Efficacy of Curcumin in Protecting the Rat Liver from CCl4-Induced Injury and Fibrogenesis. Histological and Immunohistochemical Study

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Abstract: Introduction: Liver fibrogenesis occurs as a wound-healing process after many forms of chronic or toxic hepatic injury. **Objectives:** This study was designed to evaluate the hepatoprotective effect of curcumin from CCl4-induced injury and fibrosis in rats and to assess the role of matrix metalloproteinase-2 (MMP-2). **Materials and methods:** Rats were equally divided into three groups. Group A; was the vehicle control, Group B: rats were intraperitoneally injected with CCl_4 (0.1 ml/100 gram body weight), without curcumin treatment. Group C: rats were injected with similar doses of CCl_4 and given curcumin by oral gavage at a dose of 200 mg/kg. After four weeks, liver specimens from all groups were processed and prepared to be stained with H&E, Mallory trichrome and immunohistochemically for demonstration of MMP-2. **Results:** CCl4 resulted in many forms of hepatocytes degeneration; hydropic degeneration, fatty change, apoptosis and necrosis, associated with significant increase in hepatic collagen deposition. These histopathological changes were apparently ameliorated in the curcumin-treated rats. Additionally, curcumin significantly decreased the elevated MMP-2 expression in both hepatocytes and sinusoidal cells following CCl4 injections. **Conclusions:** These findings suggest that the effect of curcumin on matrix metalloproteinase-2 might be considered as one of its mechanisms to improve hepatic injury and fibrogenesis after CCL4 administration. Based on these results, the oral use of curcumin is recommended to improve hepatic injury and fibrosis.

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1. Introduction

Liver fibrosis is a major health problem that can lead to the development of cirrhosis and hepatocellular carcinoma **(Morsy et al., 2012)**. Hepatic fibrosis is traditionally viewed as a progressive pathological process involving multiple cellular and molecular events. It is a result of an imbalance between enhanced matrix synthesis and diminished breakdown of connective tissue proteins, the net result of which is increased deposition of connective tissue proteins in the extracellular matrix (ECM) **(Rukkumani et al., 2004)**. When this process is combined within effective regeneration and repair, there is increasing distortion of the normal liver architecture, and the end result is cirrhosis **(Arthur, 1994 and Arthur et al., 1998).**

In the hepatic extracellular space, matrix degradation occurs predominantly as a consequence of the action of a family of enzymes called the matrix metalloproteinases (MMPs), also called matrixins, are a family of zinc-dependent proteases that are thought to play a central role in this process **(Mc Crudden & Iredale, 2000 and Knittel et al., 2000)**. The three most relevant MMPs are gelatinase A (MMP-2) **(Takahara et al., 1997),** gelatinase B (MMP-9) **(Winwood et al., 1995),** and stromelysin (MMP-3) **(Herbst et al., 1991)**, all of which have been studied in the liver.

Curcumin, the main active compound obtained from the plant Curcuma longa, was first isolated two centuries ago and its structure as diferuloylmethane was determined in 1910. Curcumin has shown anti-inflammatory, anti-oxidant, antifungal, antibacterial and anticancer activities **(Rivera-Espinoza and Muriel, 2010)**. The pharmacological properties of curcumin were reviewed recently and focused mainly on its anticancer properties. However, its beneficial activity on liver diseases (known centuries ago, and demonstrated recently utilizing animal models) has not being reviewed in depth until now. Curcumin attenuates liver injury induced by ethanol, thioacetamide, iron overdose, cholestasis and acute, subchronic and chronic carbon tetrachloride (CCl4) intoxication **(Priya and Sudhakaran, 2008 and Rivera-Espinoza and Muriel, 2010)**. Unfortunately, the number of studies of curcumin on liver diseases is still low and investigations in this area must be encouraged because hepatic disorders constitute one of the main causes of worldwide mortality. Accordingly, a rat model of hepatic fibrosis will be established to describe the early histological changes and to assess

the hepatic expression of matrix metalloproteinase-2 in an attempt to clarify the possible hepato- protective mechanisms of curcumin.

2. Materials and methods

1. Animals and treatment:

The rat model of hepatic injury and fibrogenesis was established by carbon tetrachloride (CCl4) using the method originally described by **Proctor and Chatamra, (1982)** and since used by many others **(Pérez Tamayo, 1983, Kobayashi et al., 2000 and Rivera et al., 2001)** with minor modifications.

Thirty adult male albino rats (150-200 g) were equally divided into three groups. ***Group A;*** was the vehicle control in which rats were not administrated CCl_4 or curcumin, but they were intraperitoneally injected with the vehicle olive oil. **Group B*;*** was the CCl_4 group in which rats were intraperitoneally injected with CCl_4, without curcumin treatment. ***Group C;*** was a treatment group in which rats were injected with CCl_4 and treated with curcumin.

Rats in groups B & C were intraperitoneally injected with a mixture of CCl_4 (0.1 ml/100 g body weight) and olive oil [1:1 (v/v)] every other day for 4 weeks. Curcumin (200 mg/kg body weight) was suspended in sterile phosphate buffered saline and given once daily by gavage to the animals of group C **(Yumei et al., 2007)**. All rats were fed with chow diet and kept at 21-25°C under a 12-h dark/light cycle.

2. Specimens: Forty-eight hours after the last CCl_4 injection, the rats of all groups were anesthetized using ether. Immediately before sacrifice, the animals were perfused through the heart apex with 100 ml isotonic saline followed by 250 ml of 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3). The rats were then sacrificed and small specimens from the liver were removed, immediately fixed in phosphate-buffered 10% formalin, embedded in paraffin, and sectioned at a thickness of 5 µm **(Yumei et al., 2007)**.

3. Stains: Slides from all groups were stained with hematoxylin and eosin and Mallory trichrome stain for collagen demonstration.

