ORIGINAL ARTICLE

Quercetin Prevents Oxidative Stress in Cirrhotic Rats

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Abstract Our aim was to study the protective effect of
quercitin on liver cirrhosis induced by carbon tetrachloride (CCl_4) in rats and its relationship with liver morphology. Thirty male Wistar rats weighing 200–250 g were randomly divided into three groups: control, CCl_4, and CCl_4 +
quercetin. Rats in the experimental groups were given CCl_4
(0.5 ml/kg i.p.), diluted 1:6 in vegetable oil (5 mmol/kg body
wt), at 10:00 p.m. every 4 days for 17 weeks. Quercetin
(500 μl/kg i.p.; 150 μmol/kg body wt) or vehicle was administered at 6:00 p.m. for the last 3 weeks of the study.
Control group rats were given only olive oil for the same
period. At the end of the 17 weeks, all rats were sacrificed.

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Blood samples were taken for determination of serum indicators (ALT, AST, total bilirubin, conjugated bilirubin, factor
V) and the livers were dissected out and divided into two
parts: one was homogenized and the supernatant was used
for measurement of superoxide dismutase (SOD), catalase,
and glutathione peroxidase (GPx) activities, as well as lipid
peroxidation. The other part was used for the histopathological study. CCl_4 caused a marked rise in serum levels of
ALT, AST, total bilirubin, and conjugated bilirubin, as well
as a decrease in factor V ($P < 0.05$). Lipid peroxidation levels were significantly increased, whereas GSH, SOD, catalase, GPx, and GST levels were decreased in the liver of
CCl_4-treated rats. Quercetin (50 mg/kg/day) successfully attenuated these effects of CCl_4. We conclude that quercetin
has beneficial effects on liver fibrosis in rats by enhancing
antioxidant enzyme activity and decreasing the pro-oxidant
effect.

Keywords Quercetin · Carbon tetrachloride · Antioxidant
enzymes · Oxidative stress

Introduction

Chronic injury leading to liver fibrosis occurs in response to a
variety of insults, including alcohol abuse, drugs, metabolic
diseases due to iron or copper overload, and autoimmune
attack on hepatocytes or bile duct epithelium. Carbon tetrachloride (CCl_4) administration is a well-known model for the
production of chemical hepatic injury [1]. Evidence of $CCl_3^\bullet$
formation from CCl_4 was provided in the 1960s by the identification of $CHCl_3$ [2] and C_2Cl_6 [3] in tissues of animals that
were given CCl_4. More recently, spin trapping experiments
have directly verified the production of $CCl_3^\bullet$ from CCl_4 by
the hepatic microsomal cytochrome P450 system [4]. The

major reactions of CCl$_3^{\bullet}$ with liver microsomes appear to be covalent binding and H$^{\bullet}$ abstraction from polyunsaturated fatty acids. The H$^{\bullet}$ abstraction results in CHCl$_3$ and PUFA$^{\bullet}$ formation. PUFA$^{\bullet}$ can enter into cross-linking reactions or lipid peroxidation, depending on the O$_2$ tension. In 1978, Packer et al. [5] reported that O$_2$ reacts very rapidly with CCl$_3^{\bullet}$ to yield CCl$_3$OO$^{\bullet}$. This radical is many orders of magnitude more reactive with PUFA than is CCl$_3^{\bullet}$ and therefore is a potent initiator of lipid peroxidation. Therefore, both CCl$_3^{\bullet}$ and CCl$_3$OO$^{\bullet}$ can cause membrane injury.

The sufficient time to develop cirrhosis by CCl$_4$ administration is variable. According to Perez-Tamayo [6], the interval between CCl$_4$ administrations must not be so long that the damaged cells cannot recover; generally, two doses per week are recommended, but this may vary depending on the model. Human dense cirrhosis, with nodule formation and portal hypertension, is usually considered irreversible, but the reversibility of cirrhosis in a number of diseases is well documented [7].

