Candesartan modulates the antioxidant effect of silymarin against CCl_4-induced liver injury in rats

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ABSTRACT

Aim: Liver fibrosis represents the final common pathway of chronic hepatic inflammation. The efficiency of single drug is limited in liver fibrosis. Consequently, combined therapy is more effective than monotherapy. Therefore, the aim of this study was to investigate the preventive effect of candesatran (CAN), silymarin (SIL) and their combination in CCL_4-induced liver fibrosis. **Methods:** Rats were divided into five groups: Control, CCL_4-treated rats, CAN-treated rats (2 mg/kg/day, orally), SIL-treated rats (100 mg/kg/day, orally) and their combination (SIL+CAN)-treated rats. All groups were treated for 7 weeks. ALT, AST and GGT were determined in serum. TNF-α, oxidative stress parameters (MDA, GSH levels and SOD activity) and NO level were measured in liver tissue. Other liver tissues were examined histopathologically. **Results:** CCL_4 induced marked elevation of ALT, AST and GGT. Moreover, CCL_4 increased liver tissue of TNF-α, MDA and NO contents and decreased GSH level and SOD activity. Administration of either CAN or SIL significantly alleviated CCL_4-induced biochemical changes. On the other hand the combined administration of CAN with SIL has an ameliorative effect which is greater than each drug alone. **Conclusion:** The combination therapy between CAN and SIL is more effective than either drug alone which is attributed to augmentation of their antioxidant effects.

Keywords: Fibrosis, Angiotensin II, TNF-α, Oxidative Stress.

1. INTRODUCTION

Hepatic fibrosis is a common pathological condition resulted from chronic liver injuries and it is characterized by progressive deposition of an altered extracellular matrix (ECM).[1] Oxidative stress plays a basic role in initiation and development of liver damage. It induces necrosis and apoptosis of hepatocytes, inflammatory response and directly activates hepatic stellate cells (HSCs), resulting in the initiation of fibrosis.[2]

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Angiotensin II (Ang II) is the main component of renin–angiotensin system (RAS). Ang II binds to two receptor subtypes, Ang II type 1 and type 2 (AT1 and AT2) receptors. Angiotensin receptor blockers (ARBs) are highly selective for the AT1 receptor and block the deleterious effects of Ang II, such as vasoconstriction, aldosterone release, retention of sodium and water, sympathetic nerve activation and cell proliferation.[3] ANG II has a significant effect in the pathogenesis of liver fibrosis, it induces proliferation and contraction of HSCs and extracellular matrix deposition in hepatic cells leading to hepatic dysfunction.[4] Based on this background, blocking the ANG II receptors may inhibit collagen type-1 synthesis, fibrogenic cytokines expression and reduce oxidative stress in fibrotic liver.

Candesartan (CAN) is a selective AT1 receptor blocker used as an antihypertensive drug. In experimental models, CAN significantly attenuates tissue fibrosis by decreasing production of ECM.[5] Using of CAN in chronic liver disease is not fully investigated.

Silymarin (SIL), is a traditional hepatoprotective drug, which has many properties including: antioxidant, antifibrotic, and anti-inflammatory effects. Silymarin has a wide clinical applications in alcoholic liver diseases, liver cirrhosis, toxic and drug induced liver diseases.[6]

Hence, renin–angiotensin system is a new target in ameliorating chronic hepatic fibrosis. Therefore, we conducted this study to investigate the preventive effect of combination therapy between CAN and SIL on CCL_4 induced liver fibrosis.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Drugs and chemicals

Silymarin (SEDICO CO, 6-October City, Egypt), Candesartan (Pharaonia-pharo pharma, Alexandria, Egypt), Ellman's reagent, L-Hydroxyproline, Reduced glutathione and Tetramethoxypropane (Sigma-Aldrich Company, St. Louis, MO, USA). All other chemicals were of analytical grade.

