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Full Length Article

Hepatoprotective Effect of Royal Jellies with Different Harvesting Time on CCl_4-Induced Liver Damage in Rats

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Abstract

Liver damage, both acute liver injury and chronic liver damage, can lead to liver fibrosis and cirrhosis. Studies have suggested that royal jelly (RJ), the vital food for queen honeybee development, is able to boost lipoprotein metabolism and lower both the serum total cholesterol (TC) and the low-density lipoprotein (LDL) levels. At 7 days after the mice were administered royal jelly as a hepatoprotective agent against paracetamol-induced liver damage, it was determined that RJ has a marked protective effect on liver tissue. Based on this information, we can ascertain that RJ may be beneficial in the treatment of liver damage. Therefore, the aim of this study was to investigate the protective effect of RJ on CCl_4 liver injury in male SD rats, and to compare the protective effect of royal jelly at different harvest times. The harvest times of royal jelly on CCl4-induced liver injury had no significant effect on LFTs, liver biological index and gross pathology, but the changes of SOD, GSH, T-AOC, LPO and MDA in liver injury caused by CCl4 were significant. Moreover, 72 h RJ had better protective effect than the 48 h harvested royal jelly. © 2019 Friends Science Publishers

Key word: Liver injury; Royal jelly; harvest times

Introduction

The liver is an organ with important metabolic functions, which can detoxify toxic substances. However, hepatic injury can occur during these metabolic reactions (Hinton *et al.*, 2001). Liver damage, both acute liver injury and chronic liver damage, can lead to liver fibrosis and cirrhosis. Some commonly seen risk factors that are major causes of chronic liver "include hepatitis C virus (HCV), hepatitis B virus, heavy alcohol consumption, and nonalcoholic fatty liver disease (NAFLD)" (Ruhl and Everhart, 2003). Liver damage can be caused by various etiologies and can usually relate to some activated inflammatory response and excess oxidative stress inflammatory cytokines, thus inducing an inflammatory cascade, which ends up in cellular death (Ahmad *et al.*, 2018; Wu *et al.*, 2018). It is an important cause of morbidity and mortality worldwide. Liver diseases remain to be a major health concern despite the progresses achieved by medical and pharmaceutical approaches. Many synthetic drugs are used to treat hepatic disorders, however, due to the fact that they all have side effects, for decades people put more attention on traditional herbal drugs, spices, fruits, vegetables, and medicinal plants because of their safety and efficacy.

Royal jelly (RJ) is mainly secreted by the worker-bee hypopharyngeal and mandibular glands from the 5th to the 15th days of their lives (Zeng, 2009). It is a compound containing a distinct combination of proteins (11.4–16.9%) (Wytrychowski *et al.*, 2013), sugars (7.9–17.9%) (Wytrychowski *et al.*, 2013), lipids (3–8%) (Takenaka and Echigo, 1980), amino acids, vitamins, and minerals. RJ contains many bioactive substances and possesses many pharmacological properties in experimental animals, including antitumor (Nakaya *et al.*, 2007), antibacterial (Fontana *et al.*, 2004), anti-allergic (Taniguchi *et al.*, 2003, Vucevic *et al.*, 2007), anti-aging (Niu *et al.*, 2013) and antihypertensive (Takaki-Doi *et al.*, 2009) properties, especially RJ's anti-oxidant (Nakajima *et al.*, 2009) and anti-inflammatory (Kohno *et al.*, 2004) capabilities. RJ can improve lipoprotein metabolism and reduces serum total cholesterol (TC) and low-density lipoprotein (LDL) levels (Guo *et al.*, 2007). The key factor of the development of hyperlipidemia, insulin resistance, and those cardiovascular

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risk factors associated with non-alcoholic fatty liver disease is a fatty acid metabolism balance in conjunction with adipose tissue, liver tissue, and systemic inflammation (Fabbrini *et al.,* 2010). Based on this information, we can ascertain that RJ may be beneficial in the treatment of liver disease such as liver damage. In fact, 7 days after the mice were administered RJ as a hepato-protective agent against paracetamol induced liver damage; the observations revealed that RJ has a marked protective effect on liver tissue (Uzbekova *et al.,* 1998; Kanbur *et al.,* 2009).

