Dietary Cholestin (Red Yeast Extract) Reduces Toxicity of Oxidized Cholesterol in Rats

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ABSTRACT

This study focused on the effects of dietary cholestin (red yeast extract) on the toxicity of oxidized cholesterol in rats. The rats were divided into eight groups and fed with or without supplement of 1% cholestin and 3% oxidized cholesterol in their diets for 8 weeks. It was found that cholestin could improve the body weight decrease and the glutathione (GSH) level in the liver, and increase of liver and kidney weight to body weight ratio, and thiobarbituric acid-reactive substances (TBARS) level in the liver of rats caused by oxidized cholesterol ($P < 0.05$). It also reduced the plasma content of alanine transaminase (ALT), aspartate transaminase (AST) and alkaline phosphatase (ALP), indicating that cholestin could prevent the hepatotoxicity induced by oxidized cholesterol ($P < 0.05$). In addition, cholestin possessed recovering effect and a short-term preventing effect on the toxicity of oxidized cholesterol in rats. Taking all these data together, cholestin may play an important role in diminishing the toxic effects of oxidized cholesterol in rats.

Key words: cholestin, oxidized cholesterol, toxic effect, rats, liver function

INTRODUCTION

Red yeast rice is common used in China for centuries to enhance the color and flavor of food, as well as a traditional medicine for digestive and vascular functions[1-4]. In the late 1990s, dietary supplement companies decided to commercialize red yeast extracts because of their ability to reduce cholesterol as efficiently as statin drugs. It was placed on market in 2001[2,4-9]. However, in the spring of 2001, the US Food and Drug Administration (FDA) banned the sale of dietary supplement containing red yeast extracts or xuezhikang[10], which was found to contain lovastatin. Because lovastatin belongs to drug category, FDA made an administrative decision to remove this dietary supplement (often sold as cholestin in earlier times) from the health food store. Cholestin is the fermented product of rice on which red yeast (*Monascus purpureus*) has been grown; and it is a dietary staple in many Asian countries with typical consumption ranging from 0.5 to 2 oz/person/day[11]. This product has been used as a food preservative for maintaining taste and color in fish and meat, and/or as functional medicine[12]. The medicinal properties of red yeast extract were described by pharmacologists of the Ming Dynasty (1368-1644) as cited by Ma *et al*.[3]. Increased levels of cholesterol and triglycerides are known to be risk factors for developing coronary artery diseases. Lipid-lowering agents that inhibit HMG coenzyme A reductase are now prominent among the drugs of choice for treating hypercholesterolemia. It is another effective way to control cholesterol level with diet and food supplements[13]. Cholestin also contains 2-6% fatty acids including palmitic acid, linoleic acid, oleic acid and stearic acid[14], some of which have been shown to have the ability to reduce blood-lipid level in animal models and humans[2,4,5,15-19]. Indeed, diets enriched with cholestin were effective in reducing cholesterol in high cholesterol rabbits and rats[20-22].

Oxidized cholesterol as well as oxidized fatty acids from food system have been a concern for diseases. A variety of oxidized cholesterols have been detected in processed foods such as meat products[23], commercial sweet baked foods[24] and seafoods[25]. Therefore, consumption of oxidized cholesterols is inevitable in

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our usual diet. The formation of oxidized cholesterols was accelerated by polyunsaturated fatty acids (PUFAs) present in lipids[26]. Oxidized cholesterols have been known to be more injurious to arterial cells than pure cholesterol and are more directly connected to the development of atherosclerosis, coronary heart disease and possibly carcinogenic effects[27-29].

In this study, an animal model was used to evaluate the protection effect of dietary cholestin on liver injury induced by oxidized cholesterol.

MATERIALS AND METHODS

I. *Preparation of Cholestin*

Cholestin is a fermented product of rice on which red yeast (*Monascus purpureus*) has been grown. *Monascus purpureus* strain BCRC 31498 was purchased from the Bioresources Collection and Research Center (Food Industry Research and Development Institute, Taiwan, R.O.C.). The fungus was maintained on malt extract broth (MEB) agar, containing 4 g/L yeast extract, 20 g/L malt extract, 20 g/L glucose, and 20 g/L agar (pH 7.0). Freshly inoculated cultures were incubated at 28°C for 5 days, kept at 4°C as stock culture and then transferred to fresh medium monthly.