2.1.2 Animals

Male Swiss albino rats weighing 170–200 g were housed in the animal house of the Faculty of Medicine, Assiut University. The rats were kept under the same environmental conditions and provided with their dietary requirements consisting of standard diet pellets. Food and water were given *ad libitum*. Animals left one week to acclimatize with environmental conditions. Experiments were conducted in accordance with the internationally accepted guidelines for animal care.

2.2 Methods

2.2.1 Experimental protocol

The animals were divided into 5 groups, each of them contain 10 rats (n=10).

Group I: Control group injected with olive oil 3 times weekly for 7 weeks.

Group II: Liver fibrosis was induced by injecting of 1.5 ml/kg, ip of diluted CCL_4 (1:7) in olive oil; 3 times per week for 7 weeks.[7]

Group III: Rats injected with CCL_4 and treated simultaneously with SIL for 7 weeks in a dose of 100 mg/kg/day, orally.[8]

Group IV: Rats injected with CCL_4 and treated simultaneously with CAN for 7 weeks in a dose of 2 mg/kg/day, orally.[9]

Group V: Rats injected with CCL_4 and treated simultaneously by SIL and CAN in the same previous doses.

At the end of 7th weeks, animals were anaesethized with light ether and blood samples were withdrawn directly by heart puncture for separation of serum. Portion of liver was kept in 10% formalin buffer for histological examination. Another part of liver was cut into small pieces then homogenized in ice-cold 0.1 M phosphate buffer saline (PBS) pH 7.4 then stored at −40°C for biochemical analysis.

2.2.2 Assessment of liver function testes

Serum aminotransferases (ALT and AST) were determined according to the method of Reitman and Frankel.[10] Serum GGT was determined according to the method Szasz.[11] All kits purchased from Diamond Co., Egypt.

2.2.3 Determination of hepatic TNF-α levels in liver tissues

Hepatic content of TNF-α was determined with enzyme-linked immunosorbent assay kit (Wkea med supplies Corp, Changchun Jilin, China).

2.2.4 Measurement of oxidative stress in liver tissue

Hepatic malondialdehyde (MDA) concentration determined according to method of Uchiyama and Mihara.[12] Reduced glutathione (GSH) measured using Ellman reagent.[13] Whereas, SOD activity measured by method of Nishikimi et al.[14] Nitric oxide (NO) liver tissue content was determined by colorimetric method described by Montgomery and Dymock.[15]

2.2.5 Histopathological examination

Paraffin tissue blocks were prepared for sectioning and the obtained sections were stained by hematoxylin & eosin stain then examined through the light electric microscope.

2.2.6 Statistical analysis

Statistical analysis was achieved using GraphPad Prism 5.0 software (GraphPad, San Diego, Ca., USA). Data were presented as mean ± SE. Multiple comparisons between any

two groups for all data were assessed by one way ANOVA with Tukey-Kramer test as multiple comparison post ANOVA test. Statistical significance was defined as $P < 0.05$.

3. RESULTS

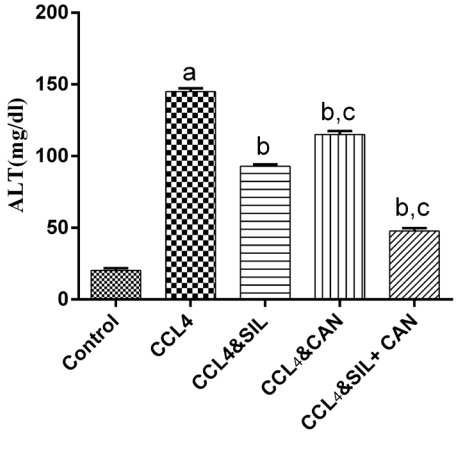
3.1 Effect of SIL or/and CAN on liver function tests

Administration of CCL_4 induced significant increase in serum levels of ALT, AST and GGT with respect to control rats. The serum levels of ALT, AST and GGT have been greatly reduced after oral administration of SIL or CAN compared to fibrotic rats but CAN was less effective than SIL. Administration of both SIL and CAN in concomitant with CCL_4 markedly decreased the serum ALT, AST and GGT levels compared to group treated with either SIL or CAN alone (Figures 1, 2 & 3, respectively).

