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Liver regeneration after partial hepatectomy in rat is more impaired in a steatotic liver induced by dietary fructose compared to dietary fat

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ABSTRACT

Hepatic steatosis (HS) has a negative effect on liver regeneration, but different pathophysiologies of HS may lead to different outcomes. Male Sprague-Dawley rats were fed a high fructose (66% fructose; H-fruc), high fat (54% fat; H-fat), or control chow diet for 4 weeks. Based on hepatic triglyceride content and oil red O staining, HS developed in the H-fruc group, but was less severe compared to the H-fat group. Hepatic mRNA expression levels of fatty acid synthase and fructokinase were increased and those of carnitine palmitoyltransferase-1 and peroxisome proliferator-activated receptor-α were decreased in the H-fruc group compared to the H-fat group. Liver regeneration after 70% partial hepatectomy (PHx) was evaluated by measuring the increase in postoperative liver mass and PCNA-positive hepatocytes, and was impaired in the H-fruc group compared to the H-fat and control groups on days 3 and 7. Serum levels of tumor necrosis factor-α, interleukin-6 and hepatocyte growth factor did not change significantly after PHx. In contrast, serum TGF-β1 levels were slightly but significantly lower in the control group on day 1 and in the H-fat group on day 3 compared to the level in each group on day 0, and then gradually increased. However, the serum TGF-β1 level did not change after PHx in the H-fruc group. These results indicate that impairment of liver regeneration after PHx in HS is related to the cause, rather than the degree, of steatosis. This difference may result from altered metabolic gene expression profiles and potential dysregulation of TGF-β1 expression.

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1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is a common clinical problem that results from a variety of metabolic disorders, including obesity, diabetes mellitus, and dyslipidemia. Fatty liver is present in 9.6–29% of autopsy cases [1,2], and moderate hepatic steatosis (30–60% fatty hepatocytes) is found in 6% of autopsies after accidental deaths [2]. The 5-year survival rate of patients with nonalcoholic steatohepatitis (NASH) which is a progressive liver disease in NAFLD, is 75% [3]. Complications of NASH, including cirrhosis and hepatocellular carcinoma (HCC), are expected to increase with the growing epidemic of diabetes and obesity [4]. However, it is unclear which cases of NAFLD are likely to progress to severe fibrosis and HCC.

Obesity has escalated to epidemic proportions worldwide and many causes have been suggested, including dietary components. Excessive caloric intake has been related to high-fat foods and increased portion sizes. The increase in consumption of high-fructose corn syrup (HFCS) has a temporal relationship with the epidemic of obesity, and overconsumption of HFCS in calorically sweetened beverages may have played a role in the epidemic [5]. Excessive fructose and sucrose (which contains 50% fructose) intake is a risk factor for developing NAFLD and may be associated with NASH [6,7]. Therefore, abnormal metabolism may be a pathophysiological feature of hepatic steatosis.

Many growth factors and cytokines such as hepatocytes growth factor (HGF) stimulate liver regeneration. In contrast, transforming growth factor (TGF)-β is a potent inhibitor of hepatocyte proliferation [8] and is involved in growth arrest once the liver has reached an appropriate functional mass [9]. Liver regeneration requires orchestrated functions of these molecules in appropriate amounts and at appropriate locations and times [10]. Hepatic steatosis is associated with an increased incidence of complications and mortality after liver resection or transplantation [11], and these outcomes are associated with a decrease in the ability of the liver to regenerate in NAFLD [12]. However, direct experimental evidence of a relationship between a high fructose or high fat diet and cytokine expression in liver regeneration is lacking.

In this study, we investigated mechanistic differences in liver regeneration in fatty liver and metabolic abnormalities caused by

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dietary fructose or fat. The results may contribute to elucidation of the mechanisms that affect the outcome of NAFLD.

