JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

Protective Effect of *Monascus*-Fermented Red Mold Rice against Alcoholic Liver Disease by Attenuating Oxidative Stress and Inflammatory Response

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ABSTRACT: *Monascus purpureus* NTU 568 fermented rice is reported to exhibit a wide variety of biological effects, including antitumor, antihypertriglyceridemia, antioxidant, and anti-inflammatory activities. However, its role in the pathogenesis of alcoholic liver disease remains obscure. In this study, the hepatoprotective effects of *Monascus*-fermented red mold rice (RMR) was evaluated in vivo using chronic alcohol-induced mice as an experimental model. The alcohol-induced mice were orally treated with RMR at 307.5 mg/kg (1-fold), 615 mg/kg (2-fold), and 1537.5 mg/kg (5-fold) for 5 weeks, whereas controls received vehicle only. Treatment with RMR significantly attenuated the increased level of serum transaminases (aspartate aminotransferase and alanine aminotransferase) and hepatic triglyceride and total cholesterol accumulation. Furthermore, RMR elevates hepatic antioxidant ability that reduced hepatic cell damage (steatosis) and decreased tissue inflammatory cytokine levels. These findings suggest that *Monascus*-fermented RMR may represent a novel, protective strategy against alcoholic liver disease by attenuating oxidative stress, inflammatory response, and steatosis.

KEYWORDS: *Monascus purpureus*, hepatoprotective, alcoholic liver disease, oxidative stress, steatosis, inflammation

INTRODUCTION

In the World Health Organization (WHO) global status report on alcohol and health (2011), the hazardous and harmful use of alcohol is a major global contributing factor to death, disease, and injury. Almost 4% of all deaths worldwide are attributed to alcohol, greater than deaths caused by HIV/AIDS, violence, or tuberculosis. Alcohol drinking is a major etiologic factor in causing fatty liver, alcoholic hepatitis, cirrhosis, and/or hepatocellular carcinoma.[1] Alcoholic liver disease (ALD) results in a fatty liver (or steatosis), a disorder in which hepatocytes contain macrovesicular droplets of triglycerides. Steatosis predisposes people who continue to drink to hepatic fibrosis and cirrhosis.[2] The important progress in this field has been the appreciation of the role of oxidative stress and inflammatory responses in the pathogenesis of alcohol liver injury.[3] These mechanisms include the activation of Kupffer cells expressing pro-inflammatory cytokines, chemokines, and reactive oxygen species (ROS) and interactions between alcohol metabolism, various hepatic cells, multiple cytokines, and the immune system.[4,5]

Monascus-fermented rice, known as red mold rice (RMR), has been proven to produce many functional secondary metabolites. In the current study, *Monascus*-fermented products such as monacolin K (antihypercholesterolemic agents), γ-aminobutyric acid (GABA) (antihypertension and antidepression agents), and dimerumic acid (antioxidant) were found.[6−8] In addition, the *Monascus*-fermented rice contains various pigments (yellow pigments, ankaflavin and monascin; orange pigments, monascorubrin and rubropunctanin; red pigments, monascorubramine and rubropuctamine) that may have biological activity.[9,10]

This research focused on the effects of oral administration of a RMR fermented by *Monascus purpureus* NTU 568 for alcohol-induced mice on liver antioxidant enzyme, pro-inflammatory cytokine, triglyceride (TG), and total cholesterol (TC) accumulation. This study examined the kidney index and kidney histopathology to investigate the safety of *Monascus*-fermented rice.

MATERIALS AND METHODS

Chemicals and Reagents. Glutathione (GSH), glutathione reductase (GRd), glutathione disulfide (GSSG), nicotinamide adenine dinucleotide phosphate (NADPH), 5,5-dithiobis[2-nitrobenzoic acid] (DTNB), ethylenediaminetetraacetic acid (EDTA), NaN_3, 1-methyl-2-vinylpyridinium trifluoromethanesulfonate (M2VP), and $MgCl_2 \cdot 6H_2O$ were purchased from Sigma Chemical Co. (St. Louis, MO). Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were purchased from BioQuant (San Diego, CA). Superoxide dismutase (SOD) assay kits were purchased from Cayman (Ann Arbor, MI). TC assay kit was purchased from Randox (Crumlin, Co. Antrim, U.K.). TG assay kit was purchased from Fortress (Fortress Diagnostics Limited, Antrim, U.K.). Interleukin (IL)-1β, IL-6, and tumor necrosis factor α (TNF-α) immunoassay kit were purchased from eBioscience (San Diego, CA). Transforming growth factor β1 (TGF-β1) immunoassay kit was purchased from R&D Systems (Minneapolis, MN).

Sample Preparation. RMR is obtained from *M. purpureus* NTU 568 fermented rice. *M. purpureus* NTU 568 strain was maintained on potato dextrose agar (PDA) slants at 10 °C and transferred monthly. The preparation of RMR was carried out under the substrate of long-grain rice (*Oryza sativa*) purchased from a local supermarket in Taiwan and using the method of solid-state culture.[11] Briefly, 500 g of rice was soaked in water for 8 h. After that, excess water was removed with a sieve.

