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ORIGINAL ARTICLE

Protective effect of aqueous extracts from *Rhizopus oryzae* on liver injury induced by carbon tetrachloride in rats

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ABSTRACT

Hepatoprotective effects of *Rhizopus oryzae*/ U-1 aqueous extract (RU) were demonstrated in carbon tetrachloride (CCl_4)-induced liver-injured rats. In order to investigate the RU effects, the rats were administered RU at a dose of 10 or 100 mg/kg of body weight for 10 days before induction of the liver injury by oral administration of CCl_4 (125 mg/kg body weight). (i) Pretreatment with RU caused a significant decrease in serum lactate dehydrogenase (LDH), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities that were increased by the administration of CCl_4. (ii) RU pretreatment (100 mg/kg) increased 5-bromo-2′-deoxyuridine incorporation at 48 h after CCl_4 treatment in hepatocytes. (iii) Histological hematoxylin and eosin staining of the liver showed that RU pretreatment reduced the damage induced by CCl_4 administration. (iv) Reverse transcriptase PCR analysis showed RU retreatment caused a transient but significant increase in hepatocyte growth factor (HGF) and a sustained and significant increase in insulin-like growth factor-I (IGF-I) gene expression in hepatocytes injured by CCl_4 treatment. From these results, we conclude that oral pre-administration of RU was effective to suppress liver injury induced by the subsequent oral CCl_4 administration, and RU-induced increase in IGF-I and HGF gene expression may be, even in part, involved in biological actions of RU in rats.

Key words: *insulin-like growth factor 1 (IGF-1), liver injury, Rhizopus oryzae.*

INTRODUCTION

Rhizopus fungi have traditionally been used in China for fermentation to produce alcohol, and to produce tempeh and fermented soy foods in Southeast Asia (Meussen *et al*. 2012). *R. oryzae*/ U-1 aqueous extract (RU) administration improves reproductive functions, such as improving fertilization rate, fecundity and hatchability in quails and chickens (Zhang *et al*. 1999), extending the mating season in rats (Higuchi *et al*. 1979), synthetically advancing ovarian steroid hormones in rats and rabbits (Higuchi *et al*. 1979; Saito *et al*. 1980; Horiuchi *et al*. 1985) and improving the rate of pregnancy in cows (Umezu *et al*. 1973). In addition, oral RU administration can protect against *Salmonella* infection by inducing activation of peripheral monocytes and improving the Th1/Th2 balance using models of *Salmonella* infection in rats (Suzuki *et al*. 2007).

Recently, while modern dairy cows have acquired high lactational performance to improve nutritional management and breeding, conception rate has decreased and calving intervals extended by their decreased reproductive efficiency (Lucy 2001). The long calving intervals cause extremely serious economic losses for dairy farmers. The major factor of decrement of reproduvtive efficiency is not resolved clearly, and it has been suggested that the cause was the loss of the energy balance by improving lactational performance and body size of modern dairy cows.

Meanwhile, many findings have focused on the influence of a hormone and cytokine secreted by liver in the genitals have been reported for several years. The GH-IGF system is the endocrine system related to reproduction. The "GH-IGF system" consists of growth hormone (GH), insulin-like growth factor (IGF) and

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IGF binding protein (IGFBP). Coordinated function of GH and IGF-1 is involved in reproductive functions such as gonadotrophic secretion and ovogenesis (Silva *et al*. 2009). Therefore, lack of IGF-1 suppresses the expression of follicle stimulation hormone receptor in the granulosa cells (Zhou *et al*. 1997) and finally causes ovulation disorder by halting follicular growth in the IGF-1 knockout mouse (Baker *et al*. 1996).

A change in the GH-IGF system has been suggested as important in periparturient or lactating dairy cows (Lucy 2001; Kawashima *et al*. 2007; Rhoads *et al*. 2008a, b). It is reported that GH receptor (GHR) and IGF-1 gene expression in the liver of delivered Holstein cows receiving artificial insemination treatment is higher in pregnant dairy cows than nonpregnant dairy cows. Because a difference was not seen to the expression of GHR gene in the uteruses of both, it seems influence on the health condition of the liver gives to a fertility rate after the artificial insemination may exist. GH sensitivity by increase of GHR induces high production of IGF-1 and activates a uterus secretion line and improves the uterus environment for an embryo to grow. In postpartum ovulation in dairy cows, follicular growth is affected by highly concentrated IGF-1 secreted by the liver but not dominant follicular size or blood flow to the ovary (Kawashima *et al*. 2007). Postpartum, the first dominant follicular ovulation is a factor in early recovery of ovarian function. It is associated with a high reproduction ability in high milk-producing dairy cows. IGF-1, which is produced in the liver, closely participates in this ovulation. These reports suggest the importance of liver function in improvement of dairy cow fertility. Although improvement in the rate of pregnancy in cows with RU has already been reported (Umezu *et al*. 1973), the mechanism for this effect has not been fully elucidated. Furthermore, to our knowledge, no study related to the potential benefit of RU on damaged liver has been reported.

Improvement effect of breeding performance by RU is already reported as described above, but the mechanism is not elucidated. We hypothesized that the actions of RU may involve not only direct but also indirect ones such as via IGF-I synthesis and release from the liver. Particularly, the liver is the major organ that synthesizes and releases IGF-I, which is known to stimulate cell proliferation. In order to assess the hypothesis, therefore, we used rats with carbon tetrachloride (CCl_4)-induced liver injury as a model case. The actions of RU pre-administration on liver injury were elucidated by analysis of the suppressing effects of RU on serum enzyme activities such as lactate dehydrogenase (LDH), alanine aminotransferase (ALT) and aspartate aminotransferase (AST), hepatocyte incorporation of 5-bromo-2′-deoxyuridine (BrdU), histological analysis, as well as gene expression of hepatocyte growth factor (HGF) and IGF-1.

