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Curative and protective effects of L-arginine on carbon tetrachloride-induced hepatotoxicity in mice

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ABSTRACT

L-arginine may aid in the liver detoxification and may benefit in the treatment of liver disorders such as liver injury. The present study was to investigate the possible protective and curative effects of L-arginine on carbon tetrachloride (CCl_4) induced hepatotoxicity. Mice received a single dose of CCl_4. L-arginine treatment was given for 6 days prior or post to CCl_4 injection. CCl_4-intoxication caused marked liver cell necrosis with inflammatory and apoptotic lesions. L-arginine treatment reduced hepatic necrosis and inflammation. CCl_4-intoxication also enhanced hepatic lipid peroxidation, decreased hepatic GSH level and inhibited the activities of antioxidant enzymes. Pre-treatment and post-treatment with L-arginine decreased lipid peroxidation and restored the antioxidant status to near normal levels. These results suggest that L-arginine administration has hepatoprotective and hepatocurative effects against CCl_4 induced hepatotoxicity in mice.

1. Introduction

Amino acid L-arginine is a precursor for the synthesis of protein, nitric oxide (NO), creatine, agmatine, and polyamines, and is an intermediate in the detoxification of ammonia. L-arginine has been linked to enhanced immunity, the release of the human growth hormone, greater muscle mass, rapid healing from injury, increased sexual potency, and helping to reverse atherosclerosis [1,2]. L-arginine may benefit in the treatment of liver disorders such as liver injury, hepatic cirrhosis, and fatty liver degeneration [3].

CCl_4 is a well-known hepatotoxin induces oxidative stress and causes liver injury by the formation of free radicals [4]. CCl_4 causes liver damage following the cleavage by cytochrome P450 to form trichloromethyl free radical ($CCl_3^{\cdot}$). This radical quickly adds molecular oxygen to form the trichloromethyl peroxyl radical ($CCl_3OO^{\cdot}$) [5]. Abstraction of hydrogen atoms from unsaturated lipids by such radicals creates carbon-centered lipid radicals [6]. These lipid radicals quickly add molecular oxygen to form lipid peroxyl radicals, thereby initiating the process of lipid peroxidation. Unless scavenged by radical scavengers, these lipid peroxyl radicals in turn abstract hydrogen atoms from other lipid molecules, thereby propagating the process of lipid peroxidation [7]. In a study of plasma amino acid levels after CCl_4 induced acute liver damage in rats, at 16 and 24 h after CCl_4 treatment, an increase in blood plasma amino acid levels and positive correlations with the dose of CCl_4 were observed for most individual amino acids. The only exception was L-arginine which decreased in a dose dependent manner [8].

Cellular protection against free radicals involves an elaborate antioxidant defense system, including several antioxidant enzymes and low molecular weight substances such as glutathione. These biochemical defenses serve to lower the steady state concentrations of free radicals, which might otherwise cause excessive damage to cell components [9]. L-arginine pre-treatment of rats 5 days before and concomitantly with cyclosporine prevented the significant increase in MDA, improved the activity of GPx enzyme and ameliorated the depletion of the GSH content. These findings may indicate a possible protective effect of L-arginine against nephrotoxicity induced by cyclosporine treatment [10]. Depletion in the antioxidant enzymes and thiol status was observed in ethylene glycol-treated rats (hyperoxaluric rats). L-arginine co-supplementation to these rats prevents the retention of calcium oxalate crystals by way of protecting the renal cells from oxidative injury [11]. Pretreatment of ischemic rats with molsidomine and L-arginine markedly attenuated renal dysfunction, morphological alterations, improved the tissue as well as urine NO contents, reduced elevated thiobarbituric acid reactive substances levels and restored the depleted renal antioxidant enzymes [12].

The present study has been conducted to evaluate the possible hepatoprotective and hepatocurative roles of L-arginine administration against CCl_4-induced hepatotoxicity in mice through investigation of lipid peroxidation and antioxidant enzymes.

