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ORIGINAL PAPER

Protective effects of curcumin, α-lipoic acid, and *N*-acetylcysteine against carbon tetrachloride-induced liver fibrosis in rats

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Abstract Liver fibrosis is a major health problem that can lead to the development of liver cirrhosis and hepatocellular carcinoma. On the other hand, several antioxidants have been shown to possess protective effect against liver fibrosis. Therefore, in the present work, the effectiveness of curcumin, α-lipoic acid, and *N*-acetylcysteine in protecting against carbon tetrachloride (CCl_4)-induced liver fibrosis as well as the mechanism(s) implicated in this protective effect was studied. The antioxidants used in this study resulted in hepatoprotective effect as evident by substantial decreases in collagen deposition in histopathological examinations in addition to significant decrease in serum levels of alanine aminotransferase, aspartate aminotransferase, gamma glutamyl transpeptidase, bilirubin, and transforming growth factor-alpha (TGF-α) as well as hepatic malondialdehyde concentration, with a concurrent increase in serum matrix metalloproteinase-13 (MMP-13) and hepatic reduced glutathione (GSH) levels as compared to CCl_4 fibrotic group. In conclusion, curcumin, α-lipoic acid, and *N*-acetylcysteine protect rats against CCl_4-induced liver fibrosis most possibly through their antioxidant activities and their capacities to induce MMP-13 and to inhibit TGF-α levels.

Keywords Curcumin · α-Lipoic acid · *N*-Acetylcysteine · Liver fibrosis

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Introduction

Liver fibrosis is a consequence of most chronic liver diseases such as hepatitis viral infection and autoimmune hepatitis. It is characterized by impaired liver function and increased production of extracellular matrix proteins, mainly collagens [1]. Carbon tetrachloride (CCl_4), a well-known hepatotoxin, is widely used in laboratory animals to induce toxic liver injuries including fibrosis. CCl_4 requires biotransformation to produce free radicals that eventually lead to membrane lipid peroxidation, and it has been established that free radicals and lipid peroxidation play a

critical role in the pathogenesis of various hepatic disorders including hepatic fibrosis. Therefore, many antioxidants have been shown as potential hepatic antifibrotic agents [14, 21].

It has been established that curcumin, α-lipoic acid, and *N*-acetylcysteine exert multiple pharmacological actions that involve antioxidant activities and thus suppress fibrogenesis in rats with CCl_4-induced liver injury [7, 16, 22]. However, their mechanism(s) of action are yet to be fully elucidated. Matrix metalloproteinases (MMPs) play an important role in tissue remodeling and repair in both physiological and pathological conditions including liver fibrosis [19]. MMP-13, the main interstitial collagenase in rodents, plays a critical role in mediating the regression of hepatic fibrosis [4]. On the other hand, Lee et al. [13] reported that transforming growth factor-alpha (TGF-α) induces activation of hepatic stellate cells which play a key role in the pathogenesis of hepatic fibrosis. Therefore, the present study was undertaken to study the antifibrotic effects of curcumin, α-lipoic acid, and *N*-acetylcysteine and to elucidate their possible mechanism(s) of action in CCl_4-induced liver fibrosis in rats.

Materials and methods

Animals

Male Wistar rats weighing 180–200 g were used after 1 week for proper acclimatization to the animal house conditions (12 h lighting cycle and 25±2°C temperature) and had free access to standard rodent chow and water. All experimental procedures were conducted according to the ethical standards approved by the Institutional Animal Ethics Committee guidelines for animal care and use, El-Minia University, Egypt.

Chemicals

Curcumin, α-lipoic acid, and *N*-acetylcysteine were purchased from Sigma–Aldrich Corp. (St. Louis, MO, USA). CCl_4 was purchased from BDH/PROLABO Chemicals, England. All other chemicals were of analytical grade and were obtained from commercial sources.

