Antioxidant and antiapoptotic effects of capsaicin against carbon tetrachloride-induced hepatotoxicity in rats

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Abstract
The aim of the study was to evaluate the potential hepatoprotective utility of capsaicin against carbon tetrachloride (CCl₄)-induced liver injury and to explore the possible mechanisms whereby this agent mediated its beneficial effects. We randomized 40 rats into four groups for treatment with corn oil, CCl₄, capsaicin and both CCl₄ and capsaicin, respectively, for 8 weeks. At the end of the experiment, blood samples were collected and used for determination of aspartylaminotransferase (AST), alanine aminotransferase (ALT), and total bilirubin, while the liver tissues were subjected to hematoxylin and eosin examination; evaluation of malondialdehyde (MDA), reduced glutathione (GSH) and active caspase-3 contents; and evaluation of superoxide dismutase (SOD), catalase (CAT) and glutathione-S-transferase (GST) activities. Animals treated with CCl₄ exhibited significant elevation in AST, ALT, total bilirubin and caspase-3 and exhibited significant decrease in activities of SOD, CAT, GST and GSH contents. The combination (both capsaicin and CCl₄) group has preserved the liver histology, liver enzymes and bilirubin close to normal, exhibited significant induction in the activities of CAT, SOD and GST, increased the liver content of GSH and active caspase-3 and conversely showed significant decrease in liver MDA content compared to CCl₄ challenged rats. Capsaicin confers an appealing hepatoprotective effect which might be explained partially via diminishing the generation of MDA, induction of antioxidant systems and inhibition of active caspase-3.

Keywords
Carbon tetrachloride, capsaicin

Introduction
Liver diseases are caused due to infections, parasites, nutrition deficiency, inborn errors, toxic substances and malignancy (Ismail et al., 2009). Carbon tetrachloride (CCl₄) induces liver cell injury via the generation of trichloromethyl free radical by the mixed function oxidase system of the endoplasmic reticulum (Brattin et al., 1985; Ismail et al., 2009). In addition to trichloromethyl free radical, other toxic products arising from peroxidative degeneration of membrane such as trichloromethylperoxy (OOCCl₃) and chlorine (Cl) free radicals are responsible for the wide spread hepatocyte damage induced by CCl₄ (Brattin et al., 1985; Wolf et al., 1980). Compared to other body organs, liver exposes to higher concentration of toxicants, such as CCl₄, and their metabolites because of the dual blood supply from the hepatic artery and the portal vein, and high liver content of microsomal cytochrome P450 metabolizing enzymes (Thirunavukkarasu and Sakhthisekaran, 2001)

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Many reports have emphasized on the importance of the interaction between nutrition and liver health, including the slowing down of liver damage especially in case of fatty liver and chemicals-induced liver damage (Escott-Stump et al., 2002; Ismail et al., 2009; Young and Woodside, 2001).

Capsaicin is the chief pungent principle found in red-hot chilli peppers derived from capsicum fruit extracts (Anandakumar et al., 2008a). Capsaicin has antioxidant, iron-binding properties and a hypolipidemic effect (Dairam et al., 2008; Manjunatha and Srinivasan, 2007; Srinivasan et al., 2004). Capsaicin has attracted research interest because of its dramatic biological effects on hyperalgesia, inflammation and peptic ulcers (Chowdhury et al., 1996; Kang et al., 1995).

Interestingly, previous studies have utilized the potent antioxidant property of capsaicin in counteracting the peroxidative changes in the pulmonary tissues of rat which were induced by toxic substances such as chloroform, CCl₄, sulfur dioxide and nitrogen dioxide (De and Ghosh, 1989, 1992). In addition to its potent antioxidant effect, capsaicin inhibits many metabolizing enzymes such as the aryl hydrocarbon hydroxylase (AHH) responsible for the metabolism of carcinogenic polycyclic aromatic hydrocarbons including benzo(a)pyrene leading to attenuation of its carcinogenic potential (Beecher, 1995; Modly et al., 1986).

In view of the above-mentioned beneficial effects of capsaicin, our aim was to evaluate the potential hepatoprotective utility of capsaicin against CCl₄-induced hepatotoxicity and to elucidate the possible mechanisms whereby capsaicin mediates its beneficial effects. Collecting these data might lead to focusing on the development of novel hepatoprotective agents that are commonly consumed in human diet.