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2. Materials and methods

2.1. Chemicals

CCl_4 and L-arginine were obtained from Sigma chemicals, Saint Louis, MO, USA. All other chemicals and solvents used were of the highest purity grade available.

2.2. Maintenance of animals

Forty female Swiss albino mice (25 ± 2 g) obtained from Theodor Bilharz Research Institute, Cairo (Egypt) were used. The animals were housed for about one month prior to experimental use. All mice were housed under constant conditions of a 12-h light/dark cycle in a temperature and humidity controlled room, and were maintained according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH) [13]. Food and water *ad libitum* were allowed.

2.3. Experimental protocol

Mice were divided into four groups of ten animals each as follows: a CCl_4 group received a 6-day repeated oral dose of tween 80 (1 ml/kg) diluted in distilled water (1%, v/v) then a single intraperitoneal (i.p.) dose of CCl_4 (20 mg/kg) dissolved in corn oil (2%, v/v) which is documented to induce acute hepatotoxicity in mice [14] and then mice were sacrificed after 24 h of CCl_4 injection [15]; a vehicle control group received the respective vehicles only (i.e. received a 6-day repeated oral dose of 1% tween 80 (1 ml/kg) then a single i.p. dose of corn oil then mice were sacrificed after 24 h of oil injection); a protection group received a 6-day repeated oral dose of L-arginine 200 mg in 1 ml of 1% tween 80/kg then a single i.p. dose of CCl_4 (20 mg/kg) and then mice were sacrificed after 24 h of CCl_4 injection; and a curative group received firstly a single i.p. dose of CCl_4 (20 mg/kg), then 24 h later received a 6-day repeated oral dose of L-arginine 200 mg in 1 ml of 1% tween 80/kg and then mice were sacrificed at the end of the 6th day from oral administration of L-arginine.

2.4. Samples

All mice from each group were sacrificed by decapitation at the end of the 8th day of the experiment and following removal, a portion of the liver of each mouse was saved for histopathological analysis. The remaining portion was rapidly rinsed with ice-cold saline and dried on filter paper. Weighed dried liver was homogenized in 0.9% NaCl solution using a homogenizer and the resultant homogenate (10%, w/v) was then centrifuged at 5000 rpm for 15 min at 4 °C and the resultant supernatant was used for determination of biochemical parameters.

2.5. Histopathological analysis

A sample of liver obtained after decapitation was washed in saline and fixed in 10% formalin for the routine haematoxylin and eosin (H&E) staining technique and histopathological examinations. Fixed tissues were processed routinely, embedded in paraffin wax, sectioned into 5 μm thick sections in a rotary microtome and then stained with H&E dye. At least three different sections were examined per sample of liver. The pathologist evaluating liver sections was unaware of the treatment the mice had received.

2.6. Biochemical measurements

2.6.1. Estimation of lipid peroxidation

The malondialdehyde (MDA) content, a measure of lipid peroxidation, was estimated by its ability to react with thiobarbituric acid forming a 1:2 adduct as described by the method of Ohkawa et al. [16].

2.6.2. Estimation of reduced glutathione

Estimation of GSH content was performed spectrophotometrically, using Ellman's reagent [17].

2.6.3. Estimation of antioxidant enzymes activities

2.6.3.1. Estimation of glutathione peroxidase. GPx activity was determined by its ability to catalyze the oxidation of GSH by *t*-butyl hydroperoxide [18].

2.6.3.2. Estimation of glutathione reductase. The activity of GR was estimated by measurement of the decrease in absorbance due to the oxidation of NADPH to $NADP^+$ accompanied with the reduction of oxidized glutathione [19].

2.6.3.3. Estimation of glutathione-S-transferase. GST activity was determined by measurement of the rate of GSH conjugation with 1-chloro-2,4-dinitrobenzene as substrate [20].

2.6.3.4. Estimation of superoxide dismutase. SOD activity was measured by the degree of inhibition of the reduction of nitroblue tetrazolium dye [21].

2.6.3.5. Estimation of catalase. catalase activity was kinetically determined by monitoring the rate of decomposition of hydrogen peroxide [22].