MATERIALS AND METHODS

Chemicals

CCl_4 was purchased from Wako Pure Chemical Inds. Ltd. (Osaka, Japan). BrdU and phosphate-buffered saline (PBS, pH 7.0) were obtained from Nacalai Tesque Inc. (Kyoto, Japan).

Fungal strain and aqueous fungus extracts

R. oryzae/ U-1 used for this study was a fungus isolated from fermentation using ground barley and bran as the culture medium. Before incubation at 24°C for 4 days, *R. oryzae*/ U-1 was inoculated into malt extract medium (20 g malt extract, 1 g polypeptone and 20 g glucose per liter). After incubation, the fungal cells were collected, suspended in water (10 g/100 mL) at 45°C and stirred for 30 min. This solution was centrifuged at 3000 × *g* for 20 min. Then the supernatant was concentrated *in vacuo* to produce a dried powder. The working solution of RU for administration to rats was produced to dissolve this powder with distilled PBS (20 mg/mL).

Animals and treatments

The animals used for this study were 4-week-old specific pathogen-free (SPF) male Slc:Wistar rats (Japan SLC, Inc., Shizuoka, Japan) with body weights of 70 ± 10 g. They were maintained on a commercial diet (CE-2; CLEA Japan Inc., Tokyo, Japan) and tap water *ad libitum* and kept at a temperature of 22 ± 3°C, with relative humidity of 55% ± 10% and a constant 12 h light : 12 h dark schedule. The 28 animals were divided into four groups mentioned below. The RU solution described above was administered orally at a dose of 0.5 (10 mg/kg) or 5 mL /kg (100 mg/kg) of body weight using a gastric tube once a day for 10 consecutive days. When RU was administrated at 0.5 mL/kg, PBS (4.5 mL/kg) was added to adjust total volume. The CCl_4-untreated control (Control) group and the CCl_4-treated control (CCl_4 Control) group were administered PBS or CCl_4, respectively. Ten days after the onset of RU treatment, liver injury was caused by oral administration of CCl_4 (125 mg/kg body weight) diluted at 25% in olive oil (Nacalai Tesque Inc.) except the Control group. BrdU (Nacalai Tesque Inc.) was injected intraperitoneally 12 h before CCl_4 treatment at a dose of 100 mg/kg body weight. At 24 or 48 h after CCl_4 administration, blood was drawn from the abdominal vein under isoflurane anesthesia. Thereafter, the liver was removed. Serum was separated by centrifugation at 2000 × *g* for 10 min and was analyzed biochemically. Several parts of each liver were snap-frozen in liquid nitrogen and stored at −80°C until analysis. For histological examination, whole livers were fixed in 20% phosphate-buffered formalin. All procedures were performed under the guidance of the Committee for Animal Experimentation at Azabu University (090305-1).

Serum biochemical analysis

Serum levels of hepatic enzymes, including LDH, ALT and AST were determined using an automatic analyzer (Hitachi 9000; Hitachi High-Technologies Corp., Tokyo, Japan).

Histology and immunohistochemistry

Rat liver specimens were fixed with 20% phosphate-buffered formalin, dehydrated in a graded series of ethanol, embedded

in paraffin, and sectioned serially at 4 μm. Histological analyses were performed on paraffin section stained by hematoxylin and eosin (Muto pure chemicals Co., Ltd, Tokyo, Japan). Immunohistochemical analyses were performed on deparaffinized sections using monoclonal antibody against BrdU (1:50; cat# X1028 (cln: MoBu-1), Exalpha Biologicals Inc., Shirley, MA, USA) with a staining kit (Vectastain Elite ABC Mouse IgG; Vector Laboratories Inc., Burlingame, CA, USA) and a peroxidase stain diamino benzidine (DAB) kit (Nacalai Tesque Inc.). Sections were then counter-stained with Mayer's hematoxylin. For statistical analysis, the total number of cells (hematoxylin staining) and BrdU-immunopositive cells on three random visual fields per section were counted under a microscope at ×400 (Eclipse 80i; Nikon Corp., Tokyo, Japan) and three experiments were performed.

RNA isolation and RT-PCR

Total RNA was extracted from the liver of rats with a High Pure RNA Tissue Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. First-strand complementary DNA (cDNA) was synthesized from 500 ng of total RNA using reverse transcriptase (PrimeScript II RTase; Takara Bio Inc., Shiga, Japan) and an oligo dT primer. PCR amplification from reverse-transcribed cDNA was conducted with primers designed specifically for HGF (forward: 5′-TTCCCGTTGTGAAGGAGATACT-3′ (nt: 1543–1564) and reverse: 5′-AGGTCCTGATCCAATCTTTT CA-3′ (nt: 2134–2155)) based on the rat HGF gene (GenBank accession no. NM017017), IGF-1 (forward: 5′-GACGCTC TTCAGTTCGTGTG-3′ (nt: 893–912) and reverse: 5′-GTCTTG GGCATGTCAGTGTG-3′ (nt: 1091–1110)) based on the rat IGF-1 gene (GenBank accession no. NM178866) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward: 5′-ATGGGAAGCTGGTCATCAAC-3′ (nt: 1034–1053) and reverse: 5′-GGATGCAGGGATGATGTTCT-3′ (nt: 1454–1473)) based on the rat GAPDH gene (GenBank accession no. AF106860). GAPDH was used as an internal control to normalize template concentrations. Briefly, for PCR, 1 μL of reverse-transcribed cDNA was added to a 50 μL reaction mixture containing each deoxynucleotide triphosphate (dNTP) at 2.5 mmol/L, 1.25 units of Taq polymerase (ExTaq; Takara Bio Inc.), and each primer at 0.2 μmol/L. Thermal cycling was performed under the following conditions: 30 cycles for HGF, IGF-1 and GAPDH at 95°C for 10 s, at 50°C (HGF and IGF-1) or 48°C (GAPDH) for 30 s, and 72°C for 50 s (PC812; Astec Co. Ltd, Fukuoka, Japan). The PCR product concentration is proportional to the starting cDNA concentration with the cycle profile above for each gene. PCR products were detected on a 1.5% agarose gel and were stained with ethidium bromide (Nacalai Tesque Inc.). Quantitative analysis of rat HGF levels was performed using scanning gels stained with ethidium bromide with a gel imager (Gel Scene; Astec Co. Ltd) and analysis with Image J software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

