Journal of Experimental and Integrative Medicine
www.scopemed.org
DOI: 10.5455/jeim.150514.or.104

Original Research

GESDAV

Attenuation of carbon tetrachloride-induced hepatic fibrosis by glycine, vitamin E, and vitamin C

Aziza A. Saad[1], El-Hassan M. Mokhamer[2], Mohamed A. Abdel Mohsen[1], Geylan A. Fadaly[3]

[1]Department of Applied Medical Chemistry, Medical Research Institute, Alexandria University, Alexandria, Egypt, [2]Department of Pharmacology and Toxicology, Faculty of Pharmacy and Drug Manufacturing, Pharos University in Alexandria, Egypt, [3]Department of Pathology, Medical Research Institute, Alexandria University, Alexandria, Egypt

Address for correspondence:
Dr. EL-Hassan M. Mokhamer, Department of Pharmacology and Toxicology, Faculty of Pharmacy and Drug Manufacturing, Pharos University in Alexandria, P.O. Box 3, Sidi Gaber, Alexandria 21311, Egypt.
E-mail: hassan.mokhamer@pua.edu.eg

Received: April 07, 2014
Accepted: May 15, 2014
Published: August 27, 2014

ABSTRACT

Objective: Mounting clinical and experimental evidence has demonstrated that even advanced fibrosis and cirrhosis are reversible. Thus, there is a considerable imperative to develop antifibrotic strategies that are applicable to liver fibrosis. In the present study, the attenuation of carbon tetrachloride (CCl_4)-induced hepatic fibrosis in rats through the co-treatment with antioxidant vitamins C and E or glycine alone was investigated. **Materials and Methods:** Sixty male Sprague-Dawley were divided randomly into control, CCl_4, vitamin E+C, and glycine groups. Except for the control group, all rats in the other groups received orally 2 ml/kg CCl_4 dissolved in olive oil (1:1, v/v) twice a week; antioxidant vitamins were supplemented orally (p.o.) at a dose of 200 IU/kg/day vitamin E and 50 mg/kg/day vitamin C. Glycine dose was 0.6 g/Kg/day (p.o.). After 9 weeks, all rats were sacrificed for biochemical and histopathological investigations. **Results:** Serum levels of hepatic transaminases, alkaline phosphatase, gamma glutamyl transferase, and β-glucuronidase strikingly increased in CCl_4 group. As a biomarker of fibrinogenesis, hyaluronic acid was significantly elevated. These were associated with drastic significant decline in serum albumin concentration level, blood glutathione (GSH) content, GSH peroxidase, catalase and superoxide dismutase enzymatic activities, as well as a striking significant increase in the concentration of serum malondialdehyde. A significant increase in the mean serum concentration levels of tumor necrosis factor-a and transforming growth factor-b1 has been observed in CCl_4-treated rats. Histopathological examination highlighted and confirmed the biochemical results. **Conclusion:** The results of this study confirmed the ameliorative effects of vitamin E plus C combination and glycine supplements against liver fibrosis in rats induced by CCl_4.

KEY WORDS: Antioxidants, carbon tetrachloride, glycine, hepatic fibrosis, oxidative stress

INTRODUCTION

Liver fibrosis is a reversible wound-healing process that occurs in almost all patients with chronic liver diseases. The sustained liver injuries in chronic liver diseases cause multiple cells and cytokines act in a dynamic and interactive way to comprise the mechanisms of fibrogenesis. Though early stage of fibrosis is usually silent in symptoms; progression to cirrhosis may cause almost all kinds of hepatic complications, including portal hypertension, ascites, hepatic encephalopathy, and impaired metabolic disturbance, therefore, it is largely responsible for mortality of hepatopathy [1,2]. Meanwhile, liver fibrogenesis is orchestrated by a heterogeneous population of profibrogenic myofibroblasts, the majority originating from hepatic stellate cells (HSCs), following a process termed "activation". Other sources of fibrogenic cells have recently been implicated, including portal fibroblasts and cells derived from the bone marrow [3].

On the other hand, carbon tetrachloride (CCl_4) is a potent hepatotoxin, and a single exposure to it can rapidly lead to severe centrizonal necrosis and steatosis [4]. In general, CCl_4 is metabolized by microsomal mono-oxygenase system (cytochrome P450 2E1) to its active metabolite, this process results in the fragmentation of the lipid peroxide radicals, lipid hydroperoxides and other products, each acting like an

active oxidizing agent [5]. Furthermore, these processes are immediately followed by the infiltration of inflammatory cells and release of various cytokines and growth factors. Thus, lipid peroxidation caused by free radicals of CCl_4 metabolism plays a vital role on the CCl_4-induced liver injury [6]. In this aspect, the level of tumor necrosis factor (TNF)-α consistently rises during the ongoing CCI_4-induced liver toxicity [7]. It is well-known that TNF-α stimulates the secretion of cytokines such as transforming growth factor (TGF)-β1 which is also an important activator of HSCs in the course of hepatic fibrogenesis [5].