**Materials and methods**

**Materials**

CCl₄ was obtained from El-Gomhorya Company, Cairo, Egypt, and capsaicin (Sigma-Aldrich) from Egyptian American Company for Laboratory Service, Cairo, Egypt.

**Animals and treatment**

We obtained male Sprague Dawley rats weighing (220 ± 5 g) from the laboratory animal colony, Ministry of Health and Population, Helwan, Cairo, Egypt. Rats were maintained under standard laboratory conditions at the animal center, Faculty of Pharmacy, Al-Azhar University, Cairo, Egypt, and were fed with basal diet and supplied with water ad libitum. After a period of adaptation, we randomized 40 rats equally into four groups. The first group was injected subcutaneously (s.c.) with corn oil and served a negative control group, the second group was treated s.c. with 2 ml/kg CCl₄ in corn oil (50% v/v) twice a week to induce chronic damage in the liver (Jayasekhar et al., 1997). The third group was treated orally with capsaicin, 20 mg/kg, dissolved in corn oil twice a week. The fourth group was treated with both CCl₄ and capsaicin using the same dose schedules mention above. After 8 weeks, we concluded the experiment and euthanized the animals. We closely monitored the rats on a daily basis for any signs of toxicity along the experiment duration and recorded the body weights once weekly. All experiments on laboratory animals were performed in accordance with the protocol approved by the University Ethics Committee.

At time of euthanasia, we collected blood samples and liver tissues from all animals, weighed the liver to calculate the relative liver weight to body weight and divided each liver sample into three parts; first part was formalin fixed, paraffin embedded, the second part was homogenized and third part was preserved frozen at −70°C.

**Histopathological examination**

The paraffin-embedded specimens were cut into sections of 46 μm thickness and stained with hematoxylin and eosin (H&E) stain according to the method of Bancroft and Stevens (1996). Two pathologists, blinded to the protocol, performed the histopathological evaluation of the stained tissue sections.

**Biochemical analysis**

Each blood sample was placed in a dry clean centrifuge tube and centrifuged for 10 minutes at 3000 revolutions per minute (rpm) to separate the serum. Serum was carefully separated into clean dry Wassermann tubes using a Pasteur pipette and used for the determination of serum liver function tests (aspartylaminotransferase [AST], alanine aminotransferase [ALT] and total bilirubin) using standard techniques (Reitman and Frankel, 1957; Sbrana et al., 2006).
Assessment of antioxidant/pro-oxidant status
Liver tissues supernatants were used for the isolation of mitochondria by the method of Johnson and Lardy (1967). The mitochondrial fractions were used for evaluation of the following parameters: protein was estimated by the method of Lowry et al. (1951), lipid peroxidation (LPO) was assayed by the method of Ohkawa et al. (1979), in which the malondialdehyde (MDA) released served as the index of LPO. Superoxide dismutase (SOD) was assayed according to the method of Marklund and Marklund (1974). Catalase (CAT) activity was assayed by the method of Sinha (1974). Reduced glutathione (GSH) was assayed by the method of Moron et al. (1979). The activity of glutathione-S-transferase (GST) was determined according to method of Habig et al. (1974), GST activity was determined with both 1-chloro-2,4-dinitrobenzene (CDNB) and 1,2-dichloro-4-nitrobenzene (DCNB) as substrates.

Evaluation of active caspase-3
Caspase-3 was evaluated immunohistochemically in paraffin-embedded liver tissue sections using caspase-3 antibody at a 1:100 dilution (Thermo Fisher Scientific Inc., Fremont, CA, USA) using a sensitive peroxidase-streptavidin method, as described previously (Krajewska et al., 1997; Yoo et al., 2001). Briefly, each block was cut into 4-μm thick sections, which was deparaffinized in xylene and rehydrated in graded alcohol and water. Endogenous peroxidase was blocked, the antigen was unmasked and non-target proteins were blocked. The slides were incubated overnight, with the primary antibody at 4°C. After extensive washing, the sections were incubated at room temperature for 10 minutes, with biotinylated anti-mouse immunoglobulin antibodies (Zymed, San Francisco, CA, USA) at a 1:20 dilution, and subsequently with streptavidin–biotin peroxidase complexes at a 1:25 dilution. The reaction products were visualized by immersing the slides in 3,3′-diaminobenzidine tetrahydrochloride and finally counterstained with hematoxylin.