Data are expressed as means ± SD. Differences among groups of rats were assessed by analysis of Dunnett's multiple comparison method. *P*-values < 0.05 were regarded as statistically significant.

RESULTS

Effects of RU administration on serum biochemical parameters in CCl_4-treated rats

Figure 1 shows the suppressing effects of RU pretreatment on serum enzyme activities indicating hepatic damage caused by CCl_4 treatment. The levels of LDH, ALT and AST in CCl_4 Control group were considerably and significantly increased compared to those of the Control group 24 and 48 h after CCl_4 treatment. RU pre-treatment (particularly at the dose of 100 mg/kg RU) significantly suppressed the increase in these enzyme activities compared to that for the CCl_4 Control group.

Evaluation of RU on hepatocyte incorporation of BrdU and histopathological observation

To clarify the role of RU on hepatocyte proliferation, BrdU incorporation was assessed (Fig. 2). BrdU-labeled cells were scarce in the Control and CCl_4 Control groups (~0.8%) even at 24 h after CCl_4 treatment (Fig. 2A,B-I,B-II). Forty-eight hours after CCl_4 treatment, a few BrdU-labeled cells were detected (~4.0%) in the CCl_4 Control group (Fig. 2A,B-V). Finally, the percentage of BrdU-labeled cells was increased in a dose- and time-dependent manner in RU treated animals. Practically, the percentage of BrdU-labeled cells was markedly increased up to 4.6% and 34.5% at 100 mg/kg RU at 24 and 48 h after CCl_4 treatment, respectively (Fig. 2A,B-VI,B-VII).

Histopathological observation showed that RU treatment (100 mg/kg) inhibited tissue damage in the liver (Fig. 3 and Table 1). The effects of RU markedly appeared at 48 h after CCl_4 treatment. Liver sections from the CCl_4 Control group 24 h after CCl_4 treatment showed slight and/or moderate hepatocellular degeneration and necrosis, as well as inflammatory cellular infiltration in the centrilobular zone (Fig. 3II). At 48 h after CCl_4 treatment, there were a few signs of liver reproduction such as hyperplasia of bile duct and interlobular connective tissue while necrosis and serious steatosis were seen in these sections in the CCl_4 Control group (Fig. 3III and Table 1). RU administration did not cause any changes in the pathological features 24 h after CCl_4 treatment, but the degradation of liver damage signs such as decrease of necrosis cells and steatosis, and the exteriorization of the liver reproduction sign such as hyperplasia of bile duct and interlobular connective tissue were observed 48 h after CCl_4 treatment (Fig. 3IV,V and Table 1).

HGF and IGF-1 mRNA expression in the liver

Expression of HGF and IGF-1 genes involved in the recovery of damaged hepatocytes was assessed by

