RESEARCH ARTICLE

Protective effects of L-carnosine on CCl4 -induced hepatic injury in rats

Mehyar Mohammad Alsheblak[1], Nehal M Elsherbiny[1], Amro El-Karef[2], Mamdouh M El-Shishtawy[1]

[1] Department of Biochemistry, Faculty of Pharmacy, Mansoura University, Mansoura, 35516, Egypt
[2] Department of Pathology, Faculty of Medicine, Mansoura University, Mansoura 35516, Egypt

Correspondence: Mamdouh M El-Shishtawy. Department of Biochemistry, Faculty of Pharmacy, Mansoura University, Mansoura, 35516, Egypt
<drnehal@hotmail.com>
<mshisht@mans.edu.eg>

To cite this article: Alchiblack MM, Elsherbiny NM, El-Karef A, El-Shishtawy MM. Protective effects of L-carnosine on CCl4 -induced hepatic injury in rats. *Eur. Cytokine Netw.* 2016; 27(1): 6-15 doi:10.1684/ecn.2016.0372

ABSTRACT. The present study was undertaken to investigate the possible protective effect of L-carnosine (CAR), an endogenous dipeptide of alanine and histidine, on carbon tetrachloride (CCl_4)-induced hepatic injury. Liver injury was induced in male Sprague-Dawley rats by intraperitoneal (i.p.) injections of CCl_4, twice weekly for six weeks. CAR was administered to rats daily, at dose of 250 mg/kg, i.p. At the end of six weeks, blood and liver tissue specimens were collected. Results show that CAR treatment attenuated the hepatic morphological changes, necroinflammation and fibrosis induced by CCl_4, as indicated by hepatic histopathology scoring. In addition, CAR treatment significantly reduced the CCl_4-induced elevation of liver-injury parameters in serum. CAR treatment also combated oxidative stress; possibly by restoring hepatic nuclear factor erythroid 2-related factor 2 (Nrf-2) levels. Moreover, CAR treatment prevented the activation of hepatic stellate cells (HSCs), as indicated by reduced α-smooth muscle actin (α-SMA) expression in the liver, and decreased hepatic inflammation as demonstrated by a reduction in hepatic tumor necrosis factor-α (TNF-α) and restoration of interleukin-10 (IL-10) levels. In conclusion, CCl_4-induced hepatic injury was alleviated by CAR treatment. The results suggest that these beneficial, protective effects are due, at least in part, to its anti-oxidant, anti-inflammatory and anti-fibrotic activities.

Key words: CCl_4-induced hepatic injury, carnosine, Nrf2, α-SMA

Liver fibrosis is a serious health problem worldwide, with significant morbidity and mortality [1]. Fibrosis is the wound healing response of the liver to repeated injury induced by many factors, including viral infection, drugs and toxins, alcoholic liver disease and autoimmune liver diseases [2]. Without elimination of these etiological agents, liver fibrosis normally progresses to cirrhosis, and distortion of the normal architecture of the liver culminating in liver failure, portal hypertension and hepatocellular carcinoma (HCC) [3].

Various processes are involved in the development of fibrotic lesions: activation of Kupffer cells by damaged hepatocytes and infiltrating macrophages [4]; release of inflammatory cytokines, growth factors and reactive oxygen species (ROS) by activated Kupffer cells [5]; activation of hepatic stellate cells (HSCs) induced by the paracrine action of pro-inflammatory and pro-fibrogenic cytokines produced by the Kupffer cells and hepatocytes [6]; as well as fibrosis evoked by activated HSCs, with accumulation of extracellular matrix (ECM) proteins, and subsequent qualitative and quantitative alterations in ECM composition [7].

Carbon tetrachloride (CCl_4)-induced liver injury is a common and validated model in rats [8]. CCl_4 is metabolized by cytochrome P450 to toxic free radicals [9]. These radicals covalently bind to cellular macromolecules and consequently lead to liver injury by causing membrane lipid peroxidation, hypomethylation of nucleic acids, disturbance of calcium homeostasis, excessive production of inflammatory cytokines, fibrosis, cirrhosis, cell death, and cancer, depending on the dose and exposure time [10].

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a redox-sensitive transcription factor that is essential for the protection of various tissues. It acts through the control of the antioxidant response element (ARE)-mediated expression of cellular phase II detoxifying enzymes, and diverse antioxidants, in response to oxidative and electrophilic stress [11]. Under normal conditions, Nrf2 expression is suppressed by Kelch-like ECH-associated protein 1 (Keap1) in the cytoplasm. Upon activation, Nrf2 is released from Keap1, escapes Keap1-mediated suppression, and translocates into the nucleus where it binds to the ARE and subsequently induces prompt expression of target cytoprotective genes that confer cellular protection and remove damage evoked by oxidative stress [12].

L-carnosine (β-alanyl-L-histidine, CAR) is a naturally occurring dipeptide that was first described in the 19th century [13]. CAR was reported to possess diverse biological activities, including antioxidant activity, neurotransmitter

doi: 10.1684/ecn.2016.0372

functions, anti-inflammatory and anti-aging, membrane protection, and pH buffering properties [14]. In the context of antioxidant activity, CAR is known to potently scavenge ROS and aldehydes, chelate pro-oxidant metals, and inhibit lipid peroxidation, protein oxidation, and advanced glycation end product (AGE) formation [15]. Therefore, it is not surprising that CAR was proposed to effectively prevent oxidative stress-induced pathological conditions, including atherosclerosis [16], diabetic complications [17], aging [18], neuronal damage [19] and Alzheimer's disease [20]. Specifically, CAR was found to effectively prevent hepatic oxidative stress due to ischemia–reperfusion, liver necrosis and alcoholic and non-alcoholic fatty liver [21, 22], thioacetamide (TAA)-induced liver damage, and liver necrosis [23]. However, the potential ameliorating effect of CAR in CCl_4-induced hepatic injury, in association with Nrf2 up-regulation, has not yet been investigated. In the present study therefore, we aimed to evaluate the protective effects of CAR on CCl_4-induced oxidative hepatic injury and inflammatory responses, and to delineate the mechanisms underlying these protective effects in rats.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats, (180-200 g) were purchased from the Urology and Nephrology Center-Experimental Animal Center, Mansoura University, Mansoura, Egypt and housed in a certified animal care facility, under controlled environmental conditions, at room temperature 22 ± 2°C and a 12-hour light-dark cycle, and were allowed access to food and water *ad libitum*. The study was conducted in compliance with the ethical guidelines for investigations in laboratory animals, and the experimental design was approved by the Ethical Committee of the Faculty of Pharmacy, Mansoura University, Mansoura, Egypt.

Experimental design

After one week of acclimatization, rats were randomized into three equivalent groups (n = 10 in each group), as follows: (1) control group: Rats received biweekly olive oil (2 ml/kg, i.p.), in addition to 5% carboxymethyl cellulose (CMC, 1 ml/kg/day, i.p.), twice weekly for six weeks to serve as a negative control group. (2) CCl_4 group: Rats received 50% (v/v) CCl_4 in olive oil (2 ml/kg, i.p.), twice a week for six weeks. (3) CAR group: Rats received CCl_4 in olive oil (2 ml/kg, i.p.), twice a week, as well as daily CAR (250 mg/kg, i.p.) for six weeks. The dose of CAR was selected based on previous studies that reported the antioxidant effects of CAR [23, 24].