The biochemical measurements were expressed and refereed to mg protein which was determined by the method of Bradford [23].

2.7. Statistical analysis

Results are presented as mean ± S.D. A statistical analysis between two groups was performed using Student's *t*-test. $P < 0.05$ was considered significant for all analysis. An IBM computer with a statistical software system instate version 2.03 (Graphpad, USA) was used for these calculations.

3. Results

3.1. Histopathological analysis

As represented in Table 1, liver sections of control mice showed no pathological changes (0); hepatic cells are arranged in cord separated by widened sinusoid, hepatic cells show low nucleo/cyto-

Table 1
Tissue damage semi-quantitative score in liver.

Animal groups	Control group	CCl_4 group	Protection group	Curative group
Score	0	+++	++	+

0: when no score is evaluated for necrotic, inflammatory or apoptotic lesion.
+ (mild): when <25% of 10 high power field is affected by necrotic, inflammatory or apoptotic lesion.
++ (moderate): when 25–50% of 10 high power field is affected by necrotic, inflammatory or apoptotic lesion.
+++ (severe): when >50% of 10 high power field is affected by necrotic, inflammatory or apoptotic lesion.

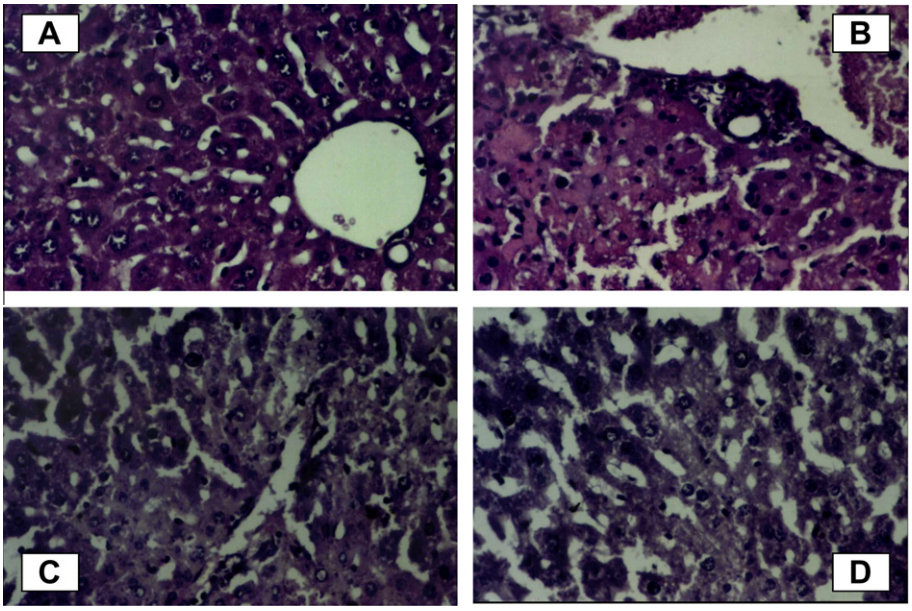

Fig. 1. Liver histopathology. (A) Section of control mouse liver showing no pathological changes in which the normal hepatic cells are arranged in cord separated by widened sinusoids. Sinusoids are lined by simple flat endothelium. Hepatic cells show low nucleo/cytoplasmic ratio. Nuclei are small round and densely stained (H&E, ×400). (B) Section of intoxicated mouse liver with CCl_4 shows hepatic necrosis with acute inflammatory cells and apoptotic changes (H&E, ×400). (C) Section of L-arginine pre-treated intoxicated mouse liver shows scattered areas of necrosis among inflammatory infiltrate (H&E, ×400). (D) Section of L-arginine post-treated intoxicated mouse liver shows improvement in the liver tissue; hepatic cells are formed with focal area of hyaline degeneration. Sinusoids are dilated and lined by flat endothelium (H&E, ×400).

