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Research Article

Red Yeast Rice Protects Hepatocytes conditions of Rats Receiving High Fat Diet

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ABSTRACT

Triglyceride (TG) is a simple and the main lipid of the daily diet. Nowadays, TG is emerging as an independent risk factor for cardiovascular disease, and increasing clinical data that indicate a high level of this simple lipid in serum may alert and play a role in liver impairment. Red yeast rice (RYR) reduces serum TG in human and animals. The rice is widely used as a natural inhibitor of HMG-CoA reductase to correct serum cholesterol level. Bioactive components of the rice are well known to have antioxidant properties. This study examined whether RYR protects hepatocytes by evaluating the serum AST-ALT, HDL-Cholesterol (HDL-C), TG levels, and the number of liver foam cells in hypertriglyceridemic rats. Twenty male Wistar rats were grouped into 5. Four groups received a high-fat diet (HFD), 40 g/animal/day for 60 days to induce hypertriglyceridemic condition. Along with the HFD treatment, three groups received 108, 54, and 27 mg/kgBW/day of RYR, respectively. Two other groups received standard and only HFD diet, respectively. Intracardiac blood was collected for measuring AST and ALT using AST or ALT activity assay Kit, respectively, serum TG and HDL-C by Enzymatic Caloric Test. Hematoxylin-Eosin–stained 4µmm thick slices of liver tissues were prepared to count foam cell number by a light microscope with 400x magnificence. Data were analyzed using the Kruskal Wallis continued by the Mann Whitney U test. The p values of < 0.05 were considered to be significant. The present study found that 108, 54, and 27 mg/kgBW/day of RYR significantly decreased serum TG, HDL-C, AST, and ALT compared with those of hypertriglyceridemic rats receiving no RYR. The decreasing levels of the parameters were in relation to the doses of RYR. The doses of 108 and 54 mg/kgBW/day resulted in complete recovery of the liver tissues suffered from steatosis ($p < 0.05$). The RYR dose of 108 mg/KgBW/day completely corrects the serum HDL-C level. In conclusion, red yeast rice may have a potency to protect hepatocytes injuries due to hypertriglyceridemia.

Keywords: Red yeast rice, foam cell, hypertriglyceridemia, AST-ALT

Introduction

Hyperlipidemia is associated with an intake of a high-fat diet (HFD) usually characterized by dyslipidemia including a high level of triglyceride (TG) and accompanied by a low level of high-density lipoprotein [1]. Long term hyperlipidemia in non-alcoholic drinkers may lead to fatty liver disease including hepatic steatosis and liver damage [2]. The disease was considered to be more susceptible to Asian people than Caucasian and Afro-American people [3]. A study on Wistar rats receiving high sucrose diet showed that postprandial hypertriglyceridemia alerts the development of insulin resistance and glucose intolerance, both may develop to type 2 diabetes mellitus (T2DM) [4]. High levels of TG and remnant cholesterol (triglyceride-rich lipoproteins) are important triggers for the development of cardiovascular impairment and its complications [5]. Hypertriglyceridemia contributes to the development of fatty liver dis-

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ease – NAFLD [6]. It was reported that red yeast rice (RYR) contains monacolin K, a natural HMG-CoA reductase inhibitor [7]. Red yeast rice also contains monascin and ankaflavin reported to have anticancer, antioxidative, and anti-inflammatory abilities [8]. These active substances of RYR increased HDL-C while monacolin K was unlikely to show the activity [9]. A meta-analysis study reported RYR contains effective bioactive components that are relatively safe to correct dyslipidemia [10]. An extract of RYR known as Xuezhikang (80 mg/kg/day) was reported to have more potencies in reducing serum LDL-C level than simvastatin (1 mg/kg/day) in male Sprague-Dawley rats [11].