2. Materials and methods

2.1. Animals and diets

Male Sprague-Daley rats of age 7 weeks were obtained from Kyudo (Kumamoto, Japan). Rats were acclimatized to laboratory conditions for at least 7 days at a constant temperature of 24 °C with a 12-h light-dark cycle, and fed a standard rodent diet (CE-2; Kyudo, Kumamoto, Japan) and water ad libitum. All animal experiments were approved by the Institutional Animal Care and Use Committee guideline of Kagoshima University.

After acclimatization, rats were placed in groups that were fed three different diets for 4 weeks ad libitum: control chow (10% of total calories as fructose, 12% as fat, and 19% as protein; control group), a high fat diet (8% of total calories as fructose, 54% as fat, and 15% as protein; H-fat group), and a high fructose diet (66% of total calories as fructose, 11% as fat, and 19% as protein; H-fruc group). The diets were obtained from Nosan Corporation (Kanagawa, Japan). All diets were prepared as a powder, and diet weights and intake energy were measured every other day.

A 70% partial hepatectomy (PHx) was performed after 4 weeks of intake of each diet. Rats were anesthetized by ether inhalation. After midline laparotomy, the median and left hepatic lobe were pushed out and ligated with 2.0 silk sutures, and resected as described previously [13]. Resected livers were weighed and used for RNA extraction, or thin slices were immersed in 10% formalin and embedded in paraffin to make 4-μm sections for routine staining with hematoxylin and eosin. After PHx, all rats were fed with a standard CE-2 diet and sacrificed on days 1, 3, and 7 after PHx. Blood was collected by vena cava puncture and centrifuged. The resulting serum was stored at −80 °C. The remaining right hepatic lobes were removed and weighed.

2.2. Evaluation of insulin sensitivity

Fasting blood glucose (FBG) and serum immunoreactive insulin (IRI) levels were determined by ELISA (Morinaga Institute of Biological Science, Kanagawa, Japan). The homeostasis model assessment-insulin resistance index (HOMA-IR) was calculated from FBG (mg/dl) and IRI (mU/ml) levels as FBG × IRI/405. After 4 weeks diet intake, rats in all groups underwent an intraperitoneal insulin tolerance test (ITT), which was performed after a 4-h fast by intraperitoneal injection of 0.5 U insulin per kg of body weight. Blood was drawn from a tail vein after injection for measurement of plasma glucose concentrations [14].

2.3. Assessment of hepatic steatosis

Hepatic steatosis was assessed using hepatic triglyceride levels and oil red O staining. Hepatic lipids were extracted with chloroform-methanol and measured enzymatically using a commercial kit (L-type Wako TGH, Wako Pure Chemical Industries, Osaka, Japan). Oil red O staining was performed to evaluate accumulation of fat droplets in hepatocytes in frozen liver sections. The ratio of the oil red O-stained area to the total area was determined using Image J software (http://rsb.info.nih.gov/ij/index.html).

2.4. Assessment of hepatic mRNA levels of genes associated with metabolism of lipids and fructose

The relative levels of specific mRNAs in resected liver on day 0 were assessed by real-time quantitative polymerase chain reaction (RT-qPCR) using Syber Premix Ex Taq (TaKaRa Bio, Shiga, Japan). Total RNA was extracted from each liver using Isogen (Nippon Gene, Tokyo, Japan). Expression levels of target genes were calculated relative to the level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which was used as an endogenous control gene to normalize the target gene expression levels. All procedures were performed according to manufacturers' instruction. mRNA levels were determined for carnitine palmitoyltransferase (CPT)-1, peroxisome proliferator-activated receptor (PPAR)-α, AMP-activated protein kinase (AMPK), fatty acid synthase (FAS), fructokinase (FK), and cytochrome P450 2E1 (Cyp2E1). Primer sequences are listed in Table 1. The PCR primers were obtained from TaKaRa Bio Inc.

Table 1
Primers used for PCR.