plasmic ratio, nuclei are small round and densely stained, and sinusoids are lined by simple flat endothelium (Fig. 1A). Injection with CCl_4 caused severe pathological changes (+++) (Table 1); widespread necrosis with acute inflammation and apoptotic changes in liver are present (Fig. 1B). L-arginine at the dose of 200 mg/kg for 6 days could reduce the hepatic injury score of inflammation, apoptosis and necrosis; pre-treatment with L-arginine showed moderate pathological changes (++) and L-arginine post-treatment showed mild pathological changes (+) (Table 1). In Fig. 1C scattered areas of necrosis among inflammatory infiltrate are still present reflecting noticed improvement in the hepatic cells architecture of mice pre-treated with L-arginine. Fig. 1D reflected better improvement in the liver pathology of mice post-treated with L-arginine; hepatic cells formed cords with focal area of hyaline degeneration and sinusoids dilated and lined by flat endothelium.

3.2. Biochemical measurements

3.2.1. Lipid peroxidation

As shown in Table 2, hepatic MDA of CCl_4-intoxicated mice was markedly increased as compared to control group. Pre-treatment or post-treatment with L-arginine significantly decreased the MDA content compared with untreated CCl_4-intoxicated mice.

3.2.2. Reduced glutathione level

CCl_4-intoxication significantly decreased hepatic GSH level and pre-treatment or post-treatment with L-arginine significantly increased its level compared with untreated CCl_4-intoxicated mice (Table 2).

3.2.3. Antioxidant enzymes activities

Table 3 shows that CCl_4 significantly inhibited liver activities of antioxidant enzymes (SOD, catalase, GPx, GR and GST) compared with those of control group. On contrary, pre-treatment or post-treatment of CCl_4-intoxicated mice with L-arginine was significantly enhanced the liver activities of these antioxidant enzymes compared with those of untreated CCl_4-intoxicated mice.

Table 2
Hepatic contents of MDA and GSH.

Animal groups	Control group	CCl_4 group	Protection group	Curative group
MDA (μmol/mg protein)	0.53 ± 0.06	2.45 ± 0.47[a]	1.18 ± 0.43[b]	0.80 ± 0.19[c]
GSH (μmol/mg protein)	6.51 ± 0.39	4.76 ± 0.24[a]	5.59 ± 0.34[b]	7.44 ± 1.01[c]

Data are reported as mean ± S.D. (n = 10 in each group).
[a] $P < 0.001$ as compared to the control group.
[b] $P < 0.01$ as compared to the CCl_4 group
[c] $P < 0.001$ as compared to the CCl_4 group.

Table 3
Activities of hepatic antioxidant enzymes.

Animal groups	Control group	CCl_4 group	Protection group	Curative group
SOD (unit/mg protein)	45.52 ± 0.83	25.79 ± 12.59[a]	61.11 ± 5.12[e]	73.55 ± 9.57[e]
Catalase (unit/mg protein)	116.4 ± 21.3	26.19 ± 11.12[b]	44.05 ± 14.99[c]	53.5 ± 25.76[c]
GPx (unit/mg protein)	5.01 ± 0.30	3.54 ± 0.34[b]	4.95 ± 0.53[e]	4.50 ± 0.66[c]
GR (unit/mg protein)	5.22 ± 1.07	3.10 ± 0.78[a]	4.63 ± 0.45[d]	4.15 ± 0.32[c]
GST (unit/mg protein)	5.36 ± 1.15	2.77 ± 0.89[a]	7.09 ± 2.88[d]	4.80 ± 1.38[c]

Data are reported as mean ± S.D. (n = 10 in each group).
[a] $P < 0.01$ versus control group
[b] $P < 0.001$ versus control group.
[c] $P < 0.05$ versus CCl_4 group
[d] $P < 0.01$ versus CCl_4 group
[e] $P < 0.001$ versus CCl_4 group.

