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Carbon tetrachloride-induced hepatotoxicity in rat is reversed by treatment with riboflavin

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ABSTRACT

Liver is a vital organ for the detoxification of toxic substances present in the body and hepatic injury is associated with excessive exposure to toxicants. The present study was designed to evaluate the possible hepatoprotective effects of riboflavin against carbon tetrachloride (CCl_4) induced hepatic injury in rats. Rats were divided into six groups. Hepatotoxicity was induced by the administration of a single intraperitoneal dose of CCl_4 in experimental rats. Riboflavin was administered at 30 and 100 mg/kg by oral gavage to test its protective effect on hepatic injury biochemically and histopathologically in the blood/liver and liver respectively. The administration of CCl_4 resulted in marked alteration in serum hepatic enzymes (like AST, ALT and ALP), oxidant parameters (like GSH and MDA) and pro-inflammatory cytokine TNF-α release from blood leukocytes indicative of hepatic injury. Changes in serum hepatic enzymes, oxidant parameters and TNF-α production induced by CCl_4 were reversed by riboflavin treatment in a dose dependent manner. Treatment with standard drug, silymarin also reversed CCl_4 induced changes in biomarkers of liver function, oxidant parameters and inflammation. The biochemical observations were paralleled by histopathological findings in rat liver both in the case of CCl_4 and treatment groups. In conclusion, riboflavin produced a protective effect against CCl_4-induced liver damage. Our study suggests that riboflavin may be used as a hepato-protective agent against toxic effects caused by CCl_4 and other chemical agents in the liver.

1. Introduction

Liver is a vital organ that plays a key role in the conjugation and detoxification of many drugs [1]. However its function is generally impaired by xenobiotics or infections. Chronic or excessive exposure of xenobiotics leads to cirrhosis or malignant lesions in untreated cases. At present, millions of people suffer from hepatic damage induced by alcohol, chemicals and infections. Thus, acute and chronic liver diseases continue to be serious health problems in the world [2]. Chemicals like paracetamol [3], carbon tetrachloride (CCl_4) [4], nitrosamines, and polycyclic aromatic hydrocarbons damage the liver significantly. There is a need to develop newer drugs or safer options from current available compounds which provide hepatoprotection. Options are limited in the modern medicine due to unreliability and limited efficiency of the available options [5].

Available literature evidence shows that extensive oxygen free radicals such as superoxide anion radical ($^{\cdot}_{2}$) and hydroxyl radical ($OH^{\cdot}$) are formed due to liver-toxic chemicals, ionizing radiations, environmental pollutants, and drug exposure [3,4] which causes hepatotoxicity [6]. CCl_4 is an extensively used chemical solvent in industry. It is a well-established hepatotoxin and it is the best-characterized animal model of xenobiotic induced free radical-mediated hepatotoxicity [7]. CCl_4 causes hepatic injury through several pathways [8]. Elevated lipid peroxidation due to increased free radical formation generated from CCl_4 is thought to be one of the mechanisms leading to hepatotoxicity [9]. CCl_4 also causes the activation of immune systems through the infiltration of inflammatory cells to the site of injury. Thus immune cells may be responsible for the release of pro-inflammatory cytokines such as TNF-α and IL-6 which further enhance hepatotoxicity through repeated cycle of inflammation.

Riboflavin, also known as vitamin B_2, is an easily absorbed micronutrient with a key role in maintaining health in humans and animals. Vitamin B_2 is required for a wide variety of cellular processes. It plays a key role in energy metabolism and is required for the metabolism of fats, carbohydrates, and proteins. It is the central component of the cofactors flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), and is therefore required by all flavoproteins such as glutathione reductase which protects cells from harmful effect of ROS [10,11]. Riboflavin deficiency causes protein and DNA damage, which leads to cell stress and increased apoptosis [11–14]. Riboflavin also affects the immune

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system, through anti-inflammatory activities [15–17]. Riboflavin reduces the mortality of mice with septic shock [18], helps in protection from bacterial infection and is involved in phagocytosis [19]. In addition to being a central component of flavoprotein, riboflavin also works as an antioxidant by scavenging ROS [20]. In animals, riboflavin deficiency results in lack of growth, failure to thrive, and eventually death [21].