4. Immunohistochemistry: (localization of Matrix Metalloproteinase -2 expression in the liver parenchyma) **(Gomez et al., 1999)**: Immunohisto-chemical staining was performed on formalin-fixed paraffin sections using the avidin-biotin immunoperoxidase technique. Briefly, unstained sections mounted on polylysine-coated slides were deparaffinized with xylene and rehydrated with decreasing concentrations of ethanol. Nonspecific binding was blocked with 1.5% normal horse serum. Avidin and biotin binding sites contained in the samples were blocked using a commercial avidin-biotin blocking kit (Vector Laboratories Inc., Burlingame, CA). Sections were then incubated for 30 minutes at room temperature with anti-MMP-2 (5 mg/ml) diluted in phosphate-buffered saline (PBS) containing normal horse serum (Oncogene Science, Cambridge, MA). The tissue sections were washed in ice-cold saline and incubated with a secondary biotinylated anti-mouse Ig G. Endogenous peroxidase activity was blocked using 0.3% H2O2 in horseradish peroxidase (Vector Laboratories, Inc.). Peroxidase activity was visualized using diaminobenzidine (Vector Laboratories, Inc.). This technique uses unlabeled primary antibody, biotinylated secondary antibody, and a preformed avidin and biotinylated horseradish peroxidase macromolecular complex. In accordance with the recommendations of the manufacturer, the avidin-biotin complex reagent contains avidin and biotinylated horseradish peroxidase reagents that were specifically prepared to form ideal complexes for immunoperoxidase staining. The slides were then rinsed in water and lightly counterstained with hematoxylin. Before the blocking procedure, the samples were preincubated with 0.1% trypsin in PBS for 12 min at 378°C, as suggested by the manufacturer.

Immunohistochemical control procedures. Negative control immunohistochemical procedures included omission of the primary antibody from the described staining protocol and its replacement with PBS plus normal horse serum (Oncogene Science).

5. Morphometric and Statistical Study: The measurements were done for all groups (10 rats each) using the Image Analyzer (Leica Q Win standard, digital camera CH-9435 DFC 290, Germany). The microscopic field of measurements is an area = 786,432.0 μ^2. For each specimen, 5 high power fields (X400) were randomly selected, Photographed and stored. The Digitalized pictures were examined by 2 investigators on a high-resolution color display. The total measurements for each rat group were 50 measurements (*each group consisted of 10 animals and 5 measures per animal specimen were taken*).

A-Area percent of collagen in different animal groups (Mallory stained sections): (blue coloured areas)

B. Area percent of MMP-2 positive cells: (brown coloured areas)

Data entry and analysis was done using the software statistical package of social science "SPSS version 16" (Chicago, IL). All data were expressed as mean ± SD. One way ANOVA and student T-test were used. Values of $p< 0.05$ were considered significant.

3. Results

1. Histology & immunohistochemistry:

A. Hematoxylin and Eosin-stained sections:

Light microscopic examination of the control rat liver revealed the presence of central veins with its endothelial lining and blood sinusoids which were lined by endothelial cells and Kupffer cells. Radiating plates of hepatocytes with vacuolated, eosinophilic cytoplasm and rounded open face nuclei were seen. Portal tract with wide portal vein branch and bile ducts was observed (Figs. 1a, 1b).

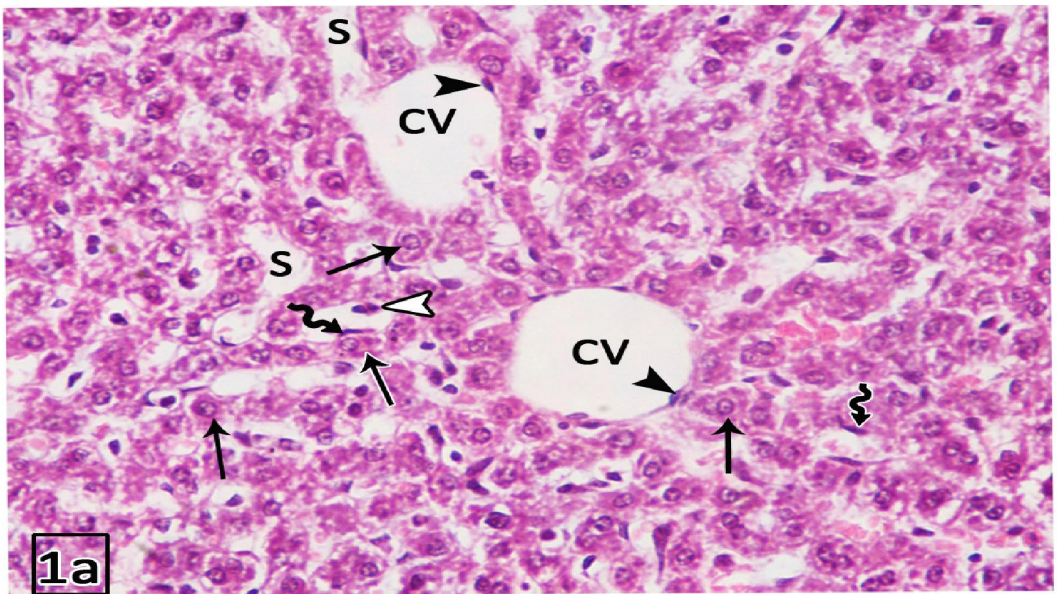


Fig. (1a): paraffin section in the control liver parenchyma showing central vein (CV) with its endothelial lining (arrow heads) and blood sinusoids (S) lined by endothelial cells (curved arrows) and Kupffer cells (white arrow heads). Plates of hepatocytes (arrows) with eosinophilic cytoplasm and rounded open face nuclei are seen. H&E X 250