M. purpureus strain BCRC 31498 was grown in liquid medium by inoculating one loop of stock culture into a 500-mL Erlenmeyer flask containing 50 mL of malt extract broth I (Blakeslee's formula) growth medium (containing 20 g of malt extract, 20 g of glucose, and 1 g of peptone in 1 L of distilled water, pH 4.7) and incubated at 30°C on a rotary shaker at 220 rpm. Lovastatin esterase activity was induced by the addition of lovastatin ammonium salt (LAS) to each flask to a final concentration of 0.5 mg/mL. The culture was then allowed to incubate for another day before it was harvested.

The harvested red yeast was extracted with boiling water at 100°C for 4 hr. The extract was then filtered through a Bűchner funnel and freeze-dried. The dried red yeast extract (cholestin) was stored at -20°C before use.

II. *Preparation of Oxidized Cholesterol*

Cholesterol (99.9% purity, Wako Chemical, Osaka, Japan) was heated at 150°C for 12 hr to produce oxidized cholesterol[30], a peroxide value (POV) of approximate 50.2 meq/kg oil, a content of the thiobarbituric acid reactive substances (TBARS) of 1.58 mg/kg oil and a content of the acid value (AV) of 1.74 mg/g oil. The values of POV, AV and TBARS in the oil were determined by AOAC method[31].

III. *Animals*

Male weanling Wistar rats were purchased from the National Laboratory Animal Center (Taiwan, R.O.C.). They were kept in air-conditioned room (23 ± 1°C, 50-60% humidity) lit for 12 hr/day (7 a.m. to 7 p.m.).

Table 1. Composition of rat diet in each group for test Cholestin and oxidized cholesterol

Ingredients	Diets			
	Basal diet (%)	Cholestin diet (%)	Oxidized cholesterol diet (%)	Cholestin + oxidized cholesterol diet (%)
Casein	20	20	20	20
Methionine	0.3	0.3	0.3	0.3
Cellulose	5	5	5	5
Corn oil	2	2	2	2
Cholesterol	3	3	0	0
Oxidized cholesterol	0	0	3	3
Choline	0.2	0.2	0.2	0.2
Mineral mix(a)	3.5	3.5	3.5	3.5
Vitamin mix(b)	1	1	1	1
Cholestin	0	1	0	1
Corn starch	65	64	65	64

(a) Minerals (per 100 g of diet): NaCl 7.4 g, $K_2C_6H_5O_7 \cdot H_2O$ 22 g, K_2SO_4 5.2 g, $CaHPO_4$ 50 g, MgO 2.4 g, $FeC_6H_5O_7 \cdot 5H_2O$ 0.6 g, $MnCO_3$ 0.35 g, $CuCO_3$ 30 mg, $CrK(SO_4)_2 \cdot 12H_2O$ 55 mg, $CoCl_2 \cdot 6H_2O$ 10 mg, KI 1 mg, $ZnCO_3$ 160 mg.

(b) Vitamin (per 100 g of diet): thiamine 100 mg, riboflavin 150 mg, pyridoxine HCl 100 mg, nicotinamide 1000 mg, D-panthenate 500 mg, folic acid 50 mg, vitamine B_{12} 0.1 mg, vitamin A 2.5×10^5 IU, vitamin E 100 mg, calciferol 2×10^4 IU, vitamin C 3.7×10^3 mg.

Experimental protocol was approved by the Institutional Animal Care and Use Committee of Toko University. After acclimatizing for 2 weeks with a commercial non-purified diet (rodent Laboratory Chow 5001, Purina Co., USA), 48 rats were divided into eight groups. Six rats in each group were assigned to receive one or two of four formulated diets during a 8 week period of one or two of four formulated diets (Table 1). The diets were synthesized as described previously by the American Institute of Nutrition (AIN)[32] and included: basal diet (control diet), cholestin diet (1% cholestin in diet), oxidized cholesterol diet (3% oxidized cholesterol) and oxidized cholesterol + cholestin diet (3% oxidized cholesterol + 1% cholestin). The diet regimen used in each group was as follows: Group A: rats were fed with basal diet for 8 weeks; Group B: rats were fed with cholestin diet for 8 weeks; Group C: rats were fed with oxidized cholesterol diet for 8 weeks; Group D: rats were fed with cholestin + oxidized cholesterol diet for 8 weeks; Group E: rats were fed with oxidized cholesterol diet for 1 month and then with cholestin diet for 1 month; Group F: rats were fed with oxidized cholesterol diet for 1 month and then with basal diet for 1 month; Group G: rats were fed with cholestin diet for 1 month and then with oxidized cholesterol diet for 1 month. Group H: rats were fed with basal diet for 1 month and then with oxidized cholesterol diet for 1 month. Water and food were freely available during the experimental period. After feeding, all rats were weighed. Blood samples were taken from the tail vein of the rats at 0, 2, 4, 6 and 8 week intervals and analyzed. The plasma of blood samples were collected by centrifugation (1,000 ×g for 15 min) and examined for alanine transaminase (ALT), aspartate transaminase (AST) and alkaline phosphatase (ALP) activities. On the 8 week, liver and kidney of the rats were excised and weighed. Ratios of liver and kidney weight to body weight were obtained. The livers were stored at -40°C for glutathione (GSH) and TBARS determinations.