Therefore, this study was undertaken to evaluate the hepatoprotective effect of riboflavin in CCl_4-induced hepatic injury in rats through biochemical and histological assessments. Our study shows for first time that riboflavin reverses CCl_4-induced changes in biochemical markers of liver toxicity and inflammation.

2. Materials and methods

2.1. Animals

In this study male Wistar albino rats weighing 200–250 g (10–12 weeks old) were used. The animals were obtained from the Experimental Animal Care Center, College of Pharmacy at King Saud University. They were housed under ideal laboratory conditions (12 h light/12 h darkness cycle, 45–55% relative humidity and temperature 23–25 °C), maintained on standard pellet diet and water ad libitum throughout the experimental period. All experiments were carried out according to the guidelines of the animal care and use committee at King Saud University.

2.2. Drugs and chemicals

Riboflavin from Sigma-Aldrich (Switzerland) and silymarin from Sigma-Aldrich (USA) were used in the study. Carbon tetrachloride, all other solvents and chemicals used for experimental work were of analytical grade.

2.3. Experimental design

Rats were divided into six groups with six animals in each group. Hepatic injury was induced in rats by intra-peritoneal (i.p.) administration of a single dose of 0.5 ml/kg CCl_4 [22]. Silymarin (45 mg/kg, p.o.) which is an antioxidant was used as a reference standard [23,24]. The experimental design was as follows: Group-I rats served as control; Group-II rats (CCl_4) were exposed to CCl_4 on day one; Group-III rats (CCl_4 + R30) were exposed to CCl_4 (0.5 ml/kg on day one) and treated with riboflavin 30 mg/kg, p.o. for seven days; Group-IV rats (CCl_4 + R100) were exposed to CCl_4 on day one and treated with riboflavin 100 mg/kg, p.o., daily for 7 days; Group-V rats (CCl_4 + S45) were exposed to CCl_4 on day one, followed by the administration of silymarin (45 mg/kg, p.o.) for seven days and Group-VI rats (R 100) served as riboflavin per se group and were treated with riboflavin 100 mg/kg, p.o., daily for 7 days. All the rats were sacrificed at the end of the study by decapitation under light ether anesthesia, as per the protocol.

Blood samples were collected in heparinized tube followed by serum separation at 3000 *g* for 10 min. Samples were then kept at −20 °C until the analysis of liver function parameters. Biochemical estimations were done in serum by an autoanalyzer (Dimension® RXL MAXTM, Siemens, USA) to assess hepatic function, whereas whole blood was used for TNF-α estimation. Livers were isolated and washed in ice cold physiological saline for the assessment of oxidative stress and histopathological changes.

2.4. Determination of lipid peroxides, measured as malondialdehyde (MDA)

Level of MDA, a product of membrane lipid peroxidation, was estimated in liver tissue by the method of Okhawa [25], using the standard calibration curve prepared with tetraethoxy propane. MDA was expressed as nmol of MDA per milligram of protein. Protein was estimated by the method of Lowry [26].

2.5. Determination of reduced glutathione (GSH)

GSH content was estimated in liver tissue by the method of Sedlack [27]. The absorbance of reaction mixture was read within 5 min of addition of dithiobis-2-nitrobenzoic acid at 412 nm using UV-spectrophotometer, against a reagent blank.

2.6. Intracellular TNF-α estimation in whole blood

Following lysis of RBC in whole blood and centrifugation at 300 ×*g* for 5 min, the supernatant was discarded and fixation/permeabilizing solution (Miltenyi Biotec, Germany) was added to the pellet followed by incubation for 10 min at room temperature in the dark. After washing, hamster anti-TNF-α monoclonal Ab conjugated to PE (BD Biosciences, USA) was added to the cells and incubated for 30 min at room temperature in the dark followed by analysis immediately on a Cytomics FC 500 flow cytometer (Beckman Coulter, USA). The stained cells were analyzed using CXP software (Beckman Coulter, USA) [28].