Experimental procedures

Animals were randomly divided into five groups of ten animals each. The first group served as the control group. The second group was intraperitoneally injected with a mixture of CCl_4 (0.1 ml/100 g body weight) and olive oil (1:1, *v*/*v*) twice/week for 8 weeks to induce hepatic fibrosis as described by Fu et al. [6] with minor modifications. Groups 3–5 received curcumin (200 mg/kg; Fu et al. [6]), α-lipoic acid (30 mg/kg; Melhem et al. [15]), and *N*-acetylcysteine (300 mg/kg; Galicia-Moreno et al. [7]), respectively. The tested drugs were suspended in 0.5% aqueous solution of carboxymethyl cellulose and were administered orally daily 1 week before and 8 weeks concurrently with CCl_4 injections. All groups received equivalent volumes of the above-used vehicles. Seventy-two hours after the last CCl_4 injection, rats were sacrificed, and blood samples were collected and centrifuged at 3,000×*g* for 10 min to obtain clear sera. The liver was excised from each animal, and a slice was taken for histological examination. Liver samples were snap frozen in liquid nitrogen, stored at −80°C, and subsequently homogenized in cold potassium phosphate buffer (0.05 M, pH 7.4) for various biochemical analyses.

Biochemical analysis

Using commercially available UV/colorimetric kits, serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma glutamyl transpeptidase (GGT), and bilirubin (HUMAN GmbH, Germany) as well as hepatic glutathione (GSH) (Biodiagnostic, Egypt) levels were quantified according to the manufacturers' guidelines. Hepatic lipid peroxidation was determined as thiobarbituric acid-reacting substance and is expressed as equivalents of malondialdehyde (MDA), using 1,1,3,3-tetramethoxypropane as standard [3]. Serum MMP-13 and TGF-α (Uscn Life Science Inc., Wuhan, China) were determined using ELISA kits according to the manufacturer's instructions.

Histological examination

Liver tissue samples were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with

Table 1 Effects of curcumin, α-lipoic acid, and *N*-acetylcysteine on serum ALT, AST, GGT, and bilirubin on CCl_4-induced liver fibrosis in rats

Group	ALT (U/L)	AST (U/L)	GGT (U/L)	Bilirubin (mg/dL)
Control	64.4±2.6	33.6±2.9	10.1±0.67	0.64±0.12
CCl_4	133±2.4*	171±8.5*	16.8±0.49*	1.62±0.13*
CCl_4 + curcumin	116±2.3*, **	69.0±3.5*, **	11.0±0.52**	0.91±0.07**
CCl_4 + α-lipoic acid	115±7.8*, **	68.8±6.6*, **	11.4±0.37**	0.88±0.12**
CCl_4 + *N*-acetylcysteine	77.9±2.0**, ***, ****	91.0±4.1*, **	13.1±0.31*, **, ***	1.01±0.14**

Data are presented as means ± SEM of ten rats

*P<0.05 compared with control, **P<0.05 compared with CCl_4, ***P<0.05 compared with curcumin, ****P<0.05 compared with α-lipoic acid

hematoxylin and eosin for histological examination and grading using light microscopy. Masson's trichrome stain was used to identify increases in liver collagenous tissue. To quantify the hepatic fibrosis, we used the Knodell index [12] with some modifications. At least five fields containing a central vein of each specimen were analyzed, and the microscopic examination was performed in a blind way.