Animal sacrifice and sample collection

At the end of the study period, animals were sacrificed and blood samples were collected via puncture of the retro-orbital venous plexus using heparinized capillary hematocrit tubes. Blood was centrifuged at 3000 rpm for five minutes, and serum samples were separated and stored at -80° C until they were analyzed. Liver tissues were isolated, rinsed in ice-cold saline and homogenized (10% w/v) in ice-cold, sodium phosphate buffer (0.01 M, pH 7.4) containing 1.15% KCl. The homogenates were centrifuged at 3000 rpm for 20 min at 4° C. The supernatant was collected and stored at -80° C until use.

Biochemical determinations

Biochemical markers of liver injury, including serum alanine aminotransferase (ALT), aspartate aminotransferase (AST, ELITech, France), γ-glutamyl transpeptidase activities (GGT, VITRO SCIENT, Germany), albumin (Diamond Diagnostics, Egypt), and total bilirubin levels (Biodiagnostics, Egypt) were determined using commercial kits according to the manufacturer's instructions.

Hepatic oxidative stress parameters

Levels of superoxide dismutase (SOD), malondialdehyde (MDA), and glutathione (GSH) in liver homogenates were assessed using kits from a biodiagnostic company (Cairo, Egypt) as follows:

The hepatic thiobarbituric acid reactive species concentration (TBARs), mainly MDA, was measured as described previously [25]. In brief, after precipitation of proteins by trichloroacetic acid, thiobarbituric acid reacts with MDA to form thiobarbituric acid-reactive substance that is measured spectrophotometrically at 532 nm.

Hepatic SOD activity was determined using the phenazine methosulfate method [26], in which the ability of the enzyme to inhibit the phenazine methosulfate-mediated reaction of nitroblue tetrazolium dye is measured as an indication of SOD activity.

The concentration of hepatic, acid-soluble thiols, mainly GSH, in the liver homogenate was measured according to the method described by Ellman, 1959 [27]. This method is based on the development of a relatively stable yellow color, when DTNB (5-5' dithionine 2-nitrobenzoic acid) is added to a sulfhydryl compound. The yellow color developed was measured spectrophotometrically at 412 nm.

Assessment of NO level

NO levels in liver tissue were determined using a kit from (Biodiagnostics, Cairo, Egypt) according to manufacturer's instructions. Briefly, NO was converted into nitrous acid in acidic medium. The nitrous acid was then diazotized with sulfanilamide. The product was then reacted with N-(1–naphthyl) ethylene diamine to produce azo dye that was measured spectrophotometrically at 540 nm.

Enzyme-linked immunosorbent assay

An ELISA was used to assess hepatic nuclear levels of Nrf2 (MyBioSource Company, San Diego, USA), and tumor necrosis factor-α (TNF-α) and interleukin-10 (IL-10) (eBioscience Inc., San Diego, CA, USA), according to the manufacturers' instructions.

Hepatic histopathological and immunohistochemical evaluations

Liver tissues obtained from the sacrificed rats were fixed in 4% paraformaldehyde, embedded in paraffin, cut into 3 um sections, and stained with hematoxylin-eosin for grading of total and individual necroinflammatory activity parameters using Ishak's activity index and the METAVIR algorithm [28].

Quantification of fibrotic areas

Quantitative analysis of collagen fiber deposition in Masson's trichrome-stained liver tissues was performed using morphometric analysis. Briefly, a total of 30 fields, representing ten fields each of medium-sized portal areas, medium-sized central veins, and parenchyma, were randomly chosen for each rat, and images were taken with a digital camera mounted on a BX51 Olympus optical microscope (Olympus Corporation, Tokyo, Japan). Collagenous areas stained with Masson's trichrome were extracted by splitting the picture using Photoshop software, into green and blue channels and analyzed using the NIH Image software (Scion Corporation), guided by the method described by Ishak *et al.* [28]. The extent of fibrosis was expressed as the percentage of the stained area relative to the total area. The percentages for portal area expansion, central vein thickening, and parenchymal fibrous areas obtained from the ten fields were each expressed as the mean ± standard deviation. The total fibrotic area of each specimen was calculated as the mean percentage of the three zones. Grading of necroinflammation and quantification of fibrosis were assessed by a pathologist (El-Karef A), blinded to the different animal groups.

Immunohistochemichal staining of activated HSCs

Samples were incubated with anti-α-smooth muscle actin (α-SMA) antibody (EPOS; DakoCytomation, Kyoto, Japan) at 4°C overnight, after heating in 1 mM EDTA solution (pH 8) at 121°C for 5 min to retrieve the antigen. The labeling was developed in DAB/H_2O_2 solution. Positive cells within the parenchyma and developing septa and their interfaces were counted in 15 randomly selected fields under a ×40 objective lens, excluding the vascular cells of the central veins and portal areas.

Statistical analysis

Experimental data were statistically analyzed using Prism software (Graph Pad, San Diego, CA, USA). Values were expressed as means ± SEM, and differences between groups were tested for significance using analysis of variance (ANOVA), followed by Tukey's *post hoc* test. Statistical significance was predefined as $p<0.05$.

RESULTS

CAR treatment attenuates CCl_4-induced liver dysfunction

Serum aminotransferases and GGT activities, as well as serum albumin and total bilirubin levels were used as biochemical indicators of liver damage. As shown in *figure 1*, serum ALT, AST and GGT activities significantly increased 8.8-, 4.8- and 2-fold in CCl_4-only-treated rats compared to the control group, respectively. Interestingly, CAR treatment significantly reduced serum ALT, AST and GGT by 82.9 %, 66.1% and 52.2%, compared to the CCl_4-onlytreated group. There were no significant differences in AST, ALT and GGT activities between the CAR-treated rats and the control rats. In parallel, marked elevations in serum albumin and total bilirubin were observed in the CCl_4-only-treated rats compared to the control group ($p<0.01$ and $p<0.001$), respectively. On the other hand,