3.2 Effect of SIL or/and CAN on hepatic TNF-α

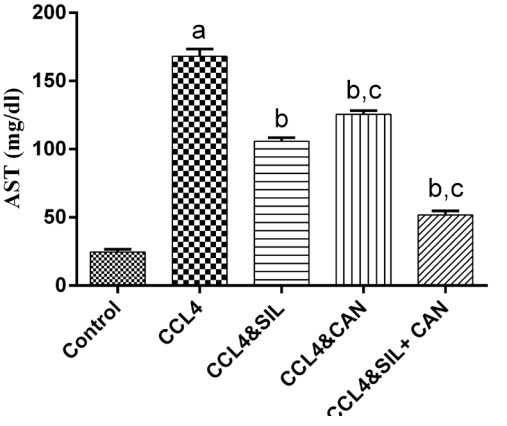
Regarding to the control group, the liver contents of TNF-α were found markedly higher in model rat. Animals given SIL or CAN significantly attenuated the level of TNF-α as compared to model CCL_4-treated rats. TNF-α levels of CAN treated rats were still significantly higher than SIL group. However, the level of TNF-α in combination treated animals (SIL plus CAN) was markedly lower than either SIL or CAN groups (Table 1).

Figure 1. Effect of SIL, CAN or their combination on serum levels of ALT



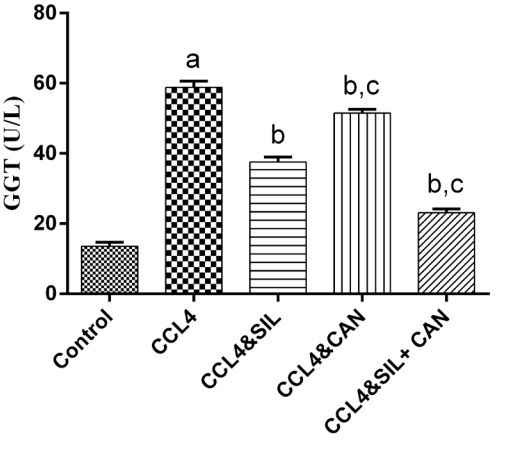
Results are expressed as mean± SE, (a, b and c) significant difference from control, CCL_4 and SIL, respectively (n=10) at $P < 0.05$.

Figure 2. Effect of SIL, CAN or their combination on serum levels of AST



Results are expressed as mean± SE, (a, b and c) significant difference from control, CCL_4 and SIL, respectively (n=10) at $P < 0.05$.

Figure 3. Effect of SIL, CAN or their combination on serum levels of GGT



Results are expressed as mean± SE, (a, b and c) significant difference from control, CCL_4 and SIL, respectively (n=10) at $P < 0.05$.

Table 1. Effect of SIL, CAN and their combination on liver content of TNF-α, MDA, GSH, SOD and NO in CCL_4-rats

Parameters	Control	CCL_4	CCL_4 + SIL	CCL_4 + CAN	CCL_4 + (SIL+CAN)
TNF-α (ng/g tissue)	25.14 ± 1.15	82.50 ± 4.89[a]	51.17 ± 1.03[b]	58.18 ± 1.41[b]	25.18 ± 1.45[b,c]
MDA (nmol/g tissue)	17.89 ± 1.87	192.1 ± 6.56[a]	81.52 ± 4.03[b]	136.7 ± 5.11[b,c]	40.25 ± 3.73[b,c]
GSH (µmol/g tissue)	8.108 ± 0.22	1.351 ± 0.1[a]	4.801 ± 0.18[b]	3.659 ± 0.136[b,c]	7.470 ± 0.29[b,c]
SOD (U/g tissue)	2320 ± 61.03	777.3 ± 44.34[a]	1697 ± 67.47[b]	1600 ± 110.5[b]	2041 ± 77.0[b,c]
NO (µmol/g tissue)	25.47 ± 1.172	94.23 ± 4.21[a]	64.36 ± 2.20[b]	75.39 ± 1.72[b]	36.77 ± 3.05[b,c]

Results are expressed as mean ± SE, (a, b and c) significant difference from control, CCL_4 and SIL, respectively (n=10), at $P < 0.05$.