Gene	Gene Bank Number	Primer sequences	
PPARα	NM_013196.1	Forward	5′-GACAAGGCCTCAGGATACCACTATG-3′
		Reverse	5′-TTGCAGCTTCGATCACACTTGTC-3′
AMPK	NM_019142.1	Forward	5′-GGCTCGCCCAATTATGCTG-3′
		Reverse	5′-AGAGTTGGCACGTGGTCATCA-3′
CPT-1	NM_031559.2	Forward	5′-CGCTCATGGTCAACAGCAACTAC-3′
		Reverse	5′-TCACGGTCTAATGTGCGACGA-3′
FK	NM_031855.3	Forward	5′-CCAGCTGTTCGGCTATGGAGA-3′
		Reverse	5′-CACAGCCAACCAGATGCTTCA-3′
FAS	NM_017332.1	Forward	5′-CACAGCATTCAGTCCTATCCACAGA-3′
		Reverse	5′-TCAGCCCAGGCACAGATGAG-3
Cyp2E1	NM_031543	Forward	5′-CCTACATGGATGCTGTGGTG-3′
		Reverse	5′-CTGGAAACTCATGGCTGTCA-3′
GAPDH	NM_017008.3	Forward	5′-GGCACAGTCAAGGCTGAGAATG-3′
		Reverse	5′-ATGGTGAAGACGCCAGTA-3′

2.5. Assessment of liver regeneration

The liver regeneration rate (%) was calculated as $100 \times \{C - (A - B)\}/A$, where A is the estimated total liver weight at the time of partial hepatectomy, B is the excised liver weight, and C is the weight of the regenerated liver at the final resection [13]. Liver samples were also stained for proliferating cell nuclear antigen (PCNA). After fixation with formalin and paraffin embedding, tissue was incubated with anti-PCNA (clone PC10; Dako, Tokyo, Japan). The PCNA proliferation index was determined in at least 1000 hepatocytes in each group. Data are expressed as the percentage of PCNA-stained hepatocytes out of the total number of hepatocytes examined.

2.6. Assessment of serum markers

Serum level of high sensitivity C-reactive protein (hs-CRP; Rat CRP ELISA, Life Diagnostics, West Chester, PA) was determined using commercially available kits. Serum levels of tumor necrosis factor (TNF)-α, interleukin (IL)-6, TGF-β1 (all R&D Systems, Minneapolis, MN) and HGF (Institute of Immunology, Tokyo, Japan) were measured on days 0, 1, 3 and 7.

2.7. Statistical analysis

Statistical comparison among groups was performed using one-way ANOVA or repeated ANOVA and a post-hoc Fisher PLSD test. A Student t-test was used for comparison of two groups. $P < 0.05$ was considered statistically significant. Data are presented as the mean ± standard error (SE) or standard deviation (SD).

3. Results

3.1. Metabolic parameters in rats fed with a high fat or high fructose diet

Total energy intake for 4 weeks and body weight (mean body weight relative to basal body weight) did not differ between the H-fat and H-fruc groups, but were significantly higher in these groups compared to the control group ($P < 0.05$) (Table 2). The serum triglyceride level was higher in the H-fruc group than in the H-fat and control groups. There were no significant differences in serum adiponectin levels among the three groups, but serum leptin levels were significantly higher in the H-fat and H-fruc groups compared to the control group ($P < 0.05$). Serum hs-CRP was significantly higher in the H-fruc group compared to the H-fat group ($P < 0.05$). FBG was significantly higher in the H-fruc group than in the control group ($P < 0.05$). IRI and HOMA-IR showed a tendency to be higher in the H-fruc group (Table 2) and the H-fruc group had significantly more severe insulin resistance based on ITT results ($P < 0.001$) (Fig. 1A).

Table 2
Metabolic parameters and serum biochemical markers after 4 weeks of diet intake.