RJ composition is affected by bee species, nectar sources, geographical environment and nutrition (Zeng *et al.,* 2006; Zheng *et al.,* 2010; Wei *et al.,* 2013). In addition, RJ has different harvesting times depending on the beekeeping practice; it can be harvested 72 h (72 h RJ) or 48 h (48 h RJ) after grafting larvae. However, does a difference exist between the biological functions of 48 h RJ and 72 h RJ? (Guo *et al.,* 2015; Guo *et al.,* 2016) studied the effect of 48 h RJ and 72 h RJ on the immune function of mice and observed the protective effect of royal jelly on bromobenzene induced oxidative damage in mice. From our current level of understanding, its traditional claim of hepatoprotective potential is lacking scientific evidential support. There is currently no research illustrating a hepatoprotective effect against carbon tetrachloride induced liver damage in rats. Therefore, this study focused on investigating the possible potential hepatoprotective effects of the RJ against CCl_4-induced hepatic injury in male SD rats.

Materials and Methods

Sample Collection

Samples of 48 h RJ and 72 h RJ were obtained from Jiangxi Agriculture University's Honeybee Institute by a without grafting larvae technique. The Italy bee (*Apis mellifera*), raised by Jiangxi Agricultural University's Bee Research Institute, controls the ovipositional patterns of the queen bee and is based on the technique of royal jelly production without grafting larvae. This technique ensures that the all larvae's production of royal jelly is from the same first day. Royal jelly was collected after 48 h and 72 h and was named as 48 h RJ and 72 h RJ, respectively. The two samples were stored at -18°C for further investigation.

Chemicals and Reagents

AST and ALT levels were analyzed using kits from Anhui Yipunuokang Biotechnology Ltd. MDA, SOD, GSH-Px and protein were analyzed using kits from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Carbon tetrachloride (CCl_4) was a commercial product obtained from the National Pharmaceutical Group Chemical Reagent Co., Ltd. (Shanghai, China). Bifendate and all other reagents used in the experiment were of analytical grade.

Experimental Animals and Feeding

Male SD rats with a body weight of 80–110 g were obtained from Hunan SLAC Experiment Animal Ltd (Changsha, China). In this experiment, a barrier housing facility was used in accordance with the National Standard Laboratory Animal Requirements of Environment and Housing Facilities (GB 14925-2010). The rats were housed in acrylic cages lined with wood

Shavings at a constant room temperature (23 ± 1^oC) and maintained on a 12 h: 12 h light/dark cycle. The care of the laboratory animals and the animal experimental operation were performed in accordance with the committee of the Jiangxi University of Traditional Chinese Medicine (2017JZ010). The rats were fed a standard diet and water *ad libitum*, and were quarantined for at least 5 days before the start of experiment. The rat body weight was monitored every week. A total of 72 S.D rats, as mentioned above, were divided into nine groups consisting of eight animals per group as follows:

Group I: normal controls, fed a standard diet.
Group II: This group was fed a standard diet for 4 weeks. On the 29^{th}day, they were treated with 1 mL/kg CCl_4 (1:1 v/v of CCl_4 in olive oil) and served as the model group.
Group III: positive group, fed a standard diet and daily administration of oral bifendate (200 $mg \cdot kg^{-1}$).
Group IV: rats fed 48 h RJ, low dose (20 mL/kg/d)
Group V: rats fed 48 h RJ, middle dose (100 mL/kg/d).
Group VI: rats fed 48 h RJ, high dose (500 mL/kg/d).
Group VII: rats fed 72 h RJ, low dose (20 mL/kg/d).
Group VIII: rats fed 72 h RJ, middle dose (100 mL/kg/d).
Group IX: rats fed 72 h RJ, high dose (500 mL/kg/d).
Group IV to Group IX received CCl_4 at an oral dose of 1 mL/kg (1:1 v/v of CCl_4 in olive oil) on the 29^{th} day.