The secondary metabolite of Monascus purpureus called citrinin is known to be carcinogenic, hepatoxic, and nephrotoxic to humans and animals [12]. The previous study strongly proposed that citrinin toxicity and genotoxicity involve oxidative stress or increased permeability of mitochondrial membranes [13]. Preliminary studies showed maximal citrinin dose without any kidney complications in humans is 0.2 µg/kgBW/day. Adults and children consuming high diet may be exposed on citrinin at a concentration ranging from 9 to 53 µg/kg grain-based products. Citrinin concentration of average diet consumers is between 19 and 100 µg/kg grain-based products [14]. Daily intake monacolin K may contain about 0.2% of the total grain-based product [15].

The present study prepared the hypertriglyceridemic rats by adopting the Murwani's HFD having 4 types each which contain the same type of lipid components but differ in its concentrations [16]. This study applied T1HFD (type 1 of Murwani's HFD) containing the lowest concentration of lipid to mimic daily high fat diet. We modified the RYR doses of Aggraeni CD (2007) [17] into a half portion to minimize the toxic effect of RYR. In respect to these conditions, the potencies of RYR in correcting serum AST-ALT, TG, HDL-Cholesterol (HDL-C), and in reducing liver foam cells development in hypertriglyceridemic rats were studied.

Material and Methods

Animal

Twenty male Wistar rats, 120 -160 g, age 8 – 12 weeks, were separated into 5 groups in equal numbers. The rats were bred at the Physiology Laboratory of Faculty of Medicine, Universitas Brawijaya. Each animal was individually housed in wood shaving-bottomed and wire-topped cages. The room was under controlled temperature (22°-25°C), humidity was 50–60%, and the light was set on for 12 hours. Animals received human care as outlined by the Animal Care – Research Ethics Committee of Universitas Brawijaya.

Preparation of hypertriglyceridemic rats and RYR

The present study used Wistar rats because the strain was susceptible to hypertriglyceridemic condition and its complications when the animals were treated with HFD [18, 19, 20]. Branded RYR was purchased from a local drugstore. The rice was thoroughly pounded then filtered to obtain fine powder. The rats were fed with 40 g/rat/day of T1HFD - the type 1 of Muwarni's HFD and free water access for 60 days to induce hypertriglyceridemia. The T1HFD contains the least percentage of cholesterol, cholic acid, and lard. It consists of 200 g branded poultry feed (purchased), 100 g wheat flour, 4 g cholesterol, 0.4 g cholic acid, 10 ml lard, and 85.6 ml water [16]. Along with the T1HFD administration, 3 groups of the rats received 108, 54, 27 mg/kgBW/day of RYR respectively in 2 ml bidestilated-water, by sonde. The RYR doses were half of those used in the previous study [17]. Two other groups were healthy rats receiving a standard diet and T1HFD-treated rats with no RYR treatment.

AST-ALT, TG, and HDL-C measurement

At the final of the experiment, the rats were given ether anesthesia by putting them in transparent glass boxes (20 x 20 x 20 cm) containing ether saturated-air. One box was used for one rat only. Unconscious rats were tested for pain responses prior to surgery. Intracardiac blood was aspirated by using 5 ml syringes, and the blood taken was put in vacutainer without EDTA. Blood samples were immediately centrifuged at 3500 rpm for 15 minutes. Serum was separated and stored overnight at -20°C. The serum AST - ALT was measured by using AST – ALT activity assay kit for rats. Briefly, the activities of AST and ALT were detected by the amount of glutamate and pyruvate, respectively. Glutamate and pyruvate were detected by using colorimetric assay at λ450 and λ570, respectively (Sigma-Aldrich). The results

were in mU/L. Serum TG was measured using enzymatic colorimetric assay kit for rat (GPO-PAP Methode-Roche), and HDL-C was determined by rat serum HDL-C Assay Kit (Crystal chem). Subsequently, the results were obtained in mg/dL.