Vitamin C is an important free radical scavenger in extracellular fluids, trapping radicals and protecting biomembranes from peroxide damage. It effectively scavenges singlet oxygen, superoxide, hydroxyl, water soluble peroxyl radicals and hypochlorous acid. It is also reported to be an excellent source of electrons and, therefore, can donate electrons to free radicals such as hydroxyl and superoxide radicals and quench their activity [8]. Meanwhile, vitamin E, a potent peroxyl radical scavenger, is a chain-breaking antioxidant that prevents the propagation of free radicals in membranes and plasma lipoproteins [9].

L-glycine is a simple, non-essential, and relatively non-toxic amino acid. Current evidences have shown that glycine provides significantly cytoprotective and anti-inflammatory activities. Its beneficial effects do not depend on its metabolism or its influence on cellular energy metabolism, but depend on its special structural characteristics, which glycine shares with other agents such as alanine, taurine, and strychnine (which appears to have similar beneficial effects at high concentrations) [10]. These beneficial effects have been found in a variety of tissues and organs including the liver [11].

Based on the abovementioned facts, the present study aimed to investigate the possible protective effects of both of vitamin C plus E combination and glycine alone on liver fibrosis induced by CCl_4.

MATERIALS AND METHODS

Chemicals

All chemicals used were of analytical grade purity and needed no further purification. Kits for the determination of serum alanine transaminase (ALT), aspartate transaminase (AST), gamma glutamyl transferase (GGT) and alkaline phosphatase (ALP) activities, as well as kit for determination of serum albumin, were purchased from BioSystem (Barcelona, Spain). CCl_4 was purchased from Aldrich (Milwaukee, WI, USA), nicotinamide adenine dinucleotide phosphate, glycine, glutathione (GSH), p-nitrophenol, p-nitrophenyl-β-D-glucuronide, thiobarbituric acid, hydrogen peroxide (H_2O_2), diethylene triamine pentaacetic acid, pyrogallol, superoxide dismutase standard and malonaldehyde bis(diethyl acetate) (1,1,3,3-tetraethoxy-propane) were purchased from Sigma (St. Louis, MO, USA). Enzyme-linked immunosorbant assay (ELISA) kits for determination of rat serum TNF-α, TGF-β1 and hyaluronic acid (HA) levels were purchased from Bender Med Systems (Vienna, Austria). Vitamin C and vitamin E were purchased from Pharco (Alexandria, Egypt).

Animals

Male Sprague-Dawley, with an average weight of 225-250 g, were purchased from Animal House, Medical Technology Center, Medical Research Institute, Alexandria University. They were maintained in 12 h light/dark cycle at constant temperature of 25°C with free access to standard pellet food and tap-water. They were treated humanely according to the National Guideline for animal care.

Induction of Liver Fibrosis and Treatment

60 male albino rats were divided randomly into four groups (n = 15 each): Group I (normal control); Group II CCl_4; Group III (vitamin E+C); and Group IV (glycine). Except for the normal control group, all rats in the other groups received orally 2 ml/kg body weight CCl_4 dissolved in olive oil (1:1, v/v), twice a week; Group III was daily administrated 200 IU/kg body weight vitamin E and 50 mg/kg body weight vitamin C (oral), respectively; rats in group IV were given daily glycine with an oral dose of 0.6 g/kg body weight.

Nine weeks post CCl_4-treatment; rats were sacrificed by cervical dislocation. Blood samples were collected by cardiac puncture, and the separated sera were kept at −80°C till biochemical investigations were carried out.

Histopathology

Liver samples were obtained from the same lobe in all animals and fixed in 10% buffered formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin.

Biomarkers of Liver Functions

Serum levels of ALT, AST, GGT, and ALP enzymatic activities, as well as serum albumin concentration, were measured using commercially available kits according to the manufacturer's instructions. Serum activity level of β-glucuronidase (β-Glase) was also measured [12].

Biomarker of Hepatic Fibrosis

ELISA kit was used to measure serum HA level according to the manufacturer's instructions.

Biomarkers of Oxidative Stress

The concentration levels of serum malondialdehyde (MDA) [13] and whole blood reduced GSH [14] were measured. Furthermore, erythrocytes levels of glutathione peroxidase (GPx), [15] superoxide dimutase (SOD) [16] and catalase (CAT) activity [17] were determined.

Hepatic Fibrosis Factors (TGF-β1 and TNF-α)

Cytokines concentration levels in the serum samples were measured by commercially available ELISA kits according to the manufacturer's instructions.

Statistical Analysis

Statistical analysis was performed with using SPSS software (version 17.0; Chicago, IL, USA). Parametric data were analyzed statistically by one-way analysis of variance followed by post-hoc test when appropriate. Significance level was defined as $P < 0.05$.

RESULTS

Liver Functions

CCl_4 significantly increased serum activities of ALT, AST, ALP, GGT, and β-Glase as compared to control ($P < 0.05$) [Table 1]. Antioxidant vitamins and glycine caused a significant decrease ($P < 0.05$) in the elevated activity levels of ALT, AST, ALP, and GGT when compared with that of CCl_4-treated group.