Received: June 28, 2011
Revised: August 23, 2011
Accepted: August 23, 2011
Published: August 23, 2011

The rice was autoclaved (HL-341 model, Gemmy Corp., Taipei, Taiwan) for 20 min at 121 °C in a "koji-dish" (the koji-dish was made of wood with dimensions of 30 × 20 × 5 cm), which is a fermented instrument tray of RMR during the fermentation process. After having been cooled, the rice was inoculated with a 5% (v/w) spore suspension. The inoculated rice was cultivated at 30 °C for 10 days. During the culturing stage, 100 mL of water was daily added to the rice from the second day to the fifth day. At the end of cultivation, the crushed and dried product with the mold was used for the experiments.

Animals and Treatments. Male C57BL/6J mice were purchased from National Laboratory Animal Breeding and Research Center (Taipei, Taiwan) at 8−10 weeks of age and housed in a 25 °C controlled room under a 12 h light/dark cycle with free access to food and water. After acclimatization, mice were divided into five groups (eight mice pre group) and fed either control Lieber−DeCarli liquid diet or a Lieber−DeCarli alcohol-containing liquid diet (Dyets Inc., Bethlehem, PA). The alcohol-fed group was allowed free access to an alcohol-containing diet with increasing content by 1% (v/v) every day until the mice were consuming diets containing 6.2% (v/v) ethanol for 5 weeks.[12] Five groups of mice were separated as follows: (1) control liquid diet fed untreated mice; (2) model alcohol-feeding mice; (3) model mice treated with different doses of RMR (1× RMR, 2× RMR, and 5× RMR, respectively). In the treatment groups, different test materials were administered orally by stomach tube every day around 2:00 p.m. for 5 weeks. The mice in normal and model control groups were given the same volume of vehicle only.

Doses and Groups. Mice were divided at random into five treatment groups, including normal, alcohol-containing diet, 1× RMR, 2× RMR, and 5× RMR groups. The dose of RMR powder was calculated in accordance with Body's formula of body surface area as recommended by the U.S. Food and Drug Administration (FDA).[13] The daily dietary dosage of RMR is usually recommended at 1.0−2.0 g for adults.[14] Therefore, 1.5 g of RMR was used as the 1-fold dosage for an adult with a body weight (bw) of 60 kg and a height of 170 cm; 307.5 mg/kg of bw (1-fold dosage) of RMR was used as a frame of reference for conversion of the dosage into a mice model, 615 mg/kg bw of RMR was used as 2-fold dosage, and 1537.5 mg/kg bw of RMR was used as 5-fold dosage relatively.

Determination of Monsacin and Ankaflavin Concentration. RMR (1 g) was extracted respectively with 10 mL of ethanol at 60 °C for 30 min. The extracts (10%, w/v) were further filtered with 0.45 μm pore size filter and analyzed by high-performance liquid chromatography (HPLC). HPLC was performed in triplicate according to the method described previously. Monascin and ankaflavin were detected using a UV detector UV2075 plus (Jasco Co., Tokyo, Japan) set at 231 nm.[15]

Determination of Citrinin Levels. A 1 g portion of dried RMR powder was extracted with 10 mL of ethanol at 65 °C for 30 min. The extracts (10% w/v) were further filtered with a 0.45 μm filter and were analyzed by HPLC. HPLC analysis was performed according to the method previously described[16] and was carried out on an HPLC system PU2089 plus (Jasco Co.) in this study. Citrinin is determined by HPLC on a C_{18} column (25 cm × 4.6 mm inner diameter, 5 μm, Luna, Phenomenex, Inc., Torrance, CA) using the mobile phase with the composition of water, acetonitrile, and trifluoroacetate (450:550:0.5). The flow rate is set at 1.0 mL/min, and the detector used is a fluorescence detector (FL-1, Rainin, Woburn, MA). The excitation and emission wavelengths were set at 330 and 500 nm, respectively.[17]

Pigment Estimation. One gram of RMR was extracted with 95% ethanol at 37 °C for 90 min. Pigment concentrations were estimated using a spectrophotometer set at 500, 470, and 400 nm for red, orange, and yellow pigments, respectively. The pigment level was expressed as optical density units per gram of dried medium multiplied by the dilution factor.[18]

Liver and Kidney Histology. Liver or kidney histology was performed by fixing a sample of tissue in 10% (v/v) neutral phosphate-buffered formalin and embedding it in paraffin. Slide-mounted tissue sections (3−4 mm) were histochemically stained with hematoylin and eosin (H&E).

Plasma Liver and Kidney Analysis. Blood samples were allowed to clot, and the sera were isolated by centrifugation at 1000*g* for 10 min and then kept at −20 °C before the following assay. The enzymatic activityies of AST and ALT were measured using commercial kits BQ042-CR and BQ006A-CR from BioQuant (San Diego, CA). Blood urea nitrogen (BUN) was measured in triplicate using an automatic biochemical analyzer (Beckman-700, Fullerton, CA).

Determination of GSH/GSSG in Liver Tissues. The concentration of reduced GSH in liver tissues was measured using GSH (0−100 μM) as the standard. The diluted sample solution or standard (10 μL) was mixed with 95 μL of the reagent (2 U/mL GRd, 200 μM NADPH, and 2 mM EDTA in 50 mM phosphate buffer, pH 7.2), followed by the addition of 100 μL of the reagent (10 mM DTNB in 50 mM phosphate buffer, pH 7.2). The reaction mixture was then incubated at room temperature, and the absorbance at 405 nm was determined every 1 min up to 5 min. The concentration was expressed as GSH (μM) in liver tissues.