For calibration of the method, tonsil tissue section and dilution buffer, instead of primary antibody, were used as positive and negative controls respectively. The immunoreactivity for caspase-3 was considered positive if predominantly cytoplasmic, with some nuclear staining.

The percentage of immunoreactive cells in this assay was calculated by counting the positive-stained nuclei (brown color) against the total number of cells in three random high-power fields for each tissue section. Three tissue sections per animal were used and six animals were included from each group. Data were presented as mean ± standard error of 10 animals per group.

Biostatistics
All data are presented as mean ± SEM (Steel and Torrie, 1980). Group differences were analyzed using one-way analysis of variance (ANOVA) followed by Tukey’s test as a post ANOVA multiple comparison test on raw data (Sigma Stat version 3; SPSS Inc., Chicago, IL, USA). Scores for the active caspase-3 protein were subjected to Kruskal–Wallis test followed by the Dunn test (Sigma Stat version 3; SPSS Inc., Chicago, IL, USA). A p value <0.05 was considered statistically significant.

Results

Effect on relative liver weight to total body weight
Figure 1 shows the relative liver weight to 100 g of body weight of different groups of rat that were euthanized at the end of the study. CCl₄-treated animals exhibited significant (p < 0.05) increase in the final relative liver weight to 100 g body weight compared to control animals. Treatment with capsaicin alone did not change the relative liver weight to body weight compared to control. Treatment with capsaicin plus CCl₄ significantly decreased the
relative liver weight to body weight compared to CCl₄-treated group.

**Biochemical results**

In the CCl₄-treated group, the serum levels of AST, ALT and total bilirubin significantly \( (p < 0.05) \) increased to 226.7 ± 8.8, 94.5 ± 3.3 and 6.8 ± 0.43, respectively, compared to the negative control group values of 92.4 ± 4.2, 21.6 ± 2.05 and 0.37 ± 0.03. Administration of capsaicin in addition to CCl₄ (combination group) significantly decreased the CCl₄-induced elevation of these marker levels \( (p < 0.05) \). The combination group exhibited the following values: 127 ± 46.8, 28.2 ± 1.4 and 1.5 ± 0.08 for AST, ALT and total bilirubin, respectively. In contrast to CCl₄ group, capsaicin alone treated group showed no significant decrease in all these marker levels compared to control animals (Figure 2A–C).

**Histological examination**

The sections of livers obtained from control rats showed the characteristic hepatic architecture of the normal central vein and surrounding hepatocytes (Figure 3A). While those obtained from CCl₄-treated group exhibited fatty change, ballooning degeneration and necrosis in most of the hepatic parenchyma. The portal area exhibited massive numbers of inflammatory cell infiltration, and the inflammatory reaction divided the hepatic parenchyma into lobules (Figure 3B). Conversely, no histopathological alteration was detected in the tissue sections of the capsaicin-treated group (Figure 3C). Interestingly, the liver sections prepared from combination group showed that the liver tissue restored its structure to almost the normal picture although rare ballooning degeneration and fatty change were detected in the hepatic parenchyma associated with few inflammatory cell infiltration in between (Figure 3D).
Compared to the control group, the CCl$_4$-treated animals exhibited significant ($p < 0.05$) increase in tissue MDA content (1033 $\pm$ 45.3% of control value; Figure 4), with concomitant decrease in the GSH content to 47.2 $\pm$ 1.26% of the control value (Figure 5A). In the same line, the activities of SOD, CAT and GST also significantly reduced to 30.5 $\pm$ 8.1%, 26.6 $\pm$ 3.5% and 35.9 $\pm$ 2.2% of the control value, respectively (Figure 5B–D).

**Oxidative state results**

Compared to the control group, the CCl$_4$-treated animals exhibited significant ($p < 0.05$) increase in tissue MDA content (1033 $\pm$ 45.3% of control value; Figure 4), with concomitant decrease in the GSH content to 47.2 $\pm$ 1.26% of the control value (Figure 5A). In the same line, the activities of SOD, CAT and GST also significantly reduced to 30.5 $\pm$ 8.1%, 26.6 $\pm$ 3.5% and 35.9 $\pm$ 2.2% of the control value, respectively (Figure 5B–D).
Compared to the CCl\textsubscript{4}-treated animals, animals treated with capsaicin along with CCl\textsubscript{4} (combination group) revealed significant amelioration of CCl\textsubscript{4} effects to near normal values. The tissue content of MDA and GSH were 162 ± 31\% and 97.3 ± 3.4\% of corresponding values in the control animal, respectively (Figures 4 and 5A), while the SOD, CAT and GST activities were 93.5 ± 7.3\%, 111.4 ± 7.2\% and 113 ± 4.2\%, respectively. However, the capsaicin alone treated animals showed significant increase in GSH (146.2 ± 8.1\%), SOD (111.3 ± 6.3\%), CAT (140.4 ± 8.4\%), GST (266.7 ± 11.8\%) and nonsignificant decrease in MDA (93.5 ± 5.1\%) when compared with the control animals (Figure 5B–D).

