Protective effect of hesperidin on oxidative and histological liver damage following carbon tetrachloride administration in Wistar rats

Aslı Çetin¹, Osman Çiftçi², Ali Otlü¹

¹Department of Histology and Embryology, Medicine Faculty, Inonu University, Malatya, Turkey
²Department of Pharmacology, Medicine Faculty, Inonu University, Malatya, Turkey

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Abstract

Introduction: In the current study, the protective effect of hesperidin (HP) on carbon tetrachloride (CCl₄)-induced hepatotoxicity in rats was investigated.

Material and methods: Twenty-eight rats were divided equally into four groups. The first group was kept as a control and given only vehicle. In the second, rats were orally administered 50 mg/kg/day HP for 10 days. Carbon tetrachloride was given in a single intraperitoneal injection at the dose of 2 ml/kg in the third group. In the fourth group, the rats were treated with equal doses of CCl₄ and HP.

Results: It was found that CCl₄ induced oxidative stress via a significant increase in the formation of thiobarbituric acid-reactive substances (TBARS) and caused a significant decline in the levels of glutathione (GSH), catalase (CAT) and superoxide dismutase (SOD) in rats. In contrast, HP blocked these toxic effects induced by CCl₄, causing an increase in GSH, CAT and SOD levels and decreased formation of TBARS ($p < 0.01$). In addition, histopathological damage increased with CCl₄ treatment. In contrast, HP treatment eliminated the effects of CCl₄ and stimulated anti-apoptotic events, as characterized by reduced caspase-3 activation.

Conclusions: The current study demonstrated that CCl₄-induced hepatotoxicity can be prevented with HP treatment. Thus, co-administration of HP with CCl₄ may be useful for attenuating the negative effects of CCl₄ on the liver.

Key words: liver, hesperidin, carbon tetrachloride, hepatotoxicity.

Introduction

Carbon tetrachloride (CCl₄) is a potent hepatotoxic chemical that produces free radicals and is widely used to induce acute hepatic injury in experimental animal models [1]. Carbon tetrachloride-induced hepatic necrosis is caused by bioactivation of the microsomal cytochrome P450-dependent monooxygenase system, resulting in the formation of a trichloromethyl radical (CCl₃) and reactive oxygen species (ROS) [2]. Reactive oxygen species consist of free radicals or oxygen free-radical-generating agents, such as a superoxide anion (O₂⁻), an hydroxyl radical (OH⁻) and hydrogen peroxide (H₂O₂) [3]. Metabolic processes are usually associated with the generation of free radicals, particularly oxy-
gen-derived radicals that oxidize and damage surrounding biomolecules [4]. The consequences of CCl4-induced lipid peroxidation include membrane disintegration, loss of membrane-associated enzymes [5, 6] and necrosis.

Hesperidin (HP) is a bioflavonoid that plays a role in plant defense and is abundant in citrus species, such as grapefruit, lemon and orange. Hesperidin is used effectively as a supplemental agent in complementary therapy protocols, since it possesses biological and pharmacological properties as an effective antioxidant, anti-inflammatory, anti-carcinogenic, and anti-hypertensive agent with lipid-lowering activity [7–9]. The antioxidant properties of HP protect testicular function from cadmium toxicity, and HP regulates hepatic cholesterol synthesis by inhibiting the activity of 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase [10–12].

Hepatotoxins, including CCl4, lead to oxidative stress and histological damage in the liver. Therefore, antioxidant agents such as HP may prevent CCl4-induced hepatotoxicity. In this study, we examined the biochemical and histological effects of HP on CCl4-induced toxicity.

Material and methods

Chemicals

Hesperidin was obtained from Sigma Chemical Co. (St. Louis, MO). Carbon tetrachloride was given by İnönü University chemistry laboratory as a gift. All other chemicals for biochemical and histological analysis were purchased from Sigma Chemical Co. (St. Louis, MO).

