PROPOLIS AND LOSARTAN HAVE PROTECTIVE EFFECT AGAINST CARBON TETRACHLORIDE-INDUCED LIVER FIBROSIS IN ALBINO RAT

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ABSTRACT
Forty five male albino rats were separated into nine groups (control, 2 model, and 6 treatment groups). Rats in two model groups were given intraperitoneal injection of 50% carbon tetrachloride (2ml/Kg twice weekly for either 4 or 8 weeks). Rats in treatment groups were given ccl4 for 8 weeks and also given losartan (10mg/kg/day twice weekly), propolis (100mg/kg/day twice weekly) or combined losartan and propolis via gastrogavage. The treatment started either simultaneously or after 4 weeks from beginning of ccl4 injection. Control group rats were given only olive oil for the same period. At the assigned time, all rats were sacrificed. Blood samples were taken for determination of serum indicators (SGOT, SGPT, ALP) and the livers were dissected out and prepared for histopathological study. There was a significant (P ≥ 0.05) reduction in fibrosis area and score and alpha-SMA positivity in all sections of the livers of animals that received treatment simultaneously with CCl4 for 8ws in comparison to ccl4 injection alone for 8ws. There was also a significant reduction in the serum levels of SGPT, SGOT and ALP, however, these levels were still significantly (P ≥ 0.05) higher than control values. When the treatment started after fibrosis has begun (after 4ws of ccl4 injection) the fibrosis process slowed or even stopped with down regulation of active HSCs as evident by reduction of alpha-SMA positive stain. However, the serum levels of en-
zymes were reduced to a lesser degree. In all periods of treatment, losartan was more effective in inhibition of HSCs and of fibrosis while propolis was more effective in decreasing enzyme levels that may indicate more protection of hepatocytes. So, combined treatment gave better reduction of both fibrosis and enzyme levels. Reversal of already present fibrosis was not evident in this study.

Key words: liver fibrosis, losartan, propolis.

INTRODUCTION

Liver fibrosis refers to the accumulation of interstitial or ‘scar’ extracellular matrix (ECM) after either acute or chronic liver injury (1). The scars occur as the liver tries to repair damaged tissue. The early deposition of fibrillar ECM (predominantly collagen types I and III) in the subendothelial space of Disse is directly responsible for the progressive reduction of liver function. Liver fibrosis leads to cirrhosis, ultimately, end-stage liver failure and increased risk for hepatocellular carcinoma (2). However, a concept of reversibility of cirrhosis has been suggested by many investigators over many decades (3). This has accelerated enthusiasm for developing antifibrotic therapies (4).

Reactive oxygen species (ROS) have a rule in liver damage and progression of fibrosis by inducing hepatic stellate cells (HSCs) proliferation and collagen synthesis (5). Native propolis, which is a resinous hive product collected by honeybees from various plant sources, contains many antioxidants. Its antioxidant activity depends on the presence of flavonoids (6; 7). The chemical composition of propolis has a striking variability depending on the site of collection from different geographic origin (8). As a result, recently almost every publication on propolis biological activity includes some kind of chemical characterization of the bee glue used (9). The constituents of the Egyptian propolis are phenolic acid esters (72.7%), phenolic acids (1.1%), aliphatic acids (2.4%), dihydrochalcones (6.5%), Chalcones (1.7%), flavanones (1.9%), flavones (4.6%) and tetrahydrofuran derivatives (0.7%) (10; 11). Although several studies demonstrated a protective effect of propolis on acute hepatotoxicity in rats induced by alcohol and/or carbon tetrachloride (12; 13;
and a decrease in chronic alcohol induced hepatocellular fatty degeneration (15). However, few studies focused on the effect of propolis on prevention of liver fibrosis induced by chronic administration of ccl4 (16) or dimethylnitrosamine in rats (17).

A rule of rennin-angiotentin system in liver fibrosis has also been suggested (18; 19; 20). Angiotensin II (AngII) induces activation of HSCs in vivo, a key event in liver fibrogenesis (21). Angiotensin-converting enzyme inhibitors and angiotensin II type 1 receptor (AT1R) blockers were tested in hepatic fibrosis induced by CCl4 and a reduction in the mean fibrosis score and the progression of hepatic fibrosis was observed (22; 23; 24; 25).