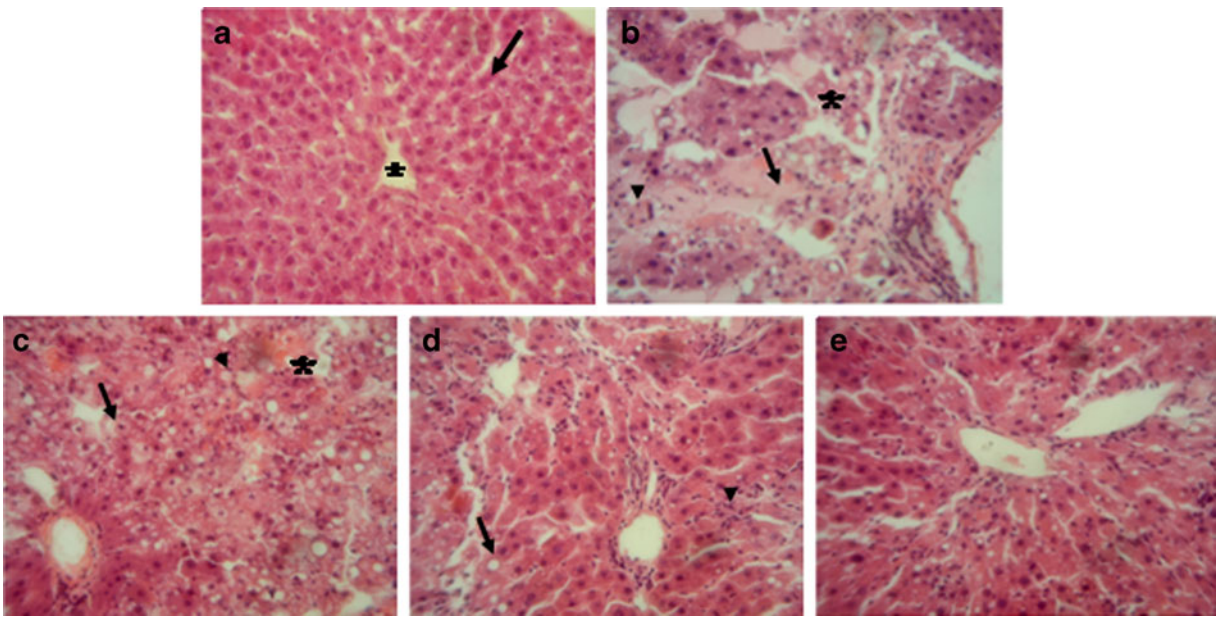

Fig. 1 Effect of curcumin, α-lipoic acid, and *N*-acetylcysteine on carbon tetrachloride-induced histopathological changes in rat liver (×40). **a** Representative photomicrograph of normal liver tissue stained with hematoxylin and eosin. Notice normal appearance of hepatocytes (*arrow*); central vein is of normal diameter (*asterisk*) showing neither dilation nor congestion. **b** Liver tissue after exposure to carbon tetrachloride for 8 weeks; areas of coagulative necrosis are scattered in the liver lobule near to the central vein (*arrow*), hepatocytes showing vacuolated cytoplasm (*arrowhead*), and some apoptotic cells could be easily distinguished in the field (*asterisks*). Notice dilatation of the central vein and sinusoids with inflammatory cell infiltration. **c** Liver tissue after treatment with curcumin; general improvement of hepatic architecture could be observed, but some hepatocytes are still showing vacuolated cytoplasm (*arrow*), and others are apoptotic (*arrowhead*). Areas of necrosis are still observed (*asterisks*). **d** Liver tissue after treatment with α-lipoic acid; the appearance is better than the previous one, less number of vacuolated cells (*arrow*), disappearance of necrotic tissue, with little inflammatory infiltration (*arrowhead*). **e** Liver tissue after treatment with *N*-acetylcysteine; notice nearly complete recovery of hepatic tissue with scarce vacuolation and inflammatory infiltration

Table 2 Effects of curcumin, α-lipoic acid, and *N*-acetylcysteine on grading of histopathological changes on CCl_4-induced liver fibrosis in rats

Group	−	+	++	+++
Control	9	1	–	–
CCl_4	0	1	2	7
CCl_4 + curcumin	3	4	3	0
CCl_4 + α-lipoic acid	6	3	1	0
CCl_4 + *N*-acetylcysteine	8	2	0	0

CCl_4 causes severe histopathological changes in the liver in the form of inflammatory cell infiltration, collagen deposition, necrosis, vacuolation, and apoptosis of hepatocytes. Improvement of these pathological changes varied with different kinds of treatment. Ten rats were used in each group.

Statistical analysis

The data are expressed as means ± SEM. Statistical analysis was performed by one-way ANOVA followed by Tukey–Kramar post analysis test for multiple comparisons with $P<0.05$ being considered as statistically significant.