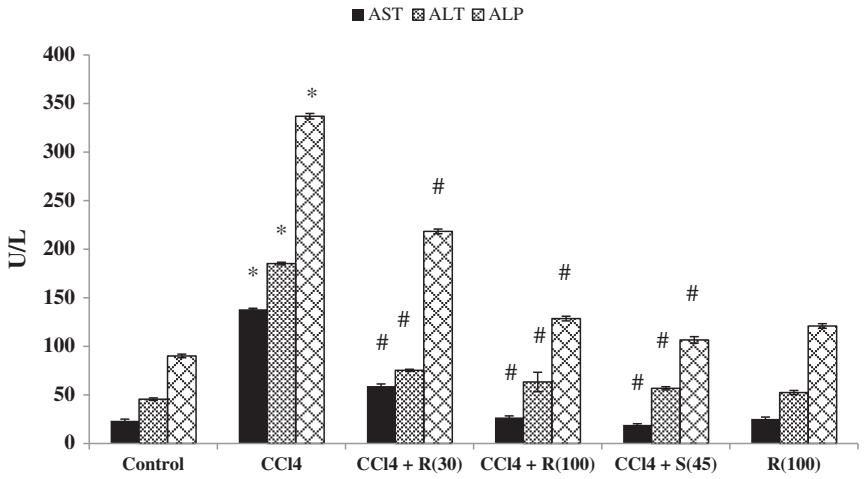

Fig. 1. Effects of riboflavin on CCl_4-induced changes on liver function parameters in serum of different experimental groups. The data are expressed as mean ± SEM ($n = 6$). *$p < 0.05$, vs control group; #$p < 0.05$, vs CCl_4 group. ANOVA followed by Tukey–Kramer multiple comparison test.

Table 1
Effects of riboflavin on CCl_4 induced changes in liver function in serum.

Groups	GGT (U/l)	ALB (g/dl)	TP (g/dl)	DB (μmol/l)
	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM
Control	5.80 ± 0.49	11.30 ± 0.19	65.52 ± 0.87	0.272 ± 0.08
CCl_4	13.20 ± 0.80*	7.14 ± 0.39*	57.78 ± 1.54*	2.32 ± 0.08*
CCl_4 + R(30)	8.33 ± 0.67#	9.03 ± 0.88#	63.87 ± 2.34#	0.43 ± 0.10#
CCl_4 + R(100)	7.80 ± 0.37#	11.06 ± 1.13#	69.50 ± 1.08#	0.41 ± 0.05#
CCl_4 + S(45)	7.28 ± 0.37#	11.62 ± 0.24#	71.76 ± 1.83#	0.35 ± 0.09#
R(100)	6.17 ± 0.79	11.92 ± 0.40	68.30 ± 2.56	0.31 ± 0.06

GGT = Gamma glutamyl transferase, ALB = albumin, TP = total protein, DB = direct bilirubin, CCl_4 = carbon tetrachloride, R = riboflavin, S = silymarin, SEM = standard error of mean. The data are expressed as mean ± SEM ($n = 6$). ANOVA followed by Tukey–Kramer multiple comparison test.
* $p < 0.05$ vs control group.
\# $p < 0.05$ vs CCl_4 group.

2.7. Histopathology

Animals were killed by cervical decapitation and the livers fixed in 10% buffer formosaline. Paraffin sections of 3–4 μm thickness were prepared and stained with hematoxylin and eosin (H&E) for histopathological examination under light microscopy.

2.8. Statistical analysis

Results were expressed as mean ± SEM. One way analysis of variance (ANOVA) followed by Tukey–Kramer multiple comparisons test was used to identify significance among groups. Values were considered statistically significant when $p < 0.05$. Statistical analysis was carried out using GraphPad Prism 3.0.