CCl_4 caused a significant reduction in the concentration level of serum albumin as compared to control rats ($P < 0.05$). Serum albumin concentration level was significantly increased in antioxidant vitamins and glycine pretreated rats ($P < 0.05$) as compared to CCl_4-treated group [Table 1].

Biomarker of Liver Fibrosis

CCl_4 caused a significant increase in serum concentration level of HA ($P < 0.05$) when compared to control the group. This increase was inhibited by the ameliorative effect of antioxidant vitamins (E and C) ($P < 0.05$) and glycine ($P < 0.05$) [Table 1].

Biomarkers of Oxidative Stress

Serum MDA concentration level was increased significantly in CCl_4 treated rats ($P < 0.05$) as compared to control the group. Reduction of serum MDA concentration was observed in antioxidant vitamins and glycine treated groups ($P < 0.05$) as compared to CCl_4 treated group [Table 2].

CCl_4 caused a significant depletion of erythrocytes GSH content as compared to control rats ($P < 0.05$). While GSH content was significantly increased in antioxidant vitamins treated group ($P < 0.05$) and glycine ($P < 0.05$) as compared to CCl_4-group [Table 2]. Furthermore, CCl_4 caused a significant reduction in the enzymatic activities of erythrocytes GPx, CAT, and SOD as compared to the control group ($P < 0.05$). These enzymatic activity levels were significantly increased in antioxidant vitamins, and glycine treated group ($P < 0.05$) as compared to CCl_4-group [Table 2].

Hepatic Fibrosis Factors (TGF-β1 and TNF-α)

CCl_4 caused a significant increase in serum levels of TNF-α and TGF-β1 ($P < 0.05$) as compared to control group [Table 2]. This increase was inhibited by the prophylactic effect of antioxidant vitamins and glycine ($P < 0.05$) [Table 2].

Histopathological Results

Normal liver architecture has been observed in the control group [Figure 1]. Histopathological changes, associated with CCl_4 intoxication for 9 weeks are shown in Figure 2. Histopathological changes in CCl_4 + vitamins E and C group are shown in Figure 3. Histopathological changes in CCl_4 + glycine group are shown in Figure 4.

DISCUSSION

CCl_4-induced toxic liver injury which is a well-characterized model for hepatic fibrosis has been extensively performed [6]. CCl_4 damages hepatocellular membrane via lipid peroxidation and this is followed by the release of inflammatory mediators from the activated inflammatory cells which are thought to potentiate CCl_4-induced hepatic injury [18]. AST and ALT, the aminotransferases in liver cells, are cytoplasmic in nature, but upon liver injury these enzymes enter into the circulatory

Table 1: Mean serum activity levels of AST, ALT, ALP, GGT and β-Glase as well as serum concentration levels of albumin and HA in the different study groups

	Control	CCl_4	CCl_4+vitamin C and E	CCl_4+glycine
AST (U/l)	53.5±2.1	568±22[a]	146±5[b]	225±12[bc]
ALT (U/l)	37±2	359±17[a]	157±5[b]	198±8[bc]
ALP (U/l)	151±3	561±19[a]	272±12[b]	345±15[bc]
GGT (U/l)	4.2±0.3	13±0.6[a]	8.1±0.5[b]	10±0.5[bc]
β-Glase (U/l)	1.06±0.06	4.62±0.21[a]	2.67±0.13[b]	3.47±0.16[bc]
Albumin (g/l)	35.5±0.88	21.4±0.79[a]	25.5±0.88[b]	25.4±1.1[b]
HA (ng/ml)	102±3	343±3[a]	189±9[b]	245±4[bc]

$P<0.05$ when compared with [a]control, [b]CCl_4, or [c]CCl_4+vitamin C and E groups; $n=15$ for each group. CCl_4: Carbon tetrachloride, AST: Aspartate transaminase, ALT: Alanine transaminase, GGT: Gamma glutamyl transferase, ALP: Alkaline phosphatase, HA: Hyaluronic acid, β-Glase: β-glucuronidase

Table 2: Mean concentration levels of serum MDA, TNF-α, TGF-β1 and GSH content as well as the mean levels of GPx, CAT and SOD activity in a different study groups

	Control	CCl_4	CCl_4+ vitamin C and E	CCl_4+glycine
MDA (nmol/ml)	0.45±0.02	1.08±0.05[a]	0.56±0.03[b]	0.65±0.02[b]
GSH (mg%)	34.3±1.2	12.5±0.8[a]	22.0±1[b]	24.8±0.59[b]
GPx (U/g Hb)	9.5±0.5	3.8±0.3[a]	7±0.3[b]	5.5±0.3[bc]
CAT (U/ml)	3049±100	929±60[a]	2584±130[b]	2403±129[b]
SOD (ng/g Hb)	14.8±0.8	5.1±0.3[a]	11±0.5[b]	9.6±0.7[b]
TNF-α (pg/ml)	15.2±0.42	26.9±0.7[a]	18.9±0.45[b]	17.5±0.52[b]
TGF-β1 (ng/ml)	0.87±0.06	5.02±0.38[a]	3.53±0.27[b]	3.80±0.17[b]