Liver foam cells counting

The liver was gently and thoroughly washed by phosphate buffer solution to remove blood, then was put into organ container in neutral buffered formalin 10% solution and stored at room temperature for 48 hours. Hematoxyline-Eosin–stained 4 µmm thick slices of liver tissue were prepared. The number of liver foam cells were observed using light microscope (400× magnificence). The detection of liver foam cells based on the non-alcoholic distribution pattern of steatosis showed 4 types of distribution namely; zone 1, zone 3, panacinar, and azonal [21]. Liver foam cell was presented as a percentage of hepatocytes in a field of view. The mean percentage of 5 different fields of views of each slice was listed to obtain the mean liver foam cells percentage of a rat. The double-blind method was done to evaluate and count the liver cells.

Statistical analysis

The data were analyzed using Kruskal-Wallis. The analysis was continued by the Mann-Whitney U test. The statistical significance level was $p < 0.05$. Data were expressed as means ± SD.

Results and Discussion

Effect of RYR on serum AST-ALT and TG of T1HFD-treated rats

Serum TG and AST-ALT of T1HFD-treated rats receiving the three doses of RYR were significantly reduced ($p < 0.05$) compared with those of T1HFD-treated rats receiving no RYR. The given doses of RYR lowered the serum TG and AST – ALT level of T1HFD-treated rats in respect with dose manner of RYR ($p < 0.05$). Unfortunately, the serum levels of TG and AST- ALT were not approximate to those of the healthy rats ($p > 0.05$) (Table 1).

Effect of RYR on liver foam cells numbers and serum HDL- C

The T1HFD induced the liver foam cells development ($p < 0.05$) (Table 2) meanwhile there were no inflammation signs which were characterized by infiltration of mixed inflammatory cell (lymphocytes, neutrophils, eosinophils, and Kupffer cells) and ballooning hepatocytes [22]. Fatty degeneration had developed if liver foam cells were more than 5%; if those cells were more than 50%, then fatty liver had been existed [23]. The RYR doses of 108 and 54 mg/KgBW/day completely inhibited liver foam cells formation in T1HFD-treated rats. ($p < 0.05$).

The given doses of RYR significantly increase serum HDL-cholesterol compared with those of T1HFD-treated rats (Table 2). The most effective dose of RYR to increase serum HDL-C is 108 mg/KgBW. The latter dose completely corrected serum HDL-C approximate to those of healthy rats ($p < 0.05$).

Muwarni and her colleagues reported the T1HFD induced hypercholesterolemia but failed to develop aortic foam cells in adult male Wistar rats. The main lipids source of T1HFD were cholesterol and lard [16]. The small intestine absorbed only 50% of dietary cholesterol; the rest will be excreted through feces [24]. Serum total cholesterol consists of dietary and biliary cholesterol absorbed in the small intestine (20%) and newly synthesized cholesterol (80%) produced in the liver and peripheral parts. The cholesterol is used to form steroid hormone and bile acid [25]. Daily cholesterol and TG absorbed from small intestine lumen will be carried in their transporters, chylomicrons. Through lacteal ducts of small intestinal villi, the chylomicrons containing cholesterol and TG reach lymphatic duct from which through subclavian vein the chylomicrons enter blood circulation [26].

The present study showed that T1HFD increases the serum TG ($p < 0.05$) (Table 1), doubles the hepatic foam cells development compared with those of healthy rats and reduces the serum HDL-C ($p < 0.05$) (Table 2), and makes serum AST-ALT to elevate ($p < 0.05$) (Table 1). The rat's body weight is significantly increased, compared with healthy rats (data were not shown). Triglyceride source of TIHFD was from lard [16]. The lipid components of the lard are TG, diacylglycerol (DAG), free fatty acid, and minor components such as phospholipids, sterols, tocopherols, carotenoids, and fat-soluble vitamins [27]. Triglyceride and DAG are absorbed from small intestine lumen as free fatty acids and monoacylglycerol (MAG), both the latter are resynthesized into TG in the en-

Table 1. Serum AST- ALT and TG of healthy and T1HFD-treated rats, and three groups of T1HFD-treated rats receiving RYR at doses of 108, 54, and 27 mg/Kg/BW/day respectively