3.3 Effect of SIL or/and CAN on hepatic oxidative stress parameters

Table 1 shows that, MDA content significantly increased in the liver of rats treated with CCL_4 compared to control. The MDA content decreased in SIL or CAN treated rats with respect to fibrotic rats. However, MDA content of SIL-treated group was significantly lower than CAN-treated rats. The administration of combination therapy to rats (SIL and CAN) in concomitant with CCL_4 markedly diminished the levels of MDA compared to group treated with SIL or CAN alone. On the other hand, the hepatic content of GSH and SOD activity notably reduced in CCL_4 challenged rats with respect to control. Treatment of rats by SIL or CAN significantly improved the levels of GSH content and SOD activity compared to CCL_4-treated rats but GSH content in rats treated by CAN still significantly lower than that treated by SIL. However, GSH and SOD levels of SIL plus CAN treated group were markedly greater than either SIL or CAN treated groups.

3.4 Effect of SIL or/and CAN on hepatic NO content

The hepatic content of NO was significantly increased in CCL_4-model group compared to control. SIL or CAN notably attenuated the hepatic NO content compared to model rats. The combination regimen notably reduce the level of NO compared to CCL_4-treated rats. This reduction was significantly higher than groups treated with SIL or CAN alone (Table 1).

3.5 Histological examination

There was normal histological structure of the central vein and hepatocytes of control liver (A). Hepatic tissue of CCl_4-treated rats showed sever necrosis, fatty changes with ballooning degeneration and mononuclear cell infiltration in the hepatocytes surrounding the dilated central vein (B). Group of rats administrated CCL_4 with SIL showed fatty changes and ballooning degeneration which were detected in most hepatocytes surrounding the central vein (C). Rats treated with CAN have a moderate mononuclear cell infiltration, ballooning degeneration, fatty changes and necrosis in the hepatocytes surrounding the dilated central vein (D). The combination group (SIL+CAN) showed normal histological structure of the central vein (CV) and hepatocytes with some hydropic degeneration (d) of the hepatocytes (E). (Figure 4).

4. DISCUSSION

The renin angiotensin system (RAS) antagonists are group of therapeutic drugs with promising pharmacological action on liver fibrosis. Here, we tried to investigate the hepatic preventive relevance of CAN and its combination with SIL against CCL_4-induced liver fibrosis and the underlying mechanism of this effect.

CCL_4 is one of experimental models used in induction of liver fibrosis however it is resemble most important properties of human liver fibrosis. The CCL_4 administration results in hepatocyte damage, necrosis, free radicals production, inflammation, and fibrosis. In the present study, the hepatotoxicity of CCL_4 in rats was confirmed by a significant elevation of AST, ALT and GGT. This might be due to the release of these enzymes from the cytoplasm, into the blood rapidly after rupture of the hepatic plasma membrane and cellular damage.[16]

TNF-α is one of pro-inflammatory cytokines in liver injuries. The results of the current study indicated that CCL4 produced significant elevation in hepatic level of TNF-α. TNF-α stimulates the expression of tissue inhibitor of metalloproteinase (TIMP) in HSCs. Therefore, it decreases metalloproteinase enzymes activity as well as decrease matrix degradation.[17] Also, TNF-α is involved in the onset of lipid peroxidation, cell membrane disruption and replacement of the necrotic area with connective tissue.[18]

Oxidative stress has been implicated in the process of liver fibrogenesis and many etiological agents of fibrogenesis. It is well documented that CCL_4 causes hepatotoxicity via generation of free radicals which cause disturbance in cellular antioxidant defenses. In the current study, CCL_4 resulted in a significant increase in the hepatic MDA concentration as well as depletion of GSH content and SOD activity. CCL_4 metabolized by cytochrome P-450 to form reactive metabolite, trichloromethyl, that initiates the lipid peroxidation of cell membrane. Lipid peroxidation has been implicated in the pathogenesis of various liver injuries and subsequent liver fibrogenesis.[19] In addition; generation of lipid peroxides in injured hepatocytes may have a direct stimulatory effect on matrix production by activated HSCs.[2]