$P<0.05$ when compared with [a]control, [b]CCl_4, or [c]CCl_4+vitamin C and E groups; $n=15$ for each group. MDA: Malondialdehyde, TNF-α: Tumor necrosis factor-α , TGF-β1: Transforming growth factor-β1, GSH: Glutathione, GPx: Glutathione peroxidase, CAT: Catalase, SOD: Superoxide dismutase, CCl_4: Carbon tetrachloride

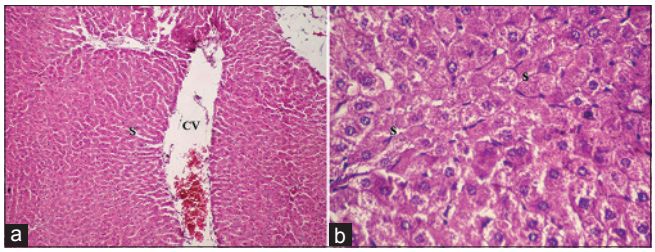

Figure 1: Paraffin section from normal control liver tissue after 9 weeks showing well-arrayed hepatic cords radiating from the centrally dilated central vein (CV) intervening sinusoids (S) are compressed with no congestion; no steatosis and no fibrosis are identified; (a) H and E, ×200, (b) H and E, ×400

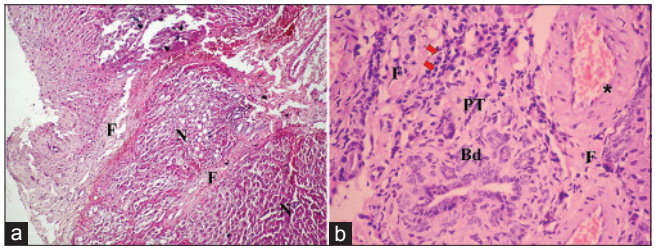

Figure 2: (a) Paraffin section from liver tissue after carbon tetrachloride (CCl_4) intoxication for 9 weeks showing severe fibrosis among hepatic parenchyma intersected by thick fibrous septae (F) forming prominent cirrhotic change (F) with intervening regenerative nodules (N) (H and E, ×200). (b) Paraffin section from liver tissue after (CCl_4) intoxication for 9 weeks showing portal tract (PT) with prominent fibrous collagenic stroma (F) and heavy chronic lymphocytic infiltrate (arrow), bile duct proliferation (Bd) is extensive with thick wall congested hepatic vein (*) (H and E, ×400)

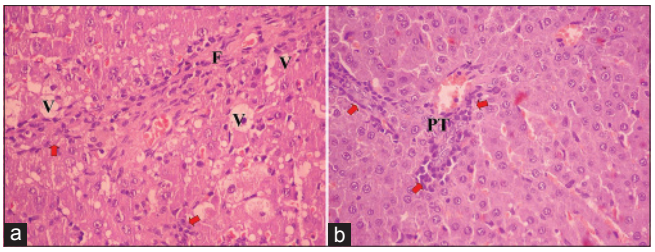

Figure 3: (a) Paraffin section from liver tissue after carbon tetrachloride (CCl_4) intoxication for 9 weeks receiving vitamins E and C showing moderate steatohepatitis (arrow) is evident with mild fibro-cellular thin septae (F) and scattered foci of macro and micro vesicular steatosis (V) (H and E, ×400). (b) Paraffin section from liver tissue after (CCl_4) intoxication for 9 weeks receiving vitamins E and C showing residual infiltration of the portal tract (PT) by chronic inflammatory cells (left arrows) with mild lobular hepatic infiltration (right arrow); no steatosis is evident (H and E, ×400)

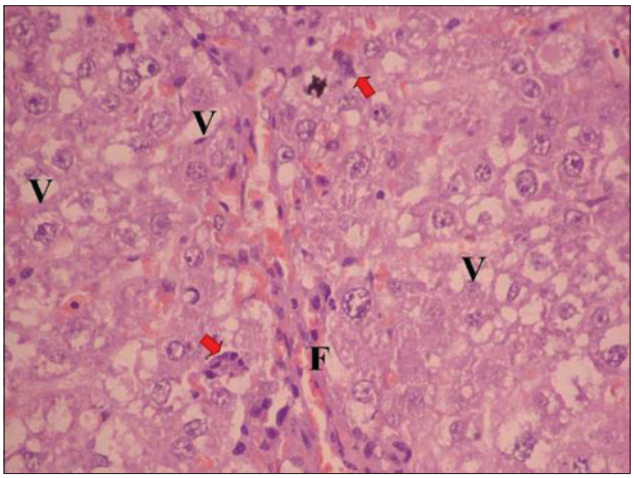

Figure 4: Paraffin section from liver tissue after carbon tetrachloride intoxication for 9 weeks receiving dietary glycine showing regression of the thick fibrous bands with residual thin interrupted ones (F) among the moderately steatotic hepatic vacuoles (V). Mild steatohepatitis (arrow) is evident (H and E, ×400)