Group	Serum AST (mU/L) (Mean ± SD) n = 4	Serum ALT (mU/L) (Mean ± SD) n = 4	Serum TG (mg/dl) (Mean ± SD) n = 4
Healthy rats	34.11 ± 1.07[a]	39.76 ± 0.05[a]	102.80 ± 10.98[a]
T1HFD-treated rats	114.00 ± 0.97[b]	121.98 ± 1.35[b]	230.75 ± 14.77[b]
T1HFD-treated rats+108	50.50 ± 1.49[c]	59.34 ± 0.68[c]	125.00 ± 4.77[c]
T1HFD-treated rats+ 54	70.27 ± 1.27[d]	80.66. ± 0.92[d]	155.25 ± 9.00[d]
T1HFD-treated rats+27	94.06 ± 0.49[e]	106.19 ± 5.80[e]	191.25± 1.75[e]

Different attributed letters showed significant differences of mean values ($p < 0.05$). All serum AST-ALT and TG mean values of T1HFD-treated rats receiving RYR are compared with those of healthy and T1HFD-treated rats with no RYR.

Table 2. Liver foam cells percentages and serum HDL-C of healthy and T1HFD-treated rats, and 3 groups of T1HFD-treated rat receiving 108, 54, and 27 mg/Kg/BW/day of RYR respectively.

Group	Foam cells (%) (Mean ± SD) n = 4	Serum HDL-C (mg/dl) (Mean ± SD) n = 4
Healthy rats	2.62 ± 0.53[a]	22.53 ± 0.82 [a]
T1HFD-treated rats	5.20 ± 1.09[b]	15. 88 ± 0.98 [b]
T1HFD-treated rats + 108	2.65 ± 0.45[a]	21.85 ± 21. 88 [a]
T1HFD-treated rats+ 54	3.18 ± 0.60[a]	19.63 ± 1. 46 [c]
T1HFD-treated rats + 27	4.86 ± 0.79[b]	17.20 ± 0.45 [c]

Mean values with different attributed letters showed significant differences ($p < 0.05$), all serum HDL-C and liver foam percentages of T1HFD-treated rats receiving RYR mean values compared with those of T1HFD-treated and healthy rats.

doplasmic reticulum of enterocytes. Apo-B containing chylomicrons transport the TG into lymph vessel, and through a subclavian vein, TG enters systemic blood [26]. The high-fat diet used in the present study (TIHFD) contains cholic acid and cholesterol [16]. Human and rat liver synthesize cholic acid-a primary bile acid from cholesterol. It conjugates with glycine, taurine, or sulfate before being secreted into the intestine. Primary bile acids namely cholic acid and chenodeoxycholic acid) are converted to secondary bile acids (deoxycholic acid (DOC) and lithocholic acid (LCA) by microbial deconjugation and fermentation in the colon [28]. Primary bile acids prepare small droplets of ingested lipids which then will be hydrolyzed by lipase [29].

It is suggested that primary bile acids, as well as metabolism of cholesterol, are involved in the pathogenesis of NAFLD [30]. Primary bile acids contain various derivatives of cholic acids, and each primary bile acid displays a certain mechanism to induce oxidative stress leading to cytotoxicity [31]. A study reported the presence of cholic acid in cholesterol-containing HFD enhanced cholesterol and fat absorption, and also inhibited the transcription of cholesterol 7α-hydroxylase (CYP7A1) suppressing conversion of cholesterol to bile acids (32). Cholesterol and cholic acids components of the atherogenic diet have distinct proatherogenic effects on gene expression [33].

A study reported the children consumed significantly higher levels of dietary cholesterol suffered NAFLD compared to children without NAFLD. The total serum cholesterol levels of children with NAFLD were significantly higher, compared with the group without NAFLD [34].