	Control	H-fat	H-fruc
Body weight (% basal BW)	140.3 (7.5)	170.7 (6.3)*	171.7 (16.4)*
Total energy intake (kcal)	2108.8 (248.4)	2530.1 (72.0)*	2419.3 (84.1)*
ALT (IU/L)	33.6 (4.5)	22.6 (1.8)	24.8 (5.0)
Triglyceride (mg/dl)	67.2 (23.36)	62.7 (21.82)	145.0 (48.19)**
Free fatty acids (mEq/L)	5.63 (3.43)	8.03 (2.55)	6.90 (3.25)
Fasting blood glucose (mg/dl)	108.0 (25.0)	116.8 (18.8)	144.8 (16.3)*
Insulin (ng/ml)	3.73 (2.37)	2.76 (1.05)	4.74 (2.84)
HOMA-IR	1.09 (0.90)	0.78 (0.21)	1.77 (1.14)
Adiponectin (μg/ml)	1.71 (0.61)	2.80 (0.62)	2.36 (0.51)
Leptin (ng/ml)	0.61 (0.15)	1.64 (0.62)*	1.45 (0.50)*
Hs-CRP (mg/ml)	0.13 (0.07)	0.12 (0.05)	0.17 (0.06) ***

Data are shown as mean (standard deviation) after 4-week intake of the three diets. ALT, alanine aminotransferase; HOMA-IR, homeostasis model of assessment for insulin resistance index; and Hs-CRP, high sensitivity C-reactive protein.
* $P < 0.05$ *vs.* Cont.
** $P < 0.01$ *vs.* Cont and H-fat.
*** $P < 0.05$ *vs.* H-fat.

3.2. Severity of hepatic steatosis in the H-fat and H-fruc groups

Micro and macrovesicular steatosis were clearly visible on HE staining of livers from H-fat and H-fruc rats (Fig. 1B). The hepatic fat area (%) calculated by oil red O staining and the hepatic triglyceride content were significantly higher in the H-fat group compared to the H-fruc and control groups (Fig. 1C and D).

3.3. Hepatic mRNA expression levels in the H-fat and H-fruc groups after PHx

Hepatic mRNA levels of FK and FAS significantly increased in the H-fruc group and significantly decreased in the H-fat group compared to the control group (Fig. 2). In contrast, mRNA levels for CPT-1, PPAR-α, AMPK and Cyp2E1 were significantly lower in the H-fruc group compared to the H-fat group ($P < 0.05$) (Fig. 2).

3.4. Liver regeneration after PHx in the H-fat and H-fruc groups

Liver regeneration and the PCNA labeling index at 3 and 7 days after PHx were significantly lower in the H-fruc group than in the