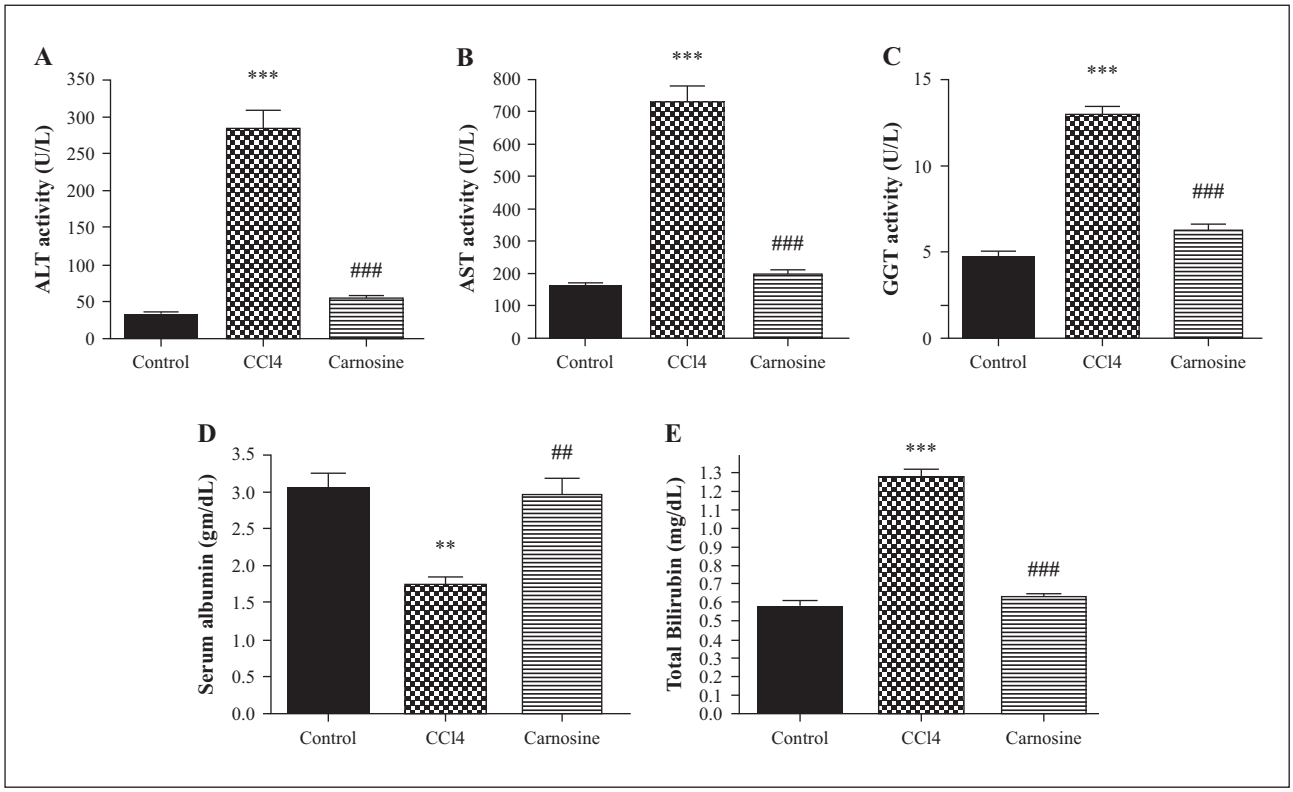

Figure 1

Effects of carnosine (250 mg/kg, i.p.) on serum activities of **(A)** alanine aminotransferase (ALT), **(B)** aspartate aminotransferase (AST), **(C)** γ-glutamyl transpeptidase (GGT), **(D)** Albumin and **(E)** total bilirubin in carbon tetrachloride (CCl_4)-induced liver injury. Data are expressed as means ± SE. Statistically significant differences are indicated as: ** $p<0.01$ and *** $p<0.001$ compared to control group; ## $p<0.01$ and ### $p<0.001$ compared to CCl_4-only-treated rats.

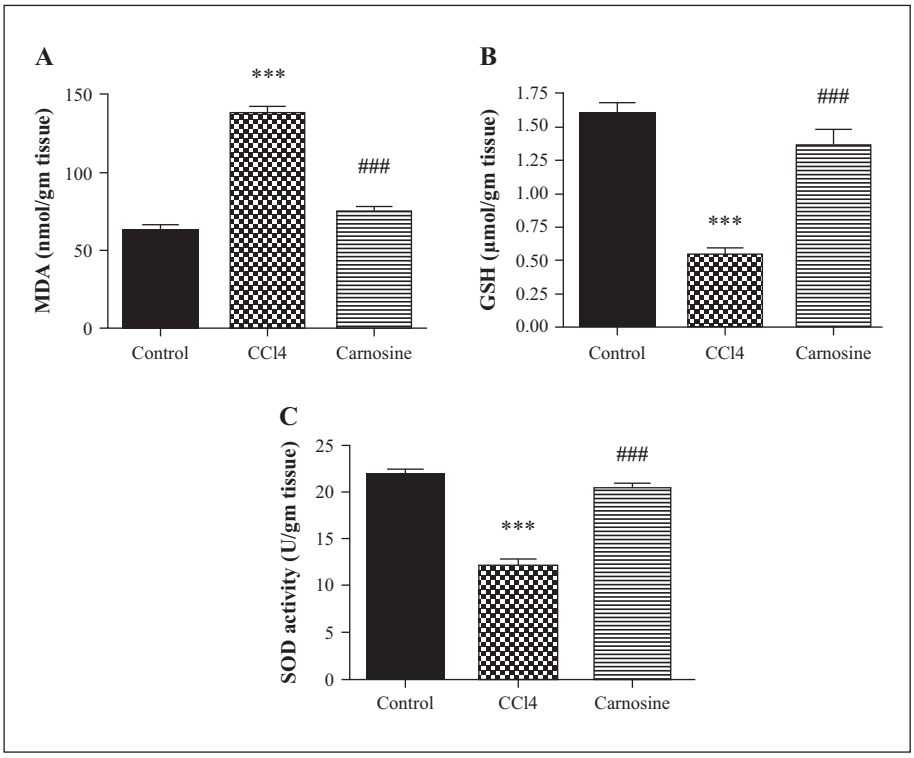

Figure 2

Effect of carnosine (250 mg/kg, i.p.) on **(A)** hepatic malondialdehyde (MDA) levels, **(B)** reduced glutathione levels (GSH) and **(C)** superoxide dismutase activity (SOD) in carbon tetrachloride (CCl_4)-induced liver injury. Data are expressed as means ± SE. Statistically significant differences are indicated as: *** $p<0.001$ compared to control group; ### $p<0.001$ compared to CCl_4-only-treated rats.

treatment with CAR significantly reduced both total bilirubin and albumin levels to the normal levels of the control, and compared to the CCl_4-only-treated group ($p<0.05$ and $p<0.001$) respectively).

CAR treatment inhibits CCl_4-induced hepatic oxidative stress

The effects of CAR on MDA and GSH levels, as well as SOD activity in liver homogenate, are shown in *figure 2*. Compared to the control group, CCl_4 treatment significantly increased hepatic MDA content ($p<0.001$), and strikingly depleted GSH levels and SOD activity ($p<0.001$and $p<0.001$) respectively, suggesting remarkable oxidative stress and lipid peroxidation in liver tissue compared to the control group. Treatment with CAR significantly attenuated oxidative stress and restored antioxidant defense in hepatic tissue compared to CCl_4-only-treated rats.

CAR treatment counteracts CCl_4-induced inflammation in the liver

As depicted in *figure 3*, CCl_4-only-treated rats showed marked hepatic inflammation as indicated by a significant increase in TNF-α levels compared to the control group ($p<0.01$). However, CAR treatment ameliorated CCl_4-induced increases in TNF-α levels when compared to CCl_4-only-treated rats ($p<0.01$). This finding was further confirmed by estimating the effect of CAR treatment on hepatic levels of anti-inflammatory cytokine IL-10. Interestingly, CAR treatment significantly restored the CCl_4-induced reduction in hepatic IL-10 levels compared to CCl_4-only-treated rats ($p<0.01$). In addition, hepatic NO levels were markedly increased in CCl_4-only-treated rats ($p<0.001$). Elevated levels of NO were significantly attenuated by CAR treatment ($p<0.01$).