The aim of this study is to test the effect of losartan (an antagonist of AT1R) and propolis (a potent antioxidant) on the prevention of liver fibrosis or the reversal of already present liver fibrosis in CCl4 treated rats and whether a combination of the two could give better results. A combination of the two drugs was not tested before.

MATERIALS AND METHODS

45 adult male albino rats, with average body weight 200gm were used in this study. They were housed in stainless steel mesh cages at room temperature. The animals were allowed free access to standard commercial diet and tap water ad libitum with a 12h light–dark cycle throughout experimental periods. All procedures involving animals were carried out in strict accordance with the international standards of animal care guidelines and were approved by the local ethical Experimental Animals Committee.

Experimental drugs:

1- Carbon tetrachloride (CCl4): Carbon tetrachloride solution was purchased from El-Gomhoria Company, Egypt. Intraperitoneal injection of 2ml/Kg sterile CCl4 dissolved in a 1:1 ratio with olive oil twice weekly was done as previously described by Iredale et al. (26).

2- Losartan (angiotensin-II receptor antagonist): Losartan tablets 50mg from (Amriya Pharm. Indu., Egypt) were dissolved in tap water, prepared as 1% solution and given orally by gavage in a dose of 10mg/kg/day, which is a clinically comparable dose for losartan (23).
3- Propolis (bee glue) powder:
Propolis powder was provided from Faculty of Agriculture, Mansoura University, Egypt. Propolis powder was dissolved in warm tap water, prepared as 10% solution and given orally by gavage in a dose of 100mg/kg/day as previously described by Seo et al. (27).

Experimental design:
Male albino rats were randomly divided into 5 groups:
• Control group (5 rats): received intra-peritoneal injection of olive oil 0.2ml/100g twice weekly for 8 weeks. These rats were sacrificed 3 days after the last injection.
• Model groups (CCl4 injected) (10 rats): received intra-peritoneal injection of CCl4 (2ml/kg dissolved in a 1:1 ratio of olive oil) twice weekly for either 4 or 8 weeks (5 rats each). These rats were sacrificed 3 days (peak fibrosis time) after the last CCl4 injection.
• Treated groups (30 rats): They received intra-peritoneal injection of CCl4 twice weekly for 8 weeks. Fifteen rats received the treatment of losartan (5 rats), propolis (5 rats) or combined losartan and propolis (5 rats) simultaneously with CCl4 injection for 8 weeks. Fifteen rats received the treatment of losartan (5 rats), propolis (5 rats) or combined losartan and propolis (5 rats) after 4 weeks from the beginning of CCl4. The rats were sacrificed 3 days after the last CCl4 injection.

Tissue processing
At the assigned times, all rats were anaesthetized with diethyl ether; samples of blood were drawn from the eye socket and collected in polyethylene tubes. The livers were rapidly removed and washed with cold normal saline. A portion of the liver was fixed in 10% phosphate buffered formalin, processed by routine histological procedures, embedded in paraffin, cut in 6µm thick sections and mounted on slides. The sections were stained with haematoxylin and eosin and sirius red that specifically stains collagen.

Immunohistochemical staining for α–SMA
Sections were subjected to deparaffinization, antigen retrieval and endogenous peroxidase blocking (3% hydrogen peroxide in methanol). Mouse anti-human α-SMA monoclonal antibody, dilution 1:100 (DAKO) which can detect rat α-SMA was applied for 60 minutes at room temper-
Immunohistochemical staining was performed by streptavidin-biotin method using LSAB+ kit (DAKO) with diaminobensidine as a chromogen and Meyer's hematoxilin for counterstaining. Negative control was performed by leaving out the primary antibody during the staining procedure.

**Image analysis of the area occupied by collagen fibers**

Quantitative assessment of liver fibrosis was performed on sirius red stained sections. The data were obtained using Leica Qwin 500 image analyzer computer system (England). Using the measuring field menu the area, area % and standard measuring frame of a standard area equal to 763882 µm² were chosen from the parameters. The percentage of the fibrosis area over the whole observed field was assessed to represent the degree of hepatic fibrosis. Several readings were obtained in each specimen (from 6 slides per animal) and at least ten random fields were measured in each slide (28).