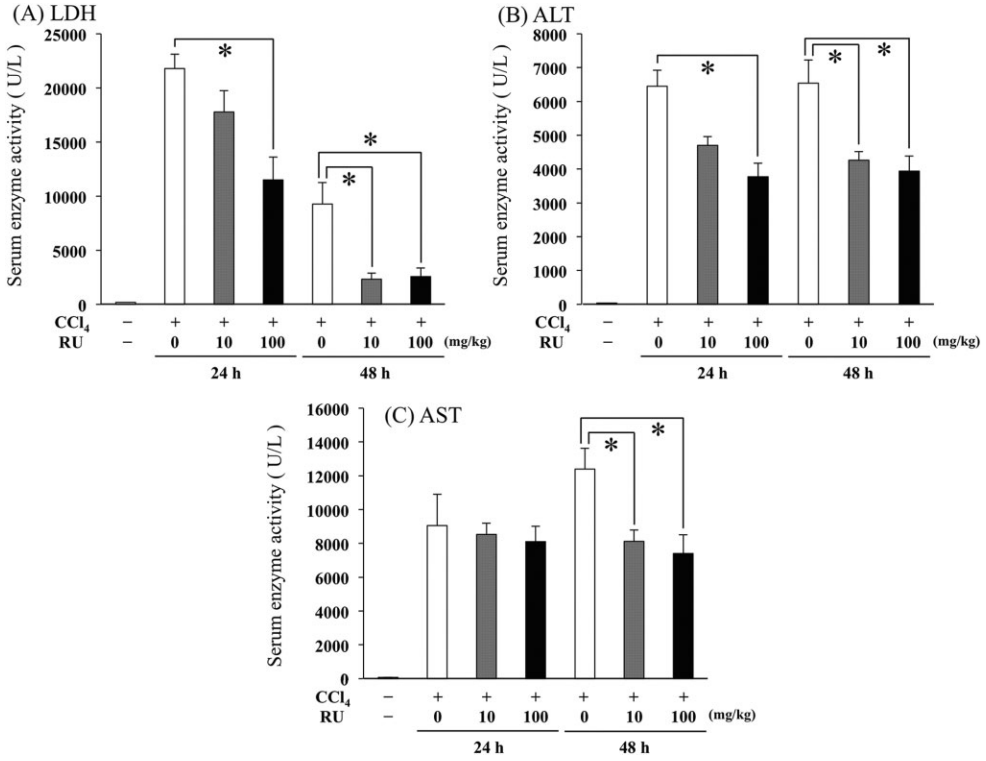

Figure 1 Suppression by *Rhizopus oryzae*/ U-1 aqueous extract (RU) pretreatment of serum activities of lactate dehydrogenase (LDH), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) increased by carbon tetrachloride (CCl_4) treatment. Serum LDH (A), ALT (B) and AST (C) activities were measured as sensitive markers for diagnosis of hepatic injury at 24 and 48 h after CCl_4 treatment in the absence or presence of RU pretreatment at 10 and 100 mg/kg. Data are expressed as means ± SD of four individuals. Data that differ significantly ($P < 0.05$) are indicated by an asterisk at the top of the bar.

semi-quantitative RT-PCR analysis (Fig. 4). At 24 h after CCl_4 treatment, HGF messenger RNA (mRNA) expression was transiently and significantly suppressed in the CCl_4 Control group only after 24 h (Fig. 4A). However, at 48 h after CCl_4 treatment, the increase in gene expression was not observed. On the other hand, expression of IGF-1 gene was significantly reduced by CCl_4 treatment 24 h afterwards (Fig. 4B). However, RU treatment caused a sustained and significant increase in gene expression at both 24 and 48 h after CCl_4 treatment (Fig. 4B).

DISCUSSION

This study showed that the aqueous extract of *R. oryzae*/ U-1 (RU) provided a protective effect for liver injury induced by CCl_4 administration in rats. (i) Pretreatment (oral administration) of RU significantly suppressed the increased serum enzyme activities of ALT, AST and LDH induced by CCl_4 (Fig. 1). (ii) Hepatocyte proliferation (as revealed by BrdU incorporation) was significantly increased by RU pretreatment in a dose- and time-dependent manner (Fig. 2). (iii) Liver reproduction signs such as hyperplasia of bile duct and interlobular connective tissue were observed by RU administration at 48 h after CCl_4 treatment (Fig. 3 and Table 1). (iv) RU pretreatment caused a sustained and significant increase in gene expression of IGF-1 in hepatocytes (Fig. 4). Results similar to these results in the present study were obtained from two separate and subsequent experiments.

In previous reports (Recknagel 1967; Ahmed *et al*. 2011), administration of CCl_4 caused hepatocellular necrosis in the intermediate region of the hepatic lobule after 6 h of injury, cellular damage (such as swelling) appeared in the centrilobular region from 12 h after administration. Hepatocellular necrosis was first observed around the central veins, later expanding to the whole hepatic lobule area after 24 h following CCl_4 administration. In parallel with these pathological changes, serum transaminase enzyme activity (including ALT, AST and LDH) in rats has been shown to reach a peak at 24–48 h after administration of CCl_4, after which it slowly decreases (Yemitan & Izegbu 2006; Ahmed *et al*. 2011). In the present study, the prominently high activities of serum ALT, AST and LDH were observed, indicating extensive liver damage induced by administration of CCl_4 (Fig. 1). Pretreatment of RU significantly decreased the activity of these markers.