GSH is the most important internal antioxidant defense and plays a critical role in regulating a variety of cellular functions. It can reduce H_2O_2 and lipid peroxide through GPx-catalyzed reactions and can conjugate and detoxify electrophiles through glutathione S-transferase catalyzed reaction.[1] SOD is a major enzyme responsible for scavenging of superoxide radicals to H_2O_2 and H_2O. Lipid peroxidation and excess free radical generation could impair the natural defense mechanism of this enzyme.[20] In the present study, the hepatic content of GSH as well

Figure 4. Histopathology of liver.

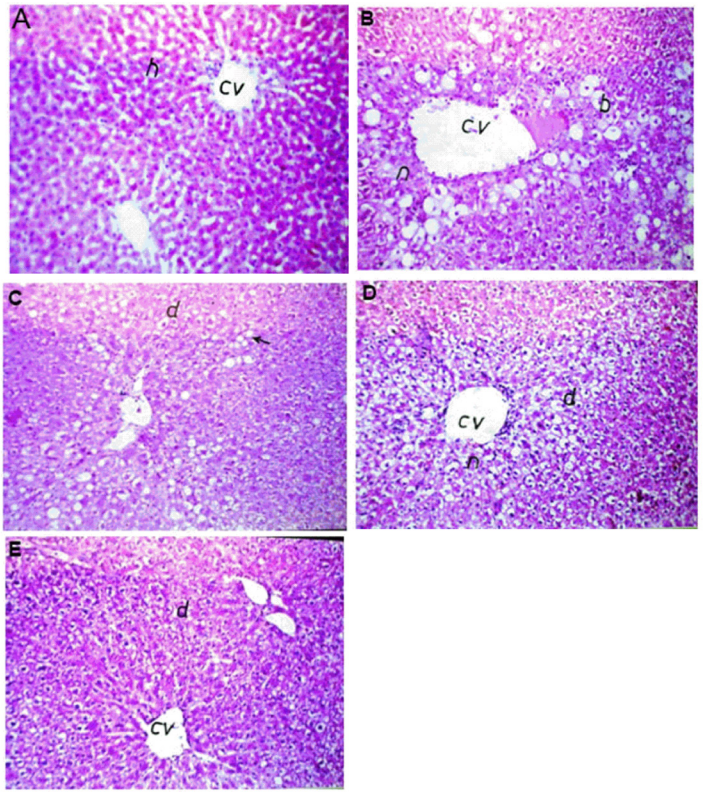


Figure 4. Photograph showing normal histological structure of the central vein (CV) and hepatocytes (h) in control rat liver (A). Fibrotic rats, showing sever necrosis (n), fatty changes with ballooning (b) degeneration and mononuclear cell infiltration in the hepatocytes surrounding the dilated central vein (CV) (B). SIL group showed fatty change (arrow) and ballooning degeneration (d) in most hepatocytes (C). Rats treated with CAN have a moderate mononuclear cell infiltration, ballooning degeneration (d), fatty changes and necrosis (n) in the hepatocytes surrounding the dilated central vein (CV) (D). The combination group (SIL+CAN) showed normal histological structure of the central vein (CV) and hepatocytes with some hydropic degeneration (d) of the hepatocytes (E).

as SOD activity were found to be decreased significantly in CCL_4-intoxicated rats as compared with control rats.

NO is created by inducible nitric oxide synthase (iNOS) which is activated by inflammatory cytokine like TNF-α and NF-ϰB.[21] The toxicity of NO starts by reacting with superoxide anion to form peroxynitrite and formation of oxidative stress, which can be dangerous through liver injury. Peroxynitrite can react directly with lipid, protein and DNA resulting in cellular dysfunction and tissue damage.[22] In our study, the level of hepatic NO in CCL_4 treated group was significantly higher than the control group.

