [17]. The weight gain of DMNA-treated animals on day 22 post-injury was 73 and 31 % of that of the control animals, for

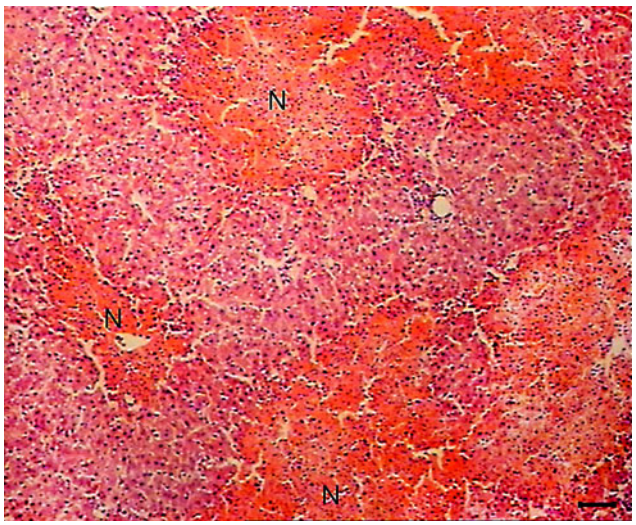

Fig. 3 Section from the liver of a DMNA + Casein treated rat with severe necrosis of the hepatocytes around a central vein and midzonal areas (N) (H and E, *scale bar* = 210 μm)

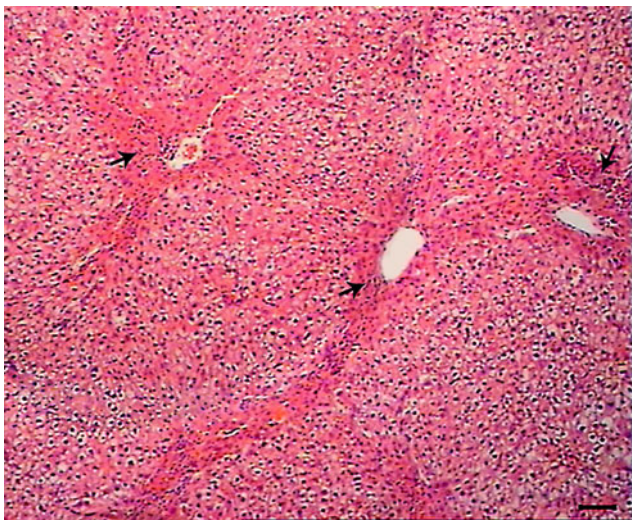

Fig. 4 Section from the liver of a DMNA + Soy treated rat. Few cells around the central veins show necrosis (*arrows*) (H and E, *scale bar* = 210 μm)

CAS + DMNA and SPI + DMNA groups, respectively. Decrease in appetite and thus reduced weight gain as a result of DMNA was also previously reported by Kusunose et al. [18]. In spite of this, weight gain and final weight were only lower in the soy protein isolate group compared to those of the other groups. This might be explained by the fact that soy protein causes lower postprandial serum insulin levels in comparison with casein [17, 19], in addition plant proteins have higher amounts of non essential amino acids such as arginine, glycine and alanine, whereas animal protein has higher amounts of the essential amino acid lysine. High ratios of arginine/lysine are associated with high serum glucagon concentration [17, 20]. The increase in serum levels of glucagon and decrease in insulin induces higher metabolic rate and thus lowers body weight seen in the SPI + DMNA group.

Low weight gain in SPI group might be due to low FER and PER of soy protein isolate, whereas PER and FER did not differ among the other groups. These findings are in accordance with those of Hoffman and Falvo [21]. At the end of the study, relative liver weights of the animals of all three groups were not significantly different. Bhathena et al. [22] also indicated similar results.

It is a known fact that the consequences of DMNA injection start with acute hepatitis and hepatocytes necrosis and these changes lead to liver dysfunction and thus, a rapid rise in cytosolic enzymes such as aminotransferases (AST, ALT), ALP and bilirubin [18, 23]. These findings are in agreement with those of Lukivskaya et al. [24] and Shin and colleagues [25] reported an increase in ALP concentration following DMNA therapy. Elevation in either AST or ALT suggests the possibility of toxic or ischemic liver injury [26–28] and the histopathological findings of the present study with the concurrent biochemical changes confirm the extent of liver damage after DMNA therapy.

Soy protein isolate group showed some decrease in elevated serum AST, ALT and total bilirubin levels following the hepatocytes damage and cytopathic effects of DMNA. Despite this potential decrease in the ALP and ALT levels, their concentrations were still higher than normal values. Soy protein isolate diet significantly increased serum albumin levels. The effect of SPI in reducing serum ALT is in accordance with the report of Sugiyama et al. [29] that indicated a great suppression in plasma AST in the animals treated with SPI.

Histopathological results of the present study showed severe necrosis of the hepatocytes with hemorrhages and inflammatory cell infiltration without signs of regeneration in the liver of the animals of the CAS + DMNA group, which is an emphasis in the increase of AST, ALT and ALP levels. Decrease of ALT levels in the SPI diet group shows the protective role of this diet on the hepato-cellular damage.

Surprisingly, SPI caused a significant increase in serum MDA compared to the other groups. This might be explained by the fact that soy protein consumption reduces hepatic fatty acid and triglyceride biosyntheses and accumulation by increasing fatty acid oxidation through activating and up regulating the transcription factor peroxisome proliferator-activated receptor-α (PPAR-α). PPAR-α is a ligand-dependent transcription factor of the receptor superfamily. It controls fatty acid oxidative metabolism through the transcriptional induction of carnitine-palmitoyl transferase 1 (CPT-1) and several enzymes for β-oxidation. Soy protein diet upregulates PPAR-α gene

expression in the liver and is associated with a higher content of CPT-1 mRNA, with respect to rats fed with casein. This pattern of gene expression is associated with an increased carbohydrate and lipid oxidation [17]. In addition, soy protein decreases the insulin/glucagons ratio which in turn reduces SREBP-1 mRNA level in the liver. Repression of SREBP-1 expression and elevation of serum glucagon levels lead to elevation in metabolic rate that might speed lipid peroxidation [17].

It has been established that fibroblasts and the derived myofibroblasts along with various cytokines work as key factors in the pathogenic process of hepatocellular damage and liver fibrosis. Transforming growth factor-β and platelet-derived growth factor are considered the prominent profibrogenic cytokines [30]. This might be the mechanism of DMNA in initiating necrosis, fatty degeneration, telangiectasis and finally hepatic fibrosis. SPI possibly prevented these processes and therefore attenuated the induction of hepatotoxicity and fibrosis by DMNA and resulted in inducing a regeneration process and ameliorated necrosis of the hepatocytes.

Considering the above results, soy protein isolated diet had potential ameliorative effect against acute liver failure by normalizing the tissue morphology and the involved enzymes and other biochemical markers (serum AST, ALT, ALP, and total bilirubin levels).

In conclusion, the main result of the present study is that soy protein isolate can prevent acute liver injury and reduce liver dysfunction induced by DMNA treatment, by inhibiting tissue necrosis and inflammatory cell infiltration.

Conflict of interest None.

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