IV. *Assays of Enzymatic Activities*

The plasma ALT, AST and ALP activities were determined using enzymatic kit with Selectra Analyser (Merck Co. Ltd., Germany).

V. *Thiobarbituric Acid-reactive Substances (TBARS) Production*

Lipid peroxidation activities in the liver were assayed by measurement of malondialdehyde (MDA), an end-product of peroxidized fatty acids and thiobarbituric acid (TBA) reaction product. The sample of 20% liver homogenate was mixed with 1.0 mL of 0.4% TBA in 0.2 N HCl and 0.15 mL of 0.2% BHT in 95% ethanol. The samples were incubated in a water-bath at 90°C for 45 min. After incubation, the TBAMDA adduct was extracted with isobutanol. The isobutanol extract was mixed with methanol (2: 1, v/v) prior to injection into a high performance liquid chromatography (HPLC) system (Hitachi) with a Model L-6200 pump (Tokyo, Japan), and used to monitor by excitation at 515 nm and emission at 550 nm Model L-4000 Hitachi fluorescence detector (Tokyo, Japan)[33].

VI. *Levels of Glutathione (GSH) Measurement*

GSH reacts non-enzymatically with 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB) to yield glutathione disulfide (GSSG) and 2-nitro-5-thiobenzoic acid (TNB). GSSG is then reduced enzymatically by NADPH and glutathione reductase (GR) to regenerate GSH. Concentrations of DTNB, NADPH and GR were chosen such that the rate of the overall reaction is linearly proportional to the concentration of total GSH. The rate of formation of TNB was followed spectrophotometrically, and the assay was calibrated using standards. If the sample reacted with 2-vinylpyridine, GSH is derivatized, and only GSSG can be detected during subsequent assay[34].

VII. *Histopathology*

Liver samples were fixed with 10% formalin phosphate-buffer, dehydrated, paraffin-embedded and archived. Sections of 2-4 μm of all zones of hepatic lobule were cut sagittally and mounted on aminopropyltriethoxysilane-coated slides (APTS, A-3648, Sigma, USA). Following deparaffinization in xylene, sections were rehydrated, stained with hematoxylin and eosin (H&E) and examined by light microscopy[35].

Table 2. The experimental diets in each group for tested cholestin and oxidized cholesterol