**The fibrosis grade**

The grade of liver fibrosis in liver sections of all animals was estimated according to Ishak's modification of HAI score (29).

**Assessment of liver functions**

The serum samples obtained by centrifugation for 10 min at 3000 g at 4°C were kept frozen at -80°C until assayed. The levels of serum glutamate-oxalate-transaminase (SGOT), glutamate-pyruvate-transaminase (SGPT) and alkaline phosphatase (ALP) were assayed according to the routine biochemical analysis system using clinical test kits (Roche, Germany) spectrophotometrically (Cobas Mira Plus, Germany).

**Statistical Analysis**

The data are presented as means ± SD. The data obtained were subjected to statistical analysis using T-test. The significance level was set at P<0.05.

**RESULTS**

**Liver parenchyma**:

The histological appearance of the liver in all rats of the control group was formed of the classical hepatic lobules. The lobules were formed of cords of hepatocytes forming flat, anastomosing plates radiating...
from the central vein and separated by hepatic sinusoids (figure 1a).

After four weeks of ccl4 injection, most of the hepatocytes had pale cytoplasm with small vacuolations (microvesicles) while some of them had large vacuoles (macrovesicles). These changes appeared mostly in the hepatocytes around central veins and most of them had condensed nuclei. Multiple areas of liver cells destruction at the interface between parenchyma and connective tissue together with infiltration of the mononuclear inflammatory cells beyond the connective tissue-parenchyma interface had been detected. Mononuclear cellular infiltration was seen in most of the portal areas. After 8ws of ccl4 injection, there was increase in the cytoplasmic vacuolation and a marked distortion of the liver architecture. Obvious nodular fibrosis with deposition of well-delineated fibrous tissue septa had been detected (figure 1b).

In liver sections of the animals that received losartan, propolis or combined losartan and propolis either simultaneously with CCl4 injection for 8 ws or after 4ws from ccl4 administration, the liver parenchyma showed mostly proper cord arrangement. However, multiple microvacuoles appeared in the hepatocytes and increased in size near the portal vein (figure 1c). The improvement in histological picture was more apparent with longer period of treatment as well as in the combined and propolis groups than in losartan group.

**Liver fibrosis:**

The hepatic lobules in control animals were demarcated by thin connective tissue formed of collagen fibers containing bile ducts, lymphatics, nerves and blood vessels. Fine collagen fibers appeared to surround the central veins (figure 2a).

After four weeks of ccl4 injection there was increase in the amount of collagen fibers in the portal areas and around the central veins. Thin short septa could be seen extending from most of the portal areas and also the central veins into the surrounding parenchyma. Fibrous tissue septa (bridging fibrosis) could be seen linking the central veins together (centro-central) (figure 2b). After 8ws of ccl4 injection, there was
marked increase in the amount of collagen fibers in the portal tracts and around the central veins. Thick well developed septa could be seen throughout the sections. These septa were extending from the portal areas and central veins connecting them together. There was marked distortion of the liver architecture with pseudolobules formation (figure 2c).

In liver sections of animals that received losartan, propolis or combined losartan and propolis simultaneously with CCl₄ injection for 8ws, there was minimal increase in the amount of collagen fibers around central veins and in the portal areas as compared with the control. Few, thin, short septa could be seen radiating from some portal tracts and central veins into surrounding parenchyma with occasional porto-central and centro-central septal bridging. However, the septa were thicker in propolis treated group and least in the combined group of losartan and propolis (figure 3).

Image analysis of sirius red stained liver sections revealed that the area occupied by collagen fibers was 3.08 ± 1.29%. The percentage of area occupied by collagen fibers and the grade of liver fibrosis according to Ishak score in the different groups are given in figures 4 & 5. Although, there is significant (P ≥ 0.05) reduction in fibrosis area and score in all treatment groups in comparison to ccl4 injection for 8ws, the groups that received treatment during the last 4ws of ccl4 injection did not show significant reduction in comparison to ccl4 injection for 4ws i.e. in comparison to the starting point. In the propolis-treated group, there was even a significant increase in the grade of fibrosis compared to the model group of ccl4 for 4ws.