Effect of CAR treatment on nuclear Nrf-2 expression in rat livers

Our results showed decreased expression levels of nuclear Nrf-2 in rat livers treated with CCl_4 as compared with the control group ($p<0.001$). However, CAR restored the expression levels of Nrf-2 in rat hepatic tissues ($p<0.05$) compared to CCl4-only-treated rats, *figure 4* .

CAR alleviates CCl_4-induced histological changes in rat hepatic tissue

Histopathological analysis of liver sections stained with hematoxylin-eosin and Masson's trichrome revealed that CCl_4 treatment induce significant liver injury characterized by marked necroinflammation and deposition of collagen. CAR treatment significantly attenuated hepatic injury compared to the CCl_4-only-treated group as indicated by a significant reduction in the necroinflammation score ($p<0.001$) compared with the CCl_4-only-treated group (*figures 5A, 6*). In addition, CAR treatment markedly reduced the fibrosis score ($p<0.001$), compared with rats receiving CCl_4 alone (*figures 5B, 6*).

Effect of CAR treatment on α-SMA expression in rat livers

Hepatic α-SMA expression was used as an indicator of HSC activation and the number of α-SMA-positive cells

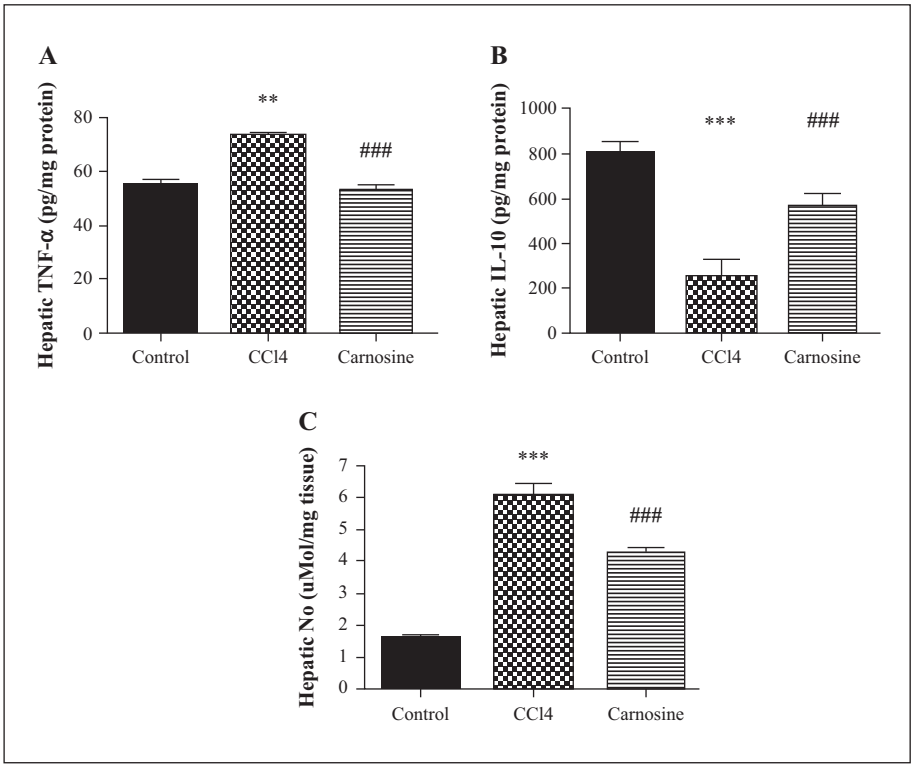

Figure 3

Effect of carnosine (250 mg/kg, i.p.) on **(A)** hepatic levels of tumor necrosis factor-α (TNF-α) and **(B)** interleukin-10 (IL-10) and **(C)** hepatic NO in carbon tetrachloride (CCl_4)-induced liver injury. Data are expressed as means ± SE. Statistically significant differences are indicated as: ** $p<0.01$ and *** $p<0.001$ compared to control group; ## $p<0.01$ compared to CCl_4-only-treated rats.

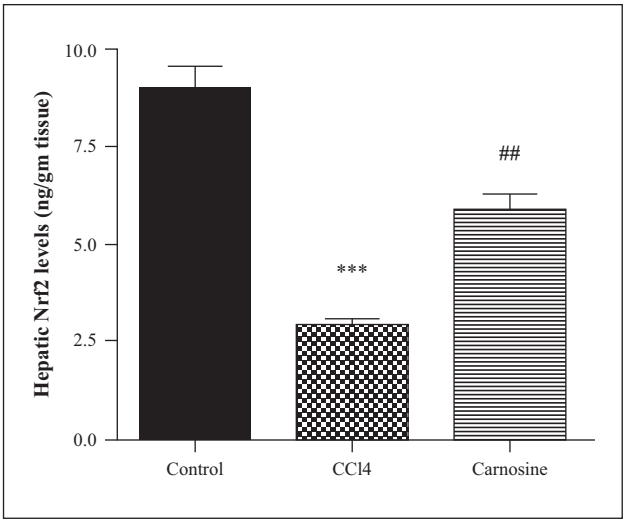

Figure 4

Effect of carnosine (250 mg/kg, i.p.) on hepatic levels of nuclear factor erythroid 2-related factor 2 (Nrf-2) in carbon tetrachloride (CCl_4)-induced liver injury. Data are expressed as means ± SE. Statistically significant differences are indicated as: ** $p<0.01$ compared to control group; # $p<0.05$ compared to CCl_4-only-treated rats.

per field was counted. CCl_4-only-treated group showed increased hepatic α-SMA expression, with increased numbers of α-SMA positive cells ($p<0.001$). CAR treatment significantly reduced α-SMA expression ($p<0.001$) as demonstrated by the decreased number of α-SMA - positive cells in the CAR-treated group compared to the group receiving CCl_4 alone (*figure 7*).

DISCUSSION

In this study, we demonstrate that CAR treatment has a preventive effect on CCl_4-induced rat liver injury. Several studies have shown that CCl_4 can induce liver dysfunction and histopathological alterations in liver tissue [29]. The present investigation clearly shows that CCl_4 increased serum levels of liver enzymes, decreased serum albumin, and induced hepatic morphological alterations that reflected damage to the hepatic cells. Meanwhile, levels of biochemical markers were markedly restored to normal by CAR treatment, which correlated well with the histopathological examination of liver samples, suggesting that CAR might have a beneficial, protective role against CCl_4-induced damage.