Liver Function Tests and Biological Assessments

After oral administration of RJ for four weeks (28 days), a single intraperitoneal injection of CCl_4 with the rate of 1mL/body weight was simultaneously administered into each group except group I on the 29^{th} day. 24 h after CCl_4 intoxication, an etherized all animals with mild ether and eye bleeding method was used for blood sample collection. Serum separation performed by centrifugation. The liver function tests on the serum were estimated *via* the standard method. Aspartate aminotransferase (AST) and alanine transaminase (ALT) were measured using the Beckman AU480 auto analyzer (Beckman Coulter, Inc. California, UN).

The animals received 8 weeks of experimental treatment were etherized with carbon dioxide and were sacrificed by cervical dislocation. Blood collection in heparinized tubes was followed by fast removal of livers, which were then washed with cold saline. After collection, the plasma and weighed liver samples were

individually snap-frozen in liquid nitrogen and stored at -80°C for biochemical analysis.

Determination of Serum Antioxidant Enzymes Activities

The serum and liver homogenate levels of glutathione (GSH), superoxide dismutase (SOD), total antioxidant capacity (T-AOC), Malondialdehyde (MDA) and lipid peroxide (LPO) were determined.

Liver Homogenate: A buffer constitutes of 50 m*M* NaCl, 20 m*M* NaH_2PO_4 $2H_2O$, and 20 m*M* Na_2HPO_4 $12H_2O$ with pH = 4 was prepared for homogenization of the liver samples (at a concentration of 200 mg/mL). The homogenate was then centrifuged at 10,000 rpm for 15 min, obtaining a supernatant for antioxidant enzyme activity determination.

Liver-histopathology: The liver samples were fixed in Bouin solution for 12 h, and then embedded into liquid paraffin. Sectioned them to 3 mm thick by micrometer followed by staining with hematoxylin-eosin dye using conventional methods. Examined the cuts under light microscopy (Leica Store Miami, Coral Gables, FL), equipped with a camera system (Canon, Tokyo, Japan).

Statistical Analysis

All data were illustrated as the mean ± standard error means (SEM). Statview5.0.1 (S.A.S. in statute Inc., U.S.A.) was used for data statistical analysis. One-way ANOVA was conducted to analyze the data with homogeneity of variance. Each group was compared to the model group *via* Dunnett's t test. Differences at $P < 0.05$ were considered statistically significant.

Results

Biological Assessments

As regards effects of RJ on body weight and relative liver weight, there was no significant difference ($P > 0.05$) in initial body weight between the groups until 4 weeks had passed. However, following the intoxication of CCl_4, some changes in body weight were noted in the 5^{th} week. Table 1 shows that the RJ or bifendate (positive) group effectively resisted body descent, which lasted until the eighth week of the experiment ($P < 0.05$). The liver index also shows that the RJ or bifendate group had little data compared with the CCl_4 group ($P < 0.05$) but no difference when compared to the normal group (Table 1).

Liver Function Tests (LFT)

Administration of CCl_4 (1 mL/kg, p.o.) induced a marked increase in the serum hepatic AST and ALT enzyme levels compared with normal controls, indicating liver damage. The *in vivo* hepatoprotective studies demonstrated that the levels of ALT, AST were significantly increased ($P < 0.05$) in the carbon tetrachloride-intoxicated rats, when compared with the animals in Group 1. It also showed that our model production was successful.

In the first set of serum hepatic AST and, ALT enzyme levels from the rats treated with RJ, all showed a significant decrease ($P < 0.05$) in all the raised enzyme parameters. In the rats that were given the royal jelly, the ALT and AST indices increased, and the difference was significant compared with the control group after administration of CCl_4, but compared with the model group, there was a gap between the ALT and AST indices, which indicated that RJ had some protective effects on function. No significant difference was noted between the 48 h RJ and 72 h RJ groups (Fig. 1A, B).

