Research Article

Curcumin ameliorates CCl_4-induced liver injury in a rat model; a deeper insight into the mechanism of action

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Abstract
Objective(s): The objective of this study was to investigate the mechanism of curcumin action in carbon tetrachloride (CCl_4)-induced liver fibrosis in rats. This approach was done by assessment of hepatic TNF-α level and oxidative stress biomarkers. **Materials and Methods:** Thirty-two male Wistar albino rats were equally divided into four groups. Group 1 serves as normal control group was administered vehicles only; Group 2 (curcumin group) was administered curcumin; Group 3 (CCl_4 group) serves as the model group of liver fibrosis by *i.p.* injection of CCl_4; Group 4 (curcumin + CCl_4 group) was administered curcumin along with CCl_4. Hepatic content of TNF-α was detected using western blotting. Hepatic malondialdehyde (MDA) and reduced glutathione (GSH) were assessed. **Results:** CCl_4 significantly increased MDA and TNF-α, and significantly decreased GSH levels. Treatment with curcumin decreased the levels of MDA and TNF-α. The levels of GSH increased significantly compared to CCl_4 group. **Conclusion:** Our data reveals that curcumin protects the liver from fibrosis. This might be accomplished via suppressing hepatic oxidative stress and inhibiting TNF-α protein expression. Therefore, curcumin acts as a potential prophylactic agent against liver injury.
Keywords: Liver Fibrosis; CCl_4; Curcumin; TNF- α.

Introduction
Decompensated liver disease, characterized by the onset of complications including ascites, hepatic encephalopathy and variceal haemorrhage, is a major cause of mortality worldwide and is associated with increased risk of hepatocellular carcinoma[1]. Hepatic fibrosis is a result of pathological deposition of extracellular matrix (ECM) in the liver. Stimulation of chronic injury may lead to acceleration of hepatic stellate cells (HSC) activation with increased ECM synthesis and impaired ECM degradation (fibrolysis), thus resulting in the disruption of the normal liver architecture[2].

In the pathological process of liver injury, numerous inflammatory cytokines are significantly up-regulated, such as tumor necrosis factor-α (TNF-α), which regulates various processes including inflammation, cellular apoptosis and vascular functions[3].

After injury, TNF-α quickly migrates into the injured tissue after vasodilatation and suppresses further cell death, activates stem cells, and promotes epithelial proliferation[4].

However, TNF-α plays a malignant role in the injured liver. It triggers a series of intracellular events that result in activation of apoptosis and accelerating hepatic cell death during liver injury[5].
CCl_4 is a potent hepatotoxin that is metabolized by microsomal mono-oxygenase system to its active metabolite.

This process results in the fragmentation of the lipid peroxide radicals, lipid hydroperoxides and other products[6]. Furthermore, these processes are immediately followed by the infiltration of inflammatory cells and release of various cytokines and growth factors[7].

Curcumin has been found to have antioxidant, anti-inflammatory, anti-hepatotoxic and anti-cancer properties[8-10]. It also decreased the expression of pro-inflammatory mediators in fibrogenesis[11,12].

Materials and Methods
Animals and experiment:
Thirty-two Wistar male albino rats (six weeks old, 200 - 250g) were purchased from the

animal house colony, National Research Center, Cairo, Egypt. All animal care procedures were in compliance with the national institutes of health guidelines for the care and use of laboratory animals. Rats were kept in stainless steel cages at room temperature of 25±3°C with a 12h dark/light cycles, and have free access to standard laboratory feed and water.

Rats were equally divided into four groups, eight rats each, as follows: Group 1 is normal control group, received 0.5% CMC solution, once daily, orally by gavage and injected with mineral oil (0.8 ml/kg), *i.p.*, twice weekly for six weeks. Group 2 is curcumin group, was administered curcumin (100 mg/kg) dissolved in 0.5% CMC solution, once daily, orally by gavage for six weeks**[13]**. Group 3 is CCl_4 group, received (0.8 ml/kg) mix of CCl_4: mineral oil (1:1), *i.p.*, twice weekly for six weeks[14]. Group 4 is (curcumin + CCl_4) group, received curcumin (same dose as group 2) and CCl_4 (same dose as group 3) for six weeks.