Oxidative stress is a key mechanism in CCl_4-hepatotoxicity [30]. CCl_4 is metabolized into highly toxic trichloromethyl ($^{\bullet}CCl_3$) and/or trichloromethyl peroxyl ($^{\bullet}OOCCl_3$) free radicals [31]. These toxic species are capable of covalent binding to proteins and lipids, initiating the lipid peroxidation process and with the consequent formation of by-products such as MDA, leading ultimately to oxidative liver cell damage. Moreover, $^{\bullet}CCl_3$ was found to bind covalently to GSH, resulting in its depletion [32]. GSH can combat oxidative stress either directly by acting as a non-enzymatic antioxidant or indirectly by involvement, as a cofactor or a coenzyme, in the enzymatic detoxification reaction for ROS [33]. Moreover, SOD is an antioxidant enzyme that can be used as an indicator of oxidative stress in cells. Raising the levels of SOD and other antioxidant enzymes can be used for the prevention and treatment of liver damage

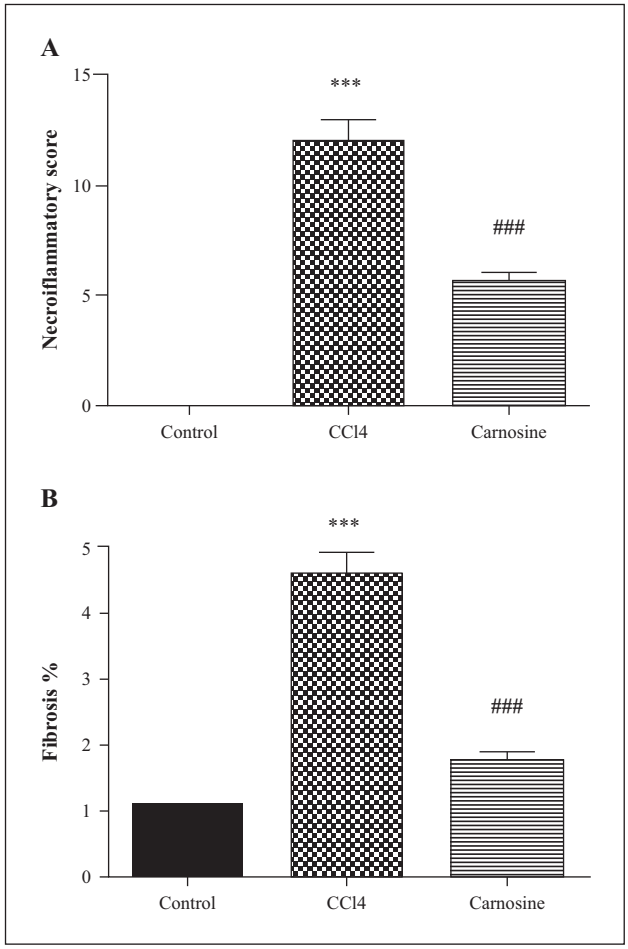

Figure 5
Effect of carnosine (250 mg/kg, i.p.) on **(A)** hepatic necroinflammatory score and **(B)** % ratio between fibrotic area to total tissue area in carbon tetrachloride (CCl4)-induced liver injury. Data are expressed as means ± SE. Statistically significant differences are indicated as: *** $p<0.001$ compared to control group; ## $p<0.01$ and ### $p<0.001$compared to CCl_4-only-treated rats.

[34]. Indeed, many studies have demonstrated that the toxic, free-radical intermediates evoked by CCl_4 lead to oxidative imbalances, with subsequent depletion of antioxidant enzymes [35, 36]. In the present study, CAR treatment markedly inhibited CCl_4-induced oxidative stress in rat livers. It seems likely that CAR relieves hepatic injury by upregulating antioxidant levels in the liver which scavenge free radicals. Of note, CAR was found to act as a potent antioxidant by the combined actions of ROS inactivation, free radical scavenging and metal chelation [37].

To gain a deeper insight into the possible antioxidant mechanism of CAR, we measured the effect of CAR on nuclear Nrf2 levels in livers of CCl_4-treated rats. It is now generally accepted that inhibition of oxidation of cellular macromolecules and upregulation of the antioxidant enzyme activity are regulated by Nrf2 [38]. Indeed, several studies have shown that CCl_4-induced liver damage is associated with dramatic decreases in Nrf-2 activity [39, 40]. Nrf2 mediates upregulation of many antioxidant enzymes in the liver [41]. Enzymatic antioxidant defense is a fundamental molecular antioxidant mechanism that eliminates ROS. Upregulation of these antioxidant enzymes results in restoration of cellular redox homeostasis in the presence of oxidative stress, and subsequent inactivation of deleterious oxidative toxicants [42]. Our results indicated that CCl_4 treatment resulted in a marked reduction of nuclear Nrf-2 levels in rat livers. However, CAR treatment restored Nrf2 expression as compared to CCl_4-only-treated group. It is well established that Nrf2 accumulation in the nucleus is an indicator of Nrf2 activation [43]. Taken together, these results suggested that CAR treatment resulted in activation of hepatic Nrf2, with subsequent inhibition of lipid peroxidation. These effects are consistent with a compensation of deficits in the hepatic antioxidant defense system after CAR treatment.

To verify further the protective impact of CAR on CCl_4-induced liver injury, we measured the levels of inflammatory markers in rat hepatic tissue. In addition to oxidative stress, inflammation is another important pathological mechanism propagating CCl_4-induced liver injury. Indeed, inflammation is considered a hallmark of early stage liver fibrosis, which leads to continuous hepatocyte damage and ultimately extensive fibrosis and cirrhosis [44]. Interestingly, it was reported that Nrf2 activation can inhibit the expression of mediators of inflammation, such as pro-inflammatory cytokines, by regulation of anti-inflammatory enzymes [45]. In agreement with that

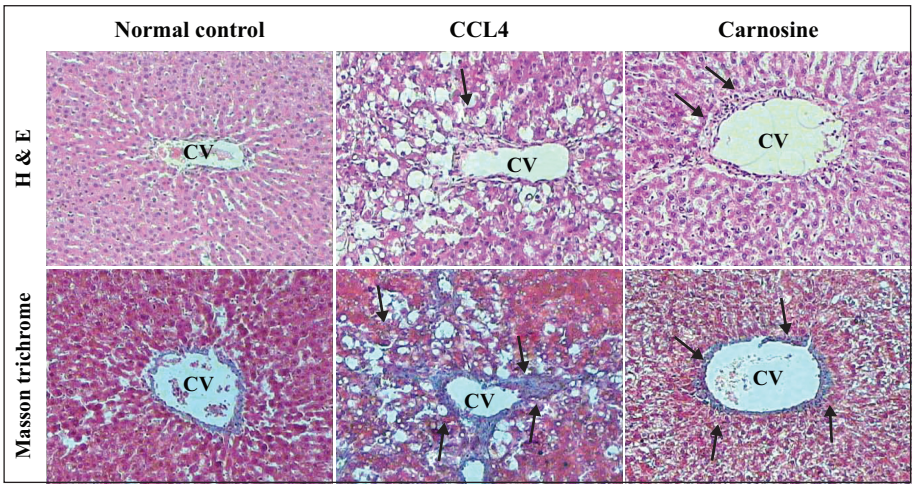

Figure 6
The upper panel showed marked necroinflammation in the CCl_4-group that became markedly reduced after treatment with carnosine. The lower panel shows increased collagen deposition in the CCl_4 group with marked reduction after treatment with carnosine (magnification ×100).