Weeks	Group							
	A	B	C	D	E	F	G	H
1-4 weeks	basal diet	cholestin diet	oxidized cholesterol diet	cholestin + oxidized cholesterol diet	oxidized cholesterol diet	oxidized cholesterol diet	cholestin diet	basal diet
5-8 weeks	basal diet	cholestin diet	oxidized cholesterol diet	Cholestin + oxidized cholesterol diet	cholestin diet	basal diet	oxidized cholesterol diet	oxidized cholesterol diet
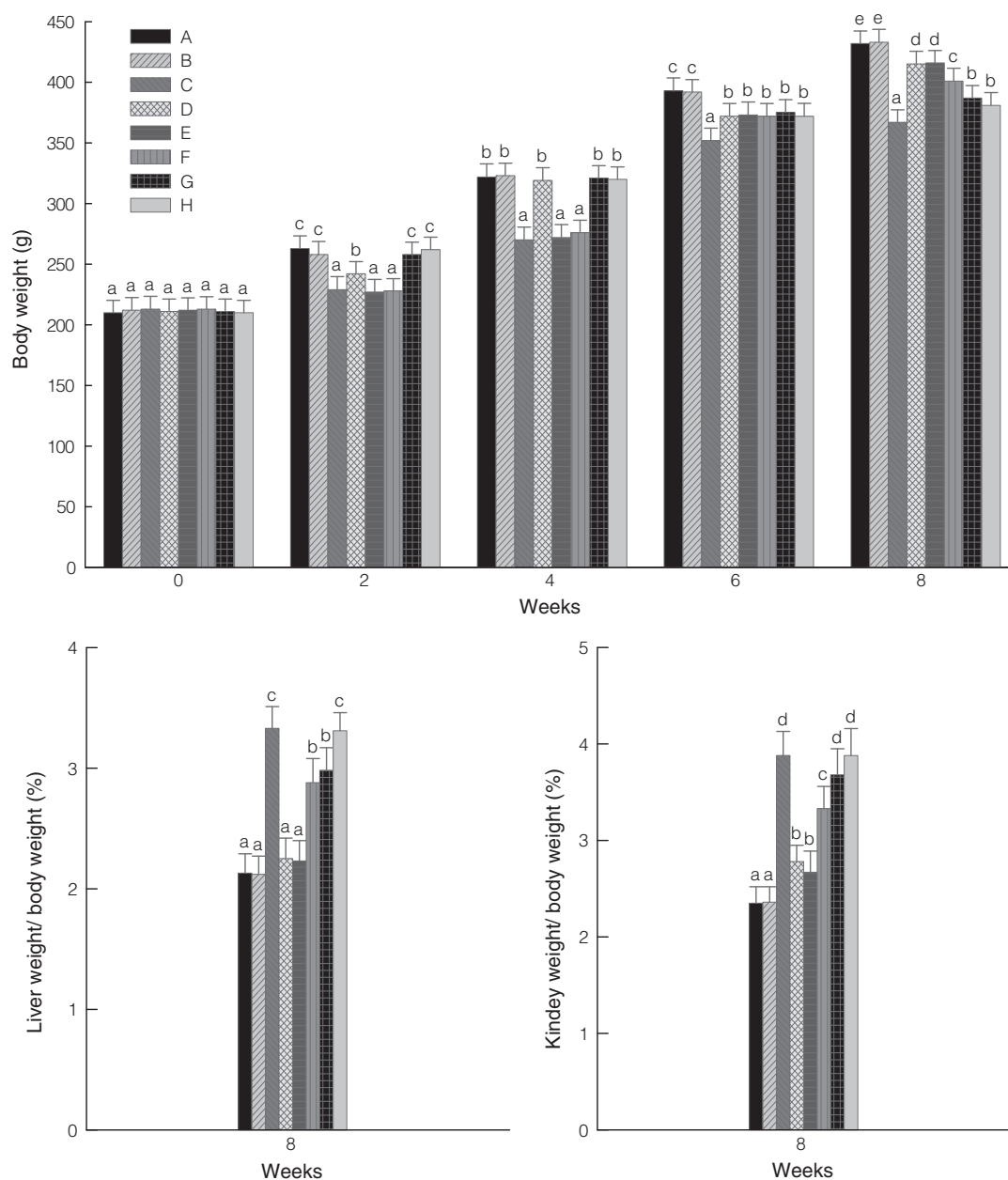
Figure 1. Effect of oxidized cholesterol and cholestin on the body weight and the ratios of liver and kidney weight to body weight of rats. a-e: values in the same week with different superscript are significantly different ($P < 0.05$). A, B, C, D, E, F, G and H mean eight groups of rats with different experimental diets. (Table 2)

VIII. *Statistical Analysis*

Statistical analysis for differences among experimental rats in the groups was performed by the 2-way analysis of variance procedure and Duncan's new multiple range test[36]. A *P* value less than 0.05 was considered statistically significant.