**Alpha-SMA immunohistochemistry:**

In control animals, alpha-SMA positive reaction was detected in the muscle layer of the portal veins and hepatic arteries within the portal tracts. The terminal hepatic venules revealed discontinous single layer of α-SMA positive cells. Hepatocytes, bile duct epithelial cells, endothelial cells lining the sinusoids and Kupffer cells were all negative for α-MA (figure 6a).
There was increase in the number of $\alpha$–SMA positive cells in the sinusoidal wall after 4ws of Ccl4 injection. Alpha-SMA positive stain could be detected around the central veins and in the thin fibrous band (figure 6b). At 8ws, alpha-SMA positive cells were distributed in the thick fibrous tissue septa (figure 6c).

In the animals that received treatment simultaneously with CCl4 for 8ws, few $\alpha$–SMA positive cells appeared only in the perisinusoidal spaces mostly in the pericentral areas. However, $\alpha$–SMA positive stain was also observed along the thin fibrous septa in propolis treated group (figure 7a, d, j). When the treatment was confined to the last 4ws of CCl4 injection period, more alpha-SMA positive cells were observed. Alpha-SMA expressing cells were seen in the portal areas, perivenular areas and adjacent perisinusoidal spaces. Alpha-SMA positive cells also appeared along the thin fibrous tissue septa (figure 7b, c, e, f, h and g).

The immunoreactive cells were more numerous in the propolis treated groups than losartan groups which in turn were more than the combined groups.

**Serum enzymes:**

The serum levels of glutamate-pyruvate-transaminase (SGPT), glutamate-oxalate-transaminase(SGOT) and alkaline phosphatase (ALP) were all within normal range in all rats of the control group (figures 8a, b and c). Marked elevation of enzyme levels followed administration of ccl4 for 4 or 8ws. The serum enzymes levels were lowered significantly ($P \geq 0.05$) in all treatment groups as compared to ccl4 injection for 8ws but still significantly ($P \geq 0.05$) higher than control. Starting the treatment simultaneously with ccl4 was more effective in lowering enzyme levels than starting it after 4 weeks from ccl4 injection. Propolis and combined treatment gave better results than losartan (figures 8a, b and c).
Figure 1: Photomicrographs of liver sections from albino rats. 1a: control group showing hepatic lobules formed of cords of hepatocytes radiating from the central vein and separated by hepatic sinusoids. 1b: CCl₄ injected group for 8ws; marked cytoplasmic vacuolations (arrows) and necrotic cells (arrow heads) are observed. Obvious nodular fibrosis with deposition of well-delineated fibrous tissue septa (*) can be detected. 1c: combined losartan and propolis simultaneously administered with CCl₄ injection for 8 ws, the liver parenchyma showed mostly proper cord arrangement. However, microvacuoles appeare in the hepatocytes and increase in size near the portal vein (arrows). (H&E X 40)

Figure 2: Photomicrographs of liver sections from albino rats. 2a: control group showing normal distribution of collagen fibers around the central vein (v) and in the portal tract (p). 2b: liver section of rat received CCl₄ injection for 4ws showing increase in the amount of collagen fibers as compared to control. Thin septa could be seen extending from the central veins into the surrounding parenchyma. Some of these septa are linking the central veins together and to the portal (arrows). 2c: CCl₄ injection for 8ws showing thick well-developed septa (arrows) throughout the section with pseudolobules (Lo) formation. (Sirius red X 40)
Figure 3: Photomicrographs of liver sections from albino rats of losartan (3a, 3d), propolis (3b, 3f) and combined losartan and propolis (3c, 3g) treated groups: minimal increase in the amount of collagen fibers (red) around central veins and in the portal areas is observed in simultaneous treatment with CCl4 injection for 8ws (3a, 3b, 3c). Few thin short septa are seen radiating into surrounding parenchyma (arrows). The septa are thicker in propolis treated group (3b). Treatment after four weeks (3d, 3f, 3g) from the beginning of CCl4 injection shows increase in the amount of fibrosis with occasional porto-central and centro-central bridging (arrows). Relatively thick septa extending into the surrounding parenchyma with multiple bridging and pseudolobules (Lo) formation are observed in propolis treated sections (3f). (Sirius red X 40).
Figure 4: Histogram of the percentage of area occupied by collagen fibers in Sirius red stained sections. For all treatment groups there is significant (* $P \geq 0.05$) decrease in percentage of area occupied by collagen fibers in comparison to CCl4 injection for 8ws. However, the percentage area is still significantly (# $P \geq 0.05$) higher relative to control. Groups that received treatment for the last 4ws of CCl4 injection are comparable to CCl4 injection for 4ws. Abbreviations: Control (C); Carbon tetrachloride (CCl4); Losartan (L); Propolis (P); Losartan plus propolis (L&P).