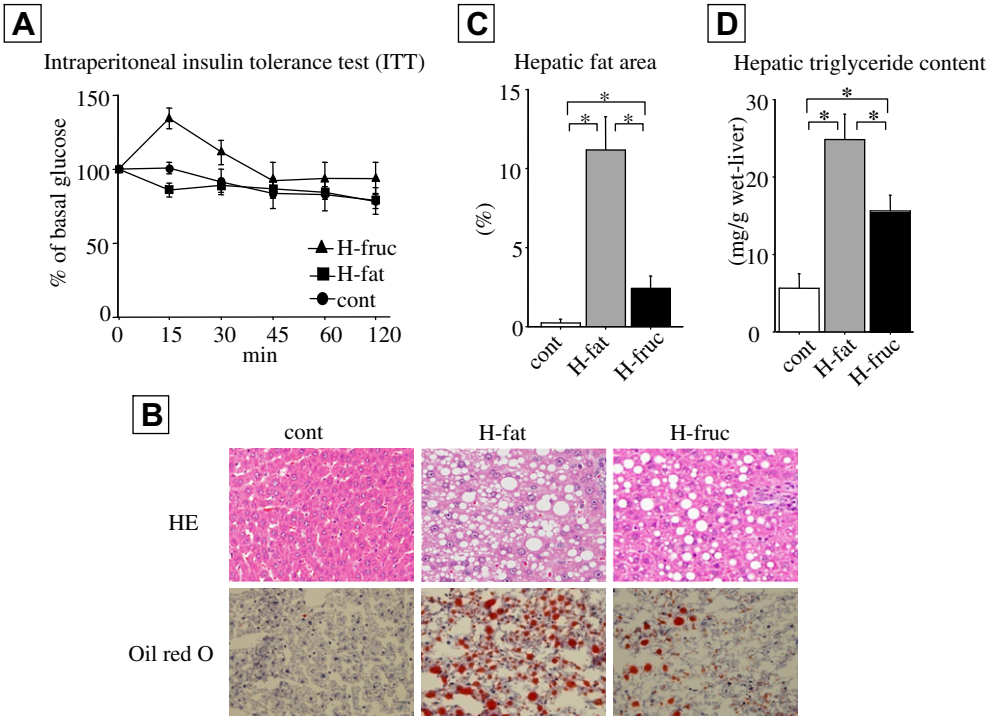

Fig. 1. Insulin tolerance test and histological evaluation of fatty liver in rats fed a chow control diet (Cont), a high-fat diet (H-fat) or a high-fructose diet (H-fruc). (A) An intraperitoneal insulin tolerance test (ITT) was performed after 4 weeks intake of each diet. After a 4-h fast, mice were injected intraperitoneally with 0.5 U of insulin per kg body weight. Glucose was measured in blood sampled from a tail vein at 0, 15, 30, 45, 60, and 120 min. H-fruc rats had significantly more severe insulin resistance compared to control and H-fat rats ($n = 5–6$, $P < 0.001$, repeated ANOVA). (B–D) Fatty liver was evaluated by hematoxilin-eosin staining (B), image analysis using oil red O staining (C), and hepatic triglyceride content (D). Fatty infiltration of the liver was most severe in the H-fat group ($n = 6$), and that in the H-fruc group ($n = 6$) was higher than in the control group ($n = 5$). *$P < 0.01$ (one way ANOVA).

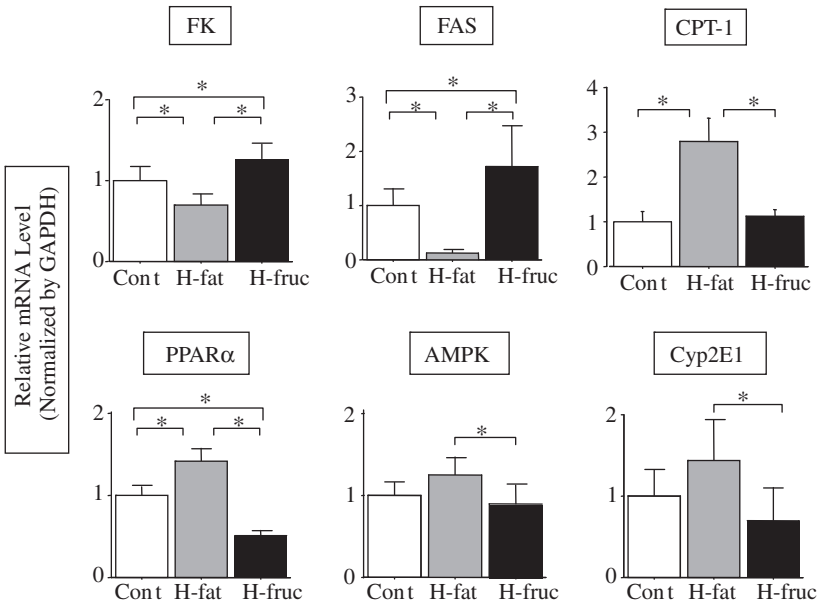

Fig. 2. Hepatic mRNA expression of genes involved in metabolism assessed by RT-qPCR (each $n = 5–6$) after 4 weeks of intake of the three diets. Expression levels of CPT-1, PPAR-α, AMPK and Cyp2E1 were significantly suppressed in the H-fruc group compared to the H-fat group. FK and FAS were significantly increased in the H-fruc group compared to the H-fat or control group. In contrast, FAS and FK were significantly decreased in the H-fat group compared to the control group. $^{*}P < 0.05$ (one way ANOVA).