Although no successful therapeutic approach to this pathogenic mechanism in liver disease has been developed, antioxidant therapies have been shown to achieve some positive effects [8–11]. Use of antioxidants may therefore have therapeutic implications for the management of liver cirrhosis. Flavonoids are phenolic phytochemicals that represent substantial constituents of the nonenergetic part of the human diet and are thought to promote optimal health, partly via their antioxidant effects in protecting cellular components against reactive oxygen species (ROS) [12]. Quercetin (3,5,7,3,4-pentahydroxy flavon) is one of the most distributed flavonoids, semiessential food components, in certain species of plants [13]. Previous studies have shown that quercetin and other flavonoids have a broad range of pharmacological properties, including carcinostatic and antiviral activities, suppression of cell proliferation, modification of eicosanoid synthesis, protection of LDL from oxidation, prevention of platelet aggregation, stabilization of immune cells, and relaxation of cardiovascular smooth muscle [8, 14].

The present study was designed to investigate the ability of quercetin to prevent CCl$_4$-induced oxidative stress and liver injury in rats.

Materials and methods

Experimental protocol

All experiments were performed in accordance with the Guiding Principles for Research Involving Animals (NAS) [15]. Male Wistar rats were obtained from the CREAL (Centro de Reprodução Animal de Laboratório) of the UFRGS, Porto Alegre, Brazil. They were kept on standard rat chow with free access to tap water, in rooms with controlled temperature and humidity, under a 12-hr light-dark cycle. Cirrhosis was induced by intraperitoneal injection of CCl$_4$ (0.5 ml, diluted 1:6 in vegetable oil) at 10:00 p.m. every 4 days for 14 weeks [11].

Quercetin (Sigma, St. Louis, MO, USA) was suspended immediately before administration in a 0.2% Tween aqueous solution. Control and cirrhotic animals received daily a 500-μl intraperitoneal injection of quercetin (150 μmol/kg body wt at 6:00 p.m.) or vehicle for the last 3 weeks of the study.

To eliminate diurnal effects, all rats were killed at the same time of day. After the animals were decapitated and exsanguinated, the livers were immediately removed and blood samples were centrifuged at 1800 g for 15 min at 4°C to obtain plasma.

Analytical procedures

Blood was removed from the vena cava to determine the serum activity of aspartate aminotransferase (AST) and alanine aminotransferase (ALT). Bilirubin concentration was estimated using commercially available kits (Boehringer, Mannheim, Germany). Activity level of clotting factor V was measured using STA compact diagnostic STAGO.

Tissue homogenate preparation

Immediately following blood collection, rats were killed by cervical dislocation and their livers were removed. The livers were cooled in ice and homogenized in 1.15% (w/v) KCl containing 1 mM PMSF. The homogenates were centrifuged at 700 g for 10 min to discard nuclei and cell debris, and the supernatant fraction obtained was frozen at -70°C for further measurements [16].

Antioxidant enzyme activities

Total superoxide dismutase (CuZnSOD + MnSOD) activity was determined in liver as the inhibition rate of autocatalytic adenocrome generation at 480 nm in a reaction medium containing 1 mM epinephrine and 50 mM glycine-NaOH (pH 10.5). Enzyme activity is expressed as units per milligram of protein [17].

Glutathione peroxidase (GPx) activity in liver was measured by monitoring NADPH oxidation at 340 nm. The reaction medium consisted of 30 nM sodium phosphate buffer, pH 7.0, 0.17 mM reduced glutathione, 0.2 U/ml glutathione reductase, and 0.5 mM tert-butyl-hydroperoxide. GPx activity is reported as nanomoles per minute per milligram of protein [18].

Catalase (CAT) activity was determined essentially using the method described by Beers and Sizer [19], in which the disappearance of H$_2$O$_2$ is followed spectrophotometrically at 240 nm. The reaction medium consisted of 50 mM sodium

phosphate buffer, pH 7.2, and 10 mM H_2O_2 [20]. The results are reported as nanomoles per milligram of protein.