The sec set of LFTs drawn showed that the difference between the RJ groups was significant compared with the model group ($P < 0.001$). The liver function damage induced by CCl_4 in the royal jelly group was no different than that in the positive control group, according to liver enzyme parameters. However, at the end of the experimental period, the rats treated with RJ all showed a significant decrease ($P < 0.001$) in all their previously raised AST levels, down to nearly normal levels; ALT also showed a significant decrease but not to near normal levels (see Fig. 1C, D). Additionally, no difference was observed between the 48 h RJ and 72 h RJ groups in the sec LFTs drawn.

Effect of Royal jelly on Oxidative Stress

Fig. 2 represents the serum and liver homogenate levels of SOD, GSH, T-AOC, LPO and MDA in all rat groups. Administration of CCl_4 (1 mL/kg, p.o.) induced a marked decrease in SOD, GSH, and T-AOC and an increase in serum and liver homogenate LPO and MDA levels compared with normal controls, indicating liver damage. When RJ or bifendate (positive group) was administered, an increase in SOD, GSH, and T-AOC was observed; these indices increased to normal levels. When comparing the 48 h RJ and 72 h RJ groups, we found that the 72 h RJ was superior to the 48 h RJ, some with a significant difference ($P < 0.05$) (Fig. 2 and Fig. 3).

Effect of RJ on Histopathology

The results of the liver histopathological study are represented in Fig. 4–6. In Fig. 4, the central vein and its surrounding hepatocytes from Groups I and III exhibited a normal histological structure. By contrast, the liver sections from group II showed hepatocellular degeneration with fibrous tissues, causing structural loosening, vascular congestion, infiltration of the lymphocytes around the central vein, and the presence of microvascular steatosis (Fig. 4B). Liver samples from the animals treated with a low dosage of RJ showed little inflammatory cell infiltration (Fig. 5A and Fig. 5B). The

Table 1: Effects of RJ on body weight and relative liver weight

group	E	6 w	7 w	8 w	Body weight after Fasting	Liver index
normal	239.88 ± 6.98	274.00 ± 7.80	310.75 ± 12.38	321.38 ± 15.57	307.00 ± 13.69	2.87 ± 0.14
CCl_4	232.81 ± 5.22	261.25 ± 8.45	303.75 ± 18.42	314.13 ± 20.05	289.38 ± 13.95	3.26 ± 0.16
Bifendate + CCl_4	240.75 ± 7.08	276.25 ± 9.50	311.13 ± 10.81	320.13 ± 11.96	307.63 ± 12.35	2.90 ± 0.26*
48 h RJ L + CCl_4	242.38 ± 7.23*	273.63 ± 6.05	317.63 ± 11.81	329.38 ± 13.18	314.38 ± 13.17	2.83 ± 0.19*
48 h RJ M + CCl_4	244.25 ± 8.62*	281.88 ± 8.44	321.83 ± 10.89	337.25 ± 12.01	314.38 ± 10.64	2.90 ± 0.31*
48 h RJ H + CCl_4	247.63 ± 9.24*	285.75 ± 7.29*	321.25 ± 10.99	338.00 ± 10.46	316.88 ± 8.98	2.83 ± 0.19*
72 h RJ L + CCl_4	250.00 ± 8.24$^{**\#}$	281.13 ± 8.90*	308.75 ± 12.73	324.50 ± 12.20	307.25 ± 12.15	2.95 ± 0.14*
72 h RJ M + CCl_4	253.00 ± 7.89**	292.75 ± 8.03*	314.38 ± 11.26	344.88 ± 11.80	326.00 ± 12.59	2.68 ± 0.22*
72 h RJ H + CCl_4	257.00 ± 7.00$^{**\#}$	295.63 ± 9.21*	325.38 ± 11.14	356.89 ± 12.93	332.50 ± 11.97	2.67 ± 0.32*