The concentration of reduced GSSG in liver tissues was measured using GSSG (0−100 μM) as the standard. The diluted sample solution or standard (70 μL) was mixed with 4 μL of M2VP. The mixture was incubated for 1 h at room temperature. The reaction mixture (10 μL) was mixed with 95 μL of the reagent (2 U/mL GRd, 200 μM NADPH, and 2 mM EDTA in 50 mM phosphate buffer, pH 7.2), followed by the addition of 100 μL of the reagent (10 mM DTNB in 50 mM phosphate buffer, pH 7.2). The reaction mixture was then incubated at room temperature, and the absorbance at 405 nm was determined every 1 min up to 5 min. The concentration was expressed as GSSG (μM) in liver tissues.

The GSH/GSSH ratio is then calculated by dividing the difference between the total GSH and GSSG concentrations (reduced GSH). GSH/GSSG = (total GSH − 2 × GSSG)/GSSG.

Antioxidant Enzyme Activities. Liver tissues were homogenized with ice-cold 20 mM Tris-HCl buffer (pH 7.4; 1:10, w/v). After centrifugation at 12000*g* for 30 min at 4 °C, the supernatant was collected for the measurement of antioxidant enzyme activities. The activity of GRd was determined using a 0.1 mL mixture of liver homogenate and 0.1 M phosphate buffer (1 mM $MgCl_2 \cdot 6H_2O$, 5 mM GSSG, and 0.1 mM NADPH, pH 7.0). The decreased absorbance at 340 nm was measured for 3 min.[19] The activity of glutathione peroxidase (GPx) was determined using 0.1 mL of liver homogenate mixed with 100 mM potassium phosphate buffer (1 mM EDTA, 1 mM NaN_3, 0.2 mM NADPH, 1 unit/mL GR, and 1 mM GSH, pH 7.0) and incubated for 5 min at room temperature; the reaction was initiated after the addition of 0.1 mL of 2.5 mM hydrogen peroxide (H_2O_2). GPx activity was calculated by the change in absorbance at 340 nm for 5 min.[20] The activity of SOD was determined using a commercially available kit (Cayman, Ann Arbor, MI). To determine the activity of catalase (CAT), 50 μL of homogenate and 950 μL of 0.02 M H_2O_2 were incubated at room temperature for 2 min. CAT activity was calculated by the change in absorbance at 240 nm for 3 min.[21]

Serum and Liver Lipid Analysis. Serum TC and TG levels were measured in triplicate using commercial enzymatic kits. These kits were as follows: TC assay kit (CH 200, Randox Laboratories Ltd.) and TG assay kit (BXC0272C, Fortress Diagnostics).

Liver tissue (0.5 g) was ground in 10 mL of ice-cold Folch solution (chloroform/methanol, 2:1, v/v) and incubated for 30 min at room temperature. The aqueous layer was aspirated and discarded, and the fixed volume of the organic layer was then evaporated to dryness. The dried lipid layer was dissolved with an equal volume of DMSO and then

Table 1. Production of Various Secondary Metabolites by *M. purpureus* NTU 568[a]

monascin (mg/kg)	ankaflavin (mg/kg)	citrinin (mg/kg)	red pigment (A_{500}/g)	orange pigment (A_{470}/g)	yellow pigment (AWg)
1385 ± 242	2166 ± 177	ND	168.5 + 6.6	109.2 + 14.0	224.1 ± 31.6

[a] Results are expressed as the mean + SD (n = 3).

Table 2. Mouse Body Weight after Various Weeks of Alcohol Diet Feeding

	body weight[a] (g)			
group[b]	initial weight	2 weeks	4 weeks	final weight
control	20.0 ± 1.7	21.9 ± 1.7	24.5 ± 1.7	26.1 ± 1.3
ALD	20.0 ± 1.3	20.9 ± 0.5	22.5 ± 0.9*	22.7 ± 0.5*
1× RMR	20.0 ± 1.1	21.5 ± 0.6	22.8 ± 1.4	23.3 ± 1.1
2× RMR	20.0 ± 0.8	21.1 ± 0.9	23.3 ± 1.5	23.8 ± 1.1
5× RMR	20.0 ± 0.7	21.6 ± 0.5	23.0 ± 1.1	23.3 ± 1.5

[a] Results are expressed as the mean ± SD (n = 8). *, $p < 0.05$ with control group. [b] ALD, alcohol diet treatment; RMR, red mold rice treatment.

used to determine the TC and TG levels using commercial enzymatic kits.

Hepatic Cytokine Levels. The levels of the inflammatory cytokine TNF-α, IL-1β, and IL-6 in liver were determined using a commercially available ELISA kit (eBioscience). The hepatic TGF-β was determined using a commercially available ELISA kit (R&D Systems), according to the manufacturer's protocol.

Statistical Analysis. All values are expressed as the mean ± SD. Statistical differences between groups were compared by two-way analysis of ANOVA software for statistical analysis. $p < 0.05$ was considered to be statistically significant.

RESULTS

HPLC Assay for Monascin, Ankaflavin, and Pigment Content of Red Mold Rice. The amounts of monascin and ankaflavin in RMR, which possess antioxidative abilities and inhibitory effects on tetradecanoylphorbol acetate (TPA)-induced inflammation in mice,[22] were 1385 and 2166 mg/kg, respectively (Table 1).