Glutathione *S*-transferase (GST) activity toward CDNB was determined spectrophotometrically at 340 nm by the method of Habig et al. [21] with slight modifications. Briefly, the assay was performed at 25°C using 100 mM potassium phosphate buffer, pH 6.5, with GSH and CDNB (dissolved in ethanol) at a final concentration of 1 mM each. The reaction was followed for 4 min and the activity was calculated from the changes in absorbance at 340 nm using the extinction coefficient of 9.6 mmol^{-1} cm^{-1} for the reaction product, dinitrophenyl-*S*-glutathione (DNP-SG). Nonenzymatic conjugation was subtracted using a blank containing buffer and the substrate, but no enzyme. One unit of GST activity was defined as the amount of enzyme catalyzing the conjugation of 1 μmol of CDNB with GSH per minute at 25°C.

Lipid peroxidation measurement

Lipid peroxidation (LPO) was measured by thiobarbituric acid reactive substances (TBARS). In the TBARS method, absorbance measurements at 535 nm were used to measure the reaction between thiobarbituric acid and the LPO products, resulting in the formation of a chromogen (Schiff's base). The homogenate was diluted to 1 mg protein/ml in 140 mM KCl and 20 mM potassium phosphate buffer, pH 7.3, and the same volume of 10% trichloroacetic acid was added (1:1). The precipitate was removed by centrifugation, and the supernatant incubated with 0.67% thiobarbituric acid for 15 min at 100°C. The results are reported as nanomoles per milligram of protein. Commercially available malonaldehyde was used as a standard [22].

Protein measurement

The protein content of the homogenate was measured by the method of Lowry et al. [23] using bovine serum albumin as a standard.

Histology

For histological studies, a piece of the liver was trimmed and fixed by immersion in Bouin's fluid. The tissues were processed according to standard histological methods. Fibrosis was semiquantitatively assessed in 3-μm sections stained with hematoxylin/eosin or Sirius red, which mainly stains collagen fibers. The histopathological fibrosis scores were as follows: 0, no fibrosis; 1, perivenular and/or pericellular fibrosis; 2, septal fibrosis; 3, incomplete cirrhosis; and 4, complete cirrhosis [24]. The tissue slices were blindly scored by two expert pathologists. The degree of fibrosis was expressed as the mean of eight fields in each slide.

Table 1 Effect of quercetin on the body weight of CCl_4-intoxicated rats

	Control	CCl_4	CCl_4 + quercetin
Body wt (g)	239 ± 3	217 ± 10[a]	229 ± 8[b]
Liver wt (g/100 g body wt)	3.7 ± 0.1	5.9 ± 0.2[a]	4.5 ± 0.3[b]

Note. Values represent the mean ± SE for six rats.

[a] $P < 0.05$, significantly different from control.

[b] $P < 0.05$, significantly different from CCl_4.

Statistical analysis

The data were compared by one-way ANOVA followed by the Student-Newman-Keuls multiple-comparison test. Results are reported as means ± SE and differences were considered to be significant at $P < 0.05$.

Results

CCl_4 significantly decreased body weight, by 10%, and increased liver weight, by 59%, compared with those in the control group. However, quercetin reversed body weight loss and liver swelling induced by CCl_4 to the levels of the control group (Table 1).

The results of the biochemical tests showed that the CCl_4 group had higher plasma levels of ALT and AST activities, total bilirubin, and conjugated bilirubin concentration and significantly lower factor V activity than the control group. Quercetin prevented the rise in bilirubin concentration and the fall in factor V, and significantly reduced the increase in transaminase activities observed in the CCl_4 group (Table 2).

The effects of quercetin on CCl_4-induced lipid peroxidation products, measured as TBARS in the liver, are reported

Table 2 Effects of CCl_4 and quercetin (Q) on serum enzyme activities, total and conjugated bilirubin, and factor V

Parameter	Group		
	Control	CCl_4	CCl_4 + Q
ALT (IU/L)	15 ± 2	415 ± 101[a]	101 ± 28[a,b]
AST (IU/L)	62 ± 4	552 ± 141[a]	103 ± 18[a,b]
Total bilirubin (mg/dl)	0.42 ± 0.02	0.55 ± 0.08[a]	0.34 ± 0.05[a,b]
Conjugated bilirubin (mg/dl)	0.10 ± 0.02	0.18 ± 0.04[a]	0.10 ± 0.02[b]
Factor V (IU/L)	4.9 ± 0.8	2.5 ± 0.4[a]	5.5 ± 0.9[b]

Note. Values are the mean ± SE for six rats.