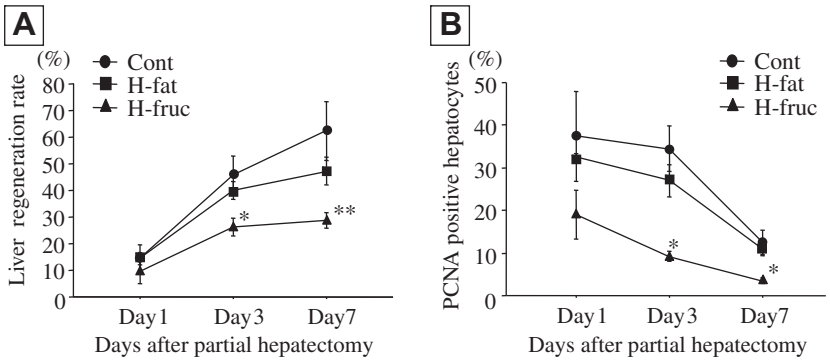

Fig. 3. Liver regeneration after 70% partial hepatectomy in rats fed three different diets (each $n = 4–6$). (A) The liver regeneration rate (%) was lowest in the H-fruc group on days 3 and 7. This rate was also lower in the H-fat group compared to the control group, but the difference was not significant. (B) PCNA labeling showed that the mitotic index was significantly lower in the H-fruc group compared to the other two groups. The PCNA proliferation index was determined in at least 1000 hepatocytes. Values are shown as the mean ± standard error ($^{*}P < 0.05$ *vs.* control and H-fat group, $^{**}P < 0.05$ *vs.* control group).

H-fat and control groups (Fig. 3A and B), but only showed a tendency to be lower in the H-fat group compared to the control group.

3.5. Serum biochemistry and cytokine levels just before and after PHx

The serum ALT level in the H-fruc group on day 1 after PHx was significantly higher than that in the H-fat group, but similar to the control group, and there was no difference among the three groups on days 0, 3 and 7 (Fig. 4A). Serum AST, ALP and albumin did not differ significantly before and after PHx. The serum TNF-α level just before PHx showed a tendency to be higher in the H-fruc group, and serum IL-6 and HGF did not differ significantly among the three groups throughout the course of the study (Fig. 4B). In contrast, the serum TGF-β1 level in the control group on day 1 and in the H-fat group on day 3 were significantly lower than the respective levels on day 0, and then gradually increased (Fig. 4B). However, serum TGF-β1 levels were not suppressed after PHx in the H-fruc group.

4. Discussion

Studies in animal models and patients with fatty liver have shown impaired liver regeneration that worsens in severe fatty liver or NASH [14–16]. However, the mechanism of impaired liver regeneration in fatty liver is not fully elucidated. In our study, we found that liver regeneration was more impaired in rats given a high fructose diet (H-fruc group) compared to those given a high fat diet (H-fat group), although the severity of hepatic steatosis in the H-fruc group was lower than that in the H-fat group. These results provide the first evidence that liver regeneration is affected by the cause, rather than the severity, of hepatic steatosis. In addition, insulin resistance, altered metabolic gene expression profiles, and abnormal expression of TGF-β1 might be involved in the mechanism of the delay in liver regeneration.

Sucrose, the major component of sugar, is hydrolyzed to glucose and fructose, and increased consumption of sugar and fructose is related to the increase in the overweight population. Fructose in soft drinks and sweetened beverages may be strongly related to