3. Results

3.1. Effects of riboflavin on CCl_4-induced changes on liver function parameters in serum

The activities of aspartate trans-aminase (AST), alanine trans-aminase (ALT) and alkaline phosphatase (ALP), total protein (TP), albumin and gamma-glutamyl transferase (GGT) were estimated in serum samples as biomarkers of liver function. In this study, single dose administration of CCl_4 to rats resulted in liver injury in rats as evidenced by a marked increase in serum AST, ALT and ALP, compared to control group. Changes in the serum liver function markers suggest increased damage to the hepatic cells by CCl_4. Treatment with riboflavin significantly ($p < 0.05$) reversed CCl_4-induced increase in AST, ALT and ALP (Fig. 1). Albumin, GGT, TP and direct bilirubin (DB) levels were also altered after the administration of CCl_4 which was reversed by treatment with riboflavin in a dose dependent manner (Table 1). Treatment with standard drug, silymarin (S) also reversed CCl_4 induced changes in biomarkers of liver function. Riboflavin per se group had no significant changes in any of the parameters compared to control group.

3.2. Effects of riboflavin on pro-inflammatory TNF-α production in whole blood leukocytes

To study the role of riboflavin on CCl_4 induced inflammation, tumor necrosis factor-α (TNF-α) was measured in systemic circulation. The administration of CCl_4 resulted in a significant increase in the production of TNF-α from blood leukocytes. In contrast, riboflavin and silymarin reduced the production of TNF-α in blood leukocytes compared with the $CC1_4$ group (Fig. 2). This data suggests that the hepatoprotective effect of riboflavin in this model may be due in part to the inhibition of TNF-α. Riboflavin per se group had no significant change in TNF-α production compared to control group.

3.3. Effects of riboflavin on CCl_4-induced changes on parameters of oxidative stress in liver

The results are summarized in Fig. 3. The administration of CCl_4 resulted in a significant ($p < 0.05$) increase in liver MDA content compared to the control group. Treatment with riboflavin showed a significant ($p < 0.05$) reversal in CCl_4-induced increase in liver MDA levels (Fig. 3). Consequently, a significant ($p < 0.05$) decrease in liver GSH level was found in CCl_4 treated rats as compared to control group which was reversed by riboflavin treatment (Fig. 3). Silymarin produced effects similar to riboflavin. Riboflavin per se group had no significant changes in oxidative stress parameters compared to control group.

3.4. Effects of riboflavin on CCl_4-induced histopathological changes in liver

Normal morphological structures of liver tissue were observed in the control group (Fig. 4a). The administration of CCl_4 caused histopathological changes in the liver such as severe centrilobular necrosis, hepatocyte ballooning, and infiltration of inflammatory cells (such as macrophages and lymphocytes) into the portal tract and sinusoid