4. Discussion

The significant impairment of hepatic GSH status associated with a substantial hepatocellular damage induced by CCl_4 suggested the determinant role of hepatic GSH in the development of CCl_4 toxicity [24]. Bhadauria et al. observed that, administration of CCl_4 caused a significant depletion in hepatic GSH with significantly enhanced hepatic lipid peroxidation in rats [25,26]. In the present study and in accordance with Hewawasam et al. [27] and with Jiang et al. [28], a significant decrease ($P < 0.001$) of the liver GSH was observed 24 h after the injection of CCl_4 compared to control. Pre- or post-treatment with L-arginine significantly increased GSH level ($P < 0.01$ and $P < 0.001$, respectively). That increase of hepatic GSH in treated mice could be due to an effect on the *de novo* synthesis of GSH, its regeneration or both [24].

The present study showed that, intoxication with CCl_4 caused extreme increase ($P < 0.001$) in the level of hepatic MDA supporting the previous observations of Manna et al. [4] and Zhu and Fung [29] who reported that single dose of CCl_4 to induce acute liver injury significantly increased the level of TBARS in mice liver homogenate. Moreover, Muriel [30] and Bhadauria et al. [26] showed that lipid peroxidation was significantly increased in the liver of CCl_4-intoxicated rats. Such results suggested that the liver is susceptible to CCl_4 treatment and that lipid peroxidation is associated with CCl_4-induced liver injury. Our results also showed that mice pretreated or post-treated with L-arginine exhibited a significant decrease in lipid peroxidation ($P < 0.01$ and $P < 0.001$, respectively). The present results are in agreement with the findings of Nanji et al. [31] who observed that animals with alcohol-induced liver injury that treated with L-arginine had about 50% decrease in the level of lipid peroxidation compared with those untreated ones. On contrary, Muriel [30] has shown that L-arginine treatment showed no significant effect on lipid peroxidation induced by CCl_4-intoxication. Zhu and Fung [29] reported that L-arginine treatment had no significant effect on the liver function of CCl_4-treated mice.

Many compounds known to be beneficial against CCl_4-mediated liver injury exert their protective action either via a decreased production of CCl_4 derived free radicals or through the antioxidant activity of the protective agents themselves [32,33]. In 2002 Lass et al. [34] have reported that L-arginine was an effective scavenger capable of scavenging O_2 and possibly other reactive oxygen species and suggested that L-arginine cardioprotective effects against oxygen radical-induced myocardial injury might be due to a direct chemical interaction of L-arginine and its derivatives with oxygen radicals. Recently, the protective effect of L-arginine on gentamicin-induced nephropathy would have resulted from its direct antioxidant properties [35]. Since, cytochrome P450 is a major contributor to lipid peroxidation in CCl_4-intoxication [5], nitric oxide is generated from L-arginine reacts with cytochrome P450 and inhibits its activity and generation of free radicals [36,37]. Hence, the mechanism for the decrease in lipid peroxidation with L-arginine may be related to the inhibitory effect of nitric oxide generated from L-arginine on cytochrome P450 activity decreasing the production of CCl_4 derived free radicals. Or may be neither related to nitric oxide ability, because of its unpaired electron, to accept other electrons and function as a free radical scavenger molecule or related to the antioxidant effects of L-arginine itself [31,36,37].

The present results showed that CCl_4-intoxication significantly decreased the activities of antioxidant enzymes (SOD, catalase, GPx, GR and GST) as compared to control mice. This confirmed the previous observations of Manna et al. [4] who showed that SOD, catalase and GST levels were decreased in CCl_4 treated mice. And was in agreement with Kang et al. [38] who observed that CCl_4-treatment was found to decrease levels of SOD, catalase and GPx significantly in rat liver homogenate and with Domitrović et al. [39] who reported that acute CCl_4-intoxication induced a significant decrease in SOD activity and GSH content in mice liver homogenate. The present results also showed that pre-treatment or post-treatment with L-arginine significantly improved all the activities of antioxidant enzymes as compared to CCl_4-intoxicated mice.