Fatty degeneration had developed if liver foam cells were more than 5% [21, 22]. Our study showed the TIHFD induced development of hepatic foam cells only up to 5.20 % with no inflammatory signs (Table 2). This condition is accompanied by the elevation of serum AST- ALT by three-fold, both TG and total cholesterol by two-fold (Table 1). Regarding these results, it was suggested that hypertriglyceridemia is prevalent as a risk factor for liver injury without a clear histologic sign of steatosis. Kim (2008) reported NAFLD patients showed an elevation of serum ALT, even though they presented no symptoms to find a medical evaluation [35]. However, another

study on NAFLD patients revealed increased serum ALT and normal range of serum TG associated with fatty liver [36]

Lipid accumulation in liver cells was caused by an imbalance between excessive lipid availability (in circulation) and lipid metabolism (especially fatty acid oxidation) causing eventual lipoperoxidative stress leading to hepatic injury [27]. The present study showed the RYR doses of 108 and 54 mg/kgBW/day completely recovered liver condition from steatosis ($p < 0.05$), the percentage mean values approximate those of the healthy rats ($p>0.05$) (Table 2). It is suggested the natural bioactive contents of these doses of RYR are sufficient to inhibit the accumulation of free fatty acid and TG in the hepatocytes. In spite of the promising results on hepatocytes condition, both doses of RYR could not reduce the serum TG approximate those of healthy rats (Table 1). It is strongly suggested that the latter condition explained why the serum levels of AST and ALT of T1HD-treated rats receiving the doses of RYR are still significantly higher compared with those of healthy rats (Table 1).

An in vitro study reported an exposure of the main fatty acids of the human body (palmitic acid and oleic acid) within a certain range of concentrations could induce the accumulation of lipids in HepG2 cells in a concentration-dependent manner, inducing late apoptosis and necrosis in HepG2 cells [37]. In our study, the T1HFD increases serum TG by two-fold (Table 1). The TG of lard in the TIHFD is rich with palmitic, oleic, and stearic acid which are predominant compared with certain other TG sources. [27]

A study enrolling healthy persons and patients with NAFLD reported serum TG and AST-ALT possess higher sensitivity than the other parameters evaluated to diagnose the disease [38].

The present study did not measure serum cholesterol and LDL-C. The previous study applying the same three doses of the same brand of RYR on rats treated with high-fat diet (HFD) reported each of the three doses of RYR reduced serum cholesterol and LDL-C compared with HFD-treated rats receiving no RYR ($p < 0.05$). The RYR dose of 108 mg/KgBW/day was the most effective dose to reduce serum cholesterol and LDL-C levels approximate to those of healthy rats ($p > 0.05$) [39]. Red yeast rice was well known to contain monacolin K– a natural HMGCo-A reductase inhibitor [7]. HMG-CoA reductase is a key enzyme in a pathway constructed by many enzymatic reactions. The pathway facilitates the synthesis of cholesterol from free fatty acid [40]. Besides containing the natural HMG-CoA reductase inhibitor, the RYR showed an anti-hypertriglyceridemic activity [41]. Other studies reported there are associations between hypertriglyceridemia and an increased risk of cardiovascular disease including excessive free fatty acid release, proinflammatory cytokines expression, coagulation factors, and fibrinolysis impairment [42].