system due to altered permeability of the membrane. In the agreement with previous studies, the data of the present work confirm the elevation in serum activity levels of AST and ALT in rats treated with CCl_4 and indicate its hepatotoxicity. Moreover, a significant increase in the serum activity levels of ALP and GGT, ectoenzymes of the hepatocyte plasma membrane [19], was observed in CCl_4 treated animals. This elevation could be attributed to CCl_4-mediated acute toxicity increased permeability of the hepatocyte membrane and cellular leakage [20]. Accordingly, it may be speculative, that ALP and GGT could be markers of hepatocyte damage or hepatic dysfunction.

Elevated activity levels of serum β-Glase were observed in rats with CCl_4-induced liver cirrhosis [21] and N-nitrosodimethylamine-induced hepatic fibrosis [22]. The increase in circulating acid hydrolases could trigger cell damage and apoptosis, which in turn raises β-Glase level in the serum. However, the increase in serum β-Glase in advanced fibrosis is mostly due to a decrease of liver lysosomal stability and the subsequent release of lysosomal enzymes into the blood stream due to the enhanced lipid peroxidation in the hepatic tissue [22]. Thus, the increased rate of release of β-Glase from the lysosomes provided evidence for increased lysosomal fragility during the pathogenesis of the CCl_4-induced hepatic fibrosis.

Both plasma albumin and blood clotting factors were mainly synthesized in the liver. When the chronic liver damage led to fibrosis, the albumin contents dropped and prothrombin time prolonged [23]; the results of the present study exhibited that CCl_4-induced chronic liver lesion in rats and there appeared a decrease of plasma albumin level. Therefore, CCl_4 impairs the capacity of the liver to synthesis albumin, so the protein content of serum decreases in such cases [20]. It should be noted that the metabolism of CCl_4 yields metabolites that are hepatotoxic and cause fragmentation of the endoplasmic reticulum and disruption of ribosomes into subunits with subsequent disengagement of the 40S subunit from mRNA [24]. It has been shown that once the protein is oxidized, it becomes highly susceptible to proteolytic degradation [25]. These findings may explain, in part, the observed decrease in the level of serum albumin in CCl_4 treated rats.

Several studies have reported an elevation in serum concentration of HA in CCl_4-treated rats [26]. It was also reported that during fibrogenesis in chronically inflamed liver, the concentration of extracellular matrix HA increases several folds [27]. Since HSCs are responsible for the synthesis of HA in liver, [28]

their activation and proliferation during fibrogenesis would have triggered an increased expression of HA along with other connective tissue components. Most of the newly synthesized hyaluronate is excreted into the extracellular space, of which a fraction can leak into the blood stream, and the process will be accelerated during liver necrosis. In addition, more than 90% of the circulating HA is degraded in the hepatic sinusoidal endothelial cells [29]. During fibrosis, the function of the sinusoidal endothelial cells is impaired due to the capillarization of sinusoids and formation of basement membrane in the space of Disse [30]. Thus, an increase in HA expression with its leakage into circulation as well as a decrease in its rate of degradation due to impairment of sinusoidal endothelial cells may explain the observed elevation in serum concentration level of HA.

The principal causes of CCl_4-induced hepatic damage are lipid peroxidation, decreased activities of antioxidant enzymes and generation of free radicals [31]. A close relationship has been reported between lipid peroxidation and fibrogenesis in rats in which fibrosis was induced by CCl_4 administration [32]. In the agreement with the previous studies, the herein results point out to the development of oxidative stress in rats treated with CCl_4. The developed oxidative stress is manifested by a significant decline in the endogenous antioxidants viz. blood GSH content as well as the activity levels of GPx, CAT, and SOD associated with a significant increase in serum concentration level of MDA. The antioxidant enzymes SOD, CAT, and GPx constitute a mutually supportive team of defense against reactive oxygen species (ROS) [33]. Depletion of GSH results in enhanced lipid peroxidation, which in turn causes increased GSH consumption.

The major mechanism by which CCl_4 induces rat liver fibrosis is that the major toxic metabolite of it, namely the trichloromethyl radical ($\bullet CCl_3$), results in a series of lipid peroxidation reactions [34]. It has been stated that lipid peroxidation, free radical-mediated process, and certain lipid peroxidation products induce genetic overexpression of fibrogenic cytokines (e.g. TGF-β1 and platelet-derived growth factor) and increase the synthesis of collagen. Free radical and MDA can stimulate the synthesis of collagen and initiate the activation of HSCs [35]. These activated HSCs secrete excess extracellular matrix (ECM) proteins, predominantly collagen Type I, leading to the accumulation of scar matrix, and ultimately hepatic fibrosis [36].