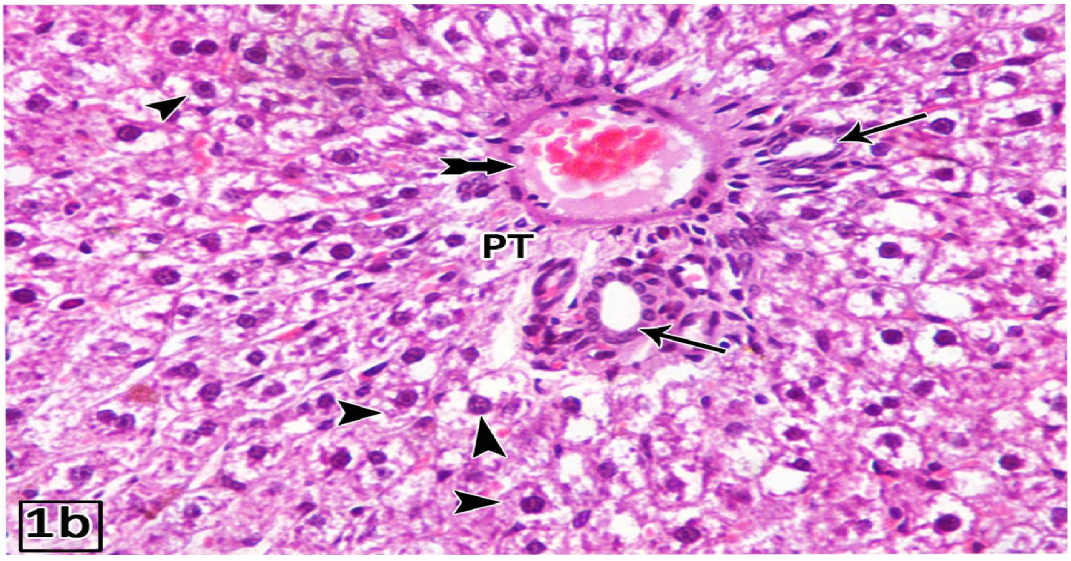
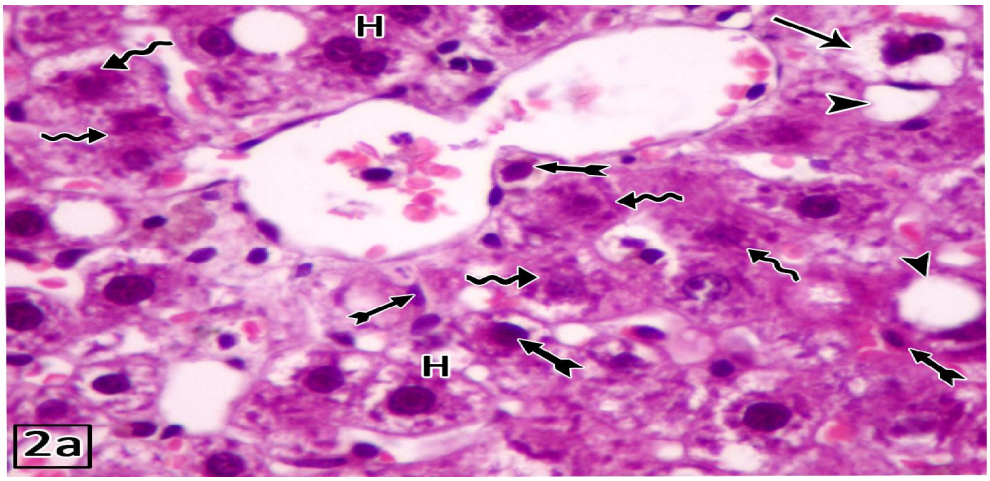


Fig. (1b): The control liver parenchyma showing portal tract (PT) with wide portal vein branch (tailed arrow) and bile ducts (arrows). Plates of hepatocytes (arrow heads) with vacuolated cytoplasm and rounded open face nuclei are seen.

Variable forms of hepatocytes degeneration were noticed in the rats of group B; 4 weeks after CCL4 injection. Some cells showed hydropic degeneration & appeared swollen with central round nucleus, while others exhibited fatty change with vacuolated cytoplasm and flattened eccentric nucleus. Hepatocyte apoptosis in the form of cell shrinkage with small dark nucleus and little eosinophilic cytoplasm was detected. Moreover, hepatocytes

necrosis with fragmentation of both the nucleus and cytoplasmic contents was seen. This was associated with fibrotic bands and fibroblasts (Figs. 2a, 2b).

In curcumin treated rats (group C), examination of the liver revealed many normal hepatocytes with vacuolated cytoplasm & open face nuclei. Only few cells exhibited fatty degeneration and necrosis with fragmented nucleus and cytoplasmic contents. Normal central veins and portal tracts were also demonstrated (Figs. 3a, 3b).