RESULTS

The effects of cholestin and oxidized cholesterol on

the growth of rats are shown in Figure 1. After feeding for 8 weeks, the weight gains of group A and group B rats were significant among all tested groups ($P < 0.05$), indicating that oxidized cholesterol retarded the growth of rats. The weights of group C rats were less than those of group D rats ($P < 0.05$) suggesting that cholestin could reduce the toxicity of oxidized cholesterol on the weights of rats. Weights of group E rats were heavier than group F rats, showing that cholestin had recovering effect on the toxicity of oxidized cholesterol. However, the weights of group G rats were not significantly different from those of group H rats. It indicated that cholestin
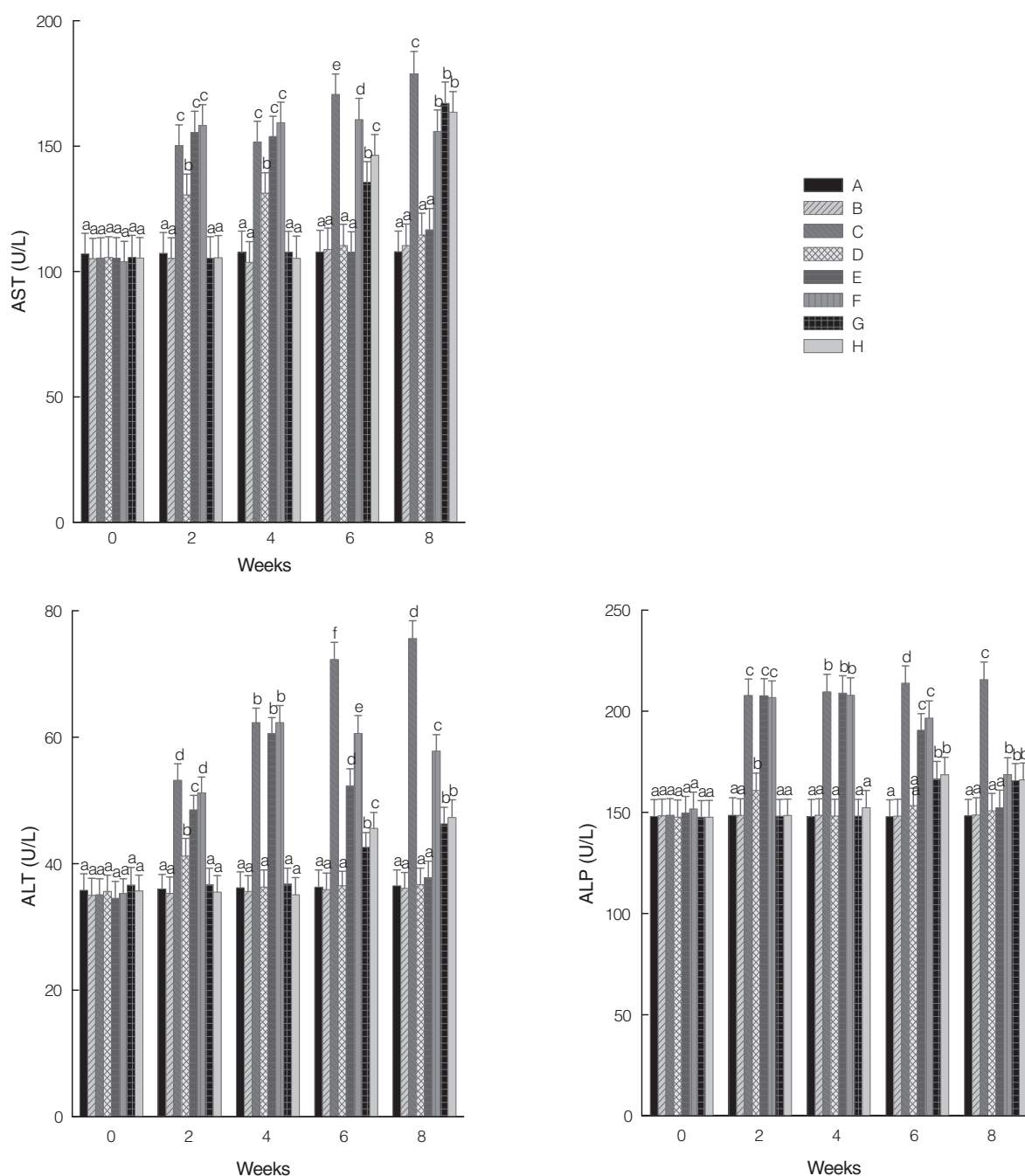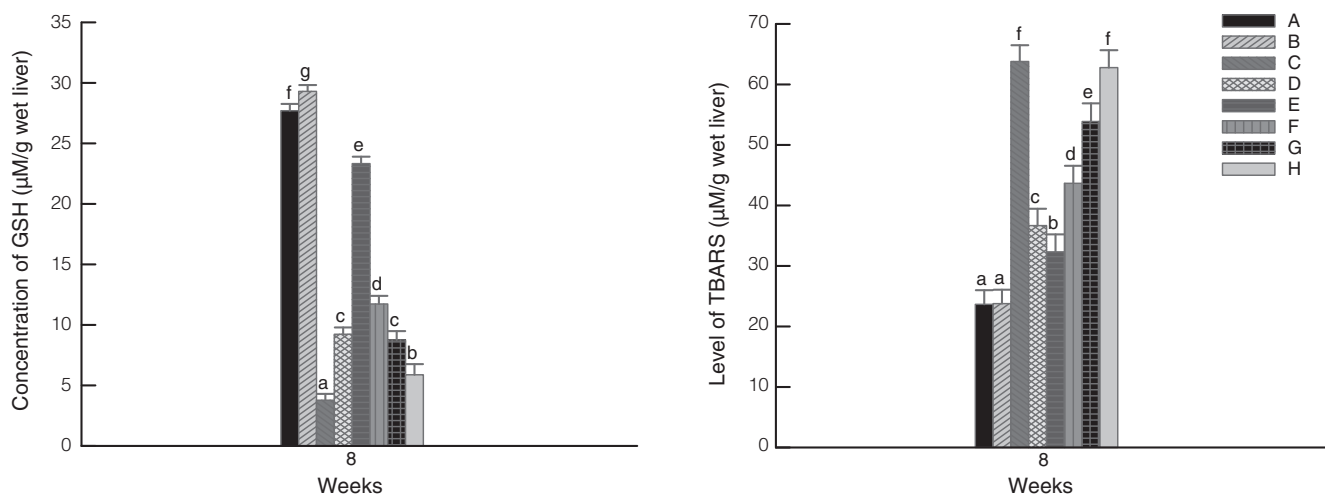
Figure 2. Effect of oxidized cholesterol and cholestin on the activities of AST, ALT and ALP in plasma of rats. a-f: values in the same week with different superscript are significantly different ($P < 0.05$). A, B, C, D, E, F, G and H mean eight groups of rats with different experimental diets. (Table 2)