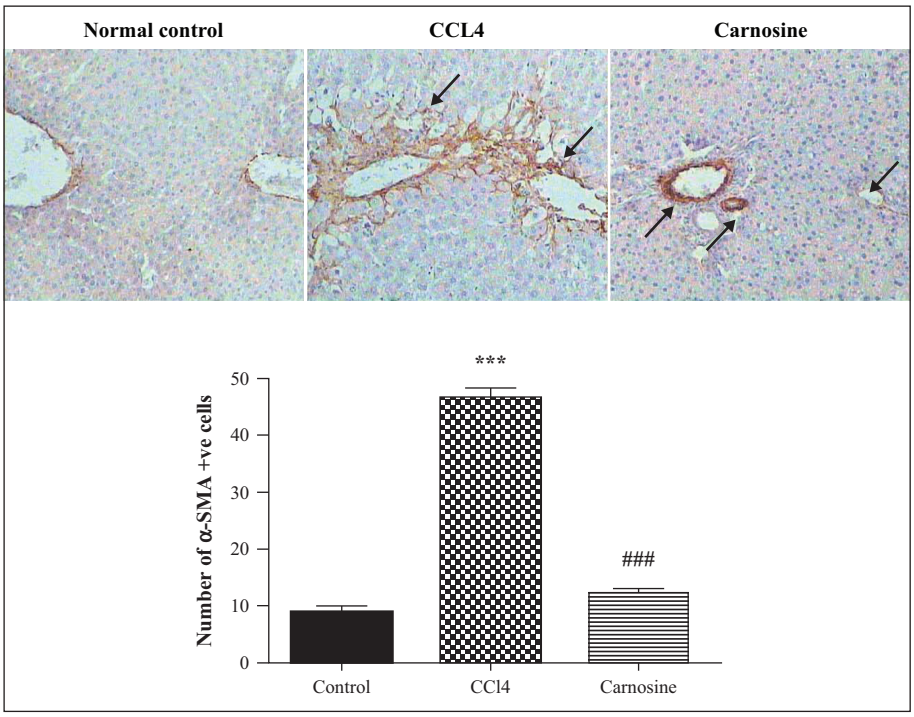

Figure 7
After CCl_4 administration, α-smooth muscle-positive HSCs and/or myofibroblasts (arrows and inset) are increased around central veins and portal areas, with marked a reduction in the carnosine-treated group compared to CCl_4-only-treated one (magnification ×100 and inset ×400).

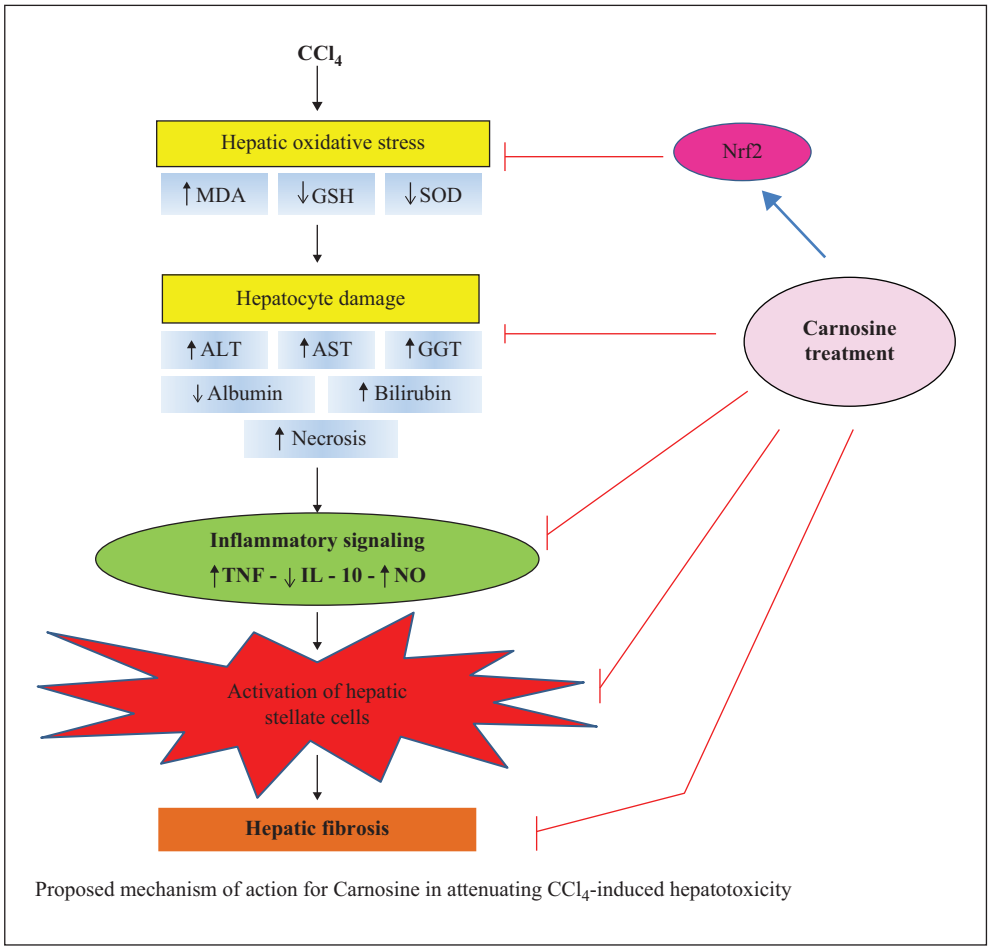

Figure 8
Proposed mechanism of action of carnosine in attenuating CCl_4-induced liver injury.

report, the present study demonstrated that CCl_4 treatment markedly increased TNF-α and suppressed IL-10 expression. On the other hand, CAR treatment counteracted these deleterious changes remarkably in hepatic tissues. IL-10 is an anti-inflammatory and immunosuppressive cytokine that decreases the production of proinflammatory cytokines, including TNF-α and IL-1β [46]. Extensive studies have reported the ability of IL-10 to combat the inflammatory response and to limit hepatotoxicity in several models of liver injury [47]. Thus, increased endogenous IL-10 expression could attenuate inflammatory bursts and subsequent liver injury induced by CCl_4 [44]. These results suggested that CAR could attenuate CCl_4-induced liver injury by way of suppressing the inflammatory response. Indeed, these results are in agreement with other studies that reported pivotal anti-inflammatory effects of CAR in different experimental models [48, 49].

NO is a highly reactive oxidant produced by inducible nitric oxide synthase (iNOS) in hepatic cells during inflammation. iNOS is an enzyme expressed in hepatocytes and inflammatory cells during both acute and chronic liver diseases [50]. iNOS-derived NO reacts with superoxide anion to form peroxynitrite, resulting in nitrosative stress [51]. Moreover, it also regulates gene expression of proinflammatory mediators, thereby contributing to the inflammatory events seen in different liver injuries [52]. The tissue damage induced by nitrosative stress could be prevented by the removal of free radicals through the activation of enzymatic and molecular antioxidants. Suppression of NO levels has been shown to attenuate CCl_4-induced liver injury [53, 54], indicating that NO signaling could be a potential target for prevention of liver injury. We suggest that activation of Nrf-2 by CAR treatment resulted in an augmentation of hepatic antioxidant defense mechanisms and hence a quenching of the nitrosative stress evoked by NO.