[a] $P < 0.05$, significantly different from control.

[b] $P < 0.05$, significantly different from CCl_4.

Table 3 Effects of CCl$_4$ and quercetin (Q) on lipid peroxidation and on antioxidant enzyme activities in liver tissue

Parameter	Group		
	Control	CCl$_4$	CCl$_4$ + Q
TBARS (nmol/mg)	24.58 ± 1.69	48.67 ± 4.20^a	31.43 ± 3.28^{a,b}
GSH (μmol/g)	4.86 ± 0.07	4.40 ± 0.06^a	4.48 ± 0.13^{a,b}
SOD (U/g)	15.64 ± 1.18	5.79 ± 0.52^a	9.87 ± 0.91^{a,b}
CAT (pmol/g)	8.29 ± 0.39	2.76 ± 0.45^c	4.88 ± 0.26^{a,b}
GPx (μmol/min · g)	0.41 ± 0.02	0.13 ± 0.01^c	0.20 ± 0.01^{a,b}
GST (μmol/min · g)	0.60 ± 0.03	0.25 ± 0.02^c	0.38 ± 0.02^{a,b}

Note. Values are the mean ± SE for six rats.

$^a P < 0.05$, significantly different from control.

$^b P < 0.05$, significantly different from CCl$_4$.

$^c P < 0.001$, significantly different from control.

Table 4 Histological grading of hepatic fibrosis

Group	Grade 0	Grade I	Grade II	Grade III	Grade IV
Control	8	0	0	0	0
CCl$_4$	0	0	0	1	7^a
CCl$_4$ + quercetin	0	5	3	0	0^{a,b}

Note. Values are for eight rats.

$^a P < 0.05$, significantly different from control.

$^b P < 0.05$, significantly different from CCl$_4$.

shown in Table 3. CCl$_4$ significantly increased the TBARS concentration, by 98%, but quercetin inhibited it by 26%, compared with that in the CCl$_4$ group.

CCl$_4$ significantly decreased the hepatic GSH concentration, by 10%, compared to that in the control group, but when quercetin was also administered, the GSH concentration was maintained at the level detected in the control group (Table 3).

Antioxidant enzyme (SOD, CAT, GPx, GST) activities were significantly different between the CCl$_4$ and the CCl$_4$ + quercetin groups (Table 3). Compared to the control group, CCl$_4$ administration significantly decreased SOD activity in the liver, by 63%, while quercetin administration increased it to 70% compared with that in the CCl$_4$ group (Table 3). Likewise, CCl$_4$ significantly decreased CAT (67%), GPx (68%), and GST (58%) activities in the liver, which were restored by quercetin administration to 77%, 54%, and 52%, respectively, compared to those in the CCl$_4$ group.

Specimens from the normal control group presented a normal structure. In specimens from the experimental group there was an apparent formation of fibrotic septa, encompassing regenerated hepatocytes into pseudo-lobules. Regenerated hepatocytes underwent severe lipoid degeneration. Specimens from the CCl$_4$ + quercetin group showed only mild fibrogenesis without pseudo-lobule formation. Statistical analysis showed that fibrogenesis in the CCl$_4$ + quercetin group was less severe than in the CCl$_4$ group (Table 4).

Discussion

Liver injuries caused by CCl$_4$ are the best-characterized system of xenobiotic-induced hepatotoxicity and commonly used models for the screening of antihepato-toxic/hepatoprotective activity of drugs [25–28]. The results of the present study demonstrate that quercetin treatment effectively protected rats against CCL$_4$-induced hepatotoxicity.