had no preventing effect from the toxicity of oxidized cholesterol on the weight of rats. The effects of cholestin and oxidized cholesterol on the relative ratios of liver and kidney weights to body weight in rats are shown in Figure 1. After 8 week of feeding, the ratios of liver and kidney weight to body weights of group C rats were more significantly increased than those of group A, group B and group E rats ($P < 0.05$). On the other hand, the ratios of liver and kidney weight to body weight in group E

rats were not significantly different from those of group A and group B rats. It meant that cholestin might reduce the toxicity of oxidized cholesterol in the rats based on the relative ratio of liver and kidney weight to body weight of rats. The ratios of liver and kidney weight to body weight in group E rats were significantly less than those of group F rats ($P < 0.05$), indicating that cholestin exhibited recovering effect on the toxicity of oxidized cholesterol. Meanwhile, the ratios of liver and kidney

Figure 3. Effect of oxidized cholesterol and cholestin on the concentration of glutathione (GSH) and level of thiobarbituric acid reactive substances (TBARS) in the liver of rats. a-g: values in the same week with different superscript are significantly different ($P < 0.05$). A, B, C, D, E, F, G and H mean eight groups of rats with different experimental diets. (Table 2)

weight to body weight in group G were not significantly different from those of group H rats. It meant that cholestin had no preventing effect from the toxicity of oxidized cholesterol based on the ratios of liver and kidney weight to body weight in rats.

The effects of oxidized cholesterol and cholestin on the activities of ALT, AST and ALP in rat plasma are shown in Figure 2. It was found that the activities of ALT and ALP in rat plasma of group C were the highest and significantly higher than all tested groups ($P < 0.05$). The activities of ALT, AST and ALP in rat plasma of group D were significantly less than those of group C ($P < 0.05$), indicating cholestin had reducing effect on the liver toxicity of oxidized cholesterol. The activities of ALT, AST and ALP in rat plasma of group E were significantly less than those of group F ($P < 0.05$). This indicated that cholestin exerted recovering effect on the hepatotoxicity of oxidized cholesterol. Furthermore, the activities of ALT, AST and ALP in rat plasma of group G were significantly less than those of group H ($P < 0.05$). However, ALT and ALP activities in rat plasma of group G were also less than those of group H ($P < 0.05$), indicating cholestin exhibited a short-term preventing effect from the liver toxicity of oxidized cholesterol on the activities of ALT, AST and ALP in plasma of rats.