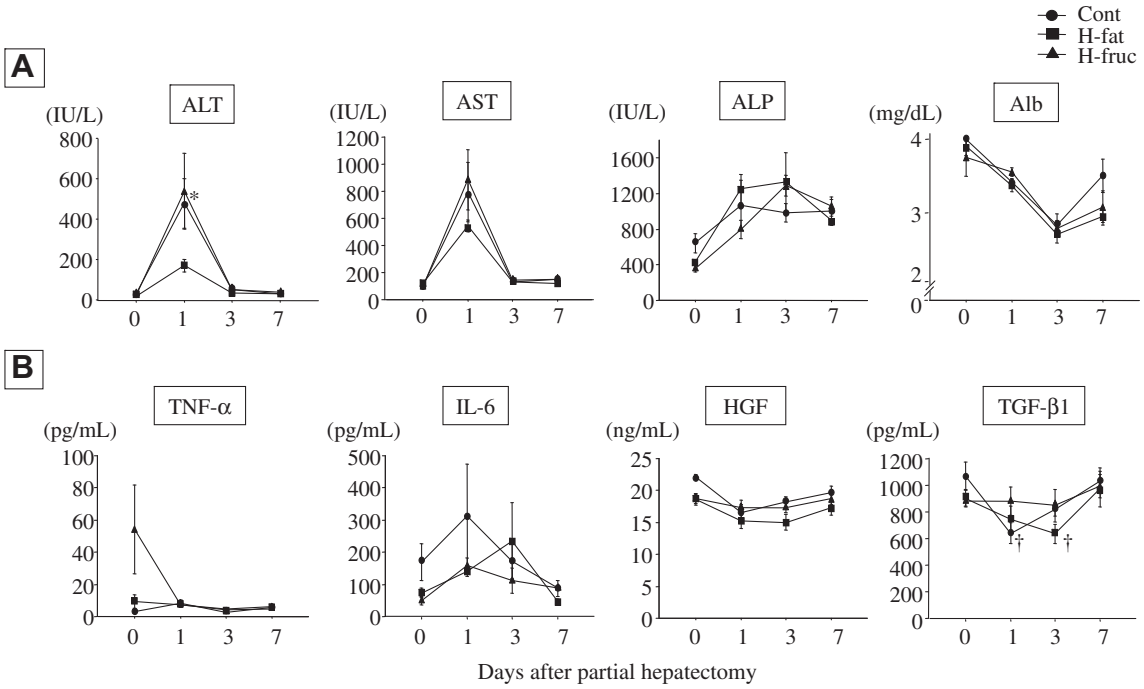

Fig. 4. Course of serum chemistry and cytokine levels during liver regeneration after partial hepatectomy (each $n = 4–6$). (A) Hepatocellular damage assessed by serum ALT in the H-fruc and control group on day 1 was higher than that in the H-fat group. However, AST, ALP and albumin did not differ among the three groups during the course of the study. (B) The serum TNF-α level just before partial hepatectomy tended to be higher in the H-fruc group. In contrast, serum TGF-β1 levels in the control group on day 1 and the H-fat group on day 3 were lower than the respective levels on day 0. Serum TGF-β1 levels in the H-fruc group did not change significantly over the course of the study. Values are shown as the mean ± standard error. $^{*}P < 0.05$ *vs.* H-fat group.

obesity [17,18], and excessive intake of sugar and fructose also cause insulin resistance [19]. An overweight status, obesity and insulin resistance are important risk factor for NAFLD, and fructose consumption has been associated with increased hepatic fibrosis in patients with NAFLD [7]. Collectively, these results suggest that sucrose, sugar and fructose are associated with the pathogenesis of NAFLD. Fructose intake is 2- to 3-fold higher in patients with NAFLD compared with gender, age and body mass index-matched controls, and hepatic mRNA expression of FK and FAS is increased in patients with NAFLD [6]. Fructose is absorbed through glucose transporter 5 (GLUT5) in the jejunum. Excess fructose is taken to the liver and phosphorylated by FK for use in gluconeogenesis and lipid production. However, since lipid production from fructose is not regulated, excessive fructose induces production of a large amount of TG [17,18]. The serum TG level and hepatic mRNA levels of FK and FAS in this study were higher in the H-fruc group compared to those in the H-fat or control groups (Table 2 and Fig. 2A). Thus, our experimental model resembles human NAFLD in these respects.

Excessive intake of sugar and fructose may have more adverse effects than excessive intake of a high fat diet. The mortality of rats with hypertension was greater after intake of a high fructose diet compared to a high fat diet [20], and a high sugar diet has been shown to induce cardiac depression and increase mortality [21]. We have also found that liver regeneration was more impaired in the H-fruc group than in the H-fat group for the first time, although these groups did not differ in total energy intake and increase of body weight. Pioglitazone, a drug that improves insulin resistance, has been shown to improve delayed liver regeneration after hepatectomy in KK-Ay mice, which develop insulin resistance [22]. In our study, insulin resistance in the H-fruc group was higher than that in the H-fat group before PHx (Fig. 1A). Thus, the different rates of liver regeneration in the two groups might have been caused by a difference in insulin resistance.