Figure 5: Histogram of the grade of liver fibrosis according to Ishak score. For all treatment groups there is significant (* $P \geq 0.05$) decrease in grade of liver fibrosis in comparison to CCl4 injection for 8ws. But the grades are still significantly (# $P \geq 0.05$) higher relative to control. Groups that received treatment for the last 4ws of CCl4 injection are comparable to CCl4 injection for 4ws except for propolis-treated group which is significantly higher.
Figure 6: Photomicrographs of liver sections from albino rats. 6a: control group showing the α-SMA positive cells in the muscle layer of vessels in portal tract (arrows). 6b: liver section of rats received CCl4 injection for 4ws, showing α-SMA positive cells in the fibrous band (arrows). 6c: CCl4 injection for 8ws, showing more α SMA positive cells distributed in the thick fibrous tissue septa (arrows).

(Immunohistochemistry for α- SMA  X 100).
Figure 7: Photomicrographs of liver sections from albino rats of losartan (7a, 7d), propolis (7b, 7f) and combined losartan and propolis (7c, 7g) treated groups: few α-SMA positive cells in the perisinusoidal wall within the liver parenchyma (arrows) are observed in simultaneous treatment with CCl4 injection for 8ws (7a, 7b, 7c). Treatment after four weeks (7d, 7f, 7g) from the beginning of CCl4 injection shows increase in the α-SMA positive cells in the sinusoidal wall around the central veins and along the thin fibrous tissue septa (arrows). α-SMA immunoreactivity is more in propolis treated groups (7b, 7f). (immunohistochemistry for α-SMA X100)
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Figure 8: Histogram of the serum enzyme levels of liver markers: serum glutamic-pyruvic transaminase (SGPT); serum glutamic oxaloacetic transaminase (SGOT) and alkaline phosphatase (ALP). Note the marked elevation of enzyme levels that followed administration of ccl4 for 4 or 8ws. The serum enzymes levels were lowered significantly (* $P \geq 0.05$) in all treatment groups as compared to ccl4 injection for 8ws but still significantly (# $P \geq 0.05$) higher than control. Starting the treatment simultaneously with ccl4 was more effective in lowering enzyme levels than starting it after 4 weeks from ccl4 injection. Abbreviations: Control (C); Carbon tetrachloride (CCl4); Losartan (L); Propolis (P); Losartan plus propolis (L&P); significant elevation in comparison to ccl4 injection for 4ws ($\$)$.
DISSCUSSION