Table 3 Effects of curcumin, α-lipoic acid, and *N*-acetylcysteine on grading of CCl_4-induced liver fibrosis in rats

Group	0	I	II	III	IV
Control	10	–	–	–	–
CCl_4	0	1	2	6	1
CCl_4 + curcumin	3	2	2	2	1
CCl_4 + α-lipoic acid	5	3	2	0	0
CCl_4 + *N*-acetylcysteine	7	1	2	0	0

0 Absence of fibrosis, *I* portal fibrous and/or fibrous portal expansion, *II* septal fibrosis, *III* bridging fibrosis (portal–portal or portal–central linkage), and *IV* cirrhosis. Ten rats were used in each group

Results

Biochemical markers

Serum levels of ALT, AST, GGT, and bilirubin were measured as markers of liver injury. All three drugs, namely curcumin, α-lipoic acid, and *N*-acetylcysteine treatments significantly decrease CCl_4-

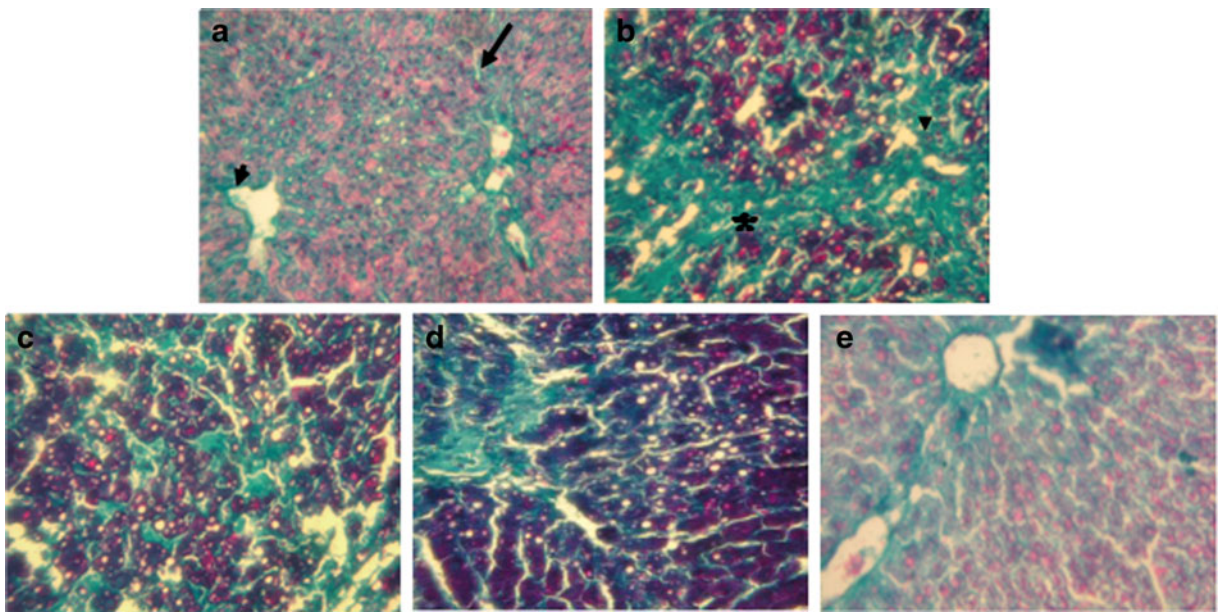

Fig. 2 Representative illustrations of rat liver tissues stained with Masson's trichrome for collagen (×40). **a** Normal liver tissue. Notice normal distribution of collagen (*green color*) around the sinusoids (*arrow*) and central vein (*arrowhead*). **b** Liver tissue after exposure to carbon tetrachloride for 8 weeks. Notice massive collagen depositions in the matrix especially around blood sinusoids (*arrowhead*) and around apoptotic hepatocytes (*asterisk*). **c** Liver tissue after treatment with curcumin; general improvement of hepatic architecture and decrease in the amount of collagen could be observed. **d** Liver tissue after treatment with α-lipoic acid; the appearance is better than the previous one with little amount of collagen deposition. **e** Liver tissue after treatment with *N*-acetylcysteine. Notice nearly complete recovery of hepatic tissue with pattern of collagen distribution back to normal