By considering the hepatosomatic indexes (HIS), note that the increase in liver weight during induction in the CCl$_4$ model was reduced by quercetin. Such data are in agreement with those reported by Jeon et al., who observed increased HSIs in animals receiving CCl$_4$ and reduced HSIs in those receiving chitosan (an oligosaccharide derived from alkaline deacetylation of chitin, superoxide, and hydroxyl radical scavenger) [28]. Other authors showed that, in animals receiving CCl$_4$ [24, 36] and in animals with biliary duct obstruction, there was an increase in liver weight [34, 37] as measured by the HSI in these models. However, Pastor et al. [37] administered *N*-acetylcysteine and observed liver weight reduction in animals with biliary duct obstruction.

LPO determines alterations in structures and cell membranes by two mechanisms: (1) covalent binding with cell macromolecules and (2) action on lipids [38]. The animals receiving CCl$_4$ presented elevated LPO (70%). This is a result of CCl$_4$ metabolization by cytochrome P-450, which yields two such highly toxic radicals as CCl$_3^{\bullet}$ and CCl$_3$O$_2^{\bullet}$ [39]. Administration of quercetin to the animals determined a 26% reduction in LPO. Quercetin's possible mechanism of action is through the improvement of damage caused by CCl$_4$, preventing injuries by the generated radicals. Quercetin can act as a scavenger of CCl$_3^{\bullet}$.

GSH is a major antioxidant and its levels can be altered by various chemical agents such as CCl$_4$. GSH is produced in the liver and is kept at high levels in most tissues. Normally there is more GSH and GSSG within the cell than in the plasma, something which does not occur during liver injury processes [41]. In our work, GSH levels were found to be decreased in cirrhotic animals, similar to reports by other authors [34, 42–45]. The administration of quercetin was able to restore GSH levels to those in the control group. These results are close to those reported by Lee et al. [46], who administered quercetin orally in order to reduce dimethylnitrosamine-induced liver injury. Those authors noted that quercetin produced an

increase in serum albumin and liver GSH levels and a decrease in lipid peroxidation, data similar to those found in our work.

In our study, SOD and CAT were significantly decreased [63%] compared to those in controls and were elevated in animals receiving quercetin, an increase of 70% for SOD and of 77% for CAT. These results match those of previous work [47–49] showing a significant increase in the activity of these enzymes when CCl$_4$-intoxicated animals were treated with antioxidants. Other authors have observed an increase in these enzymes also when PMC (2,2,5,7,8-pentametyl-6-hydroxychrome), a derivative of α-tocopherol, was administered [47]. Other antioxidant enzymes were evaluated in this work, such as GPx and GST. Under oxidative stress conditions, the GSH consumed by these enzymes tries to eliminate CCl$_4$ toxicity. In this case, GPx and GST activities are decreased. In animals treated with quercetin, a reduction in oxidative damage was observed, with regeneration of GSH, and an increase in the activity of antioxidant enzymes. GPx activity is significantly reduced in animals with CCl$_4$-induced cirrhosis compared with the control group, possibly because of the increase in superoxide and hydrogen peroxide anions resulting from the action of xenobiotics.

GST favors protection against lipid peroxides, promoting the conjugation of toxic radicals with electrophilic characteristics with glutathione [51].

CCl$_4$-induced cirrhosis is considered a classic, replicable model used to study the different morphophysiological and histological aspects present in the complex structure of the human liver. A normal lobular structure was observed in the liver of control animals in the present study. The main structural modifications observed in the cirrhotic liver are those related to nodular disorganization, whose structure is characterized by fibrotic septa that cross the parenchyma or form variously sized nodules [47, 52]. In this work, when the animals received quercetin, little portal and periportal fibrosis was observed, with occasional portal-portal bridges, nevertheless without clearly outlining the formation of nodules, without the presence of steatosis, results which suggest an improvement of liver histology.

In conclusion, chronic liver diseases, no matter the etiology, frequently determine similar cellular alterations as observed in this experimental model—cellular damage, inflammation, and necrosis—often with fibrosis and cirrhosis, leading to a gradual loss of the hepatic function despite the use of immunosuppressive agents and antiviral and anti-inflammatory drugs. The finding of a substance with well-tolerated antifibrotic therapeutic action is urgently needed to block this progression and to maintain the hepatocyte capacity of synthesis. The greatest obstacle to the development of drugs is the slow progression of significant fibrosis, which takes years or even decades in humans [53].

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