Effects of Red Mold Rice on Body and Organ Weight Change. Body weight was monitored every 2 weeks, as shown in Table 2. Animals fed an alcohol diet for 5 weeks gained weight at a slower rate than the control mice did. However, the mean of the differences between the weights of alcohol-induced disease (ALD) mice fed different doses of RMR and those not fed RMR were not statistically significant.

The liver weight per body weight in the ALD group was higher than that of the normal control group, but ALD mice fed 5× RMR recovered normal liver weight per body weight (Figure 1A). The difference between kidney weight per body weight in all alcohol-induced groups and the control group was not statistically significant (Figure 1B).

Effects of Red Mold Rice on Liver and Kidney Histopathology of ALD Mice. Histology of liver sections from the control animals exhibited normal liver architecture with well-preserved cytoplasm and prominent nucleus and nucleolus (Figure 2A), whereas extensive macrovesicular steatosis was observed in the livers of the ALD mice (Figure 2B). However, there was an obvious improvement in liver morphology compared to that of the ALD group that had not been fed RMR when the ALD mice were

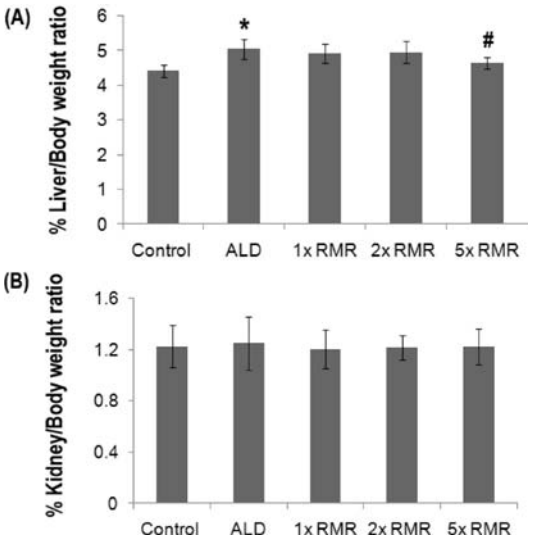

Figure 1. Effects of RMR on liver and kidney: (A) liver and body weight ratio; (B) kidney and body weight ratio. Results are expressed as the mean ± SD (n = 8). *, $P < 0.05$ with control group; #, $P < 0.05$ compared with ALD group. ALD, alcoholic liver disease; RMR, red mold rice treatment.

simultaneously fed 1× or 2× RMR (Figure 2C,D). Particularly, the hepatocytes of the 2× RMR group demonstrated mild steatotic change, but there was no visible change in the histopathology of the 5× RMR group (Figure 2E).

In histology of the kidney, there were no macroscopic changes at necropsy in sections obtained from the control or ALD mice (Figure 3A,B). Similarly, no significant differences in microscopic pathological changes were observed between the kidneys of the treated and control groups, as shown in Figure 3C−E.

Red Mold Rice Improved Alcohol-Induced Hepatic Injury. Table 3 illustrates the protective effect of RMR on the ALD mice as assessed by serum levels of ALT and AST and the AST/ALT ratio. Chronic daily feeding of alcohol caused liver injury as determined by serum markers for liver damage and hepatic histopathological changes. In the control group, serum ALT and AST levels were 55.6 ± 4.9 and 81.3 ± 11.0 U/L, respectively. After 5 weeks, administration of alcohol significantly increased the levels of serum AST and the AST/ALT ratio compared to that of the control group, but there was no significant change in ALT levels. Serum AST levels were significantly reduced with 2× RMR supplementation, whereas supplements of 5× RMR in mice on alcohol treatment significantly blunted the alcohol-induced increase in AST levels (from 104.0 ± 11.3 to 75.1 ± 9.4 U/L) and the AST/ALT ratio (from 1.9 ± 0.3 to 1.5 ± 0.2 U/L). In addition, there was no difference in BUN (kidney index) between the control and ALD mice.

Red Mold Rice Increased Antioxidant Enzyme Activities. In states of oxidative stress, GSH is converted into GSSG and becomes depleted, leading to lipid peroxidation. Table 4 shows the