*with model group $P < 0.05$; $^{\#}$ with positive group $P < 0.05$

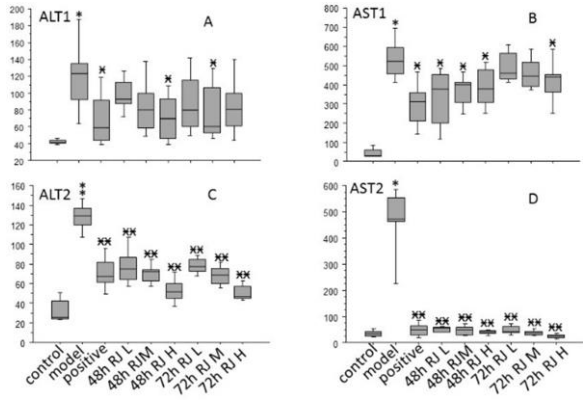

Fig. 1: Effects of 48 h RJ and 72 h RJ on CCl4-induced serum hepatic enzyme (AST, ALT) levels
*VS. control group $P < 0.05$; ^{x} VS. model group $P < 0.05$; ^{xx} VS. model group $P < 0.01$

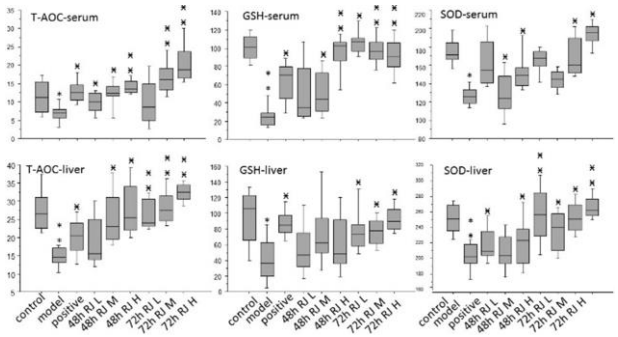

Fig. 2: Effects of 48 h RJ and 72 h RJ on CCl4-induced serum and liver homogenate levels of SOD, GSH, and T-AOC
*VS. control group $P < 0.05$; ^{x} VS. model group $P < 0.05$; ^{xx} VS. model group $P < 0.01$
Y-axis :The activites of (U/L)

histopathological architecture of liver sections obtained from the rats treated with mid and high dosages of 48 h RJ (Fig. 6A and 6B) and 72 h RJ (Fig. 6C and Fig. 6D) did not show any histological changes compared to group I. These results are in concordance with results obtained from biochemical analysis (liver function).

Discussion

The hepatic damage animal models are the basis of experimental and clinical hypothesis (Tuñón *et al.*, 2009). It is important to determine the cause and mechanism of liver disease, the screening of drugs and the judgment of curative effect, and the development of a vaccine. CCl_4 has been extensively used as a model for hepatic damage and is used as an indicator of protective activity of newly discovered drugs (Gilani *et al.*, 1998; Brautbar and Williams, 2002). The hepatotoxicity of $CC1_4$ arises from its oxidation by hepatic enzymes, which produces damaging free radicals. Structural changes of membranes (such as endoplasmic reticulum), loss of metabolic enzyme activation, as well as reduction of protein synthesis, can all be triggered by $CC1_4$ induced lipid peroxidation, leading to liver damage (Weber *et al.*, 2003). An enzyme called cytochrome P450 converts CCl_4 to a toxic metabolite $CC1_3$ radical, which reacts with oxygen to generate chloromethyl peroxy radicals. These radicals then form covalent bonds with macromolecules, causing peroxidative degradation of the hepatocellular lipid membrane (Thanh *et al.*, 2015). To evaluate liver damage, the present study measured ALT and AST levels in serum; levels of AOC, GSH, SOD, LPO and MDA, were indicators of lipid peroxidation. We also evaluated whether RJ had a protective effect, minimizing liver damage.