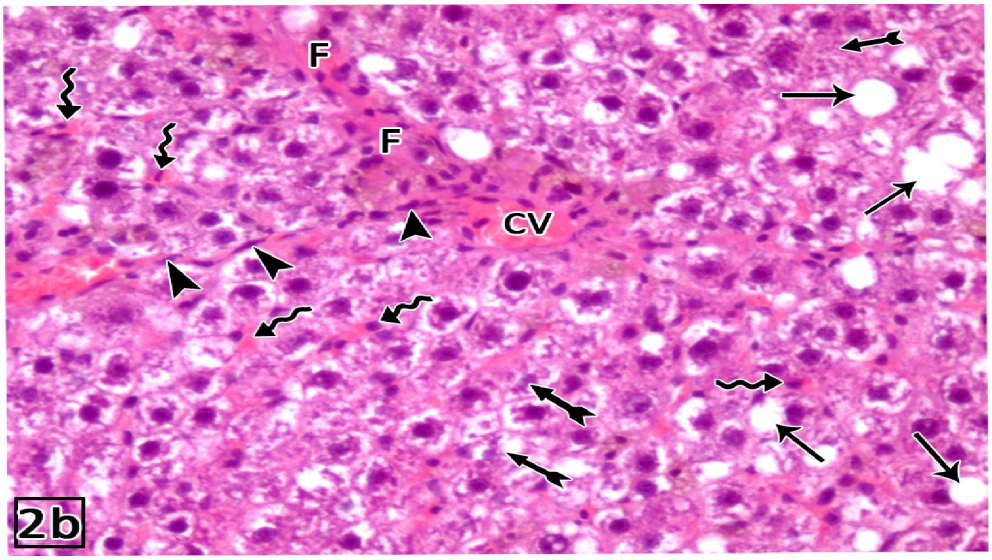


Fig. (2a): The liver parenchyma of group B showing variable forms of hepatocytes degeneration; some show hydropic degeneration & appear swollen with central round nucleus (arrow) and others show fatty change with vacuolated cytoplasm and flattened eccentric nucleus (arrow heads). The tailed arrows point to the shrunken apoptotic cells with small dark nucleus and little eosinophilic cytoplasm, while the curved arrows refer to cells with fragmented cytoplasmic contents. Few hepatocytes appear normal (H). H&E X 400

Fig. (2b): The liver parenchyma of group B showing central vein (CV) surrounded by fibrotic bands (F) and fibroblasts (arrow heads). The arrows point to numerous cells with fatty degeneration and the curved arrows to the numerous apoptotic cells. Many hepatocytes with fragmented nucleus and cytoplasmic contents (tailed arrows) are seen. H&E X 250

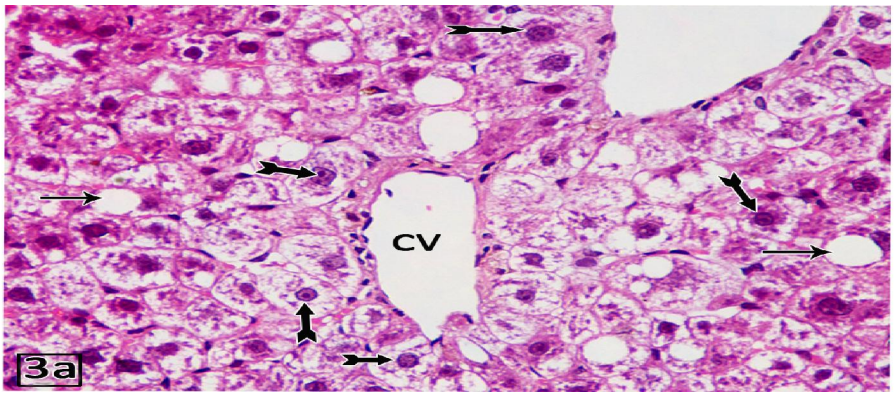


Fig. (3a): The liver parenchyma of group C showing many normal hepatocytes with vacuolated cytoplasm & open face nuclei (tailed arrows) surrounding central vein (CV). Few cells exhibit fatty degeneration (arrows). H & E X 400

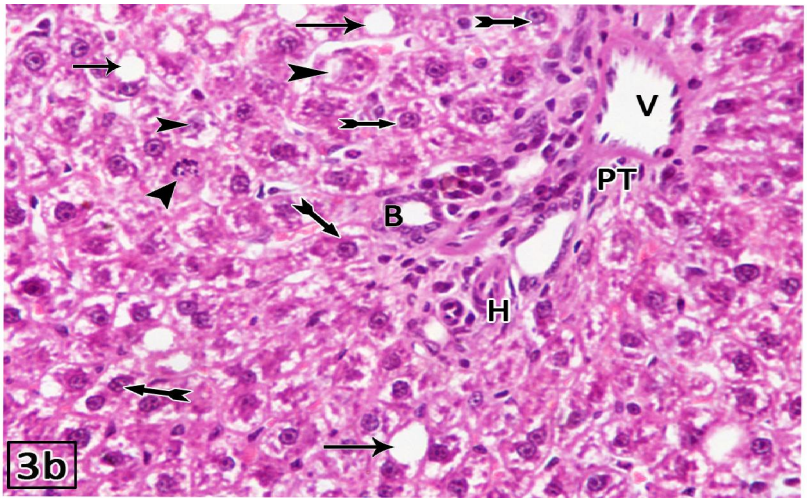


Fig. (3b): The liver parenchyma of group C showing portal tract (PT) with portal vein branch (V), hepatic artery branch (H) and bile ducts (B). Hepatocytes in the periportal area appear normal (tailed arrows). Some hepatocytes show fragmented contents (arrow heads) and few cells show fatty change (arrows). H & E X 250

B. Mallory trichrome-stained sections:

There were no detectable collagen bundles in the liver parenchyma of the control rats (Figs. 4a, 4b). On the other hand, blue coloured collagen bundles were observed around central veins and in between the plates of hepatocytes in the parenchyma of group B, 4 weeks after CCL4 injection (Figs. 5a, 5b). Whereas, in curcumin treated rats only little amount of collagen was observed (Figs. 6a, 6b).

C. Matrix Metalloproteinase-2 immuno-reactivity:

Light microscopic examination of the control liver revealed that few hepatocytes, either in the centrilobular or periportal areas, exhibited faint MMP-2 immunoreactivity (Figs. 7a, 7b). While, in group B, Strong positive MMP-2 immunoreactivity was observed in the degenerating hepatocytes around central veins and in the periportal areas. This was associated with remarkable increase in immunoreactivity in sinusoidal cells (Figs. 8a, 8b). Whereas, in animals of group C, relatively few immunopositive cells were detected in sinusoidal cells and in some hepatocytes around the portal tract (Figs. 9a, 9b).