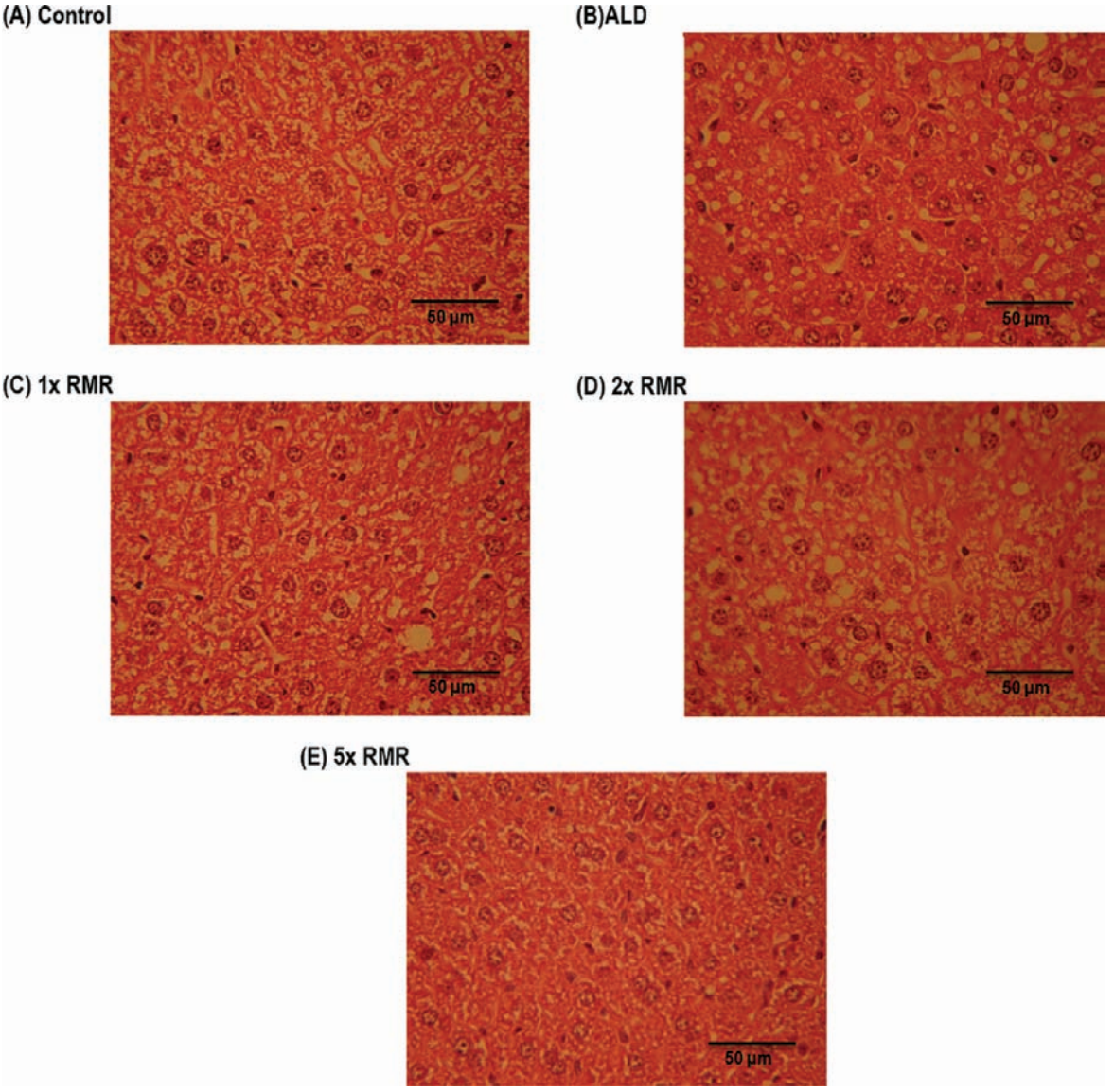

Figure 2. Effects of RMR on liver histopathology of ALD mice using H&E staining: (A) liver tissue of control mice; (B) ALD group; (C) 1× RMR group; (D) 2× RMR group; (E) 5× RMR group (bar = 50 μm).

effects of RMR on GSH/GSSG levels in ALD mice. Administration of 5× RMR to the mice resulted in significant elevation of GSH/GSSG in the liver, whereas low doses did not result in elevation of liver GSH/GSSG. Activities of liver CAT, SOD, GRd, and GPx in the ALD mice were significantly lower compared to those of the control mice. Activities of the antioxidant enzymes CAT, SOD, GRd, and GPx in the liver were increased in all treatment groups compared to those of the ALD mice. Notably, significant elevations in liver CAT, SOD, GRd, and GPx activities were observed in the 5× RMR group, which demonstrated increases of 1.59-fold, 56.6%, 30.5%, and 35.3%, respectively.

Red Mold Rice Attenuated Alcohol-Induced TG and TC Accumulation. Panels A and B of Figure 4 illustrate the effect of chronic alcohol ingestion on hepatic triglycerides and total cholesterol in ALD mice. Hepatic TG and TC levels in the ALD mice increased to match those of the control mice. Hepatic TG accumulation in all treatment groups was significantly attenuated, but there was significant attenuation in hepatic TC accumulation in the 2× and 5× RMR mice.

Panels C and D of Figure 4 illustrate the serum TG and TC contents in chronic alcohol-fed mice. Serum TG content in the ALD mice was significantly elevated compared to that of the control mice, but there was no significant elevation of serum TC. Similarly, the serum TC content in all treatment groups showed no significant change after 5 weeks of alcohol feeding, whereas there was significant attenuation in serum TG accumulation in all treatment groups.

Red Mold Rice Attenuated Alcohol-Induced Pro-inflammatory Cytokines in the Liver. Measurement of the role of RMR in hepatic IL-6, IL-1β, TNF-α, and TGF-β production under chronic alcohol exposure is illustrated by the hepatic cytokine protein values shown in Figure 5.

After 5 weeks, chronic alcohol administration significantly increased hepatic IL-6, IL-1β, and TNF-α production, and this increase was attenuated by RMR supplementation. In addition, hepatic TGF-β production increase was attenuated by RMR supplementation, but the 1× RMR group did not demonstrate significantly attenuated TGF-β production.

