Effects of Dietary Protein of Proso Millet on Liver Injury Induced by D-galactosamine in Rats

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In this paper, we examined the effects of dietary protein from proso millet on liver injury induced by D-galactosamine or carbon tetrachloride in rats using serum enzyme activities as indices. D-galactosamine-induced elevations of serum activities of aspartate aminotransferase, alanine aminotransferase, and lactate dehydrogenase were significantly suppressed by feeding the diet containing 20% protein of proso millet for 14 days as compared with those of rats fed a 20% casein diet, but not in the case of carbon tetrachloride. The results showed that proso millet protein is effective at lower dietary protein levels than that of dietary gluten reported previously. Therefore, the findings reported here may suggest that proso millet protein is considered to be another preventive food for liver injury.

Key words: proso millet; liver injury; D-galactosamine

The liver is the largest organ and plays a central metabolic role with nutrients within the body.1,2) Hepatitis has been shown to develop in response to many different causes such as virus, alcohol, and various kinds of chemicals, and these liver diseases tend to increase with alcohol consumption. Recently there has been much concern about preventive foods for lifestyle-related diseases.3,4) Therefore, it would be important to study the effects of food and food components or beverages on liver injury.

D-galactosamine-induced hepatitis has been well studied as a model of viral hepatic disease5-7) because it is indicated that the symptoms of D-galactosamine-induced liver injury resemble those of viral hepatitis8) although there is an argument against this viewpoint.9) Intraperitoneal injection of carbon tetrachloride also leads to hepatic injury by the disruption of hepatocyte membranes due to the free radical products provided by liver drug-metabolizing enzymes.10) Effects of amino acids or drugs on these hepatic injuries have been reported by many papers.11-19) Especially, Fischer et al. have first shown the effects of branched-chain amino acids on hepatic encephalopathy.20)

On the other hand, in regard to effects of foods or food components on liver disease, Sugiyama et al. have reported effects of green tea or coffee on hepatic damage.5-7,21) Recently, the effects of compounds from avocado have also been reported on liver injury induced by D-galactosamine.22) However, little information is available on the effects of dietary protein on the pathogenesis of hepatitis.23,24)

Millets are important cereals for the diets of human in Africa, Asia, and India.25,26) In Japan, the consumption has recently increased because some people think that these cereals may have a health benefit such as a replacement cereal for allergy therapy. We have reported that dietary protein of proso millet (Panico miliaceum L.) is more effective for lipid metabolism.27,28) However, no study on the effect of dietary millet protein on liver injury has been done.

This is the first report about the effects of proso millet protein on liver injury induced by D-galactosamine and carbon tetrachloride in rats using serum activities of aminotransferases and lactate dehydrogenase as indices. A protein diet of proso millet showed a clearly protective effect on D-galactosamine-induced liver injury but it failed to prevent liver damage induced by carbon tetrachloride.

Materials and Methods

Materials. The reagents were purchased as follows: carbon tetrachloride and D-galactosamine hydrochloride, assay kits for aspartate aminotransferase (AST, EC 2.6.1.1), alanine aminotransferase (ALT, EC 2.6.1.2) and lactate dehydrogenase (LDH, EC

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1.1.1.27) from Wako Pure Chemical Industries Ltd (Osaka, Japan). Proso millet, which was harvested in 1999, was obtained from a farmer in Joboji, Iwate. Carbon tetrachloride was dissolved in olive oil (1:3, volume/volume). The aqueous solution of D-galactosamine used for injection was prepared by adjusting the pH to 7.0 and concentration to 300 g/l followed by the sterilization with filtration before use.

Animal and diet. Male Wistar rats aged 5 weeks were obtained from Clea Japan Inc. (Tokyo) and were kept in an air-conditioned room at 22±1°C with a 12-h light-dark cycle (6:00~18:00). After they were fed with a diet containing 20% casein for 2 days, they were divided into three groups of 5 animals each and fed on the experimental diets (Table 1). The diets of casein and proso millet protein contained 22.2 g per 100 g of casein (Oriental Yeast Co., Tokyo) and 29.4 g per 100 g of protein concentrate of proso millet, respectively. These diets will subsequently be referred to as 20 C or 20 PM. Animal received the diet and water ad libitum. The 20 PM diet was supplemented with 1.9% L-lysine monohydrochloride and 0.3% L-threonine to simulate the amino acid composition of casein. The protein concentrate of proso millet was prepared according to the method in a previous paper with a modification. In brief, a mixture of α-amylase and glucoamylase (Ractase SR-40 and Entiron GA-4, Rakuto Kasei Industry, Ohtsu, Shiga, Japan) was used to digest the starch at 60°C for 24 h (pH 7.0), and then the resultant material was defatted and freeze-dried. The composition of the protein concentrate was as follows (g/100 g): moisture, 8.1; protein, 66.9; lipid, 2.5; ash, 0.9; total dietary fiber, 4.3; non-fibrous carbohydrate by difference, 17.3. Table 2 shows the amino acid composition of the protein concentrate of proso millet. As compared to that of casein, alanine, glutamic acid, cystine, methionine, and leucine in the protein concentrate were much higher.