HSCs are a liver-specific type of pericyte and the main source of ECM proteins. Activation of HSCs represents a fundamental event in a sequence of hepatic injury that links the inflammatory and repair phases of liver damage [55]. Activated HSCs undergo phenotypic trans-differentiation into fibrogenic myofibroblasts, characterized by several changes, including an increase in proliferation, ECM accumulation and expression of α-SMA [56]. Our results showed significantly more immunostaining for α-SMA in the CCl_4-treated group than in the control group. On the other hand, low levels of α-SMA were observed in the CAR-treated group. These results correlate with the results of histopathological studies, which showed a remarkable attenuating effect of CAR on CCl_4-induced hepatic fibrosis, as indicated by fibrosis scoring. Of note, our results confirmed previous reports of the anti-fibrotic effect of CAR in experimental models of lung and liver fibrosis [57, 58].

In conclusion, the present study demonstrated hepatoprotective effect of CAR in CCl_4-induced liver injury, *figure 8*. Thus, CAR might be promising as an anti-oxidative, anti-inflammatory, and anti-fibrotic medication to minimize toxin/chemical-induced hepatic injuries caused by oxidative stress and inflammatory reactions.

Disclosure. *Financial support: none. Conflict of interest: none.*

REFERENCES

1. Huang X, Wang X, Lv Y, Xu L, Lin J, Diao Y. Protection effect of kallistatin on carbon tetrachloride-induced liver fibrosis in rats via antioxidative stress. *PLoS One* 2014; 18: 9.
2. Balsano C, Alisi A, Nobili V. Liver fibrosis and therapeutic strategies: the goal for improving metabolism. *Curr Drug Targets* 2009; 10: 505-12.
3. Luedde T, Schwabe RF. NF-κB in the liver–linking injury, fibrosis and hepatocellular carcinoma. *Nat Rev Gastroenterol Hepatol* 2011; 8: 108-18.
4. Gäbele E, Brenner DA, Rippe RA. Liver fibrosis: signals leading to the amplification of the fibrogenic hepatic stellate cell. *Front Biosci* 2003; 1: 69-77.
5. Zhou WC, Zhang QB, Qiao L. Pathogenesis of liver cirrhosis. *World J Gastroenterol* 2014; 20: 7312-24.
6. Brenner DA. Molecular pathogenesis of liver fibrosis. *Trans Am Clin Climatol Assoc* 2009; 120: 361-8.
7. Tan Z, Qian X, Jiang R, *et al*. IL-17A plays a critical role in the pathogenesis of liver fibrosis through hepatic stellate cell activation. *J Immunol* 2013; 191: 1835-44.
8. Zhao X, Song JL, Kil JH, Park KY. Bamboo salt attenuates CCl4-induced hepatic damage in Sprague-Dawley rats. *Nutr Res Pract* 2013; 7: 273-80.
9. Yeh YH, Hsieh YL, Lee YT. Effects of yam peel extract against carbon tetrachloride-induced hepatotoxicity in rats. *J Agric Food Chem* 2013; 61: 7387-96.
10. Weber LW, Boll M, Stampfl A. Hepatotoxicity and mechanism of action of haloalkanes: carbon tetrachloride as a toxicological model. *Crit Rev Toxicol* 2003; 33: 105-36.
11. Kumar H, Kim IS, More SV, Kim BW, Choi DK. Natural product-derived pharmacological modulators of Nrf2/ARE pathway for chronic diseases. *Nat Prod Rep* 2014; 31: 109-39.
12. Scannevin RH, Chollate S, Jung MY, *et al*. Fumarates promote cytoprotection of central nervous system cells against oxidative stress via the nuclear factor (erythroid-derived 2)-like 2 pathway. *J Pharmacol Exp Ther* 2012; 341: 274-84.
13. Ditte Z, Ditte P, Labudova M, *et al*. Carnosine inhibits carbonic anhydrase IX-mediated extracellular acidosis and suppresses growth of HeLa tumor xenografts. *BMC Cancer* 2014: 14-358.
14. Zhang Z, Miao L, Wu X, *et al*. Carnosine Inhibits the Proliferation of Human Gastric Carcinoma Cells by Retarding Akt/mTOR/p70S6K Signaling. *J Cancer* 2014; 5: 382-9.
15. Aydin AF, Küskü-Kiraz Z, Doğru-Abbasoğlu S, Güllüoğlu M, Uysal M, Koçak-Toker N. Effect of carnosine against thioacetamide-induced liver cirrhosis in rat. *Peptides* 2010; 31: 67-71.
16. Rashid I, van Reyk DM, Davies MJ. Carnosine and its constituents inhibit glycation of low- density lipoproteins that promots foamcell formation in vitro. *FEBS Lett* 2007; 581: 1067-70.
17. Riedl E, Pfister F, Braunagel M, *et al*. Carnosine prevents apoptosis of glomerular cells and podocyte loss in STZ diabetic rats. *Cell Physiol Biochem* 2011; 28: 279-88.
18. Hipkiss AR, Brownson C, Carrier MJ. Carnosine, the anti-ageing, anti-oxidant dipeptide, may react with protein carbonyl groups. *Mech Ageing Dev* 2001; 122: 1431-45.
19. Bellia F, Vecchio G, Cuzzocrea S, Calabrese V, Rizzarelli E. Neuroprotective features of carnosine in oxidative driven diseases. *Mol Aspects Med* 2011; 32: 258-66.

20. Hipkiss AR. Could carnosine or related structures suppress Alzheimer's disease? *J Alzheimers Dis* 2007; 11: 229-40.

21. Artun BC, Küskü-Kiraz Z, Güllüoğlu M, Cevikbaş U, Koçak-Toker N, Uysal M. The effect of carnosine pretreatment on oxidative stress and hepatotoxicity in binge ethanol administered rats. *Hum Exp Toxicol* 2010; 29: 659-65.

22. Giriş M, Doğru-Abbasoğlu S, Kumral A, Olgaç V, Koçak-Toker N, Uysal M. Effect of carnosine alone or combined with α-tocopherol on hepatic steatosis and oxidative stress in fructose-induced insulin-resistant rats. *J Physiol Biochem* 2014; 70: 385-95.

23. Mehmetçik G, Ozdemirler G, Koçak-Toker N, Cevikbaş U, Uysal M. Role of carnosine in preventing thioacetamide-induced liver injury in the rat. *Peptides* 2008; 29: 425.

24. Aydín AF, Küskü-Kiraz Z, Doğru-Abbasoğlu S, Uysal M. Effect of carnosine treatment on oxidative stress in serum, apoB-containing lipoproteins fraction and erythrocytes of aged rats. *Pharmacol Rep* 2010: 733-9.

25. Elsherbiny NM, Abd El Galil KH, Gabr MM, Al-Gayyar MM, Eissa LA, El-Shishtawy MM. Reno-protective effect of NECA in diabetic nephropathy: implication of IL-18 and ICAM-1. *Eur Cytokine Netw* 2012; 23: 78-86.

26. DeChatelet LR, McCall CE, McPhail LC, Johnston Jr. RB. Superoxide dismutase activity in leukocytes. *J Clin Invest* 1974; 53: 1197-201.

27. Ellman GL. Tissue sulfhydryl groups. *Arch Biochem Biophys* 1959; 82: 70-7.

28. Ishak K, Baptista A, Bianchi L, *et al*. Histological grading and staging of chronic hepatitis. *J Hepatol* 1995; 22: 696-9.