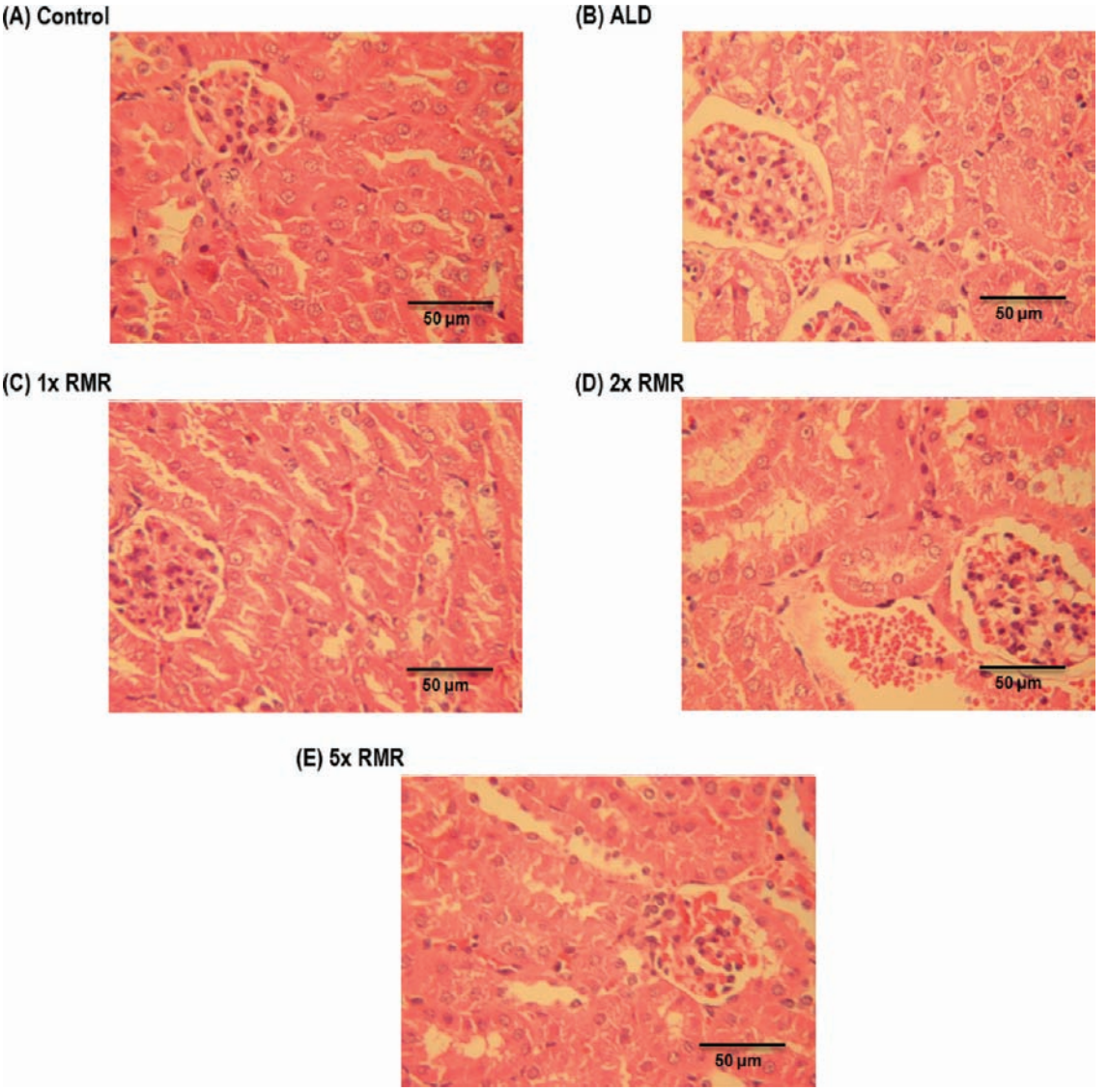

Figure 3. Effects of RMR on kidney histopathology of ALD mice using H&E staining: (A) liver tissue of control mice; (B) ALD group; (C) 1× RMR group; (D) 2× RMR group; (E) 5× RMR group (bar = 50 μm).

Table 3. Effect of RMR on Serum Enzymes and BUN in Alcohol-Induced Liver Damage in Mouse[a]

group[b]	AST (U/L)	ALT (U/L)	AST/ALT ratio	BUN (mg/dL)
control	81.3 ± 11.0	55.6 ± 4.9	1.5 ± 0.2	20.4 ± 2.6
ALD	104.0 ± 11.3*	57.8 ± 8.6	1.9 ± 0.3*	20.5 ± 4.4
1× RMR	96.7 ± 12.2	53.8 ± 4.6	2.0 ± 0.3	21.2 ± 3.1
2× RMR	82.7 ± 10.3#	52.2 ± 3.5	1.7 ± 0.2	23.8 ± 3.9
5× RMR	75.1 ± 9.4#	51.4 ± 1.7	1.5 ± 0.2#	19.5 ± 3.7

[a] Results are expressed as the mean ± SD (n = 8). *, $p < 0.05$ with control group; #, $p < 0.05$ compared with ALD group. AST, aspartate aminotransferase; ALT, alanine aminotransferase; BUN, blood urea nitrogen. [b] ALD, alcohol diet treatment; RMR, red mold rice treatment.