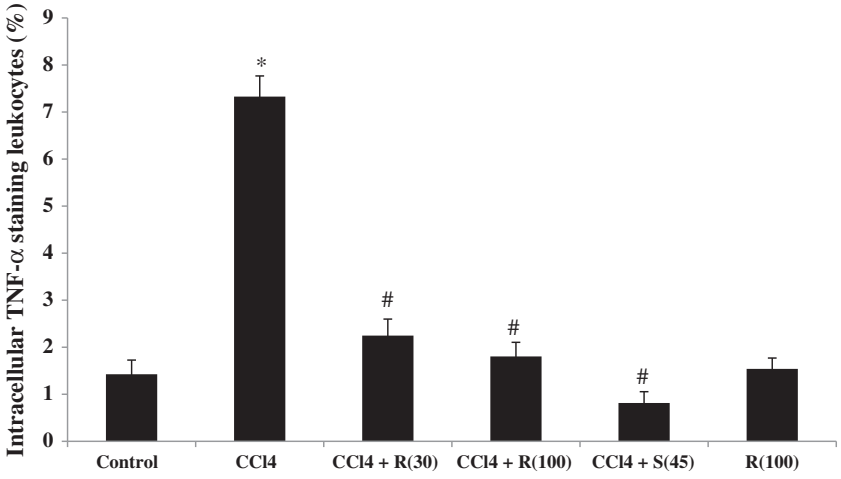

Fig. 2. Effects of riboflavin on pro-inflammatory TNF-α production in whole blood of different experimental groups. The data are expressed as mean ± SEM ($n = 6$). *$p < 0.05$, vs control group; #$p < 0.05$, vs CCl_4 group. ANOVA followed by Tukey–Kramer multiple comparison test.

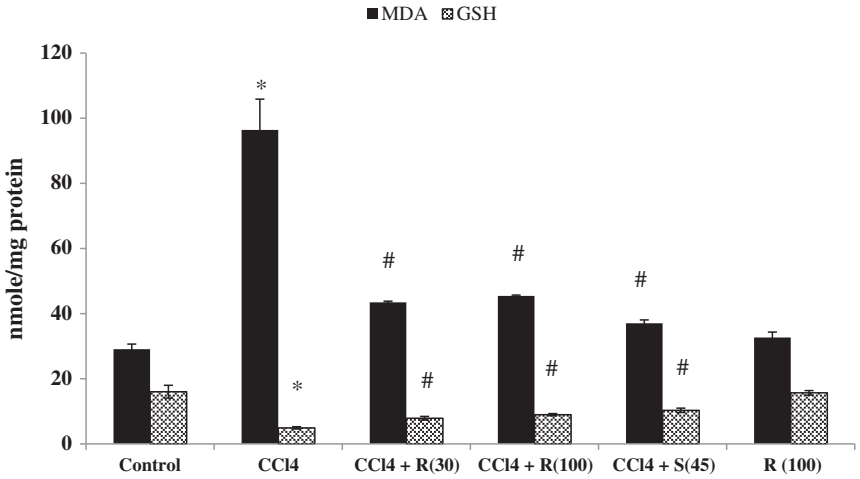

Fig. 3. Effects of riboflavin on CCl_4-induced changes on parameters of oxidative stress in the liver of different experimental groups. The data are expressed as mean ± SEM ($n = 6$). *$p < 0.05$, vs control group; #$p < 0.05$, vs CCl_4 group. ANOVA followed by Tukey–Kramer multiple comparison test.

(Fig. 4b). Treatment with riboflavin (30 and 100 mg/kg), dose-dependently, reversed the hepatic lesions produced by CCl_4 (Fig. 4c–d). Hepatoprotection of riboflavin was particularly evident from the absence of cellular necrosis and inflammatory infiltrates in the liver section of rats treated with the highest dose. The effect of riboflavin (100 mg/kg) was almost comparable to that of the silymarin (Fig. 4e) treated group. Riboflavin per se group was similar to control group (Fig. 4f).

4. Discussion

Our study showed for the first time that treatment with riboflavin ameliorated CCl_4-induced toxicity and showed the normalization of serum hepatic enzymes (like AST, ALT and ALP) and oxidant parameters (like GSH and MDA) which were further confirmed by histological findings. There are various etiological factors such as hepatotoxins [29],