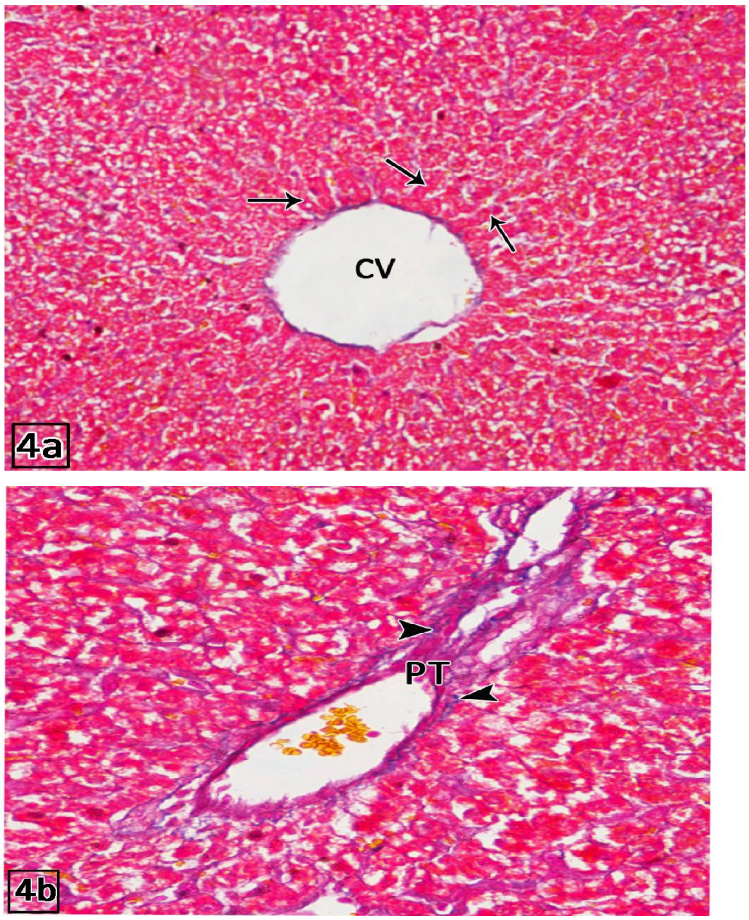


Fig. (4a): The control liver parenchyma showing central vein (CV) and radiating plates of hepatocytes (arrows) with no detectable collagen. Mallory trichrome X 250

Fig. (4b): The control liver parenchyma showing portal tract (PT) with little amount of collagen (arrow heads). Mallory trichrome X 250

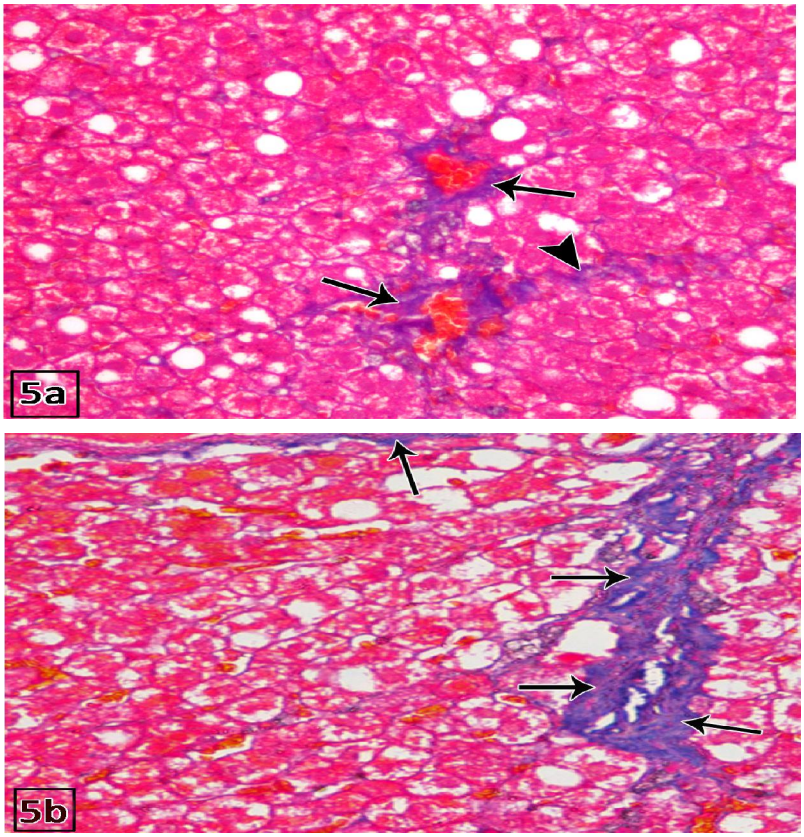


Fig. (5a): The liver parenchyma of group B showing abundant collagen bundles (arrows) around central vein and little collagen in between the plates of hepatocytes (arrow head). Mallory trichrome X 250

Fig. (5b): Thick collagen bundles (arrows) in between the plates of hepatocytes of group B rats. Mallory trichrome X 400

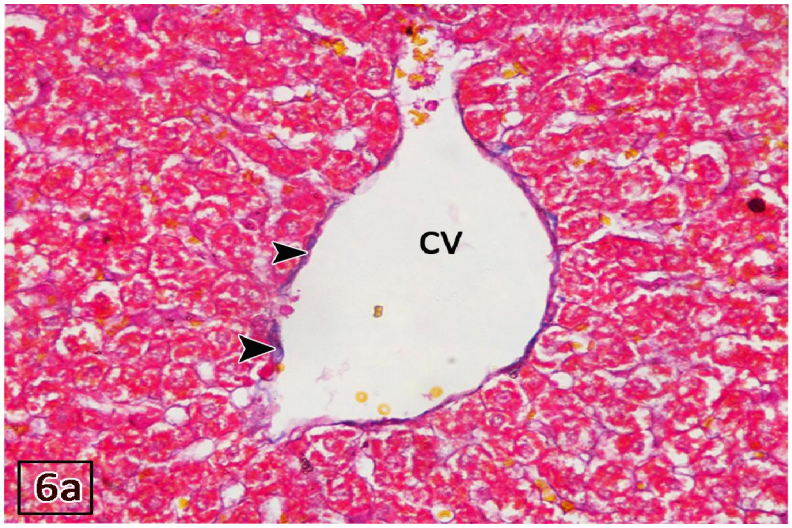


Fig. (6a): The liver parenchyma of group C showing central vein (CV) surrounded with a thin rim of collagen (arrow heads). Mallory trichrome X 400