Hepatic fibrosis is strongly associated with oxidative stress, increased transforming growth factor beta, hepatocyte death, and chronic inflammation (30). Chronic damage to the liver in conjunction with the accumulation of ECM proteins results in fibrosis, which is a characteristic of most types of chronic liver diseases (2). Activated hepatic stellate cells (HSCs) are the main ECM-producing cells in the injured liver. They migrate and accumulate at the sites of tissue repair, secreting large amounts of ECM and regulating ECM degradation (31). HSCs are supposed to be one of the most important cell targets of the pathogenetic action of angiotensin II for liver fibrosis and portal hypertension by accelerating the activation process of these cells (21). Key components of angiotensin system are locally expressed in normal liver tissue and upregulated in chronically injured livers (19) and activated HSCs de novo generate angiotensin II (20). Because the angiotensin II type 1 receptors (AT1R) antagonists have been used clinically for cardiovascular disorders, and have been confirmed as safe and without major side effects, the study of the effects of AT1R antagonists on liver fibrosis opens new possibilities for the prevention and treatment of hepatic fibrosis (32; 33). In the present study, losartan (an AT1R antagonist) was given to animals simultaneously with ccl4 for 8 weeks or after 4 weeks from start of ccl4 treatment. Alpha-SMA positive stain was markedly diminished in all sections of the livers of animals that received losartan treatment simultaneously with CCl4 for 8ws with a significant reduction in fibrosis area and score in comparison to ccl4 injection alone for 8ws. When the treatment started after fibrosis has begun (after 4ws) the fibrosis process slowed or even stopped with down regulation of active HSCs as evident by reduction of alpha-SMA positive stain. The active HSCs were more than control as evident by more alpha-SMA positive stain but significantly less than ccl4 injection alone for 8 weeks. The fibrosis area and score were also significantly decreased. Previous studies also demonstrated the inhibiting effect of losartan on liver fibrosis (32; 24; 25). Losartan has also been tested in some clinical trials as Sookoian et al. (34) and Yokohama et al. (35) who evaluated the safety and efficacy of chronic administration of lo-
sartan on hepatic fibrosis in chronic hepatitis C and non-alcoholic steato-hepatitis and reported that losartan significantly decreased the fibrosis stage with remarkable decrease in activated HSCs. Angiotensin II type 1 receptor antagonist induces its effect on HSCs and in turn on liver fibrosis by either of two mechanisms of action: first, inhibits activated HSCs by blocking AT1R expressed on the surface of HSCs; second, it suppresses the activation of HSCs as a result of the decrease in transforming growth factor beta (TGF-β1) (36). TGF-β1 activates directly HSCs, stimulates synthesis of multiple ECMs, inhibits ECM degradation by stimulating the production of tissue inhibitors of metalloproteases and increasing its own synthesis (30).

Oxidative stress is associated with liver fibrosis and with HSCs activation in vivo. Hepatocytes undergoing oxidative stress release factors which are fibrogenic for HSCs (37). Many antioxidants such as vitamin E, silymarin, phosphatidylcholine, and S-adenosyl-L-methionine have been shown to inhibit HSC activation, protect hepatocytes from undergoing apoptosis, and attenuate experimental liver fibrosis (31). Propolis has proved to possess strong antioxidant and scavenging abilities and significantly decreased lipid peroxidation processes (LPO) in plasma, liver, lungs, and brain of mice (38). It inhibited oxidative stress induced by CCl4 when the extract was added to the isolated hepatocytes in vitro (39). Its antioxidant activity depends on the presence of flavonoids (6; 7). Many flavonoids were tested in liver fibrosis and proved to be effective in reducing fibrosis (40; 41). A protective effect of propolis on hepatic fibrosis induced by either dimethylnitrosamine or CCl4 in rats was shown by Gergerlioglu et al., (17) and Bhatdaria (16). In the present study, propolis protected the liver parenchyma and significantly reduced the fibrosis area and score and alpha-SMA positive stain when introduced simultaneously with CCl4, however, its effect on fibrosis was less than that of losartan and combined treatment. Starting propolis treatment after 4ws from CCl4 injection did not improve the picture than the starting point.

In this study, the markers of liver injury SGPT (ALT), SGOT (AST) and ALP were significantly reduced in the treated groups as compared to CCl4 injection for 8ws, however, these lev-
els were still significantly higher than control values. Starting the treatment after 4 weeks of CCl4 injection reduced the serum levels of enzymes to a lesser degree. The reduction in the serum levels of the enzymes was more prominent with propolis and combined treatment than with losartan alone. Previous studies also showed that prophylactic treatment with propolis had a significant effect on reduction of the elevated ALT, AST and lipid peroxides and elevation of the reduced Glutathione (GSH) in blood and liver after acute hepatotoxicity \cite{12, 13, 14} and chronic liver injury \cite{16, 17}.

In this study, in all periods of treatment, losartan was more effective in inhibition of HSCs and of fibrosis while propolis was more effective in decreasing enzyme levels that may indicate more protection of hepatocytes. Combined treatment of the two drugs that have different mechanism of action gave better reduction of both fibrosis and enzyme levels. Reversal of liver fibrosis by treatment with losartan and propolis was not evident in this study.

**REFERENCES**


toxicity induced by CCL4 in rats. Journal of Hepatology; 34 (0): 30-36.


sions in rats ± usefulness and reliability of this animal model. Exp. Pathol.; 34:229-36.


36. Ahmad, A. and Ahmad, R.