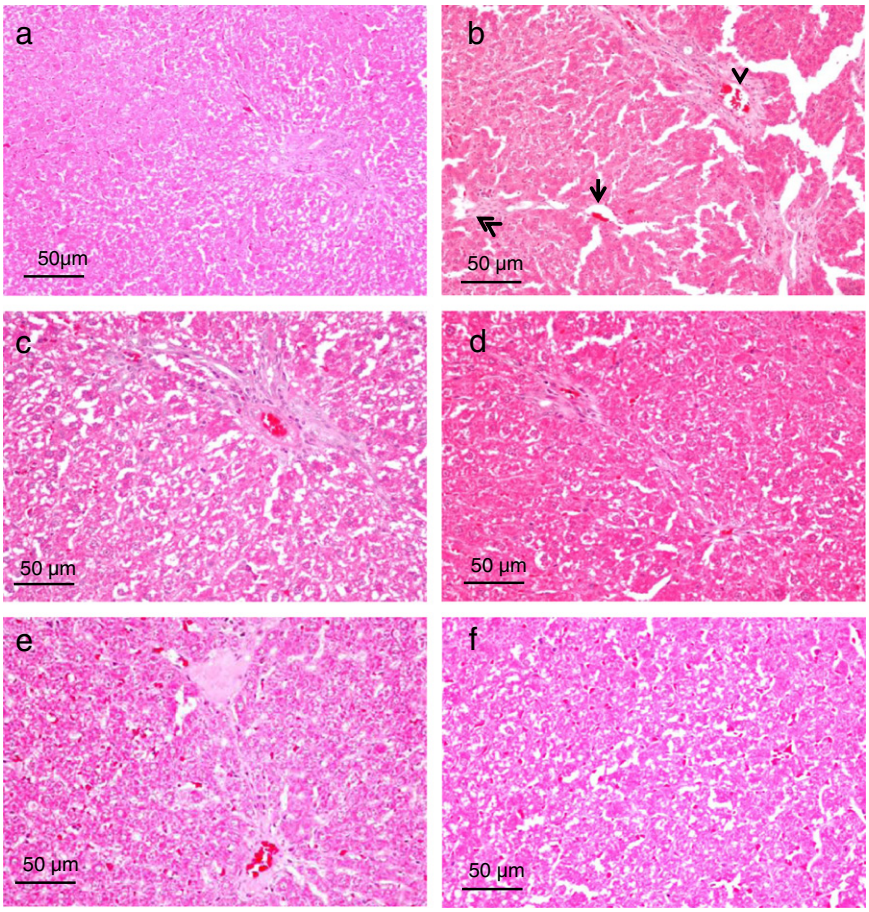

Fig. 4. Effects of riboflavin on CCl_4-induced changes in liver histopathology of different experimental groups. a) Control; b) CCl_4; c) R(30) + CCl_4; d) R(100) + CCl_4; e) Silymarin(45) + CCl_4; and f) R(100). ($n = 6$ per group; magnification = 20×). Arrow, arrow head and double arrow heads indicate hepatocyte ballooning, necrosis and inflammation of central vein, and pericellular fibrosis in the liver parenchyma respectively.

drug/chemical exposure like paracetamol [3] or CCl4, metabolic diseases [30] and alcoholism which contribute to the liver damage, often leading to severe necrosis. It is difficult to treat hepatotoxicity with the currently available drugs due to their side effects and inherent toxicities. Thus, there is a need to develop an efficient alternative for managing the liver treatment with efficacy and safety. The hepatoprotective drug should have the ability to restore the normal architecture of the liver and preserve the normal physiological mechanisms which have been distorted by the hepatotoxins [31]. Therefore, we tested riboflavin for its protective effects in a hepatic injury model.

CCl_4-induced liver injury is the preferred model as it causes the hepatic changes identical to cirrhosis/hepatitis [32,33], mononuclear cell infiltration, and steatotic foamy degeneration of hepatocytes [34]. Carbon tetrachloride induced toxicity is characterized by the generation of reactive intermediate trichloromethyl radical and trichloromethyl peroxy radicals [35] which alkylate cellular proteins and other macromolecules with a simultaneous attack on polyunsaturated fatty acids [36]. They are believed to produce lipid peroxides in the form of conjugated dienes, lipid hydro-peroxides, malonaldehyde like substances, and other short-chain hydrocarbons which eventually leads to hepatotoxicity [37].