DISCUSSION

Alcohol misuse is one of the most common public health problems in the world. Alcohol misuse has resulted in high socioeconomic waste and serious individual health damage. In Western societies, >10% of the general adult population may be classified as harmful users. In China, the problems caused by alcohol misuse have become salient following the Westernization of lifestyles. Alcohol misuse is a problem now and is the second highest cause of liver disease in the country after the hepatitis virus.[23]

For many years, investigators have studied the impact of ethanol feeding on various liver mechanisms in attempts to describe the chronic alcohol animal model response leading to alcoholic liver injury. Of all the mechanisms studied, oxidative stress plays a very important role in the pathogenesis of alcohol-induced cellular injury.[5,24,25] It has been suggested that ROS are generated during alcohol metabolism mainly by the catalytic action of alcohol-inducible cytochrome P450 2E1 in microsomes. In addition, chronic alcohol consumption permeabilizes the gut, leading to the accumulation of endotoxins in the liver. These endotoxins activate Kupffer cells, producing ROS and a variety of soluble factors and cytokines (e.g., TNF-α) and precipitating liver injury.[26]

Therefore, ALD progresses from ROS generation during alcohol metabolism; at the same time, hepatic endotoxins and

Table 4. Antioxidase and GSH Activities of Liver in Control and Experimental Animals[a]

group[b]	CAT (nmol H_2O_2/min/mg protein)	GPx (nmol NADPH/min/mg protein)	GRd (nmol NADPH/min/mg protein)	SOD (U/mg protein)	GSH/GSSG ratio
control	317.0 ± 90.8	19.2 ± 6.1	17.1 ± 6.2	23.0 ± 3.3	33.7 ± 6.5
ALD	97.0 ± 35.7*	13.3 ± 3.4*	11.8 ± 3.2*	19.6 ± 2.2*	13.7 ± 8.6*
1× RMR	207.4 ± 68.0 #	16.9 ± 0.7 #	14.1 ± 3.8 #	23.5 ± 4.1 #	19.3 ± 15.7
2× RMR	218.9 ± 83.8 #	18.2 ± 1.8 #	15.3 ± 3.7 #	24.4 ± 7.2 #	22.3 ± 8.2
5× RMR	251.5 ± 59.3 #	18.0 ± 4.2 #	15.4 ± 4.7 #	30.7 ± 12.8 #	23.8 ± 8.7#

[a] Results are expressed as the mean ± SD (n = 8). *, $p < 0.05$ with control group; #, $p < 0.05$ compared with ALD group. CAT, catalase; GPx, glutathione peroxidase; GRd, glutathione reductase; SOD: superoxide dismutase; GSH, reduced glutathione; GSSG, oxidized glutathione. [b] RMR, red mold rice treatment; ALD, alcohol diet treatment.

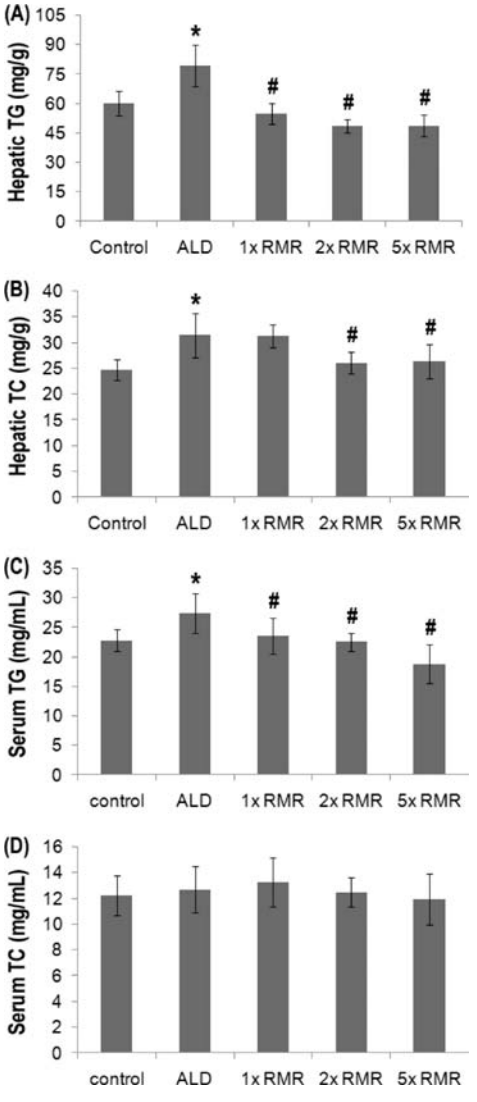

Figure 4. RMR alleviates alcohol-induced liver fatty level: (A) hepatic triglyceride content; (B) hepatic total cholesterol content; (C) serum triglyceride content; (D) serum total cholesterol content. Results are expressed as the mean ± SD (n = 8). ∗, $p < 0.05$ with control group; #, $p < 0.05$ compared with ALD group. TG, triglyceride; TC, total cholesterol.