First, due to the fact that AST and ALT are markers of hepatocyte damage (and thus indicating the severity of liver injury), their drastically increased levels observed in this experiment are in support of the proposition that CCl_4 is the cause of the development of significant hepatic damage in rats. After CCl_4 administration, the serum ALT and AST levels in those rats were significantly higher than those in control group; the observation of RJ being able to reduce these levels reflects that it can protect the rats from acute liver injury *in vivo* induced by CCl_4 by preventing hepatocytes damage. We did not observe a difference between the 48 h RJ and 72 h RJ groups in the sec set of LFTs.

Sec, because of the effects of oxidation on the animal body cell metabolism, active oxygen (ROS) can induce a series of oxidative stress. T-AOC, SOD and GSH play an important role in maintaining oxidation and antioxidation and play an important role in the repair of oxidative stress caused by injury. This describes the oxidation and antioxidant homeostasis in the blood of a relatively independent index. Reactive oxygen species attack polyunsaturated fatty acids as lipid peroxidation, forming products causing structure change of membranes and functional (Ruhl and Everhart, 2003). Antioxidant defense

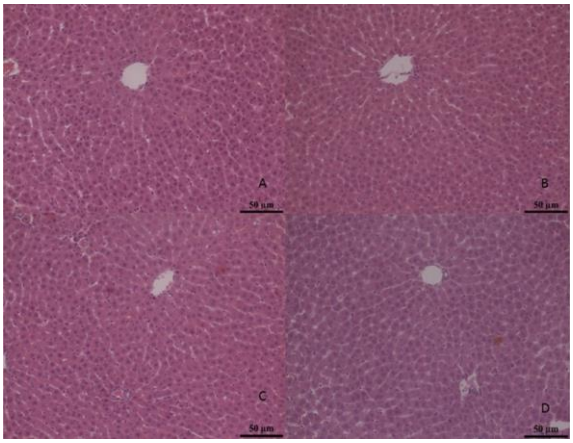

Fig. 6: The histopathological architecture of liver sections obtained from rats treated with mid and high dosages of 48 h RJ (A and B) 72 h RJ (C and D) did not show histological changes in comparison with group I

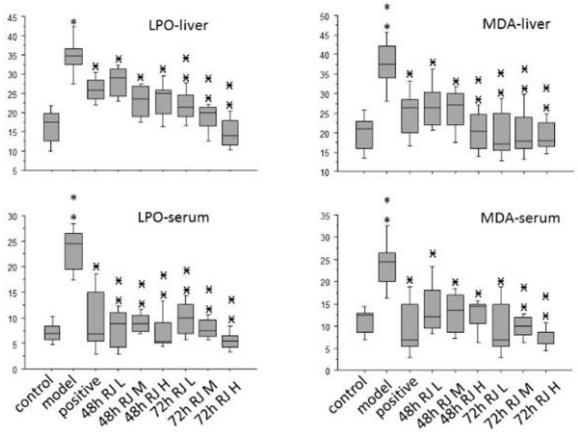

Fig. 3: Effects of 48 h RJ and 72 h RJ on CCl4-induced serum and liver homogenate levels of LPO and MDA
**VS.* control group $P < 0.05$; ^{x} *VS.* model group $P < 0.05$; $^{x\,x}$*VS.* model group $P < 0.01$
Y-axis:The activites of (U/L)

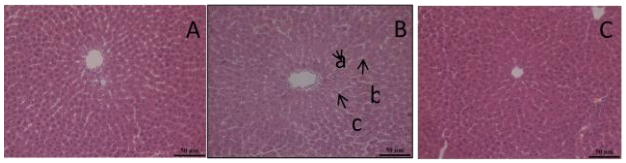

Fig. 4: (**A**) The liver sections of group 1 showed a normal histological structure of the central vein and surrounding hepatocytes. (**B**) The liver sections of group II showed hepatocellular degeneration with structural loosening (a), nuclear disappearance (b), vascular congestion (c), infiltration of the lymphocytes around the central vein, and the presence of microvascular steatosis. (**C**) The liver sections of group III showed a normal histological structure of the central vein and surrounding hepatocytes