Regarding, the hepatoprotective effect of SIL against CCL_4-induced liver injuries in rats, It was found that treatment with SIL leads to significant decrease in serum ALT, AST and GGT levels than in CCL_4-treated rats. This indicates that SIL tends to stabilize the membrane permeability lading to suppressing the leakage of enzymes through membranes, exhibiting hepatoprotective activity.[23] Furthermore, SIL exhibited more improvement in pathological changes in the form of reduced the fatty changes, hepatic degeneration and necrosis in the liver. These effects are related to antioxidant activities of SIL.

The current study showed that level of TNF-α was decreased in rats treated with SIL as compared with group treated with CCL_4 alone and this was probably due to the inhibitory effect of SIL on inflammatory cytokines. It inhibits neutrophil migration, stabilizes mast cells and affects the synthesis of prostaglandins and leukotrienes.[24] Kim et al.,[25] reported that silibinine, a component of SIL, is able to inhibit the production of TNF-α through inhibition of NF-ϰB pathway.

SIL significantly abolished the lipid peroxidation and enhanced GSH content and SOD activity. The protective effect could be explained on the bases that SIL acts as free radical scavenger depending on its phenolic structure. It inhibits free radical generation, which can prevent hepatic glutathione depletion, lipid peroxidation and

improves the SOD activity.[6] Furthermore; SIL caused a significant reduction in NO level in fibrotic rats. This effect is explained by the ability of SIL to inhibit expression of NOS and cause elevation of GSH level.[26]

Of interest, CAN administration corrected the levels of serum aminotransferases (ALT & AST) and GGT as compared to that of fibrotic rats. Simultaneously, CAN attenuated the hepatic fibrosis in treated rats. This result was further supported with histological evaluation.

ANG II increases TNF-α expression through activation of the NF-κB/TNF-α pathway depending on ROS signaling mechanism through AT1 receptor activity.[27] Alternatively, CAN treatment decreases TNF-α level in liver which is due to the decrease in TNF-α expression, reduction of NF-κB activation and reduced ROS production. The protective effect of CAN in hepatic fibrosis is likely to be mediated by its anti-inflammatory action.

ANG II is reported to stimulate the formation of ROS in hepatic cells and mediates its fibrogenic effect.[28] Our results showed that CAN suppressed the level of MDA and prevent the depletion of hepatic GSH content and SOD activity in animals given CCL_4. CAN anti-oxidative effect was attributed to blockade of angiotensin receptor and inhibition of ANG II-induced generation of ROS and oxidative stress, so our data suggest that angiotensin receptors are involved in generation of ROS. In this context, it is likely that prevention of inflammation by CAN contributes to the observed anti-oxidant effect.

An important result of elevated ANG II is promoted activation of NADPH-oxidase, which is a major source of ROS. NADPH-oxidases may lead to an increase in superoxide ($O2^{\bullet-}$) generation, which causes cellular damage.[29] In addition, NADPH-oxidases expressed in the activated HSCs and play a role in liver fibrosis.[29] Therefore, ARBs would inhibit the ANG II-dependent oxidation pathway and thus decrease production of ROS with a resultant reduced lipid peroxidation and increase in both GSH content and SOD activity.

NO level in rats given CCL_4 with CAN was suppressed compared with CCL_4 treated group. This result supports the anti-inflammatory and antioxidant effect of CAN. ARBs, partially, decreased NO production by reducing iNOS expression and re-establishes the redox status.[30]

The complicated pathogenesis of liver fibrosis suggests that combination therapy may have greater benefits for the treatment of liver fibrosis than monotherapy. Combination therapies can target different sites of action phases of fibrogenesis. Consequently, we hypothesized that a combination therapy comprising SIL and RAS inhibitors might improve the conditions of liver fibrosis.

5. CONCLUSION

Therapy with ARB has limited effects, but combination therapy of these agents with SIL further reverses fibrosis markers in a rat model of liver fibrosis and potentiates the effect of each other. In conclusion, the antioxidant action of SIL is potentiated by CAN co-administration through its antioxidant effect that depends on RAS inhibition.

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