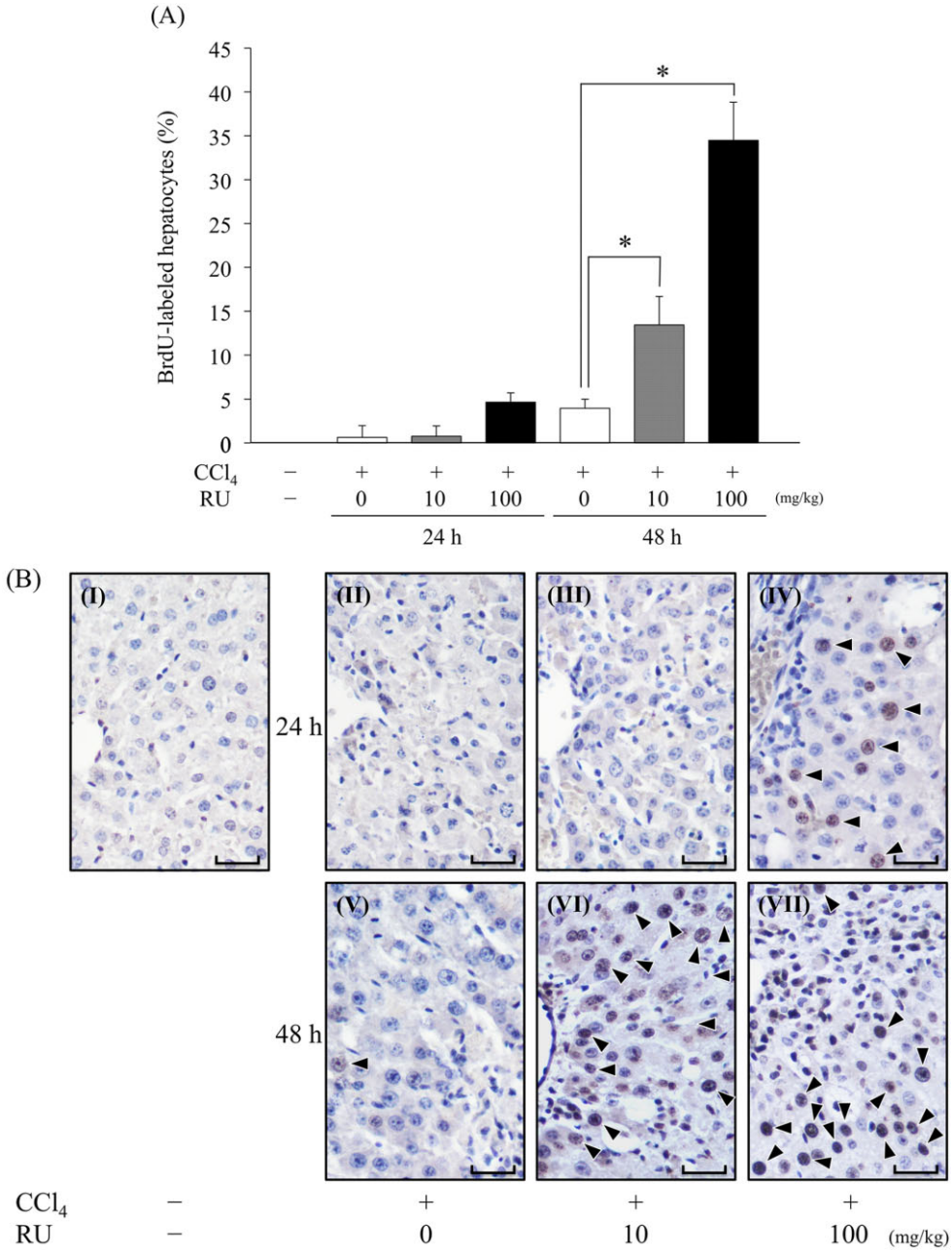

Figure 2 Increase in the incorporation of BrdU by *Rhizopus oryzae*/ U-1 aqueous extract (RU) pretreatment in hepatocytes injured by carbon tetrachloride (CCl_4) treatment. (A) Hepatocyte incorporation is shown as the ratio of 5-bromo-2′-deoxyuridine (BrdU)-immunopositive cells per total number of cells on three random visual fields per section. Data are expressed as means ± SD of four individuals. Data that differ significantly ($P < 0.05$) are indicated by an asterisk at the top of the bar. (B) Immunohistochemical detection of BrdU in the liver. Hepatocytes that had incorporated BrdU at 24 h or 48 h after CCl_4 treatment in the absence (II, V) or presence (III, IV, VI, VII) of RU pretreatment (10 or 100 mg/kg body weight). BrdU-positive cells were dyed by bistre (indicated by arrow heads). Scale bar: 50 μm.

The liver is generally known as an organ in which the proliferative ratio is extremely low in normal hepatocytes. However, the proliferative activity can be increased remarkably when the organ is injured (such as by a loss of liver mass, virus infections or toxic reagents). Indeed, hepatocellular DNA synthesis peaks 24 h after partial hepatectomy in rats (Zarnegar *et al*. 1991; Michalopoulos & DeFances 1997; Taub 2004). Hepatocellular DNA synthesis peaks at 32–40 h in the liver after injury by CCl_4 (Asami *et al*. 1991; Yamada & Fausto 1998). In the present study, administration of RU significantly increased BrdU incorporation in

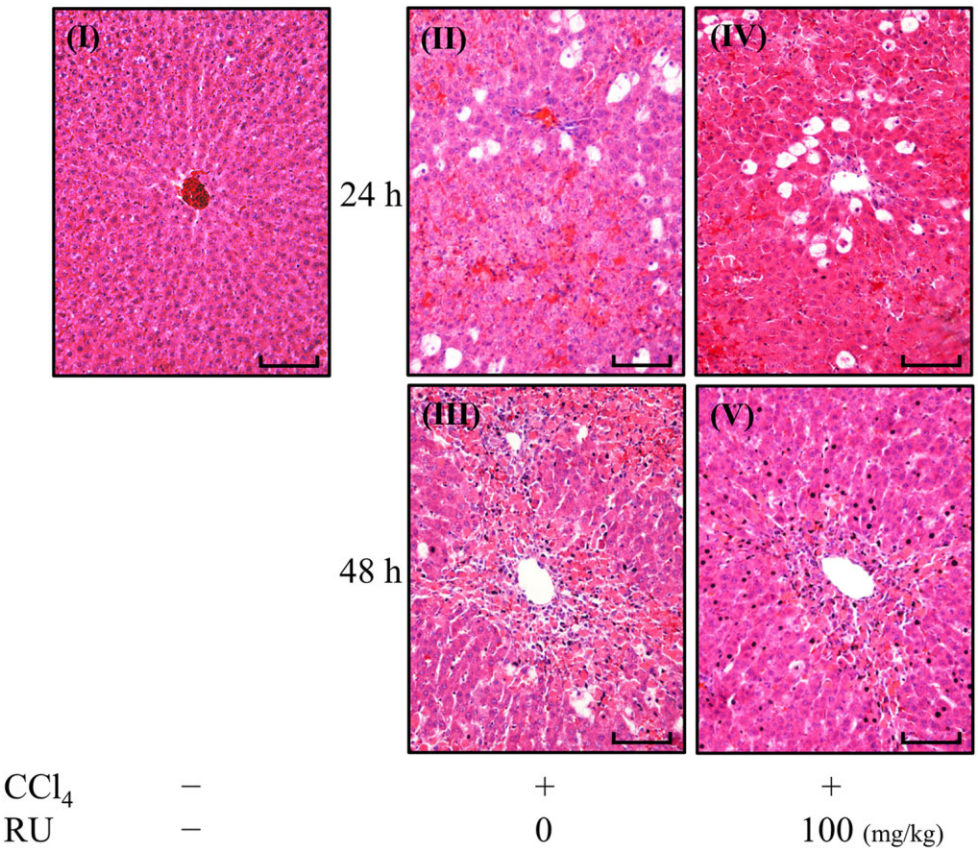

Figure 3 Recovery effects of *Rhizopus oryzae*/ U-1 aqueous extract (RU) pretreatment on histological change in hepatocytes injured by carbon tetrachloride (CCl_4) treatment. Hepatocytes were stained by hematoxylin and eosin at 24 h or 48 h after CCl_4 treatment in the presence (IV, V) or absence (II, III) of RU pretreatment (100 mg/kg body weight). Scale bar: 100 μm.