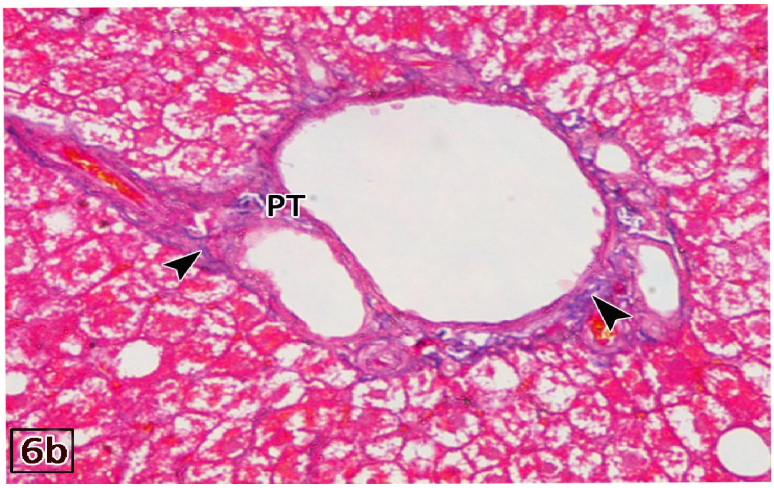


Fig. (6b): The liver parenchyma of group C showing portal tract (PT) and little amount of collagen (arrow heads). Mallory trichrome X 400

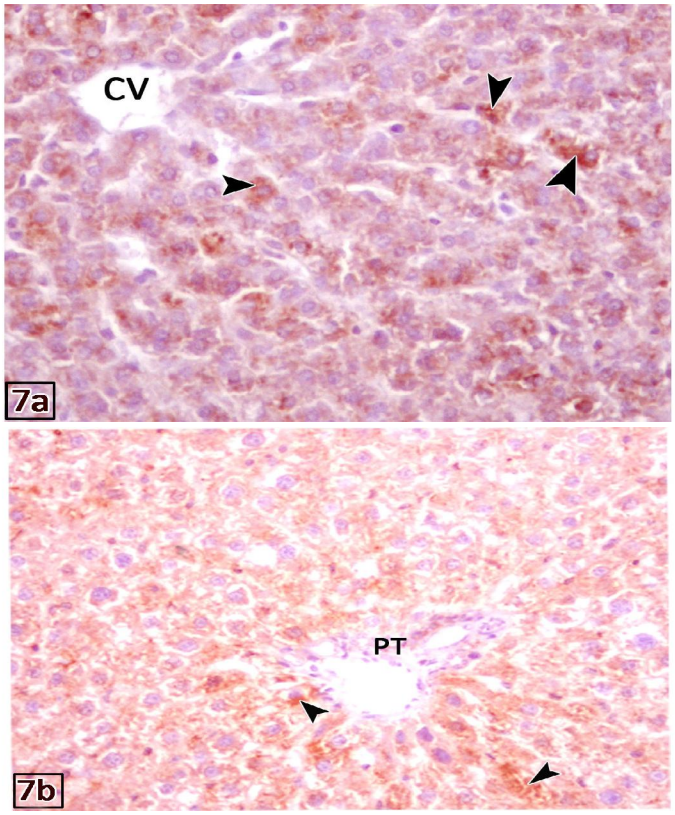
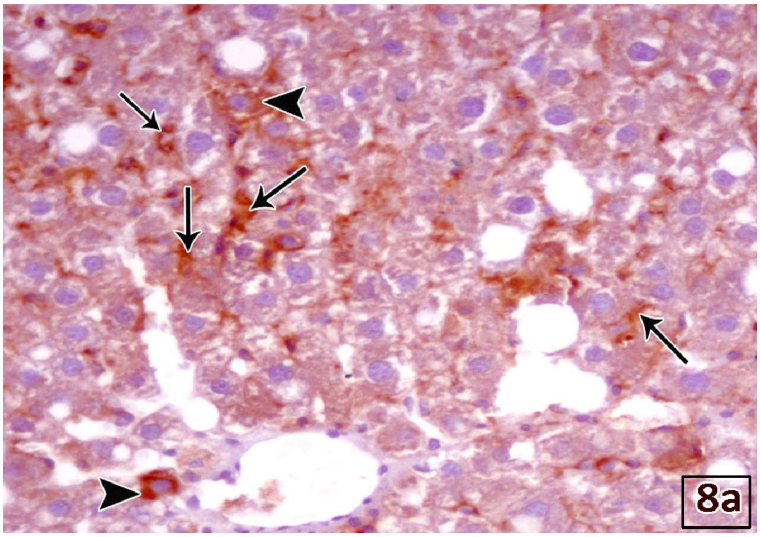


Fig. (7a): The control liver parenchyma showing few hepatocytes with faint MMP2 immunoreactivity (arrow heads) surrounding central vein (CV). Anti MMP-2 immunostaining X 250

Fig. (7b): The control liver parenchyma showing portal tract (PT) and only few hepatocytes with very faint MMP2 immunoreactivity (arrow heads). Anti MMP-2 immunostaining X 250