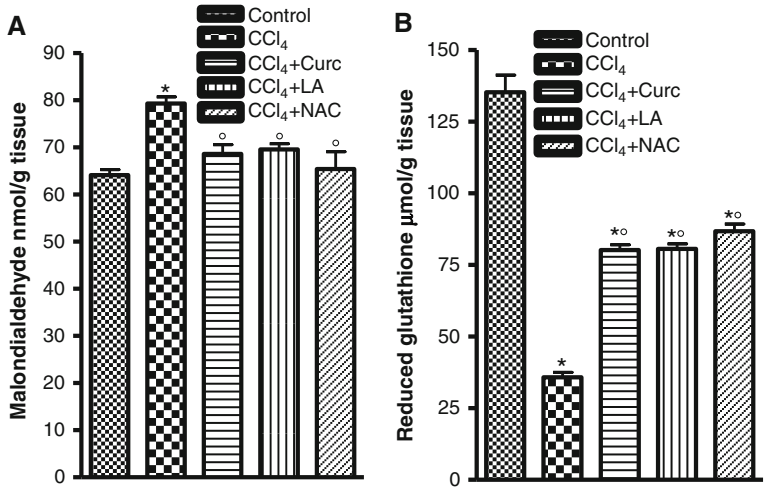

Fig. 3 Effect of curcumin (*Curc*), α-lipoic acid (*LA*), and *N*-acetylcysteine (*NAC*) on hepatic malondialdehyde (**a**) and reduced glutathione (**b**) levels after 8 weeks of carbon tetrachloride (CCl_4) administration in rats. Data are the mean ± SEM of ten rats. *,°Significantly different from control and CCl_4 groups, respectively, at $P<0.05$

induced elevation in serum levels of the tested parameters (Table 1).

Histopathological analysis

Histological changes were screened to support the tested biochemical markers of liver injury. The histopathological results showed that the tested drugs ameliorated these changes. In the tested doses, *N*-acetylcysteine is more effective than curcumin and α-lipoic acid in restoring normal architecture (Fig. 1 and Table 2).

Pretreatment with curcumin, α-lipoic acid, or *N*-acetylcysteine effectively limits the development and progression of rat liver fibrosis induced by CCl_4 as they improved the pattern of collagen distribution in hepatocytes. Once more in this respect, within the tested doses, *N*-acetylcysteine seems to be superior to curcumin and α-lipoic acid (Fig. 2 and Table 3).

Hepatic MDA and GSH

Oxidative stress plays an important role in the development of hepatic fibrosis. So, MDA, the main final product of lipid peroxidation, was determined in rat liver to determine the membrane lipid oxidative damage. In addition, GSH, the most abundant thiol antioxidant in cells, was measured in rat liver. As shown in Fig. 3, treatment with all tested drugs significantly decreases hepatic MDA (Fig. 3a) with concurrent increases in hepatic GSH (Fig. 3b) levels as compared to CCl_4 fibrotic group.

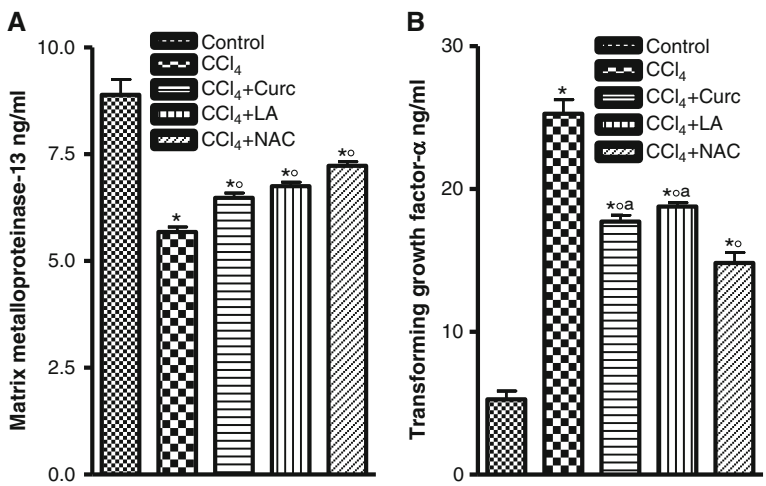

Fig. 4 Effect of curcumin (*Curc*), α-lipoic acid (*LA*), and *N*-acetylcysteine (*NAC*) on serum matrix metalloproteinase-13 (**a**) and transforming growth factor-α (**b**) levels after 8 weeks of carbon tetrachloride (CCl_4) administration in rats. Data are the mean ± SEM of ten rats. *, °,[a]Significantly different from control, CCl_4, and NAC groups, respectively, at $P<0.05$

Serum MMP-13 and TGF-α

Figure 4 shows the effects of curcumin, α-lipoic acid, and *N*-acetylcysteine on serum levels of MMP-13 and TGF-α. Pretreatment with the tested drugs significantly increases MMP-13 (Fig. 4a) with concomitant decreases in TGF-α (Fig. 4b) levels compared with CCl_4 fibrotic group.