At the end of the six weeks, animals were anesthetized by thiopental (50 mg/kg)[15] and sacrificed. The liver was isolated, embedded into liquid nitrogen and stored at −80°C.

Assessment of liver GSH level:

For determination of GSH level, a spectrophotometric kit was used. Briefly, the method is based on that the sulfhydryl group of GSH reacts with 5,5-dithio-*bis*-2-nitrobenzoic acid (Ellman's reagent) and produces a yellow colored 5-thio-2-nitro-benzoic acid which was measured colorime-trically at 405 nm using Beckman DU-64 UV/VIS spectrophotometer. Results were expressed as μmol/g liver tissue[16].

Assessment of liver lipid peroxides level:

Liver lipid peroxidation was determined as thiobarbituric acid reacting substances (TBARS) and is expressed as equivalents of MDA, using 1,1,3,3 tetramethoxypropane as standard. Results were expressed as nmol/g liver tissue[17].

Western blotting:

For determination of TNF-α expression level, western blotting analysis was performed. Liver was homogenized using Lab GEN 7 homogenizer (Cole-Parmer Co.) in homogenization buffer [20 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 0.5% Triton X-100, 0.1% SDS and 1 mM EDTA], protease inhibitor cocktail (1:200) was freshly added (Nacalai Tesque, Japan). The homogenate was centrifuged at 12,000 rpm for 10 min at 4ºC, and supernatant was resolved. A constant volume of supernatant was mixed with SDS-sample buffer (62.5 mM Tris-HCl (pH 6.8), 2% SDS, 0.02% BPB, 10% glycerol and 5% β-mercaptoethanol), separated by SDS-PAGE under reducing conditions. Protein bands were electroblotted to a nitrocellulose membrane using a semi-dry blotter (Bio-Rad) with the aid of a blotting buffer (100 mM Tris, 192 mM glycine, and 10% methanol). The blot was blocked with 5% skim milk in TBS-T buffer (25 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% Tween-20), for 1 h at room temperature. The blocked blot was incubated overnight at 4°C with primary antibody (Rabbit polyclonal antibody to TNF- α, Chongqing Biospes Co., China; or Mouse monoclonal antibody to β-actin, Sigma-Aldrich, Egypt). The next day, the blot was incubated with alkaline phosphatase (AP)-coupled secondary antibody (anti-rabbit or anti-mouse antibody, respectively) for 1 h at room temperature. Blots were finally analyzed using BCIP/NBT colorimetric detection method. Protein band densities were quantified using Image-J software, and then statistical analysis was performed using Prism-6 software.

Statistical analysis

Results were expressed as mean ± standard error of the mean (SEM). Differences among groups were statistically analyzed by one-way analysis of variance (ANOVA), followed by Tukey's Kramer post hoc test for multiple comparisons. Results were considered significant if $p < 0.05$. Data analyses were performed using Image-J and Graph Pad Prism-6 softwares.

Results:

Effect of curcumin on oxidative stress:

As an indicator of oxidative stress, liver GSH was measured. It is observed that CCl_4 group showed a significant decrease in the liver GSH compared to normal control level. In

comparison with CCl_4 group, curcumin administration significantly increased GSH level as shown in **Table 1**.

Measurement of MDA can be used to assess lipid peroxidation. Liver MDA content increased significantly upon CCl_4 administration when compared to normal control group. Our results revealed that curcumin treatment significantly suppressed MDA production compared to CCl_4 group as shown in **Table 1**.