Two separate experiments were done as follows. In experiment 1, the effects of dietary proteins on D-galactosamine-induced liver injury were investigated. After feeding the experimental diet for 14 days, diet was withheld for 4 h and D-galactosamine of 800 mg/kg of body weight was injected intraperitoneally. Twenty hours after the administration, blood was drawn from the vena cava under anesthesia with diethyl ether and the liver was quickly excised. In experiment 2, the effects of dietary proteins on carbon tetrachloride-induced liver injury were investigated. After feeding the experimental diet for 13 days, diet was withheld for 12 h and carbon tetrachloride of 2 ml/kg of body weight was administered to the rats by a stomach tube. Twenty hours after the dosation, blood was collected and the liver removed as described above. Normal rats were administered with saline or olive oil alone. The animal experiments were done in accordance with criteria established by the Animal Care and Use Committee of the Faculty of Agriculture, Iwate University.

Analysis. The activities of AST, ALT, and LDH were measured using commercial kits according to the manufacturer’s protocol.

Statistical analysis. An analysis of variance was done on the experimental data, and the differences between means were considered to be significant at p<0.05 by the Tukey Multiple Comparison Test. These analyses were done by InStat ver. 2.03

<table>
<thead>
<tr>
<th>Table 1. Composition of Experimental Diets (%)</th>
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<tr>
<td>Dietary protein</td>
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<td>Casein*</td>
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<tr>
<td>Salt mixtureb</td>
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<td>Vitamin mixturec</td>
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<tr>
<td>Corn oilc</td>
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<tr>
<td>Choline bitrarate</td>
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<td>Cellulose3</td>
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<tr>
<td>Corn starch</td>
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<tr>
<td>dL-Methionine2</td>
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<tr>
<td>L-Lysine HCld</td>
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<tr>
<td>L-Threoninee</td>
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<td>Protein content</td>
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</tbody>
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* Oriental Yeast Co., Tokyo, Japan.
b AIN-76 composition.
c Ajinomoto Co., Tokyo, Japan.
d Wako Pure Chemical Industries Ltd, Osaka, Japan.
e Nihon Kayaku Co., Tokyo, Japan.

<table>
<thead>
<tr>
<th>Table 2. Amino Acid Composition of Protein Concentrate of Proso Millet (mg/g N)</th>
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<tbody>
<tr>
<td>Casein*</td>
</tr>
<tr>
<td>Asp</td>
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Values show means for 4 measurements.
Results

Growth and liver weights

Tables 3 and 4 show the effects of feeding 20 C and 20 PM diets on food intakes, body and liver weights, and serum enzyme activities. The food intake was similar through experiments 1 and 2 (Tables 3 and 4). When rats were fed with the experimental diets and thereafter treated with D-galactosamine, significant changes in growth of animals were not observed among the dietary groups, but the relative liver weights of rats given the 20 C diet were lower than in normal animals and in rats receiving the 20 PM diet (Table 3). In experiment 2 with administered carbon tetrachloride, the growth was lower in rats fed the 20 PM diet than in normal and carbon tetrachloride-treated animals fed the 20 C diet, but the relative liver weights were higher than in other two groups (Table 4).

Serum aminotransferase and LDH activities

Several serum enzymes such as AST, ALT, and LDH have been used as biochemical markers for the acute hepatic damage. Table 3 shows the effects of dietary proteins on these enzyme activities when D-galactosamine was injected into the rats after feeding the 20 C or 20 PM diet for 14 days. Serum AST, ALT and LDH activities of the rats were markedly increased by injection of D-galactosamine as compared to those of normal rats. The increases of these serum activities were, however, significantly suppressed by feeding 20PM diet as compared with those of rats fed 20 C diet, respectively (p<0.05). Table 4 shows the effects of dietary proteins on serum enzyme activities when rats were given the 20 C or 20 PM diet for 13 days and thereafter carbon tetrachloride was administered. Unlike experiment 1, significant changes in serum AST, ALT, and LDH activities were not observed between the dietary groups of 20 C and 20 PM.