In the present study, CCl_4 induced severe hepatic injury which was demonstrated by marked elevation of ALT and AST, ALP. These are usually considered as hepatic biomarkers. Damage to hepatic cells causes a leakage of liver-specific enzymes, causing increased level of these enzymes in serum. The increased serum enzyme levels like ALT and AST are indicators of cellular damage and functional integrity of liver cell membrane [38]. Zimmerman et al. [39] stated that CCl_4-induced increase of serum ALT and AST levels are due to cell membrane and mitochondrial damage of liver cells. Other studies have also reported that these enzyme activities are significantly elevated after CCl_4 treatment [40–43]. Treatment of rats with riboflavin had a significant protective effect against CCl_4-induced hepatotoxicity in rats, as evidenced by decreased serum ALT, ALP, and AST levels (Fig. 1). Previous studies have shown similar results on hepatoprotective agents in CCl_4-induced acute liver injury model [2,24,44]; however our study has shown the effect of riboflavin on CCl_4-induced liver damage for the first time.

Albumin, gamma-glutaryl transferase (GGT), total protein (TP) and direct bilirubin (DB) levels were also altered by the administration of CCl_4 which were reversed by treatment with riboflavin in a dose dependent manner. Serum bilirubin level which is a dominant marker in liver injury indicates secretory mechanism of hepatocytes. Animals treated with riboflavin showed a decrease in serum bilirubin level suggesting protection of hepatocytes from CCl_4 mediated damage. Similar results have been reported earlier [24,44].

It has already been shown through previous studies that one of the main causes of CCl_4-induced hepatotoxicity is the generation of lipid peroxides by free radical derivatives of CCl_4. Thus, the anti-oxidant activity or the inhibition of the generation of free radicals could be one of the mechanisms in the protection against CCl_4-induced hepatoxicity. The increased serum levels of hepatic biomarkers could be [45,46] due to lipid peroxidation caused by free radical derivatives of CCl_4 leading to the leakage of these enzymes from hepatocytes [47,48]. Indeed, CCl_4-administration resulted in a significant increase in liver MDA contents compared to the control group. Treatment with riboflavin showed a significant reversal in CCl_4-induced increase in liver MDA levels. The reduction in MDA caused by riboflavin shows the free radical scavenging property of riboflavin. GSH is the main redox regulator of extracellular as well as intracellular compartment. It can detoxify ROS or free radicals directly by scavenging free radicals or by being part of glutathione redox system which include glutathione peroxidase and glutathione reductase. A significant decrease in liver GSH level was found in CCl4 treated rats as compared to control group which was reversed by riboflavin treatment. Our results are in agreement with earlier studies [2,24,44]. Our data suggest that direct free radical scavenging as well as being a part of flavoproteins such as glutathione reductase may have contributed to the antioxidant function of riboflavin.

Monocytes, lymphocytes, neutrophils and Kupffer cells are known to be activated by different stimuli such as endotoxin and CCl_4 [49]. Increased oxidative injury produced by derivatives of CCl_4 also activates Kupffer cells in the liver which may be responsible for increased release of TNF-α from inflammatory cells recruited to the liver [50]. Riboflavin prevented CCl_4-induced liver injury by the inhibition of pro-inflammatory cytokine TNF-α release from leukocytes. This data suggests that the hepatoprotective effect of riboflavin in this model may be due in part to the inhibition of TNF-α.

Biochemical improvements after riboflavin treatment were paralleled by histopathological findings. Treatment with riboflavin reversed the hepatic lesions produced by CCl_4 in a dose dependent manner. It was evident from the absence of cellular necrosis, inflammatory infiltrates and normalization of cellular structures in the liver section. Our results are in agreement with earlier reports showing hepatoprotection against chemical induced liver damage [9,38,44]. Our data suggests that antioxidant and anti-inflammatory actions of riboflavin are responsible for the normalization of hepatic function at the biochemical and structural level.

5. Conclusion

Current study shows that riboflavin prevents CCl_4-induced hepatic injury through a decrease in hepatic oxidative stress and pro-inflammatory cytokine TNF-α release from leukocytes.

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