In CCl_4 group, in accordance with the previous report, a significant increase in serum concentration levels of TNF-α and TGF-β1 when compared to that in the control group. TNF-α, pleiotropic pro-inflammatory cytokine produced rapidly by macrophages in response to tissue injury, has been implicated in liver fibrosis [37]. However, the role of TNF-α on HSCs activation and liver fibrosis is complicated and remains controversial. However, it has been reported that TNF-α enhances the expression of tissue inhibitor of matrix metalloproteinase-1 in hepatic HSCs which in turn leads to the inhibition of extracellular matrix degradation by the matrix metalloproteinase family of enzymes [38]. TGF-β1 is the most potent fibrogenic cytokine in the liver; its expression increases during fibrogenesis and it is the dominant stimulus which induces HSCs to increase ECM synthesis [39].

The histopathological examination in the present study highlights and confirms the biochemical results since it is proved that the liver tissue after CCl_4 intoxication for 9 weeks was showing severe histopathological changes that manifested by severe fibrosis among hepatic parenchyma intersected by thick fibrous septae forming prominent cirrhotic changes with intervening regenerative nodules [Figure 2a]. Portal tract (PT) with prominent fibrous collagenic stroma and heavy chronic lymphocytic infiltration, bile duct proliferation is extensive with thick wall congested hepatic vein [Figure 2b].

In Groups III and IV, treatment with antioxidant vitamins E and C together or glycine alone resulted in an improvement in biomarkers of liver function. A significant reduction in the mean serum activity levels of AST, ALT, ALP, GGT, and β-Glase as compared to that in CCl_4-treated group was observed. Furthermore, a significant increase in mean concentration level of serum albumin and a significant decrease in the mean concentration level of serum HA were observed as compared to that in CCl_4 treated group. Moreover, treatment with antioxidant vitamins E and C or glycine 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 concentration level, was accompanied by an increase in the antioxidant capacity level as seen by a significant increase in GSH concentration and GPx, CAT, and SOD enzymatic activity levels. Furthermore, the mean serum concentration levels of TNF-α and TGF-b1 were significantly reduced on supplementation with vitamins E and C together or glycine alone.

It has been reported that vitamin C has hepatoprotective property which in turn is linked to its antioxidative action. Vitamin C was reported to attenuate hepatic damage induced by some chemical agents especially in animals. It was reported that vitamin C normalized levels of ALT, AST, ALP, blood hydroperoxide, and MDA in liver of CCl_4-intoxicated rats [40].

The hydroxyl group of tocopherol reacts with the peroxyl radical to form the corresponding lipid hydroperoxide and the tocopheryl radical (Vit E-O•). The tocopheryl radical reacts with vitamin C (or other hydrogen donors), thereby oxidizing the latter and returning vitamin E to its reduced state [41]. The interaction of vitamins C and E has led to the idea of "vitamin E recycling," where the antioxidant function of oxidized vitamin E is continuously restored by other antioxidants.

The synergism between vitamin C and other antioxidants has been reported. One of the visible synergies is between vitamin C and vitamin E. All of these studies have pointed out to the ameliorative effect of the co-administration of vitamin C and vitamin E against hepatic intoxication by different agents [42,43]. Moreover, they have pointed out to the normalization of the liver functions biomarkers such as ALT, AST, and some endogenous antioxidants as a result of vitamin C and vitamin E co-administration.

All of the above-mentioned studies are in agreement with results of the present study where co-administration of vitamin C and vitamin E resulted in an improvement in liver functions, reduction in oxidative stress and effective inhibition of TNF-α production from CCl_4-stimulated Kupffer cells. This form of therapy may be attractive because it down-regulates pro-inflammatory cytokine production without total inhibiting all TNF-α bioactivity, some of which may be beneficial to the host.

For Group III, the histopathological examination showed that antioxidants treatment for 9 weeks ameliorated the necrotic and fibrotic changes caused by CCl_4. Histopathological results showed residual infiltration of the PT by chronic inflammatory cells with mild lobular hepatic infiltration. Moderate steatohepatitis is evident with mild fibrocellular thin septae and scattered foci of macro and microvesicular steatosis [Figure 3].

It is most likely that the cytoprotective role of glycine is attributed on its ability to inactivate the Kupffer cells via glycine receptors [44]. The mechanism involved may be related to inhibition of the release of pro-inflammatory cytokines by Kupffer cells induced by glycine. *In vivo* and *in vitro* experiments have found that glycine inhibits the secretion of TNF-α and interleukin-6 in Kupffer cells [45]. All of these together may, in part, explain the improved liver functions and decreased oxidative stress. Accordingly, it has been suggested that glycine may be an effective agent which could provide a future strategy for therapeutic intervention in the treatment of liver injuries induced by activated Kupffer cells.

The present histological examination confirmed the biochemical findings which demonstrated dilated PT with moderate lymphocytic infiltration and mild fibrosis separating parenchymal hepatocytes, diffuse macrovesicular steatotic changes with mild to moderate steatohepatitis [Figure 4].