Table 1: Levels of the hepatic reduced glutathione (GSH) and malondialdehyde (MDA) in the studied groups. Data are expressed as mean ± SEM.**:**

Parameter	Normal control group	Curcumin group	CCl_4 group	Curcumin+CCl_4 group
GSH (µmol/g liver tissue)	1.93 ± 0.21	2.040 ± 0.037	0.508 ± 0.13	0.767 ± 0.073
MDA (nmol/g liver tissue)	47.17 ± 0.032	49.17 ± 0.473	90.17 ± 0.773	60.67 0.514

Effect of curcumin on hepatic TNF-α:
TNF-α protein level was determined in the liver homogenate by Western blotting. As shown in **Figure 1**, the content of TNF-α in normal control group was low. The results showed a concomitant increase in TNF-α in livers derived from CCl_4 group compared to normal control group. The level of TNF-α in curcumin group was significantly diminished compared to CCl_4 group.

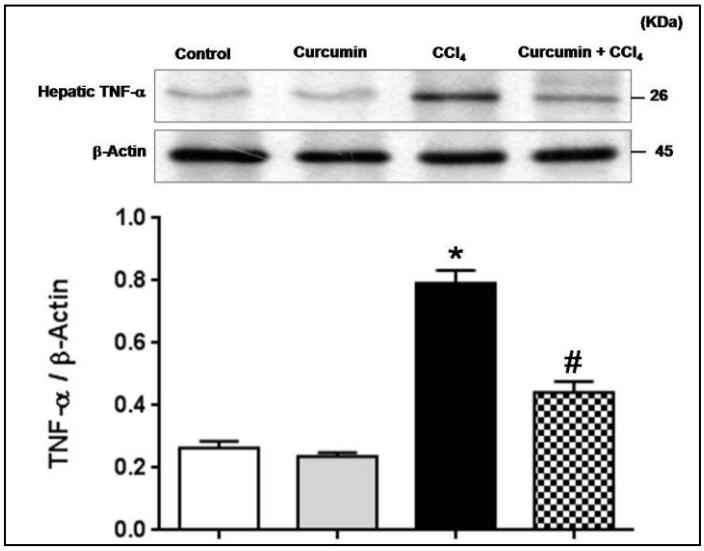

Figure 1: **Western blotting analysis of TNF-α expression in liver tissue of different groups.** Bands of TNF-α and β-actin in liver tissues are shown. Band densities were analyzed using Image J software. Each bar represents the mean ± SEM. * significantly different from normal control group at $p < 0.05$; # significantly different from CCl_4 group at $p < 0.05$.

Discussion

The principal mechanism by which CCl_4 causes hepatic damage are lipid peroxidation, decreased activities of antioxidant enzymes and generation of free radicals[18]. In this context, GSH as well as MDA were assessed as parameters of oxidative stress.

The oxidative stress in CCl_4 group is manifested by a significant decline in GSH accompanied by an increase in MDA level which reflect the oxidative stress induced damage**[19].** Treatment with curcumin improved the oxidative status induced by CCl_4. In this context, a decrease in ROS production, reflected by a significant reduction in the mean MDA level, was accompanied by an increase in the antioxidant capacity level as seen by a significant increase in GSH level as compared to that in CCl_4 group. These findings suggest that antioxidant activity of curcumin and its ability to scavenge free radicals may be involved in the protective mechanism against CCl_4-induced liver toxicity. These results are in accordance with prior studies[20]**.**

It is well known that inflammation is an important element in the initiation and progression of liver fibrosis. Chronic hepatic inflammation is accompanied by the up-regulation of TNF-α which is a pro-inflammatory cytokine produced rapidly by macrophages in response to tissue injury**[21].** In accordance with the previous reports**[22],** a significant increase in liver level of TNF-α occurred in CCl_4 group when compared to normal control group. Increased oxidative injury produced by derivatives of CCl_4 also activates kupffer cells in the liver which may be responsible for increased release of TNF-α from inflammatory cells recruited to the liver**[23].** Curcumin prevented CCl_4-induced liver injury by the inhibition of TNF-α release. This data suggests that the hepatoprotective effect of curcumin in this model is in part due to the inhibition of TNF-α. Findings of the present study are parallel with those obtained by Reyes-Gordillo et al., 2007**[24].**

Conclusion

Our data reveals that curcumin protects the liver from fibrosis via suppressing hepatic oxidative stress and inhibiting TNF-α protein expression.

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