Table 1 Histopathological manifestation in the liver by administration of RU

	CCl_4	RU	Histopathological manifestation				
			Focal and diffuse necrosis	Steatosis	Hyperplasia of Interlobular connective tissue	Hyperplasia of bile duct	Infiltrating neutrophil
24 h	+	–	+	+	–	–	+
	+	+	+	+	–	–	+
48 h	+	–	+	++	+	+	–
	+	+	–	–	++	++	+

Histopathological manifestation such as degree of damage, inflammation and regeneration in the liver were shown.
Each item is evaluated with 3 stages (+, ++ or +++), and it depends on 3 individual experiments per each group. RU, *Rhizopus oryzae*/ U-1 aqueous extract; CCl_4, carbon tetrachloride.

hepatocytes at 48 h after CCl_4 administration. Consequently, RU pretreatment showed potent accelerative ability of cell division after liver injury (Fig. 2).

Hepatocellular division requires various mitogens. HGF (Nakamura *et al*. 1987; Gohda *et al*. 1998), epidermal growth factor (EGF) (McGowan *et al*. 1981), transforming growth factor-alpha (TGF-α) (Luetteke *et al*. 1988) and other agents are known to be accelerators of the cell cycle from G1 phase to S phase in hepatocytes. Additionally, HGF is known to be a major or more potent mitogen of hepatocytes (Nakamura *et al*. 1987). HGF binds to c-met, which is located at the cell membrane and which promotes signaling via the PI3-kinase/ Akt and mitogen-activated protein kinase (MAPK) pathways, and which increases hepatocyte proliferation (Nakamura *et al*. 2011; Matsumura *et al*. 2013). In the RU-H group, HGF mRNA expression following 24 h of injury was increased significantly to fivefold of the CCl_4 control group (Fig. 4A). Hyperexpression of HGF mRNA in this early stage might be involved in significant hepatocyte proliferation.

RU reportedly activates phagocytic activity in monocytes (Suzuki *et al*. 2007). Therefore, phagocytic activity by macrophages or Kupffer cells in the liver