The effects of cholestin and oxidized cholesterol on the concentrations of TBARS and GSH in the liver of rats are shown in Figure 3. After feeding for 8 weeks, the levels of TBARS in the livers of group C and group H rats were higher than other groups ($P < 0.05$). It indicated that cholestin revealed reducing effect on the toxicity of oxidized cholesterol based on the concentration of TBARS. The level of TBARS in the livers of group E rats was less than group F, indicating cholestin exerted recovering effect on the toxicity of oxidized cholesterol. No difference was found between levels of

TBARS in the livers of group G and group H rats ($P > 0.05$), showing cholestin had no preventing effect from the toxicity of oxidized cholesterol based on the TBARS level. The concentration of GSH in the livers of group B rats was the highest among all tested groups (Figure 3). It indicated that cholestin played a role in increasing the GSH level in the liver of rats. The concentration of GSH in the livers of group E rats was less than group F ($P < 0.05$), indicating that cholestin exhibited recovering effect on the toxicity of oxidized cholesterol. The concentration of GSH in the livers of group G rats was higher than group H ($P < 0.05$), showing cholestin revealed preventing effect from the toxicity of oxidized cholesterol. But this functional effect was low because the level of GSH in the liver of rats increased only slightly. Judging from the above data, cholestin might play an important role in reducing the toxic effect of oxidized cholesterol in rats. Cholestin could increase the body weight of rats and GSH level of liver in rats, and decrease the enlargement of liver and kidney, the enzymatic activities of ALT, AST and ALP in the plasma and TBARS level in the liver of rats. These results showed that cholestin had good function in reducing toxicity of oxidized cholesterol in rats when fed diet with the supplement of oxidized cholesterol and cholestin at the same time, or fed diet with the supplement of oxidized cholesterol and then cholestin. Cholestin also had a short-term preventing effect in reducing the toxicity of oxidized cholesterol.

Histopathological changes were assessed by observing liver sections for necrotic and swollen hepatocytes. The histological finding is shown in Figure 4. Swollen cells showing enlargement and ruptured plasma membrane were identified. Morphological alterations involving all zones of hepatic lobule were observed in group C, F, G and H treatment, revealing necrosis,
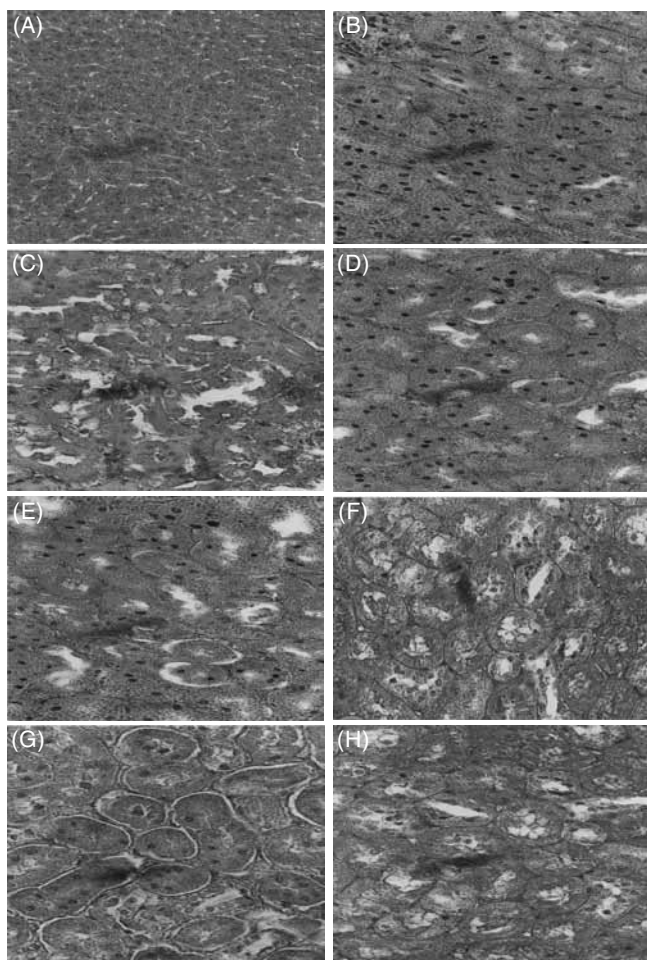
Figure 4. Microscopic cross section of liver lobules in rats after 8 weeks (400x, H&E). Bar represents 0.01 mm. A, B, C, D, E, F, G and H mean eight groups of rats with different experimental diets. (Table 2)

degeneration and enlargement of the tubular or peritubular tissues.

