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Liver Injury Following Long-Term Administration of Large Doses of Sake to Rats

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ABSTRACT

The hepatotoxic effect on rats of long-term (55 weeks) administration of sake (a rice wine, 17% ethanol by volume) at large doses (average 12.6 g ethanol/kg body weight/day) was investigated in order to gain an insight into the reasons for the high incidence of liver fibrosis in Japanese alcoholics. Rats grew favorably under the experimental conditions, and daily variations in blood ethanol and acetaldehyde levels ranged from 3.8 to 21.1 mM and from 0 to 3.5 μM, respectively. Fatty and fibrotic liver was shown histologically and biochemically in sake-administered rats.

The major form of alcoholic liver disease in Japan is liver fibrosis[3], whereas in European countries alcoholic hepatitis is more frequent[12]. This geographic difference may be due to variation in the type of alcoholic beverage consumed. Alcoholic liver injury in rats can generally be induced under an isocaloric condition with a liquid diet containing 35% ethanol by caloric content[8]. Giving an ethanol solution (15%, sweetened with 25% sucrose) in place of drinking water can also be used for this purposed[14]. However, attempts to produce cirrhosis in rats by the administration of alcohol supplements to an adequate diet have invariably been unsuccessful.

Our previous observations indicate that sake, but not whiskey or ethanol, in a liquid diet induces hepatic collagen accumulation as well as a slight but significant elevation in hepatic collagenase activity in rats[13]. The results suggest that sake might cause a greater change in collagen metabolism in the liver than ethanol itself. In the present study, the hepatotoxic effects on rats of long-term administration of large doses of sake, instead of ethanol, were investigated in hopes of developing a better animal model of alcoholic liver injury.

MATERIALS AND METHODS

Male Sprague-Dawley rats (Clea Japan, Inc., Tokyo), weighing about 200 g each, were fed a basal diet (CE-2, Clea Japan, Inc., Tokyo) and drank sake (Gekkeikan, first class, original concentration of 17% ethanol, Okura Shuzo Co., Ltd., Nada) in place of drinking water for 55 weeks ad libitum (sake group). Control rats were given tap water containing a small amount of glucose (50 g/liter) and casein (5 g/liter) (control group) as drinking water, since sake contains small amounts of sugar (50 g/kg), protein (5 g/kg) and amino nitrogen (0.31 g/kg). All animals were housed in individual, wire-bottomed cages in air-conditioned rooms (24°C) with an alternating 12 hr dark-light cycle.

Rats were given fivefold-diluted sake (3.4% ethanol) as drinking water for one week, and then given twofold-diluted sake (8.5% ethanol) for one more week.

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Table 1. Nutritional Condition of Control and Sake Groups in the 6th Month of the Experiment

Group	Total calory intake (kcal/kg body weight/day)	Ethanol ingested (g/kg body weight/day)	Percent nutrient calories among total calories			Lipotropic vitamins		
			Protein	Fat	Ethanol #	Choline chloride (mg/day)	Vitamin B_{12} (μg/day)	Inositol (mg/day)
Control	298	0	27.4	11.5	0	221	2.8	46.4
	± 81		± 9.2	± 2.6		± 61	±0.7	± 5.9
Sake	303	12.6	19.3	8.1	29.5	159	2.0	33.2*
	±101	± 3.9	±10.2	± 2.2	±10.6	± 72	±0.5	± 8.0

No. of rats = 4-6. # Calculation is based on 7.1 kcal/g ethanol. Upper and lower figures are the mean and SD of the mean, respectively. Vitamin composition of the basal diet (CE-2) (/100 g): vitamin A-acetate 1,000 IU, 7-dehydrocholesterol 200 IU, tocopherol 4.1 mg, thiamine HCl 1.2 mg, riboflavin 1.4 mg, pyridoxin HCl 1.3 mg, cyanocobalamine 3.2 μg, ascorbic acid 80 mg, d-calcium pantothenate 3.0 mg, nicotinic acid 12.4 mg, folic acid 0.2 mg, choline chloride 256 mg, biotin 26 μg and inositol 53.7 mg. * $p < 0.05$.

Thereafter, undiluted sake was given for 53 weeks. The animals drank sake constantly 2 months after the start of the experiment. The average intake of food and ethanol during a day in the 6th month of the experiment is summarized in Table 1. The daily amount of the basal diet (solid food) ingested was 86.4 and 61.9 g/kg body weight (on average) in the control and sake groups, respectively ($p<0.02$). Ethanol-derived calories were 29.5% of the total calories on the average. Therefore, in the present study, control rats were not isocalorically pair-fed with rats of the sake group.

Variations in blood ethanol and acetaldehyde concentrations in sake-administered rats were measured in one day in the 12th months of the experiment. Venous blood was drawn from the jugular vein at 6:00, 12:00, 18:00 and 24:00 o'clock, and the levels were routinely measured by a head space technique[4]. Serum glutamate pyruvate transaminase (GPT) and ornithine carbamoyl transferase (OCT) activities were determined with a GPT-Kit and OCT-Kit (Wako Pure Chemical Co., Tokyo), respectively. Serum and liver triglyceride (TG) contents were determined by a routine laboratory method[19], and liver hydroxyproline contents according to Rojkind et al[16]. The remaining livers were further processed for light microscopic studies (hematoxylin and eosin, Mallory's aniline blue and Gomori silver impregnation stains).