In conclusion, the present study revealed that co-administration of vitamin C and E or glycine improves the liver functions, oxidative status, and fibrinogenesis activity. The mechanism underlying these manifestations may be not only to their antioxidant properties but also through modulating the pro-inflammatory cytokines pathways. Since more effective new therapeutic options are lacking, patients with serious liver diseases should be encouraged to take vitamin E + vitamin C + glycine supplements, which are safe and affordable.

REFERENCES

1. Hernandez-Gea V, Friedman SL. Pathogenesis of liver fibrosis. Annu Rev Pathol 2011;6:425-56.
2. Mas VR, Fisher RA, Archer KJ, Maluf DG. Proteomics and liver fibrosis: Identifying markers of fibrogenesis. Expert Rev Proteomics 2009;6:421-31.
3. Iredale JP. Models of liver fibrosis: Exploring the dynamic nature of inflammation and repair in a solid organ. J Clin Invest 2007;117:539-48.
4. Boobis AR, Fawthrop DJ, Davies DS. Mechanisms of cell death. Trends Pharmacol Sci 1989;10:275-80.
5. Lamireau T, Desmoulière A, Bioulac-Sage P, Rosenbaum J. Mechanisms of hepatic fibrogenesis. Arch Pediatr 2002;9:392-405.
6. Nadkarni GD, D'Souza NB. Hepatic antioxidant enzymes and lipid peroxidation in carbon tetrachloride-induced liver cirrhosis in rats. Biochem Med Metab Biol 1988;40:42-5.
7. Simeonova PP, Gallucci RM, Hulderman T, Wilson R, Kommineni C, Rao M, *et al*. The role of tumor necrosis factor-alpha in liver toxicity, inflammation, and fibrosis induced by carbon tetrachloride. Toxicol Appl Pharmacol 2001;177:112-20.
8. Adikwu E, Deo O. Hepatoprotective effect of vitamin C (Ascorbic acid). Pharmacol Pharm 2013;4:84-92.
9. Traber MG, Atkinson J. Vitamin E, antioxidant and nothing more. Free Radic Biol Med 2007;43:4-15.
10. Bi W, Wang F, Bi Y, Wang T, Xue P, Zhang Y, *et al*. Renal ischemia/reperfusion injury in rats is attenuated by a synthetic glycine derivative. Eur J Pharmacol 2009;616:256-64.
11. Rentsch M, Beham A, Sirek S, Iesalnieks I, Geissler EK, Anthuber M, *et al*. Glycine but not gadolinium chloride or methyl palmitate reduces postischemic white blood cell accumulation and early graft nonfunction after liver transplantation in the rat. Transplant Proc 2002;34:2389-90.
12. Fishman WH, Kato K, Anstiss CL, Green S. Human serum beta-glucuronidase; its measurement and some of its properties. Clin Chim Acta 1967;15:435-47.
13. Satoh K. Serum lipid peroxide in cerebrovascular disorders determined by a new colorimetric method. Clin Chim Acta 1978;90:37-43.
14. Beutler E, Duron O, Kelly BM. Improved method for the determination of blood glutathione. J Lab Clin Med 1963;61:882-8.
15. Paglia DE, Valentine WN. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. J Lab Clin Med 1967;70:158-69.
16. Marklund S, Marklund G. Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. Eur J Biochem 1974;47:469-74.
17. Beers RF Jr, Sizer IW. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. J Biol Chem 1952;195:133-40.
18. Edwards MJ, Keller BJ, Kauffman FC, Thurman RG. The involvement of Kupffer cells in carbon tetrachloride toxicity. Toxicol Appl Pharmacol 1993;119:275-9.
19. Wolfgang GH, Jolly RA, Donarski WJ, Ochoa R, Petry TW. Inhibition of carbon tetrachloride-induced lipid peroxidation by novel antioxidants in rat hepatic microsomes: Dissociation from hepatoprotective effects in vivo. J Biochem Toxicol 1990;5:167-74.
20. Lin HM, Tseng HC, Wang CJ, Lin JJ, Lo CW, Chou FP. Hepatoprotective effects of Solanum nigrum Linn extract against CCl(4)-induced oxidative damage in rats. Chem Biol Interact 2008;171:283-93.
21. Abraham P. Lysosomal enzyme activity during development of carbon tetrachloride induced cirrhosis in rats. Indian J Physiol Pharmacol 2004;48:206-12.
22. George J. Elevated serum beta-glucuronidase reflects hepatic lysosomal fragility following toxic liver injury in rats. Biochem Cell Biol 2008;86:235-43.
23. Bodakhe SH, Ram A. Hepatoprotective properties of *Bauhinia variegata* bark extract. Yakugaku Zasshi 2007;127:1503-7.
24. Hicks SJ, Drysdale JW, Munro HN. Preferential synthesis of ferritin and albumin by different populations of liver polysomes. Science 1969;164:584-5.
25. Lin WC, Lin WL. Ameliorative effect of *Ganoderma lucidum* on carbon tetrachloride-induced liver fibrosis in rats. World J Gastroenterol 2006;12:265-70.
26. Wang R, Yu XY, Guo ZY, Wang YJ, Wu Y, Yuan YF. Inhibitory effects of salvianolic acid B on CCl(4)-induced hepatic fibrosis through regulating NF-?B/I?Ba signaling. J Ethnopharmacol 2012;144:592-8.
27. George J, Tsutsumi M, Takase S. Expression of hyaluronic acid in N-nitrosodimethylamine induced hepatic fibrosis in rats. Int J Biochem Cell Biol 2004;36:307-19.
28. Vrochides D, Papanikolaou V, Pertoft H, Antoniades AA, Heldin P. Biosynthesis and degradation of hyaluronan by nonparenchymal liver cells during liver regeneration. Hepatology 1996;23:1650-5.
29. Ueno T, Inuzuka S, Torimura T, Tamaki S, Koh H, Kin M, *et al*. Serum hyaluronate reflects hepatic sinusoidal capillarization. Gastroenterology 1993;105:475-81.
30. Xie SB, Yao JL, Zheng SS, Yao CL, Zheng RQ. The levels of serum fibrosis marks and morphometric quantitative measurement of hepatic fibrosis. Hepatobiliary Pancreat Dis Int 2002;1:202-6.
31. Kuzu N, Metin K, Dagli AF, Akdemir F, Orhan C, Yalniz M, *et al*.