Discussion

This study showed obviously that the intake of proso millet protein significantly reduced the elevation of serum activities of AST, ALT and LDH induced by D-galactosamine (Table 3), but did not affect the activities increased by administration of carbon tetrachloride (Table 4). These results were similar to those shown by Sugiyama et al., who demonstrated that green tea has a preventive effect on liver injury induced by D-galactosamine but not by carbon tetrachloride. As one of the reasons for the difference between the results of treatment with D-galactosamine or carbon tetrachloride, it is pointed out that absorption of tea catheccins from the intes-

tine would be low in the case of an in vivo experiment. However, in the case of this study we do not have any experimental evidence by which these inconsistent results by D-galactosamine and carbon tetrachloride can be explained.

In regard to the improvement of liver disease, the beneficial effects of branched-chain amino acids and their ketooacids on hepatic encephalopathy have been well known. The ratio of branched-chain amino acids to aromatic amino acids of protein concentrate of proso millet used in this study is 3.1, which is in an ideal range for clinical treatment of hepatitis. Thus, proso millet protein might be useful for hepatic encephalopathy upon chronic liver failure. However, since the case of chronic liver disease is different from that of acute liver injury, it is unlikely that the suppressive effect of proso millet protein on liver injury induced by D-galactosamine is due to the ratio of branched-chain amino acids to aromatic amino acids in proso millet protein.

Manabe et al. have shown that elevated serum ac-
Activities of aminotransferase induced by D-galactosamine is effectively prevented by a high gluten diet.\textsuperscript{23,24} Gluten contains much of glutamine. Therefore, they have assumed that the effectiveness of glutamine on the enzyme activities would have been mediated by glycogen metabolism rather than by uridine metabolism.\textsuperscript{10} Further, Wang et al. have shown that D-galactosamine-induced liver injury could be effectively prevented by supplementation with glutamine, glutamic acid and serine, while they have indicated concurrently that either alanine or leucine does not have any effectiveness.\textsuperscript{19} On the other hand, it has been reported that a high dose of alanine reduces experimental liver damages in rats and in primary cultured hepatocytes.\textsuperscript{12,13} These studies are, however, done using diets with high amino acid levels or in high doses of alanine, and moreover these results about the influence of alanine have discrepancies.

The contents of serine and alanine in 20 PM used in these experiments were low (1.5 and 3.1%, respectively) and the amount of glutamic acid (or glutamine) in 20 PM is mostly the same as 20 C. Thus, it would be unlikely that these results may arise from functions of glutamine, glutamic acid, and serine or alanine. Furthermore, effects of dietary arginine on D-galactosamine-induced liver injury has been shown.\textsuperscript{16} But the efficacy would be also less likely because the arginine content in protein of proso millet is lower than that of casein (Table 2).

Sugiyama et al. have reported the influence of various types of dietary fiber on liver injury.\textsuperscript{20} However, in regard to efficacy on the hepatic injury represented in this paper it does not seem also likely to consider dietary fiber as an effective component because the dietary fiber content from the protein concentrate of proso millet used in this study is estimated to be 1.2% in the experimental diet.

Since the role of cytokines in hepatitis has been shown\textsuperscript{9} and it has been suggested that green tea might suppress experimental liver injury through the inhibition of tumor necrosis factor-\alpha-induced apoptosis of hepatocytes,\textsuperscript{7} the role of cytokines may have to be taken into account, but cytokines were not measured in this study.

Concerning the mechanism by which protein of proso millet suppresses a rise in aminotransferase activities induced by D-galactosamine, our recent in vitro experiment found that digestion of proso millet protein was slow compared to that of casein (data not shown). This may suggest that release of amino acids from proso millet protein in intestine could be delayed and lasting. Therefore, an effective amino acid(s) or the ratio of amino acids may act on the liver for a longer time than casein. This would imply a clue to understanding the mechanism which is underlying. More detailed study should be done to identify the mechanism.

In conclusion, the findings reported here demonstrated clearly that the elevation of serum AST, ALT, and LDH activities by D-galactosamine was attenuated by the ingestion of proso millet protein. Since D-galactosamine-induced liver injury is a model of viral hepatic disease, proso millet protein may be useful as a preventive food for a certain kind of hepatitis. Further, these results showed the effectiveness at a lower level (20%) of dietary protein than that (40%) of dietary gluten reported previously. Therefore, proso millet protein may be considered to be another preventive food for liver injury.

Acknowledgments

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