SOD enzyme catalyze the reduction of the superoxide radical to hydrogen peroxide keeping the intracellular steady state concentrations of superoxide radical low. While, catalase and GPx detoxify hydrogen peroxide generated from the dismutation of superoxide radical by catalyzing its reduction to water. Catalase is primarily a peroxisomal enzyme that catalyzes the enzymatic decomposition of hydrogen peroxide into water and molecular oxygen. Other organic hydroperoxides (ROOH, R is alkyl or acyl groups) are also substrates for catalase. In this case the corresponding ROH is generated along with water and molecular oxygen. The cytosolic and mitochondrial GPx enzymes complement the intracellular localization and function of catalase in the metabolism of hydrogen peroxide by catalyzing the decomposition of hydrogen peroxide or ROOH in the presence of GSH into water or ROH with a concomitant production of GSSG [40]. The catalytic activity of GST promotes the nucleophilic attack of GSH toward an organic hydroperoxide (ROOH), leading to the sulfenic acid of glutathione (GSOH). Following an addition of another molecule of GSH, GSSG is resulted [41]. Since GR catalyzes the reduction of GSSG to GSH using NADPH as a source of electrons, the resulting GSSG is then recycled to GSH by NADPH-GR [42]. GSH plays an important role in the protection against toxicities by reactive oxygen compounds [43]. Therefore, our observations suggest that L-arginine administration decrease lipid peroxidation by increasing the activities of antioxidant enzymes which in turn scavenge free radicals.

The present histopathological studies confirmed observations of Wu et al. [44], Leal et al. [45], Lv et al. [46] and Srilaxmi et al. [47] who reported that administration of CCl_4 to rats caused severe hepatic damage, as demonstrated by classic histological changes including hepatocyte necrosis or apoptosis, inflammation, haemorrhage and fatty degeneration. Also, Roomi et al. [48] reported that CCl_4 administration caused an intense degree of liver necrosis.

Moreover, histopathological studies in the present study provided a supportive evidence for its biochemical studies where L-arginine administration at the dose of 200 mg/kg for 6 days reduces the hepatic injury score of inflammation, apoptosis and necrosis. Oral supplementation of L-arginine in acute liver injury model significantly improved the state of the liver injury [49]. L-arginine improved resistance of hepatic cells to damaging action of CCl_4 [50]. Chamulitrat et al. [51] implied that nitric oxide (NO) produced in experimental CCl_4 plus lipopolysaccharide-treated rats plays a protective role in the metabolism and removal of CCl_4. In another study using perfused rat livers, NO improved microcirculation and led to decreased hepatic damage in ethanol-induced hepatic injury [52]. These studies have suggested that NO plays a protective role in hepatic injury. Low-level NO acts as an antioxidant and higher level as a pro-antioxidant. It was proposed that the mechanism of low concentration of NO's protection may involve diminished metal-catalyzed lipid peroxidation and the high concentration of NO's potentiation of oxidative stress may involve mitochondrial dysfunction [53]. Furthermore, L-arginine administration probably through the generation of NO, leads to improvement in liver pathological changes such as fatty liver, necrosis, inflammation, and fibrosis. Since NO, in addition to having antioxidant effects, can also behave as a pro-oxidant, the amount of L-arginine as nitric oxide-donor used in the treatment of inflammatory liver diseases needs to be carefully titrated [33].

In conclusion, the results of the present study showed that, L-arginine administration at a dose of 200 mg/kg for 6 days decreases hepatic lipid peroxidation induced by CCl_4-intoxication,

enhances the activities of hepatic antioxidant enzymes (SOD, catalase, GR, GPx and GST) and leads to improvement in liver pathological changes, suggesting that L-arginine is somehow combating CCl_4 induced liver hepatotoxicity. L-arginine exerts its hepatoprotective and hepatocurative action against CCl_4-induced alterations in the liver may be through combination between increasing the activities of antioxidant enzymes which in turn scavenge free radicals, decreasing the production of CCl_4 derived free radicals and the antioxidant effects of L-arginine itself and its derivatives. Finally, these results indicated that, L-arginine administration showed hepatoprotective and hepatocurative effects against CCl_4 induced hepatotoxicity in mice.

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