Animals and treatment

A total of 28 healthy adult male Wistar albino rats (2–3 months of age, 250–300 g) were obtained from the Experimental Animal Research Institute (Malatya, Turkey). Animals were housed in sterilized polypropylene rat cages, under a 12/12-h light/dark cycle, at an ambient temperature of 21°C. Food and water were provided ad libitum. Mice were kept in 0.3% H2O2 for 7 min and afterward washed with PBS. Sections were counterstained with Mayer’s hematoxylin for 1 min, rinsed in tap water, and dehydrated. The caspase-3 kit was used according to the manufacturer’s instructions.

Biochemical assay

The levels of homogenized tissue TBARS, as an index of lipid peroxidation, were determined by thiobarbituric acid reaction using the method of Yagi [13]. The product was evaluated spectrophotometrically at 532 nm and results are expressed as nmol/g tissue. The glutathione (GSH) content of the liver homogenate was measured at 412 nm using the method of Sedlak and Lindsay [14]. The GSH level was expressed as nmol/ml. Superoxide dismutase (SOD) activity was measured by the inhibition of nitroblue tetrazolium (NBT) reduction due to O2- generated by the xanthine/xanthine oxidase system [15]. One unit of SOD activity was defined as the amount of protein causing 50% inhibition of the NBT reduction rate. The product was evaluated spectrophotometrically at 560 nm. Results are expressed as IU/mg protein. Catalase (CAT) activity of tissues was determined according to the method of Aebi [16]. The enzymatic decom-
position of H$_2$O$_2$ was followed directly by a decrease in absorbance at 240 nm. The difference in absorbance per unit time was used as a measure of CAT activity. Tissue protein content was determined according to the method developed by Lowry et al. [17] using bovine serum albumin as standard.

**Statistical analysis**

All values are presented as mean ± SD. Differences were considered to be significant at $p < 0.01$ for biochemical changes. The computer program SPSS 18.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. For biochemical values, statistical analyses were performed using one-way ANOVA and post hoc Tukey’s honestly significant difference test. For histological evaluation, the microscopic score of each tissue was calculated as the sum of the scores given for each criterion. Scores were given as absent (0), slight (1), moderate (2), and severe (3) for each criterion. Statistical analysis was performed with SPSS 13 and MedCalc programs. All groups were compared by the nonparametric Kruskal-Wallis test. Exact $p$-values were given where available, and $p < 0.0001$ was accepted as statistically significant. All results are expressed as means ± standard error (SE).

**Results**

**Histological evaluation**

All figures demonstrate the histological changes in the livers of rats of each group. In the control (Figure 1 A) and HP (Figure 1 B) groups, we observed normal liver architecture and hepatocytes with well-preserved cytoplasms and nuclei. In the CCl$_4$ (Figure 2) and CCl$_4$ + HP (Figures 3 A, B) groups, we observed distortion of the hepatic cords, hepatocellular necrosis, hemorrhage (Figures 2 A, C), mononuclear cell infiltration (Figures 2 B, D), vascular congestion (Figures 2 D), eosinophilic and pyknotic nuclei hepatocytes (Figures 2 C, E), as well as vacuolated hepatocytes (Figure 2 F), which were not as extensive as in the CCl$_4$ group, indicating an improved histological appearance in the liver tissue. The microscopic damage score for each group was determined in the histological section, and the results are given in Table I.

Caspase-3-stained cells were not observed in the control (Figure 4 A) or HP (Figure 4 B) groups but were abundant in the CCl$_4$ group (Figure 4 C). The density of caspase-3-positive cells was decreased in the CCl$_4$ + HP group (Figure 4 D).

**Biochemical evaluation**

Carbon tetrachloride administration led to a significant increase in thiobarbituric acid-reactive substance (TBARS) levels compared with the other groups. Moreover, HP treatment caused a significant decrease in elevated TBARS levels when administered together with CCl$_4$, compared with the CCl$_4$ group (Table II). Glutathione, CAT and SOD levels were decreased significantly by CCl$_4$ treatment compared with the other experimental groups, and these parameters were elevated significantly by HP treatment when compared with the CCl$_4$ group (Table II). There were no significant differences between the control and HP groups, except for the CAT values, which were decreased significantly by HP treatment compared with the other groups.