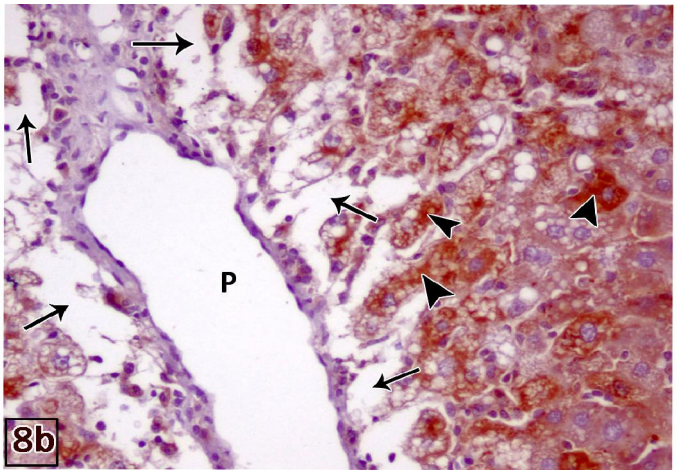


Fig. (8a): Strong positive MMP2 immunoreactivity in the cytoplasm of hepatocytes (arrow heads) and in the sinusoidal cells (arrows) of group B rats. Anti MMP-2 immunostaining X 250

Fig. (8b): The liver of group B showing portal tract with wide portal vein branch (P). Periportal hepatic parenchyma appears distorted with wide empty spaces (arrows). Degenerated hepatocytes show strong positive immunoreactivity (arrow heads). Anti MMP2 immunostaining X 250

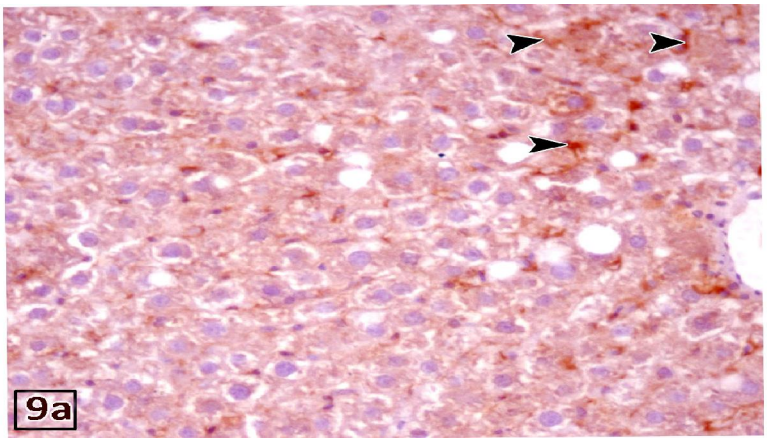


Fig. (9a): Few immunopositive sinusoidal cells in between plates of hepatocytes of group C (arrow heads). Anti MMP-2 immunostaining X 250

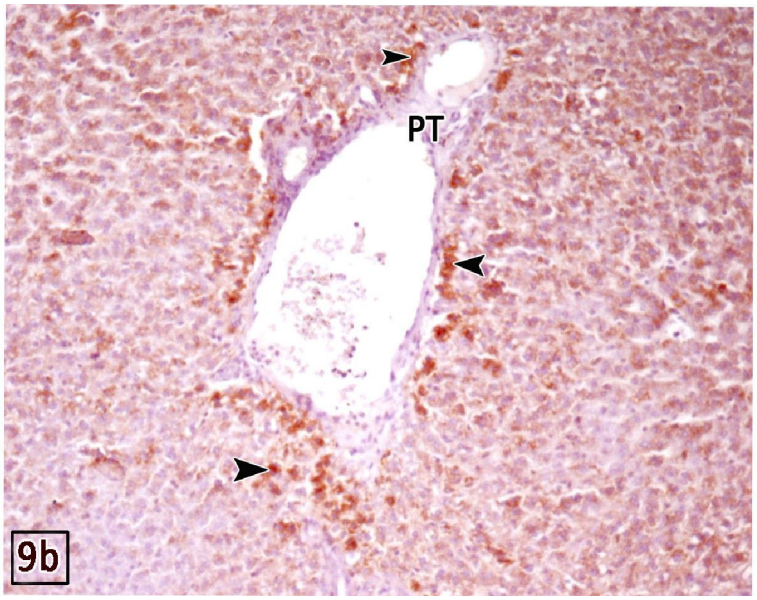


Fig. (9b): Few immunopositive hepatocytes (arrow heads) around the portal tract (PT) of group C. Anti MMP-2 immunostaining X 100

2. Statistical results:

A. Area percentage of collagen in the liver parenchyma:

Group A (control)		Group B (CCl4-group)		Group C (CCl4 rats treated with curcumin)	
Centrilobular	Periportal	Centrilobular	Periportal	Centrilobular	Periportal
0.31 ± 0.16	0.38 ±0.14	1.72±0.47 *P* =(0,00)	2.10±0.49 *P* =(0,00)	0.34±0.17 *P* =(0,333)	0.42±0.14*P* =(0,144)

- Significant increase in area % of collagen in both the centrilobular and periportal areas of group (B) as compared with the control (group A) and curcumin treated rats (Group C).
- Non significant change in area % of collagen in group (C) as compared with the control.

B.Area percentage of MMP2 immunoreactivity in the liver parenchyma:

Group A (control)	Group B (CCl4-group)	Group C (CCl4 rats treated with curcumin)
2.41 ± 1.03	4.88 ± 1.50 *P* =(0,00)	2.48±1.02 *P* =(0,741)

- Significant increase in area % of MMP2 immunoreactivity in the liver parenchyma of group (B) as compared with the control (group A) and curcumin treated rats (Group C).
- Non significant change in area % of MMP2 immunoreactivity in group (C) as compared with the control.

4. Discussion

Hepatic fibrosis caused by CCl_4 has been extensively used in experimental models in rats. Hepatic responses in rats to chronic CCl_4 stimulation are shown to be superficially similar to human cirrhosis **(Pérez Tamayo, 1983)**. CCl_4 metabolism in the liver results in the stimulation of lipid peroxidation and the production of free radicals **(Basu, 2003)**, which causes hepatic injury and fibrosis. In the current study, variable forms of hepatocytes degeneration were recorded in the early stages of CCl4-induced hepatic fibrogenesis; Hydropic degeneration, fatty change, apoptosis and necrosis. This was accompanied with significant increase in the area percentage of collagen as compared with the control rats. This was previously supported by other investigators **(Yumei et al., 2007 and Wu et al., 2010)**.