Among anti-hyperlipidemic drugs, the fibric acid derivates showed the best potency in lowering TG. It plays a role as an agonist for peroxisome proliferator-activated receptor α (PPARα) receptor; in addition, the drugs raise HDL cholesterol levels [43]. Fibric acid derivate (the fibrates) is known to cause myopathy in prolonged treatment [44]. Doses of 108 and 54 mg/kgBW/day of RYR significantly decreased the mean serum level of TG ($p < 0.05$), but the mean serum level values were not approximate to those of healthy rats ($p < 0.05$). Both doses lowered the mean serum level of AST-ALT ($p < 0.05$), but those of the AST and ALT serum level were still 2 and 1.5-fold respectively compared with those of the healthy rats ($p < 0.05$). Accordingly, it is suggested that serum free fatty acids liberated from the TG of T1HFD digestion may cause hepatocytes injury; nevertheless, the two doses of RYR completely prevents the lipid depotion of hepatocytes (Table 2). A study on the biochemistry of serum and liver of HFD-treated male Wistar rats reported that free fatty acids were more toxic for liver cells than the accumulation of TG in the cells [45]. Cytoplasmic lipid accumulation in liver cells was in relating to the gradual increase of serum free fatty acid concentration [29]. Considering the results of this study, it is suggested to explore the optimal RYR doses to inhibit or prevent the free fatty acid negative effects on hepatocytes marked by serum AST and ALT elevation. The optimal doses should consider the minimal negative effect of citrinin. Study on mice showed the major target organ of citrinin toxicity is the kidney, followed by other target organs such as liver and bone marrow [46]. Flajd and Peraica, (2010) reported some commodities intended for human consumption from some different West and East countries contained a natural occurrence of citrinin [47]. It had been proved that

citrinin is thermally unstable in aqueous solution, a study reported that the concentration of citrinin in the extract of *Monascus* decreases by 50 % after boiling in water for 20 minutes [48]. The well-known species of genus *Monascus* (*M. purpureus*, *M. ruber*, and *M. pilosus*) are often used to produce RYR for food supplement and coloring. The genus synthesized monacolin and mycotoxin citrinin [49].

Zn-deficiency male Wistar rats were animal models for hepatic inflammatory, using the models a study proved that RYR has an antioxidant activity suppressing the productions of inflammatory cytokines and ROS in the liver of the animal models [50]. The latter studies and anti-hypertriglyceridemic activity of RYR are strongly suggested to be the underlying mechanism of the serum AST-ALT level reduction in the RYR treated T1HFD rats of the present study. In addition, RYR reported containing six azaphilon derivates which could play an important role in RYR anti-inflammatory activities [51]

There are 2 types of liver foam cells, namely microvesicular and macrovesicular. A clinical study of biopsy evaluation reported that there are 4 distribution patterns of steatosis; zone 3 steatosis (foam cells are dispersed around central vein), zone 1 steatosis (foam cells are distributed around portal triad), panacinar steatosis (foam cells are evenly distributed in hepatic lobules), and azonal steatosis (foam cells are distributed around central vein and portal triad) [21]. Foam cells of our samples exhibited to distribute around central vein (zone 3 steatosis) and are macrovesicular. It was reported that macrovesicular steatosis was caused by the imbalance between the synthesis of lipids and their release from the hepatocytes. The most frequently affected hepatocytes are those of zone 3 [22]. A study reported patients with statin-induced myopathy should not use RYR to lower cholesterol [52]. Both simvastatin and RYR caused diffuse myalgia and elevated creatinine kinase levels. As soon as discontinuing these substances, the symptoms resolved [53]. Treatment with ethanolic extract of RYR enhanced the gastric absorption of Verapamil in about 2-fold [54]. Monacolin K and citrinin have a certain mechanism leading to kidney swelling or necrosis in animal studies [55]. Besides, the clinical and laboratory evidence of RYR potential in lowering blood lipids and protecting the liver from TG negative effects, it should always be alert for any unwilling reactions when the rice is used for therapeutic purpose.

Local branded RYR used in this study is registered at BPOM of Indonesia Health Ministry as MD (National industry). Indonesia national products of RYR are marketed in packaging such as in capsules, in foil packs, in tea bags, and in bottles as fluid. As far as we know, all products of RYR in Indonesia are sold freely.

Conclusion

The doses 108 and 54 mg/kg BW of RYR completely inhibited liver foam cells development. The doses 108, 54, and 27 mg/kg BW significantly reduced the serum TG and AST-ALT even though the serum levels of the parameters do not reach those of the healthy rats. The free fatty acids of the TG maybe toxic for hepatocytes by which may give a reasonable explanation that the serum AST-ALT level could not be sufficiently corrected. Optimal RYR doses need to be explore to protect hepatocytes injury due to high fat diet by considering any side effects of RYR.

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