Figure 1. In the liver, a normal histological appearance was observed following hematoxylin and eosin staining of the (A) control and (B) hesperidin (HP) groups.

VC = vena centralis; 20×.
Discussion

Carbon tetrachloride is a well-established hepatotoxic agent that causes severe liver damage and produces liver fibrosis and biochemical patterns that resemble human liver cirrhosis. The present study was designed to establish the protective effects of HP, a citrus bioflavonoid, on CCl₄-induced liver damage. The results demonstrated that HP ameliorated biochemical and histological evidence of CCl₄-induced liver damage.

Oxidative stress is caused by an imbalance between free radicals, such as TBARS, and the...
activity of the antioxidant defense system, including SOD, CAT, and GSH levels, which leads to lipid peroxidation and enzymatic inactivation [18]. TBARS are the final metabolites of peroxidized polyunsaturated fatty acids and are considered a late biomarker of oxidative stress [19]. Carbon tetrachloride treatment in rats markedly changed antioxidant enzyme activities, which was prevented by the co-administration of rutin, supporting a role for oxidative stress in CCl₄-induced liver damage [20].

The liver contains many drug metabolizing enzymes that metabolize toxic chemicals in the liver. Carbon tetrachloride is metabolized by a cytochrome P450 enzyme to produce highly toxic CCl₃ and CCl₃O₂ free radicals that damage hepatocytes [21–24]. Both CCl₃ and CCl₃O₂ bind to proteins or lipids and extract a hydrogen atom from an unsaturated lipid, initiating lipid peroxidation and liver damage. Therefore, increased TBARS in CCl₃-treated rats may result from enhanced membrane lipid peroxidation by free radicals and the failure of antioxidant defense mechanisms that prevent formation of excessive free radicals [25, 26]. Similarly, we found that CCl₃ significantly induced oxidative damage, increased TBARS levels, and decreased GSH levels and the activities of antioxidant enzymes, including SOD and CAT, in the liver. Another study showed that the balance between ROS production and antioxidant defenses mediates oxidative stress during CCl₃-induced hepatotoxicity. In addition, decreased SOD and CAT activities in the livers of CCl₃-treated rats may be due to free radicals generated by CCl₃, or inactivation of the antioxidant enzymes [27]. Another study demonstrated that administration of CCl₃ to rats caused oxidative stress in the liver and was associated with significantly lower antioxidant activities of GSH, CAT and SOD. Therefore, the available literature confirms our results [28–30].

Our study further demonstrated that HP treatment reversed the oxidative effects of CCl₃ via a significant reduction in elevated TBARS levels and induction of the antioxidant defense system. Only one other study has described the effects of HP against CCl₃ toxicity, but that study did not address any histological changes [1]. That group concluded that HP could prevent CCl₃ toxicity, which is in agreement with our results. There are a few studies describing the protective effects of HP on general liver injury [31, 32]. For example, Bentli et al. [33] determined that HP protected the liver against dioxin toxicity and claimed that it can be used to prevent liver injury. In addition to those findings, Chen et al. determined that HP reduced indicators of oxidative stress, such as ROS and lipid peroxidation, in a dose-dependent manner [34]. Heffner and Repine [35] suggested that HP offers protection by terminating lipid peroxidation side chains rather than scavenging extracellular non-lipid radicals that initiate lipid peroxidation. This supports our conclusion that HP protects liver tissue against many toxic agents, such as CCl₃, and these effects may be due to HP’s antioxidant and radical scavenging properties.