DISCUSSION

In this study, the activities of ALT, AST and ALP in plasma of rats were shown to be significantly affected by oxidized cholesterol. In the clinical examination, the increased levels of ALT, AST and ALP activities in plasma served as biomarkers for liver injury[37]. The activities of AST were significantly elevated in rats fed with oxidized cholesterol for 8 weeks meaning that oxidized cholesterol might injure liver function. Cholestin significantly reduced the enzymatic activities of ALT, AST and ALP in plasma of rats, indicating that the liver injury by oxidized cholesterol could be ameliorated by cholestin. In the experimental period, the food consumption of rats was found to significantly decrease in those groups of rats fed oxidized cholesterol diet. However, it was accompanied by adverse effects such as

nephrotoxicity and hepatotoxicity[38]. The palatability of the diet might also be affected. Therefore, the oxidized cholesterol diet may induce liver injury and reduce diet palatability in rats. TBARS was an end-product of lipid peroxidation. The level of TBARS in the liver was increased when the rats were fed with oxidized cholesterol. It means that the liver injury caused by feeding with oxidized cholesterol was due to the induction of the lipid peroxidation of liver cells. The level of TBARS in the liver of rats was significantly reduced when the rats were fed with the supplement of cholestin. These results were the same as that reported previously[1,39,40]. Therefore, it was reasonable to assume that cholestin may act as scavenger in reducing the production of lipid peroxidation[41]. Clinical trials using red yeast extract in patients with hyperlipidemia[5,42], coronary heart disease[43], and HIV-related dyslipidemia[44] as well as in inhibitory effect of tumor promotion[45] have also demonstrated an improvement in lipid profiles. In recent reports, fermented red yeast rice preparation effectively improves by its potential lipid-lowering effects[46-50]. This product possesses promising activity as a new hypolipidemic drug launched worldwide[9]. Meanwhile, the function of GSH to protect biological organisms from xenobiotic injuries was well known[25,51-53].

Lipid peroxidation is a chemical mechanism which is capable of disrupting the structure and function of the biological membranes that occurs as a result of free radical attacking on lipids. When reactive oxygen species (ROS) begin to accumulate, hepatic cells exhibit a defensive mechanism by various antioxidant enzymes. The main detoxifying systems for peroxides are catalase and GSH[54]. Catalase is an antioxidant enzyme, which destroys H_2O_2 that can form a highly reactive hydroxyl radical in the presence of iron as a catalyst[55]. By participating in the glutathione redox cycle, GSH together with GSH-Px convert H_2O_2 and lipid peroxides to nontoxic products. Reduced activity of one or more antioxidant systems due to the direct toxic effect of oxidized cholesterol leads to increased lipid peroxidation, oxidative stress, and hepatotoxicity. In the current study, oxidized cholesterol depleted GSH reservoir and reduced catalase and GSH-Px activities. These results were in harmony with other investigation[56]. For example, oxidized cholesterol induced hepatotoxicity was exacerbated by GSH depletion. In the current study, the depletion of GSH reservoir can account for the inhibition of GSH-Px activity. In addition, high levels of peroxides may explain catalase activity inhibition[57].

In our study, cholestin supplementation significantly mitigated oxidized cholesterol inducing oxidative stress and hepatotoxicity. It was clearly manifested by the improvement in all the biochemical variables determining oxidized cholesterol hepatotoxicity. In addition, cholestin inhibited lipid peroxidation, diminished the decrease in catalase and GSH-Px activities, and abrogated GSH depletion induced by oxidized cholesterol.

Cholestin had been demonstrated as a direct antioxidant that scavenges or quenches oxygen free radicals, thus inhibiting lipid peroxidation, and as an indirect antioxidant that prevents the increase in membrane permeability resulting from oxidant injury in many tissues including liver and plasma[40]. Cholestin might stimulate *S*-nitrosylation of GSH producing *S*-nitrosoglutathione, which is approximately 100 times more potent than the classical GSH[41]. In addition, *S*-nitrosylation of cysteine residues from nitrosoglutathione can inactivate caspase-3, thus preventing hepatic cell apoptosis[58]. Moreover, cholestin might lessen oxidized cholesterol inducing oxidative injury and be capable of lowering or slowing down oxidative stress[40]. In summary, the data presented in this paper showed that administration of cholestin was a safe and effective way to of lowering oxidized cholesterol toxicity.

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