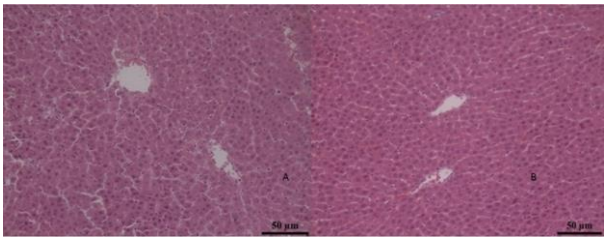

Fig. 5: The liver sections obtained from animals treated with a low dosage of RJ showed histopathological structure of liver slices that was basically normal but little inflammatory cell infiltration (A and B)

mechanisms in cells are effective to prevent and neutralize the free radical-induced damage. Once the equilibrium between lipid peroxidation reactant generation and this antioxidant system is broken, oxidative stress would arise, which disrupts the regulation of cellular functions.Among the main endogenous enzymatic defense, SOD is an antioxidant enzyme acts as a catalyst in the reaction converting O_2•- to H_2O_2, therefore preventing reactive oxygen species from both endogenous and exogenous pathways. T-AOC is a heme-containing enzyme acting as a catalyst to the decomposition of H_2O_2, and is found widely in subcellular organelles, such as peroxisomes, preventing oxidative damage by degrading H_2O_2 and OH levels. A class of enzymes is usually involved in the reduction of H_2O_2, phospholipid-hydro peroxide and other organic hydro peroxides. As a member of them, GSH reduces cellular H_2O_2 level by coupling H_2O_2 reduction with the oxidation of reduced glutathione. It can also reduce fatty acid hydro peroxides. Experiment showed that CCl_4-treated animals experienced a decrease in enzymatic activity, and their reversal to near normalcy in rats treated with CCl_4. As these data suggested, it is probable that RJ can reduce oxidative stress in rat serum and liver as well as increasing the activity of antioxidant enzymes.

Oxidative stress can cause lipid metabolism. Lipid peroxidation produces aldehydes with high activity and diffusiveness as its scary products, which can bind covalently to other cellular compounds (Zheng *et al.*, 2010). The peroxidation of polyunsaturated fatty acids can generate highly active and toxic aldehydes called MDA, which is a commonly used biomarker for lipid peroxidation assessment (Takaki-Doi *et al.*, 2009). As the most mutagenic product of lipid peroxidation, MDA "can alter membrane permeability as well as impair the fluidity of the membrane lipid bilayer" (Takaki-Doi *et al.*, 2009). LPO and MDA are the products of lipid oxidation and are often used as a measure of oxidative stress. They can also be treated as markers of lipid peroxidation. This process is a direct result of the antioxidant activity of these enzymes.

The test results showed that RJ can significantly improve the serum and liver T-AOC, SOD and GSH activity and decrease the amount of LPO and MDA; all values were close to or reached the normal range. From this experiment, a significant increase of LPO and MDA levels *in vivo* was seen with the administration of CCl_4. After treated with RJ, the rats were able to rescue their LPO and MDA levels to nearly normal levels.

Conclusion

The RJ is potentially capable of generating hepatoprotective action against CCl_4-induced hepatic damage in rats. There is no significant difference between 48 h RJ and 72 h RJ. The present study thus justified the traditional use of RJ in liver disease treatments and pointed out that RJ warrants future detailed investigation as a promising hepatoprotective agent. However, the mechanism of RJ effect remains unknown and is to be studied forward. Is the effect of royal jelly on potent liver damage achieved by enhancing the body's immune function or because of its own anti-inflammatory properties? Furthermore, RJ has a protective effect against CCl_4-induced acute hepatic damage in rats. The harvest times of royal jelly on CCl4-induced liver injury had no effect on LFTs, liver biological index and gross pathology, but the changes of SOD, GSH, T-AOC, LPO and MDA in liver injury caused by CCl4 were significant, while 72 h RJ showed better hepatoprotective effect as compared to those of 48 h RJ.

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