**Effects on hepatic active caspase-3 content**

To confirm the possibility of liver cell death through apoptosis and possible protection using capsaicin, we evaluated the active caspase-3 content in liver sections using immunohistochemical technique. The CCl\textsubscript{4}-treated group exhibited a significant increase in the content of active caspase-3 (354 ± 8.3\% of control group value; \( p < 0.05 \)). Capsaicin alone induced nonsignificant decrease in caspase-3 content (92.5 ± 1.9\% of control value), conversely the combination group showed a significant suppression in caspase-3 content compared to CCl\textsubscript{4}-treated group (135 ± 1.9\%; \( p < 0.05 \); Figure 6).

**Discussion**

Drug- or chemical-induced liver injury represents a major impediment in the modern science. Aiming at preventing liver toxicity using capsaicin, we adopted the animal model of CCl\textsubscript{4}-induced chronic hepatotoxicity in rats to investigate the hepatoprotective functions and exploring the potential mechanisms involved.

Many reports have utilized the antioxidant status as a useful tool in estimating the risk of chemicals-induced liver damage (Ismail et al., 2009; Recknagel et al., 1991; Wu et al., 2008). Enzymatic and non-enzymatic antioxidants constitute a supportive team of defense against reactive oxygen species (ROS). Enzymatic antioxidants such as SOD, CAT and GST synergistically scavenge ROS and prevent LPO. SOD disrupts superoxide radicals to hydrogen peroxide, which CAT converts to harmless water and oxygen, and hence these enzymes protect the cells against superoxide- and hydrogen peroxide-mediated LPO (Ekambaram et al., 2008). GST is a group of multifunctional proteins involved in tasks ranging from catalyzing the detoxification of electrophilic compounds to protection against peroxidative damage (Sener et al., 1979). GSH comprises the major non-enzymic antioxidant system that protects the cells against free radicals and ROS (Sies, 1986).

MDA is a major reactive aldehyde produced from the peroxidation of polyunsaturated fatty acid in the biological membranes (Vaca et al., 1988). Increased levels of LPO products play a major role in hepatotoxicity and in the early phases of tumor growth (Kolanjiappan et al., 2002; Rice-Evans and Burdon, 1993). Naturally, there is a dynamic balance between the amount of free radicals generated in the body and antioxidant defense system.

Intoxication with CCl\textsubscript{4} not only overproduces the ROS to exhaust cellular SOD, CAT, GST and GSH (Beyer, 1994; Young and Woodside, 2001) but also results in ROS attacks on unsaturated fatty acids of phospholipids in the hepatocyte membrane to subsequently begin a chain reaction of LPO (Basu, 2003; Weber et al., 2003). The cascade of oxidative stress from CCl\textsubscript{4} intoxication could disrupt the structural integrity of the hepatic cell membrane and lead to leakage of AST and ALT, hepatic cellular enzymes, into the blood from dead hepatocytes and possible
hepatic dysfunctions manifested by increased level of serum total bilirubin, thus producing signs of hepatotoxicity (Ikeda et al., 2007; Sun et al., 2001). In the present study, the observation of elevated level of hepatic MDA, and serum levels of ALT, AST and total bilirubin in the group of rats treated with CCl₄ alone is consistent with this hypothesis and might explain the inflammation manifested by H&E examination results and increased ratio of liver weight to body weight in CCl₄-treated group.