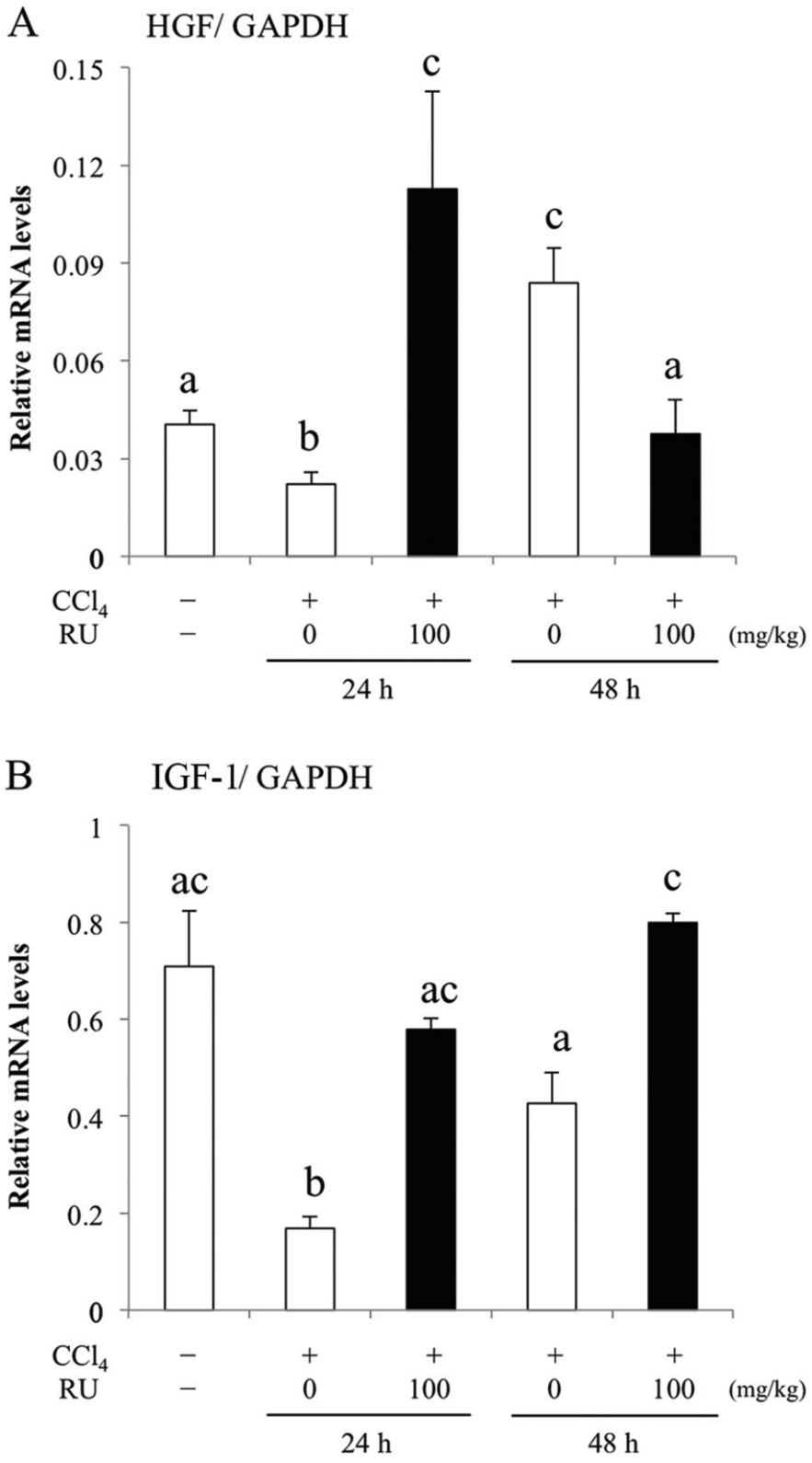

Figure 4 Effects of *Rhizopus oryzae*/ U-1 aqueous extract (RU) pretreatment on the expression of hepatocyte growth factor (HGF) and insulin-like growth factor-I (IGF-I) messenger RNA in hepatocytes injured by carbon tetrachloride (CCl_4) treatment. Relative expression levels of HGF (A) and IGF-1 (B) were calculated as a percentage relative to levels of the control gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Data are expressed as means ± SD of four individuals. Data that differ significantly ($P < 0.05$) are indicated by different letters at the tops of the bars.

might be a primary response to dramatic decreases in the quantities of *S. enteritidis* in the liver, as described in a previous report (Suzuki *et al*. 2007). Exclusion of *Salmonella* by administration of RU has been recognized only in the liver. Meanwhile, it is reported that the cells responsible for production of HGF in the liver are stromal cells (including Kupffer cells, fibroblasts and Ito cells) (Weidner *et al*. 1991). The HGF-producing stimulation in these cells is regarded as proinflammatory cytokine such as interleukin (IL)-1 and TNF-α (Tamura *et al*. 1993) or various components of bacterial cells (Sugiyama *et al*. 1996). These stimulants induce phagocytic activation. Therefore, RU may have ability of phagocytic activation and will have the possibility to drive HGF gene expression because RU is a fungal extract.

In rat models of CCl_4-treated liver injury, the serum IGF-1 level reportedly decreases in a time-dependent manner and is minimized after 24 h of CCl_4 treatment (Scharf *et al*. 2004). However, the present study showed that IGF-1 mRNA expression at the dose of 100 mg/kg was increased to 76% of the Control group at 24 h after CCl_4 treatment, and finally to the similar level at 48 h (Fig. 4B). Therefore, the data reported herein indicate that RU pretreatment caused a sustained and significant increase in IGF-1 mRNA expression, which might have major contributions to the hepatocyte proliferation in this model. Further examination should be undertaken to explore which compounds in RU are responsible for involving the mechanisms that accelerate the gene expression of these factors.

In conclusion, RU pretreatment effectively recovered liver functions injured by CCl_4 administration in rats. In addition, RU pretreatment caused a sustained and significant increase in gene expression of IGF-I, which is known as an accelerator of cell proliferation. Finally, these results imply that RU pretreatment exerts its effects via increase in these hepatic factors, such as IGF-I involved in cell proliferation.

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REFERENCES

Ahmed AF, Mahmoud MF, Ouf MA, El-Fathaah EA. 2011. Aminoguanidine potentiates the hepatoprotective effect of silymarin in CCL4 treated rats. *Annals of Hepatology* **10,** 207–215.

Asami O, Ihara I, Shimidzu N, Shimizu S, Tomita Y, Ichihara A, Nakamura T. 1991. Purification and characterization of hepatocyte growth factor from injured liver of carbon tetrachloride-treated rats. *Journal of Biochemistry* **109,** 8–13.

Baker J, Hardy MP, Zhou J, Bondy C, Lupu F, Bellvé AR, Efstratiadis A. 1996. Effects of an Igf1 gene null mutation on mouse reproduction. *Molecular Endocrinology* **10,** 903–918.

Gohda E, Tsubouchi H, Nakayama H, Hirono S, Sakiyama O, Takahashi K, *et al*. 1998. Purification and partial characterization of hepatocyte growth factor from plasma of a patient with fulminant hepatic failure. *Journal of CIinical Investigation* **81,** 414–419.

Higuchi T, Seto K, Hayashi R, Kojima H, Saito H, Yoshimatsu K, Saito H. 1979. Influence of the treatment with gonadotrophin and *Rhizopus* extract on the LH action in proestrus rats. *Medicine and Biology* **99,** 145–147.

Horiuchi C, Tabe M, Ushikoshi A, Ushikoshi S, Ushikoshi K, Ushikoshi I, Seto K. 1985. The influence of the treatment with gonadotrophin and *Rhizopus* extracts on LH action in DI rats. *Medicine and Biology* **110,** 27–29.

Kawashima C, Fukihara S, Maeda M, Kaneko E, Amaya Montoya C, Matsui M, *et al*. 2007. Relationship between metabolic hormones and ovulation of dominant follicle during the first follicular wave *post-partum* in high-producing dairy cows. *Reproduction* **133,** 155–163.

Lucy MC. 2001. Reproductive loss in high-producing dairy cattle: where will it end? *Journal of Dairy Science* **84,** 1277–1293.

Luetteke NC, Michalopoulos GK, Teixido J, Gilmore R, Massague J, Lee DC. 1988. Characterization of high molecular weight transforming growth factor alpha produced by rat hepatocellular carcinoma cells. *Biochemistry* **27,** 6487–6494.