In the liver, ATP is produced as an energy source by carbohydrate metabolism. ATP is also produced by β-oxidation of fatty acids. Fatty acids are oxidized by mitochondria or peroxisomes and are finally transformed to acetyl-CoA. In β-oxidation, ATP is produced from fatty acid. In this process of fatty acid oxidation, CPT-1 is the rate-controlling enzyme, and nuclear receptor transcription factors such as PPAR-α and AMPK regulate expression of CPT-1. In our study, expression of CPT-1 was decreased in rats fed a high fructose diet, and expression of PPARα and AMPK also decreased. The decreased expression of PPAR-α, AMPK and CPT-1 may reduce ATP production [23,24]. On the other hand, expression of FK in the liver increased in H-fruc rats, and this increases consumption of ATP and leads to ATP depletion in the liver [6,25]. Thus, a high fructose diet induced a decrease in ATP production due to a decrease in fatty acid β-oxidation and an increase in ATP consumption due to increased FK expression. These changes produce an overall decrease in ATP, which is essential for liver regeneration, compared to high-fat diet. Delayed liver regeneration has also been shown in PPAR-α null mice [26] and may involve decreased expression of cyclin D1 and c-Myc, which are G1/S phase regulators, and increased expression of IL-1β. Thus, the greater impairment of liver regeneration in the H-fruc group compared to the H-fat group was due to alteration of gene expression profiles.

Liver regeneration after administration of carbon tetrachloride is impaired via abnormal expression of IL-6 and TNF in leptin-deficient ob/ob mice [27]. Picard et al. examined liver regeneration after PHx in methionine-low, choline-deficient diet-fed rats and Zucker fatty rats, and suggested that abnormal function of the leptin receptor might be related to impaired liver regeneration, rather than fatty liver itself [28]. We did not examine leptin receptor expression in this study, but the serum leptin and adiponectin levels did not differ between the H-fruc and H-fat groups. This suggests that changes in the leptin pathway were

not involved in the different rates of liver regeneration in the two groups.

Serum TGF-β1 levels were slightly but significantly lower on day 1 in control rats and on day 3 in H-fat rats, compared to the respective levels just before PHx. However, serum TGF-β1 in the H-fruc group did not change in liver regeneration from day 0 to day 7 (Fig. 4). TGF-β1 is a potent inhibitor of cell proliferation. In a small-for-size liver graft model, hepatic TGF-β1 increased slightly after transplantation of full-size grafts and then decreased rapidly. TGF-β1 progressively increased after transplantation of quarter-size grafts and hepatocyte proliferation was markedly inhibited [10]. TGF-β1 enhanced by LPS also plays an important role in the mechanism of hepatic failure due to infection after hepatectomy, especially in inhibition of liver regeneration [29]. In contrast, inhibition of TGF-β/Smad signaling improves regeneration of small-for-size rat liver grafts, and a single dose of anti-TGF-β1 monoclonal antibody enhances liver regeneration after partial hepatectomy in biliary-obstructed rats [30]. It is possible that dysregulation of TGF-β expression was one of the causes of delayed liver regeneration in the H-fruc rats. However, there were only small differences in serum TGF-β1 levels in control and H-fat rats after PHx. In addition, we did not examine the TGF-β/Smad signaling pathway in the liver of these rats. Therefore, a further study is needed to elucidate the association of dysregulation of TGF-β expression and delayed liver regeneration in the H-fruc rat.

In conclusion, fatty liver was less severe in the H-fruc group than in the H-fat group, but liver regeneration was more impaired in the H-fruc group. These results suggest that impaired liver regeneration in fatty liver is related to the cause, but not necessarily to the degree, of hepatic steatosis. Dysregulation of genes associated with metabolism or ATP production and potential dysregulation of TGF-β1 expression may contribute to impairment of liver regeneration after PHx in hepatic steatosis induced by a fructose diet.

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