Discussion

Hepatic fibrosis can lead to the development of hepatic cirrhosis with risk of liver failure and hepatocellular carcinoma [1]. In liver fibrosis, normal hepatic tissue is replaced with collagen-rich extracellular matrix. Within the tested doses, a comparison between the antioxidants used in this study indicates that they have different capacities to prevent collagen accumulation and to improve the histological architecture of the liver, *N*-acetylcysteine being the most effective. This is in agreement with the findings of Galicia-Moreno et al. [7] and Pereira-Filho et al. [17] who found that administration of *N*-acetylcysteine preserved the normal levels of collagen in CCl_4-intoxicated rats. On the other hand, the development of CCl_4-induced liver fibrosis is usually associated with oxidative stress and lipid peroxidation [14]. Bedossa et al. [2] reported that hepatocyte lipid peroxidation plays a major role in the regulation of collagen α_1(I) gene expression and that it may be a link between hepatocyte injury and hepatic fibrosis. As shown in the present study, *N*-acetylcysteine, an antioxidant and GSH precursor, decreased hepatic lipid peroxidation and GSH depletion. GSH plays an important role in antioxidant defense directly through scavenging reactive oxygen species and indirectly through functioning as a cofactor of antioxidant enzymes [5].

Remodeling of fibrillar collagen in rodents has been widely ascribed to the action of MMP-13. The antioxidants used in the current study resulted in increase in serum MMP-13 levels. This result is supported by the findings of Pinlaor et al. [18] who found that curcumin reduces periductal fibrosis in liver fluke-infected hamsters after long-term treatment by a pathway including induction of tissue resorption via MMP-13 overexpression. Moreover, Fallowfield et al. [4] reported that resolution of CCl_4-induced hepatic fibrosis was retarded in MMP-13-deficient mice. Furthermore, telmisartan, an angiotensin II type 1 receptor antagonist, prevented liver fibrogenesis and pre-neoplastic lesions by a mechanism involving an increase in MMP-13 expression [10]. In addition, Velasco-Loyden et al. [20] revealed that the aspartate salt of adenosine IFC305 suppresses the activation of hepatic stellate cells, the main extracellular matrix-producing cells in the fibrotic liver, by inhibiting the production of collagen α_1(I) mRNA, and increasing the expression of MMP-13 mRNA, which may result in an important decrease of collagen deposition. Besides, collagen-I degradation is critical to hepatic stellate cell apoptosis and hepatocyte regeneration during recovery from liver fibrosis [8].

Various growth factors have been shown to play important roles in the development of liver fibrosis. The ability of the antioxidants used in this study to decrease serum TGF-α concentrations is in agreement with the findings of Lee et al. [13] who reported that hepatic stellate cell activation, a critical step in hepatic fibrogenesis, by TGF-α was blocked by antioxidants, such as d-α-tocopherol. In addition, Kato et al. [11] suggested that ethanol-induced TGF-α may contribute to the development of hepatic fibrosis in alcoholic liver diseases. Moreover, Ito et al. [9] reported that the antibiotic nitrofurazone induces hepatocyte proliferation with a pathway involving increase in TGF-α, and this increase was blocked by concomitant administration of *N*-acetylcysteine.

In conclusion, the antifibrotic effects of the drugs under investigation appear to stem from (1) their antioxidant activities as indicated by protection against increased lipid peroxidation and increased GSH contents and (2) their abilities to induce MMP-13 and to inhibit TGF-α levels. The rest of the studied biochemical and histopathological parameters indicate a status of structural and functional integrity of liver cells and provide further support to the suggestive mechanism of action.

Conflict of interest The authors declare that there are no conflicts of interest.

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