Protective role of genistein in acute liver damage induced by carbon tetrachloride. Mediators Inflamm 2007;2007:36381.
32. Uskokovic-Markovic S, Milenkovic M, Topic A, Kotur-Stevuljevic J, Stefanovic A, Antic-Stankovic J. Protective effects of tungstophosphoric acid and sodium tungstate on chemically induced liver necrosis in wistar rats. J Pharm Pharm Sci 2007;10:340-9.
33. Aoyama K, Matsumura N, Watabe M, Nakaki T. Oxidative stress on EAAC1 is involved in MPTP-induced glutathione depletion and motor dysfunction. Eur J Neurosci 2008;27:20-30.
34. Yao HW, Li J, Chen JQ, Xu SY. Inhibitory effect of leflunomide on hepatic fibrosis induced by CCl4 in rats. Acta Pharmacol Sin 2004;25:915-20.
35. Calò L, Giacon B, Davis PA, Pagnin E, Piccin A, Riegler P, *et al*. Oxidative stress and TGFbeta in kidney-transplanted patients with cyclosporin-induced hypertension. Effect of carvedilol and nifedipine. Clin Nephrol 2002;58:103-10.
36. Friedman SL. Hepatic stellate cells: Protean, multifunctional, and enigmatic cells of the liver. Physiol Rev 2008;88:125-72.
37. Brouckaert P, Fiers W. Tumor necrosis factor and the systemic inflammatory response syndrome. Curr Top Microbiol Immunol 1996;216:167-87.
38. Osawa Y, Hoshi M, Yasuda I, Saibara T, Moriwaki H, Kozawa O. Tumor necrosis factor-a promotes cholestasis-induced liver fibrosis in the mouse through tissue inhibitor of metalloproteinase-1 production in hepatic stellate cells. PLoS One 2013;8:e65251.
39. Gressner AM, Weiskirchen R, Breitkopf K, Dooley S. Roles of TGF-beta in hepatic fibrosis. Front Biosci 2002;7:d793-807.
40. Bashandy AS, Alwasel SH. Carbon tetrachloride-induced hepatotoxicity and nephrotoxicity in rats: Protective role of vitamin C. J Pharmacol Toxicol 2011;6:283-92.
41. Neuzil J, Witting PK, Stocker R. Alpha-tocopheryl hydroquinone is an efficient multifunctional inhibitor of radical-initiated oxidation of low density lipoprotein lipids. Proc Natl Acad Sci U S A 1997;94:7885-90.
42. Tawfik M, Al-Badr N. Adverse effects of monosodium glutamate on liver and kidney functions in adult rats and potential protective effect of vitamins C and E. Food Nutr Sci 2012;3:651-9.
43. Uboh FE, Ebong PE, Akpan HD, Usoh IF. Hepatoprotective effect of vitamin C and E against gasoline vapour induced liver injury in male rats. Turk J Biol 2012;36:217-23.
44. Yin M, Ikejima K, Arteel GE, Seabra V, Bradford BU, Kono H, *et al*. Glycine accelerates recovery from alcohol-induced liver injury. J Pharmacol Exp Ther 1998;286:1014-9.
45. Garcia-Macedo R, Sanchez-Muñoz F, Almanza-Perez JC, Duran-Reyes G, Alarcon-Aguilar F, Cruz M. Glycine increases mRNA adiponectin and diminishes pro-inflammatory adipokines expression in 3T3-L1 cells. Eur J Pharmacol 2008;587:317-21.

Source of Support: Nil, Conflict of Interest: None declared.