Differences between mean values were tested for significance using Student's *t*-test. Data were expressed as the mean ± SD.

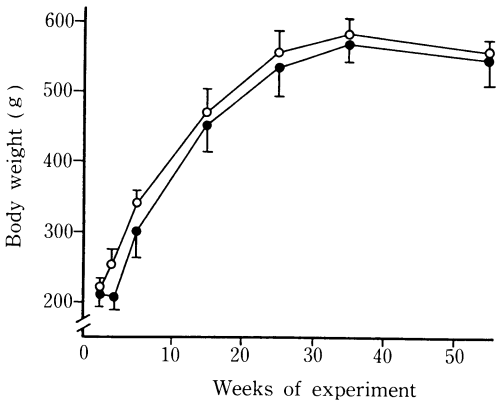

Fig. 1. Body weight of the two groups (control and sake groups). ○—○, Control group (6 rats). ●—●, sake group (4 rats). Vertical lines indicate SD of the mean.

Table 2. Blood Ethanol and Acetaldehyde Concentrations in the Sake Group

Time	Ethanol (mM)	Acetaldehyde (μM)
6:00	3.8±0.6	Not detected
12:00	7.5±2.2	0.9±0.3
18:00	21.1±8.9	3.5±1.9
24:00	8.9±3.4	1.8±0.9

The experiment was performed during the 12th week of the experiment (February, 1985). No. of rats = 4.

RESULTS

Since rats were accustomed to sake during the initial 3 weeks, body weights decreased slightly during the initial 2 weeks but gradually increased thereafter. The average ratio of the fi-

Table 3. Effect of Long-Term Administration of Sake to Rats on Liver Weight, and Serum and Liver Biochemistries

Group	Body weight	Percent liver weight	Serum			Liver	
			GPT	OCT	TG	Hydroxy-proline	TG
	(g)	(%)	(IU/L)		(mg/dl)	(μmol/g)	(mg/g)
Control	550	3.4	18	2.7	31	0.44	4.7
	± 49	±0.5	± 5	± 1.5	±11	±0.14	± 1.4
Sake	566	3.5	17	12.2*	46	0.86*	14.8*
	± 33	±0.2	± 5	± 4.2	±24	±0.41	± 5.8

* $p < 0.05$. No. of rats = 4-6. The experiment was performed during the 55th week. Upper and lower figures are the mean and SD of the mean, respectively.

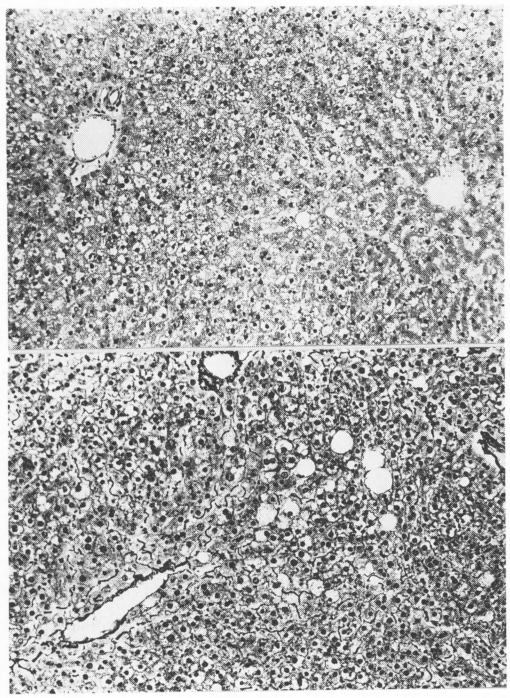

Fig. 2. Light microscopic view of liver in sake-ingested rats. Large and small fat gobules are visualized as empty spheres. Pericellular and central fibrosis is observed. A. Hematoxylin and eosin stain, × 40. B. Gomori silver impregnation stain, × 40.

nal body weight (at 55 weeks) to the peak body weight (at 35 weeks) was 0.95 (Fig. 1). There was no significant difference between the control group and sake group. The liver weight/body weight ratio (%) was not different between the two groups either. No nutritional problem was externally observed in the sake-administered rats.

Daily variations in blood ethanol and acetaldehyde levels in sake-ingested rats are summarized in Table 2. The ethanol concentration reached a maximum (21.1 mM on the average) at 18:00 in the evening, and then gradually decreased. In the early morning at 6:00, the levels were at a minimum (3.8 mM). Blood acetaldehyde levels changed similarly, but the absolute levels were much lower (up to 3.5 μM).