29. Maheshwari DT, Yogendra Kumar MS, Verma SK, Singh VK, Singh SN. Antioxidant and hepatoprotective activities of phenolic rich fraction of Seabuckthorn (Hippophae rhamnoides L.) leaves. *Food Chem Toxicol* 2011; 49: 2422-8.

30. Singh N, Kamath V, Narasimhamurthy K, Rajini PS. Protective effect of potato peel extract against carbon tetrachloride-induced liver injury in rats. *Environ Toxicol Pharmacol* 2008; 26: 241-6.

31. Shim JY, Kim MH, Kim HD, Ahn JY, Yun YS, Song JY. Protective action of the immunomodulator ginsan against carbon tetrachloride-induced liver injury via control of oxidative stress and the inflammatory response. *Toxicol Appl Pharmacol* 2010; 242: 318-25.

32. Weber LW, Boll M, Stampfl A. Hepatotoxicity and mechanism of action of haloalkanes: carbon tetrachloride as a toxicological model. *Crit Rev Toxicol* 2003; 33: 105-36.

33. Ma JQ, Ding J, Zhang L, Liu CM. Protective effects of ursolic acid in an experimental model of liver fibrosis through Nrf2/ARE pathway. *Clin Res Hepatol Gastroenterol* 2015; 39: 188-97.

34. Martin-Aragón S, de las Heras B, Sanchez-Reus MI, Benedi J. Pharmacological modification of endogenous antioxidant enzymes by ursolic acid on tetrachloride-induced liver damage in rats and primary cultures of rat hepatocytes. *Exp Toxicol Pathol* 2001; 53: 199-206.

35. Feng Y, Siu KY, Ye X, *et al*. Hepatoprotective effects of berberine on carbon tetrachloride-induced acute hepatotoxicity in rats. *Chin Med* 2010; 18: 5-33.

36. Al-Olayan EM, El-Khadragy MF, Aref AM, Othman MS, Kassab RB, Abdel Moneim AE. The potential protective effect of Physalis peruviana L. against carbon tetrachloride-induced hepatotoxicity in rats is mediated by suppression of oxidative stress and downregulation of MMP-9 expression. *Oxid Med Cell Longev* 2014; 2014: 381413.

37. Kim MY, Kim EJ, Kim YN, Choi C, Lee BH. Effects of α-lipoic acid and L-carnosine supplementation on antioxidant activities and lipid profiles in rats. *Nutr Res Pract* 2011; 5: 421-8.

38. Kumar H, Kim IS, More SV, Kim BW, Choi DK. Natural product-derived pharmacological modulators of Nrf2/ARE pathway for chronic diseases. *Nat Prod Rep* 2014; 31: 109-39.

39. Xu W, Hellerbrand C, Köhler UA, Bugnon P, *et al*. The Nrf2 transcription factor protects from toxin-induced liver injury and fibrosis. *Lab Invest* 2008; 88: 1068-78.

40. Wu S, Yue Y, Tian H, *et al*. Carthamus red from Carthamus tinctorius L. exerts antioxidant and hepatoprotective effect against CCl-induced liver damage in rats via the Nrf2 pathway. *J Ethnopharmacol* 2013; 148: 570-8.

41. Li JP, Gao Y, Chu SF, *et al*. Nrf2 pathway activation contributes to anti-fibrosis effects of ginsenoside Rg1 in a rat model of alcohol- and CCl4-induced hepatic fibrosis. *Acta Pharmacol Sin* 2014; 35: 1031-44.

42. Li N, Xia T, Nel AE. The role of oxidative stress in ambient particulate matter-induced lung diseases and its implications in the toxicity of engineered nanoparticles. *Free Radic Biol Med* 2008; 44: 1689-99.

43. Niture SK, Kaspar JW, Shen J, Jaiswal AK. Nrf2 signaling and cell survival. *Toxicol Appl Pharmacol* 2010; 244: 37-42.

44. Rocha SW, de França ME, Rodrigues GB, *et al*. Diethylcarbamazine reduces chronic inflammation and fibrosis in carbon tetrachloride- (CCl_4-) induced liver injury in mice. *Mediators Inflamm* 2014; 2014: 696383.

45. Shin DS, Kim KW, Chung HY, Yoon S, Moon JO. Effect of sinapic acid against carbon tetrachloride-induced acute hepatic injury in rats. *Arch Pharm Res* 2013; 36: 626-33.

46. O'Shea JJ, Murray PJ. Cytokine signaling modules in inflammatory responses. *Immunity* 2008; 28: 477-87.

47. Arai T, Hiromatsu K, Kobayashi N, *et al*. IL-10 is involved in the protective effect of dibutyryl cyclic adenosine monophosphate on endotoxin-induced inflammatory liver injury. *J Immunol* 1995; 155: 5743-9.

48. Son DO, Satsu H, Kiso Y, Totsuka M, Shimizu M. Inhibitory effect of carnosine on interleukin-8 production in intestinal epithelial cells through translational regulation. *Cytokine.* 2008; 42: 265-76.

49. Sahin S, Oter S, Burukoğlu D, Sutken E. The effects of carnosine in an experimental rat model of septic shock. *Med Sci Monit Basic Res* 2013; 19: 54-61.

50. Al-Shabanah OA, Alam K, Nagi MN, Al-Rikabi AC, Al-Bekairi AM. Protective effect of aminoguanidine, a nitric oxide synthase inhibitor, against carbon tetrachloride induced hepatotoxicity in mice. *Life Sci* 2000; 66: 265-70.

51. Coleman JW. Nitric oxide in immunity and inflammation. *Int Immunopharmacol* 2001; 1: 1397-406.

52. Chen T, Zamora R, Zuckerbraun B, Billiar TR. Role of nitric oxide in liver injury. *Curr Mol Med* 2003; 3: 519-26.

53. Teraoka R, Shimada T, Aburada M. The molecular mechanisms of the hepatoprotective effect of gomisin A against oxidative stress and inflammatory response in rats with carbon tetrachloride-induced acute liver injury. *Biol Pharm Bull* 2012; 35: 171-7.

54. Xiao J, Liong EC, Ching YP, *et al*. Lycium barbarum polysaccharides protect mice liver from carbon tetrachloride-induced oxidative stress and necroinflammation. *J Ethnopharmacol* 2012; 139: 462-70.

55. De Minicis S, Rychlicki C, Agostinelli L, *et al*. Semaphorin 7A contributes to TGF-β-mediated liver fibrogenesis. *Am J Pathol* 2013; 183: 820-30.

56. López-Reyes AG, Arroyo-Curras N, Cano BG, *et al*. Black bean extract ameliorates liver fibrosis in rats with CCl4-induced injury. *Ann Hepatol* 2008; 7: 130-5.

57. Soliman KM, Abdel Aziz M, Nassar YH, Abdel-Sattar S, El-Ansary A. Effects of carnosine on bilharzial infestation in hamsters: biochemical and histochemical studies. *Comp Biochem Physiol B Biochem Mol Biol* 2002; 131: 535-42.

58. Cuzzocrea S, Genovese T, Failla M, *et al*. Protective effect of orally administered carnosine on bleomycin-induced lung injury. *Am J Physiol Lung Cell Mol Physiol* 2007; 292: 1095-104.