Upon histological evaluation, we determined that CCl₃ treatment caused severe histological

| Groups | Microscopic damage (mean ± SD) |
|--------|--------------------------------|
| 1 Control | 0.39 ±0.49* |
| 2 CCl₃ | 2.13 ±0.74* |
| 3 HP | 0.70 ±1.29* |
| 4 CCl₃ + HP | 1.64 ±0.70* |

The differences between the mean values bearing different superscript letters within the same column are statistically significant (p ≤ 0.0001). SE – standard deviation.

Figure 3. Histological findings were decreased in the CCl₃ + hesperidin (HP) group (A: H + E; 20×, B: H + E; 40×)
Protective effect of hesperidin on oxidative and histological liver damage following carbon tetrachloride administration in Wistar rats

Table II. Levels of SOD, CAT, GSH and TBARS in liver tissue (mean ± SD)

| Group         | TBARS [nmol/g tissue] | Reduced GSH [nmol/ml] | CAT [kU/mg protein] | SOD [U/mg protein] |
|---------------|------------------------|------------------------|---------------------|-------------------|
| Control       | 7.54 ±0.39a            | 181.6 ±22.9a           | 0.93 ±0.11a         | 15.2 ±2.25a       |
| CCl₄          | 11.9 ±0.87b            | 112.3 ±14.1b           | 0.42 ±0.09b         | 9.41 ±1.04b       |
| HP            | 8.08 ±1.40a            | 197.1 ±36.5c           | 0.93 ±0.09a         | 16.7 ±1.90ac      |
| CCl₄ + HP     | 10.1 ±0.93c            | 158.5 ±17.5b           | 0.75 ±0.07c         | 14.2 ±2.27c       |

Means bearing different superscripts within same column are significantly different (p < 0.01).

damage including distortion of hepatic cords, necrosis, vascular congestion, vacuolated hepatocytes, hepatocellular necrosis, eosinophilic and pyknotic nuclei, as well as mononuclear cell infiltration in liver tissues of rats. We also found a significantly larger number of caspase-3-stained cells, which were indicative of liver apoptosis, in the CCl₄ group compared with the HP + CCl₄ group. This demonstrates that HP protected the liver against cell death. Ebaid et al. [36] reported that in CCl₄-injured mice, the cytoplasm was significantly reduced and the nuclei became atrophic, suggesting that CCl₄ induced severe liver cell injury. Another study showed that CCl₄ causes hepatic injury, including hepatocytic necrosis, steatosis, and inflammation [38]. These findings paralleled and confirmed our results describing histological damage. Moreover, our observations indicate that histopathological damage was ameliorated by HP.
treatment. A previous study by Bentli et al. (2013), which described the effect of HP treatment against liver injury, confirmed our findings, since they reported that HP treatment protects the liver against dioxin toxicities. Das Neves et al. also found that HP and lipoic acid exhibit protective effects against sodium arsenite-induced acute toxicity in the liver and kidneys of mice [39]. The histological effects of CCl\textsubscript{4} on liver tissue were correlated with and caused by oxidative stress. Therefore, strong antioxidant agents such as HP can protect the liver by scavenging free radicals.

In conclusion, in the current study, we confirmed that a single dose of 2 ml/mg CCl\textsubscript{4} is toxic to rats, causing increased oxidative stress and histological changes indicative of liver damage. Also, we found that the use of HP at the dose of 50 mg/kg/day for 10 consecutive days in combination with CCl\textsubscript{4} minimized its hepatotoxicity, which was evident from decreasing TBARS levels, histological changes in tissue and increasing antioxidant enzyme activities (SOD, CAT) and GSH levels. The beneficial effects of HP against CCl\textsubscript{4}-induced liver damage may be due to its antioxidant, anti-inflammatory and free radical scavenging properties. Therefore, it appears that HP, a citrus flavonoid, can prevent and protect against many toxicological situations including CCl\textsubscript{4} toxicity caused oxidative stress. In this context, it is suggested that HP may be clinically used in human health as a radical scavenger agent.

Conflict of interest
The authors declare no conflict of interest.

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