Hepatic fibrosis occurs as a consequence of net accumulation of matrix proteins (particularly collagen types I and III) in liver. Hepatic stellate cells (HSCs) play a key role in liver fibrogenesis **(Geerts, 2001)**. The cells undergo dramatic morphological and functional changes, a process called 'activation', during which the star-shaped HSC are converted to myofibroblastic cells with increased expression of α-

smooth muscle actin and decreased retinoid storage. In fibrogenesis, the normal extracellular matrix (ECM) is switched to fibrillar, contractile ECM **(Arenson et al., 1988 and Benyon and Arthur, 2001)**. Meanwhile, there is increasing evidence to indicate that in liver fibrosis, altered matrix degradation may also play a significant role. Thus, it is reasonable to speculate that a proteolytic degradation of the normal ECM may occur at the onset of liver fibrogenesis. Extracellular degradation of matrix proteins is regulated by matrix metalloproteinases **(Arthur et al., 1994)**. Compelling evidence has documented that MMPs and their tissue inhibitors are expressed prior to the onset of HSC activation in liver fibrogenesis **(Herbst et al., 1991)**. Moreover, a comprehensive study measured MMP and TIMP expression in liver injury and fibrosis and reported that after a single dose of CCl_4, MMP-13, MMP-2, MMP-9, MMP-3 and MMP-10 were all increased **(Knittel et al., 2000).** Additionally, in rat hepatic fibrosis induced by bile duct ligation, there was increased activity of MMP-2 and MMP-9 (**Kossakowska et al., 1998).**

In the present study, MMP2 immunostaining was performed in control rat liver and in rats injected for 4 weeks with CCL4. Only faint positive immunoreactivity was noticed in the cytoplasm of few control hepatocytes and in some sinusoidal cells. This was in accordance with previously performed investigations **(Monsky et al, 1993; Arthur, 1994 and Takahara et al., 1997)**. Additionally, **Monsky et al. (1993)** recorded that MMP-2 was seen in the rER of hepatic stellate cells, Kupffer cells, hepatocytes, capillary endothelial cells and fibroblasts, suggesting that MMP-2 might be produced by these cells. After CCl4 injection, a highly significant increase in MMP-2 immunoreactivity was reported in the cytoplasm of hepatocytes and in the sinusoidal cells as compared with the control liver. Previous similar studies **(Knittel et al., 2000 and Huang et al., 2009)** reported that Liver injury is accompanied by profound changes in hepatic MMP/TIMP expression. They added that, single toxic injury resulting in complete restoration was characterized by a sequential induction of MMPs and TIMPs suggesting initial matrix breakdown and matrix restoration thereafter.

Curcumin, the yellow pigment of turmeric in curry is a potent antioxidant **(Ruby et al., 1995)**. Besides its role as a dietary spice, turmeric has been used for centuries as an anti-inflammatory remedy in Chinese medicine. Curcumin has received attention as a promising dietary supplement for cancer prevention **(Ruby et al., 1995)** and liver protection **(Chuang et al., 2000)**. Accordingly, the effect of curcumin on the liver of albino rats injected with CCl4 was investigated in the current work to assess its role as a hepato-protective agent against experimentally-induced liver fibrogenesis. In addition, its effect on MMP-2 expression was demonstrated. All forms of hepatic parenchymal degeneration; hydropic degeneration, fatty change, apoptosis and necrosis, were markedly reduced in CCl4-injected rats after intragastric administration of curcumin. Moreover, collagen deposition was significantly reduced as compared with the animals of group B. This was in accordance with the results of previous study (Wu et al., 2010). They reported that curcumin ameliorated liver injury and reduced fibrosis in rats exposed to CCl4 injection.

Many previous investigations tried to explain the possible hepato-protective mechanisms of curcumin. Its antifibrotic effect was attributed to induction of apoptosis in hepatic stellate cells **(He et al., 2006 and Lin et al., 2009).** Meanwhile, it was reported that, it might either revert hepatic stellate cells back to quiescent state, initiate its apoptosis or both **(Priya and Sudhakaran, 2008)**. Moreover, it was suggested to reduce expression of cytokines; TGF- beta, NO and TNF-alpha **(Reyes-Gordillo et al., 2008)** and to decrease lipid peroxidation with subsequent antioxidant effect **(Morsy et al., 2012).**

Currently, curcumin was found to significantly decrease MMP-2 immunoreactivity in both the hepatocytes and sinusoidal cells. This might explain its antifibrotic and hepatoprotective effect. Similarly, previous investigators demonstrated that curcumin exerted its antifibrotic effect through influencing MMPs and TIMPs expressions **(Rukkumani et al., 2004 and Rajagopalan et al., 2010).** Thereby, alteration of expression of matrix metalloproteinases might be considered a strong regulatory mechanism of curcumin in preventing extracellular deposition of collagen and ameliorating the histopathological changes in the liver parenchyma following injection with CCl4.

In conclusion, oral administration of curcumin ameliorates the histopathological changes induced in the rat liver after CCl4 injection. Hydropic degeneration, fatty change, apoptosis and necrosis of hepatocytes produced by CCl4 were apparently corrected. Moreover, deposition of collagen was significantly decreased. This was associated with significant decrease in MMP-2 immunoreactivity in both hepatocytes and sinusoidal cells. Therefore, it could be suggested that curcumin might exert its hepatoprotective effect through inhibition of MMP-2 immunoreactivity. Based on these results, the oral use of curcumin is recommended to improve hepatic injury and slow the development of liver fibrosis.

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