Matsumura A, Kubota T, Taiyoh H, Fujiwara H, Okamoto K, Ichikawa D, *et al*. 2013. HGF regulates VEGF expression via the c-Met receptor downstream pathways, PI3K/Akt, MAPK and STAT3, in CT26 murine cells. *International Journal of Oncology* **42,** 535–542.

McGowan JA, Strain AJ, Bucher NL. 1981. DNA synthesis in primary cultures of adult rat hepatocytes in a defined medium: effects of epidermal growth factor, insulin, glucagon, and cyclic-AMP. *Journal of Cell Physiology* **108,** 353–363.

Meussen BJ, de Graaff LH, Sanders JP, Weusthuis RA. 2012. Metabolic engineering of *Rhizopus oryzae* for the production of platform chemicals. *Applied Microbiology and Biotechnology* **94,** 875–886.

Michalopoulos GK, DeFances MC. 1997. Liver Regeneration. *Science* **276,** 60–66.

Nakamura T, Nawa K, Ichihara A, Kaise N, Nishino T. 1987. Purification and subunit structure of hepatocyte growth factor from rat platelets. *FEBS Letters* **224,** 311–316.

Nakamura T, Sakai K, Nakamura T, Matsumoto K. 2011. Hepatocyte growth factor twenty years on: much more than a growth factor. *Journal of Gastroenterology and Hepatology* **26,** 188–202.

Recknagel RO. 1967. Carbon tetrachloride hepatotoxicity. *Pharmacological Reviews* **19,** 145–208.

Rhoads ML, Meyer JP, Kolath SJ, Lamberson WR, Lucy MC. 2008a. Growth hormone receptor, insulin-like growth factor (IGF)-1, and IGF-binding protein-2 expression in the reproductive tissues of early postpartum dairy cows. *Journal of Dairy Science* **91,** 1802–1813.

Rhoads ML, Meyer JP, Lamberson WR, Keisler DH, Lucy MC. 2008b. Uterine and hepatic gene expression in relation to days postpartum, estrus, and pregnancy in postpartum dairy cows. *Journal of Dairy Science* **91,** 140–150.

Saito H, Seto K, Kojima H, Higuchi H, Hayashi R, Ando S, Kawakami M. 1980. Influence of the treatment with gonadotrophin and *Rhizopus* extract on the FSH action in proestrus rats. *Medicine and Biology* **100,** 101–103.

Scharf JG, Dombrowski F, Novosyadlyy R, Eisenbach C, Demori I, Kubler B, Braulke T. 2004. Insulin-Like Growth Factor (IGF) -binding protein-1 is highly induced during acute carbon tetrachloride liver injury and potentiates the IGF-I-stimulated activation of rat hepatic stellate cells. *Endocrinology* **145,** 3463–3472.

Silva JR, Figueiredo JR, van den Hurk R. 2009. Involvement of growth hormone (GH) and insulin-like growth factor (IGF) system in ovarian folliculogenesis. *Theriogenology* **71,** 1193–1208.

Sugiyama A, Arakaki R, Ohnishi T, Arakaki N, Daikuhara Y, Takada H. 1996. Lipoteichoic acid and interleukin 1 stimulate synergistically production of hepatocyte growth factor (scatter factor) in human gingival fibroblasts in culture. *Infection and Immunity* **64,** 1426–1431.

Suzuki T, Fukuoka H, Ushikoshi S, Morita H. 2007. Potency of water extracts of *Rhizopus oryzae* on the Salmonella infection rat. *Food Science and Technology Research* **13,** 235–240.

Tamura M, Arakaki N, Tsubouchi H, Takada H, Daikuhara Y. 1993. Enhancement of human hepatocyte growth factor production by interleukin-1 alpha and -1 beta and tumor necrosis factor-alpha by fibroblasts in culture. *Journal of Biological Chemistry* **268,** 8140–8145.

Taub R. 2004. Liver regeneration: from myth to mechanism. *Nature Reviews Molecular Cell Biology* **5,** 836–847.

Umezu M, Takeuchi S, Iwase S, Chiba N. 1973. Increment of pregnant rats by RV administrations in cow of reproductive disease. *Journal of Animal Reproduction* **19,** 67–76.

Weidner KM, Arakaki N, Hartmann G, Vandekerckhove J, Weingart S, Rieder H, *et al*. 1991. Evidence for the identity of human scatter factor and human hepatocyte growth factor. *Proceedings of the National Academy of Science of the United States of America* **88,** 7001–7005.

Yamada Y, Fausto N. 1998. Deficient liver regeneration after carbon tetrachloride injury in mice lacking type 1 but not type 2 tumor necrosis factor receptor. *American Journal of Pathology* **152,** 1577–1589.

Yemitan OK, Izegbu MC. 2006. Protective effects of *Zingiber officinale* (Zingiberaceae) against carbon tetrachloride and acetaminophen-induced hepatotoxicity in rats. *Phytotherapy of Research* **20,** 997–1002.

Zarnegar R, DeFrances MC, Kost DP, Lindroos P, Michalopoulos GK. 1991. Expression of hepatocyte growth factor mRNA in regenerating rat liver after partial hepatectomy. *Biochemical and Biophysical Research Communications* **177,** 559–565.

Zhang J, Sugahara K, Ushikoshi I, Ushikoshi S, Seto K, Kodama H. 1999. Effect of water extract of Rhizopus delemar on the stimulus coupled responses of neutrophils and their modulation by various protein kinase inhibitors. *Clinica Chimica Acta* **282,** 89–100.

Zhou J, Kumar TR, Matzuk MM, Bondy C. 1997. Insulin-like growth factor I regulates gonadotropin responsiveness in the murine ovary. *Molecular Endocrinology* **11,** 1924–1933.