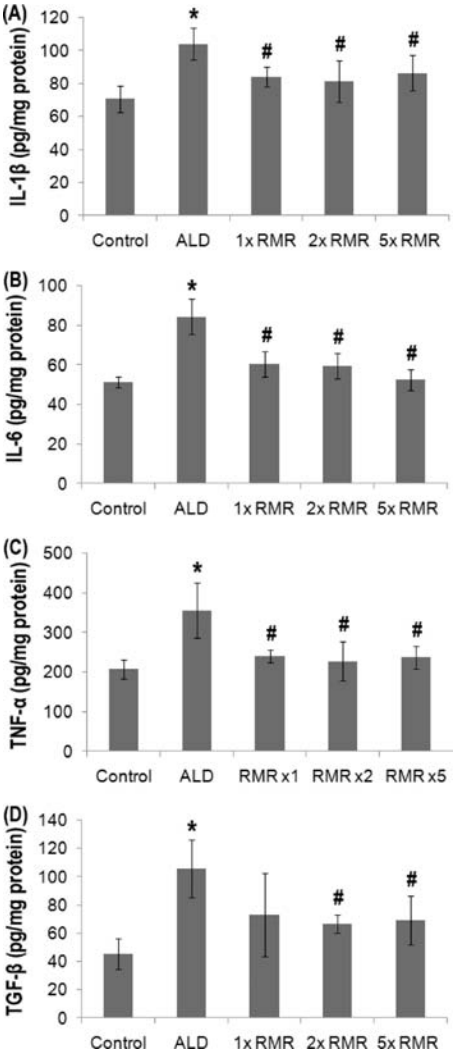

Figure 5. Effects of RMR on inflammatory cytokine expression in the liver from experimental animals. RMR attenuates (A) IL-1β, (B) IL-6, (C) TNF-α, and (D) TGF-β in experimental animals. Results are expressed as the mean ± SD (n = 8). ∗, $p < 0.05$ with control group; #, $p < 0.05$ compared with ALD group.

fatty levels increase, following a harmful course of inflammation that leads to cellular injury. Although the histologic abnormalities of ALD, that is, steatosis, hepatitis, fibrosis, and cirrhosis, have been well documented,[27] the exact mechanisms of pathogenesis of this devastating disease are still unknown, and useful treatments are still lacking at present.

To investigate the possible protective qualities of RMR, we established the chronic alcohol-fed mouse model under experimental

conditions through a 5 week alcohol diet, which can also lead to steatosis, inflammation, and necrosis.

An increased AST level and AST/ALT ratio are conventional indicators of liver injury. In the present study, alcohol also caused histopathological damages to the mouse liver (Figure 2A) and increased serum levels of hepatic enzymes, that is, AST and the AST/ALT ratio (Table 3). The liver is the primary organ of oxidative drug metabolism, so it is particularly susceptible to oxidative damage caused by increased ROS and GSH depletion. Moreover, previous studies indicated that GSH depletion of ≥20% in the liver results in impaired cell defense and tissue injury.[28] There is substantial evidence that ethanol toxicity in the liver is directly linked to the depletion of GSH.[29,30] In addition, CAT, SOD, GPx, and GRd (Table 4) play crucial roles in maintaining the redox balance in cells, and alcohol toxicity is related to the depletion of important enzymes possessing antioxidative actions.[31] In this study, RMR treatment markedly prevented ethanol-induced elevation of serum AST levels and the AST/ALT ratio compared to that of the ALD mice. These findings can be further corroborated with histopathological studies. The histopathological examination clearly revealed that macrovesicular steatosis was attenuated and hepatic cells in the RMR groups were almost normal compared to those of the alcohol-treated group. In addition, alcohol-induced lipogenesis and subsequent fatty liver are among the earliest stages of ALD, characterized by predominant TG and TC accumulation in hepatocytes as a consequence of alcohol-mediated metabolic disturbances.[32−34] The hepatic TG and TC contents in the RMR treatment groups were attenuated compared to that of the ALD mice (Figure 4A,B), but there was no significant difference in serum TC content compared to that of the controls (Figure 4C,D).

Currently, most researchers favor the hypothesis suggesting that the inflammatory reactions associated with alcoholic liver injury are a consequence of an alcohol-induced increase in the absorption of gut-derived endotoxins. The subsequent activation of Kupffer cells releases pro-inflammatory cytokines and chemokines (TNF-α and IL-1β), ROS, and NO. In human beings, levels of TNF-α and IL-6 tend to be increased in alcohol-related hepatitis and cirrhosis, whereas levels of IFN-α tend to be reduced.[35] Furthermore, activated HSC also releases a large number of cytokines during liver injury, among which TGF-β is considered to be the main fibrogenic cytokine. De novo expression of pro-inflammatory cytokines and chemokines further enhances hepatic fibrogenesis by recruiting leukocytes and perpetuating the inflammatory response.[36,37] Finally, it is also generally assumed that soluble mediators secreted by hepatocytes contribute to the stimulation and activation of HSC in chronic liver diseases.[38] These cytokines contribute to the intrahepatic recruitment and activation of granulocytes that are characteristically found in acute severe alcoholic hepatic inflammation.[39] In the RMR treatment groups, hepatic cytokine levels, including IL-1β, IL-6, TNF-α, and TGF-β, were significantly attenuated compared to those of the ALD mice (Figure 5).

In this chronic alcohol-induced liver injury model, we found that RMR possesses a hepatoprotective ability that not only decreased pro-inflammatory cytokine, TG, and TC contents but also increased antioxidative enzyme levels. Collectively, our results indicate that RMR alleviates alcoholic liver injury by inhibiting oxidative stress and inflammation. Therefore, RMR may represent a novel functional food to prevent alcoholic liver disease.

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ABBREVIATIONS USED

IL , interleukin; TNF , tumor necrosis factor; TGF, transforming growth factor.

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