The serum GPT activity was not significantly different between the control and sake-administered rats (Table 3). However, the OCT activity, a sensitive indicator of alcoholic injury of the mitochondria in hepatocytes, increased significantly in sake-administered rats. Significant increases in hepatic triglyceride and hydroxyproline contents were observed in sake-administered rats over control group. Gomori silver stain of liver specimens demonstrated a slight but obvious increase in collagen fibers, predominantly among central vein and pericellular areas (Fig. 2). Fatty liver was also observed, and its degree varied from mild to moderate (centrilobular small globular fat storage) as assessed by hematoxylin and eosin. No hepatocellular necrosis or inflammation was found. The biochemical data closely paralleled the histological findings; triglyceride and hydroxyproline contents in the liver corresponded to fat and fibrosis in the histological sections.

DISCUSSION

Rats can drink sake for longer than a year at a dose which corresponds to 630 g ethanol daily for 34 years in a 50-kg human (the average life span of rats and humans is assumed to be 2 and 60 years, respectively). The drinking style is also similar to that of patients with alcohol dependence, who drink all day long. In the present study, sake-administered rats took a smaller

amount of calories, protein, fat, vitamins and minerals from solid food than rats of the control group. Therefore, in this model, the role of factors besides ethanol, particularly nutritional factors, remains undetermined. However, the daily intake of choline chloride (control group, 221.2 mg = 74 mg/100 kcal, and sake group, 158.5 mg = 52.3 mg/100 kcal) and other vitamins were sufficiently over the daily requirement, and lipotropy (40 mg per 100 kcal or more) was sufficient to promote growth and to prevent abnormal accumulation of hepatic fat[1]. Therefore, the conditions of the present study seem unlikely to have caused the rats to be severely deficient in vitamins, in particular, lipotropic vitamins (vitamin B_{12}, inositol and biotin) (Table 1). The combination of the consumption of large doses of alcohol and the unsupplemented basal diet in rats provides a model which duplicates the relative deficiency of dietary nutrients, common in heavy alcoholics, which is one factor leading to alcoholic liver disease in humans.

Alcoholic patients have higher blood acetaldehyde levels (a greater increase in acetaldehyde than in ethanol levels) after an ethanol load than non-alcoholic subjects[5]. This fact may indicate that the capacity to metabolize acetaldehyde is impaired due to the decreased activity of aldehyde dehydrogenase, as observed in the cytosole fractions of hepatic needle-biopsy specimens[17]. Such an abnormality may predispose the patient to alcoholic liver injury. In the present study, blood ethanol concentrations ranged from 4 to 21 mM, and acetaldehyde levels from 0 to 4 μM. The ratio of blood acetaldehyde levels (μM) to ethanol levels (mM) ranged from 0.12 to 0.20 (within normal limits), indicating that no abnormal metabolism of acetaldehyde existed in the liver[20]. It is not known why impaired metabolism of acetaldehyde and severe liver dysfunction does not occur in rats even after large doses of ethanol. Different intracellular distribution of aldehyde dehydrogenase may be involved. The enzyme is mainly located in the mitochondria in rats, but in humans, it is located in the cytosole fraction of the hepatocytes[18], which allows to play a more important role in acetaldehyde oxidation.

Liver injury obtained in the present experiment included fatty and fibrotic liver, but neither hepatitis nor cirrhosis. The previous reports on ethanol-induced liver injury in experimental animals are conflicting. Ethanol feeding of rats for 6 months resulted in fatty liver but no or only a mild increase in hepatic collagen content[11]. Porta et al[14] reported that the consumption of appreciable amounts of alcohol (sweetened by 25% sucrose) for 7 months did not produce liver damage in rats given adequate dietary protection. Lieber et al[9] demonstrated that chronic feeding of 50% of the calories of a liquid diet as ethanol to baboons resulted in the development of hepatic cirrhosis in one-third of the animals studied, suggesting that the toxic effect of ethanol itself is the principal cause of cirrhosis in alcoholism. In this experiment, however, ethanol may have induced malabsorption or produced an increased nutritional requirement (choline[6] and methionine[2]), thus leading to a relative deficiency in certain nutrients such as choline. Mezey et al[10] reported that monkeys receiving large doses of alcohol and a nutritionally adequate diet for 4 years developed only slight fatty infiltration without fibrotic change of the liver. Hepatic collagen accumulation was not observed biochemically or electron microscopically. Similarly, monkeys fed a diet having fivefold higher choline content (500 mg of choline chloride/liter) than Lieber's diet (100 mg choline chloride/liter) developed only mild fatty infiltration[15]. The higher choline content of the monkey diet may protect against ethanol-induced development of fibrosis and cirrhosis.

Acetaldehyde has been implicated in the pathogenesis of liver injury associated with ethanol consumption. Fatty acid ethylesters[7] and macrophage-derived cytotoxic macromolecules[22] have been proposed to act during the initial stage in the pathogenesis of alcoholic liver disease. Our previous studies demonstrated collagen accumulation in livers from sake-administered rats, but not in livers from whiskey and ethanol-treated rats. Although the mechanisms are not known, one possible explanation for sake-induced hepatic fibrogenesis is the presence of higher alcohols contained in sake, e.g., β-phenetyl alcohol (75 mg/kg sake) and n-propanol (120 mg/kg). β-Phenetyl alcohol potentiated carbon tetrachloride hepatotoxicity[21]. However, further study will be needed to elucidate the

detailed mechanism of sake-induced liver fibrosis.

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