Several studies have demonstrated that antioxidant supplements may be an excellent prevention strategy for many diseases, including liver injury, liver fibrosis, aging, cancer and diabetes (Anandakumar et al., 2008a; Ekambaram et al., 2008; Ismail et al., 2009; Rodrigo et al., 2007; Wu et al., 2008). Furthermore, several reports have documented the decreased activities or content of SOD, CAT, GST and GSH in various toxicity conditions, indicating their implication in pathogenesis and hence targeting them in prevention of many toxicity conditions exploiting antioxidants (Anandakumar et al., 2008b; Kamaraj et al., 2007; Rodrigo et al., 2007). Our results supported this notion in which capsaicin prevented the CCl₄-induced hepatotoxicity through antioxidant activities. In the current study, capsaicin played detoxifying roles through enhancement of SOD, CAT and GST activities and GSH content to scavenge the overproduction of ROS generated from CCl₄ intoxication. Consequently, by interfering with the production of these initial ROS, capsaicin was able to abrogate the MDA levels and thereby prevent the cell membrane breakdown by ROS. At the same time, our results showed that capsaicin along with CCl₄ effectively reduced the leakage of AST and ALT from hepatocytes, an effect likely, partially owing to the above described antioxidant activities. Interestingly, histopathological evaluation of liver sections confirmed these notion however considerably less signs of liver injuries were observed in liver sections collected from rats treated with both capsaicin and CCl₄ compared to CCl₄ alone treated rat.

Figure 5. Evaluation of antioxidants defense system in rat liver after the treatment with carbon tetrachloride (CCl₄), capsaicin or both capsaicin and CCl₄. (A) Reduced glutathione (GSH) content, (B) superoxide dismutase (SOD), (C) catalase and (D) glutathione-S-transferase (GST). Data were calculated as percentage of corresponding control value. Data are presented as mean ± standard error of 10 animals per group. a, b and c indicate significant difference from control, CCl₄ or capsaicin, respectively, at p ≤ 0.05 using Tukey’s test as post analysis of variance (ANOVA) test.
The regulation of apoptosis is another potential mechanism through which many agents such as flavonoids, for example anthocyanins, may prevent toxicity and cancer (Srivastava et al., 2007). Noteworthy, Weber et al. (2003) indicated the ability of CCl₄ to induce severe apoptosis in hepatocytes. Consequences from the toxin-induced excessive oxidative stress, depletion of antioxidant enzymes and induction of membrane lipid peroxidation may prompt the extrinsic or intrinsic apoptotic pathways (Kaplowitz, 2002; Zhang et al., 2003). These pathways eventually lead to the activation of caspase pathway for apoptosis that ends up with caspase-3 activation, the real executioner of apoptosis once triggered, the active caspasas initiated cell apoptosis (Guicciardi and Gores, 2005; Riordan and Williams, 2003).

In the same line with the previous studies, our results revealed significantly increased active caspase-3 content in liver tissue samples collected from CCl₄-treated rats in comparison with controls. The significant elevation of active caspase-3 content was significantly abrogated close to normal in sections obtained from animals treated with a combination of capsaicin and CCl₄, indicating that capsaicin could inhibit apoptosis in hepatocytes which might support and at the same time explain our histological examination results and liver function results. We suggest that the induction of antioxidant enzymatic and non-enzymatic defense systems and suppression of MDA by capsaicin could be effective in preventing apoptosis activation by caspase cascades triggered by CCl₄ which might be supported by previous finding (El-Mahdy et al., 2007; Guicciardi and Gores, 2005; Riordan and Williams, 2003).

Of particular interest, the usual daily human consumption of capsaicin varies from a country to other, for example the average intake of capsaicin by high-level consumers, people of Mexico, is estimated to be 90–250 mg/day, while low-level consumers intake is up to 29.9 mg/day (López-Carrillo et al., 2003). In Thailand and Korea, the daily consumption of capsaicin/day is 8 mg/kg (McKenna et al., 2002; Zhang et al., 1997) which is in general lower than the dose used in this study. From clinical point of view, it would be pertinent for people who might use capsaicin as hepatoprotective agent as a supplement in a suitable pharmaceutical dosage form such as oral capsule to achieve the concentration used in the current study after completing the required studies.

**Conclusion**

CCl₄ induce liver toxicity via disturbing the antioxidant system, generation of LPOs and activation of caspase-3. Capsaicin confer an appealing hepatoprotective property against CCl₄-induced liver toxicity which might be explained partially via diminishing the generation of free radicals, induction of antioxidant defense systems and inhibition of active caspase-3 expression. Capsaicin may constitute a novel target for hepatoprotective therapeutic modality utilizing natural products.

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**Conflict of interest**

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