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Telmisartan inhibits the non-canonical TGF-β/JAK2/STAT3 signaling pathway and prevents carbon tetrachloride-induced liver fibrosis in rats

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Abstract This research purpose was to assess the potential inhibitory action of telmisartan on IL6/JAK2/STAT3 pathway in carbon tetrachloride (CCl4)-induced liver toxicity in rats. Carbon tetrachloride (1ml/kg; 50% in corn oil) was injected two times/week for 8 weeks in male Sprague-Dawley rats. Intoxicated rats with CCl4 were concurrently treated daily with telmisartan (10 mg/kg) for eight weeks. Telmisartan treatment markedly ameliorated the biochemical and the histological deviations in CCl4 intoxicated rats. As, telmisartan treatment mended the elevated serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities in CCl4 intoxicated group. Additionally, telmisartan significantly reduced oxidative stress parameters as lipid peroxidation and markedly increased total antioxidant capacity in liver tissue of CCl4 treated rats. Also, telmisartan treatment reduced inflammation as evidenced by the distinct decrease in hepatic interleukin-6 (IL-6) level. These biochemical results were further supported by the improvement in the altered histopathological architecture of liver tissue. Interestingly, telmisartan treatment down-regulated hepatic expression of JAK2/STAT3 in CCl4-intoxicated animals. Conclusively, the hepatoprotective action of telmisartan against CCl4-induced liver toxicity might be through mitigating oxidative stress, inflammation, and targeting IL-6/JAK2/STAT3 pathway.

Keywords: Telmisartan; Carbon tetrachloride; JAK2; STAT3; Oxidative stress

1. INTRODUCTION

Liver is the main site in which metabolism of ingested drugs, alcoholics, and other agents takes place after absorption, therefore it is mostly susceptible to chemical-induced injury. Pathological degree of hepatic injury differs from insignificant nonspecific alterations in liver structure and function to sever hepatic damage, fibrosis, and even hepatic cancer[1]. Currently, one of the therapeutic goals in both clinical and experimental researches is focused on reversing or blocking liver fibrosis, as its progression will end with hepatic liver failure and death. Many molecular and signaling pathways contribute in hepatic fibrosis pathogenesis[2]. The Janus Kinase/Signal Transducer and Activator of Transcription (JAK/STAT) pathway is amongst the distinguished signaling pathways involved in pathogenesis of liver fibrosis[3]. JAK/STAT is responsible for different genes expressions, these genes are involved in inflammatory reaction, immune responses, and apoptosis [4,5]. Different ligands activate JAKs and enhance their kinase activity resulting in activation of STATs[6], including interleukin (IL)-6[7]. Generally, activation of JAK protein by different cytokines causes activation of tyrosine residue receptors. The activated tyrosine sites consequently form precise "docking sites" with the surrounding sequences of amino acid, then the STAT protein is engaged to this "docking site",

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where it is immediately phosphorylated, and moves to the nucleus to bind to a specific gene and modifies its transcriptional mechanism[3,8]. Thus, utilization of therapeutic lines that focus on modulating JAK/STAT signaling in liver fibrosis is essential. Undeniably, renin-angiotensin aldosterone system (RAS) has been documented to principally contribute in the liver fibrosis pathogenesis[9]. In addition, angiotensin II (Ang II), the key actor of RAS, is one of the central ligands that activate JAK/STAT pathway[10,11]. Thus, pharmacotherapies targeting RAS are promising candidates for ameliorating hepatic fibrosis. Telmisartan is an Ang II type 1 receptor blocker (ARB) that is used to treat high blood pressure, heart failure, and diabetic kidney disease and has versatile actions against inflammation, oxidative stress, and apoptosis[12,13]. Telmisartan has demonstrated antifibrotic action against different experimental model of liver fibrosis[14,15]. However, its potential effect on JAK/STAT signaling as a molecular antifibrogenic mechanism remains uninvestigated. Therefore, this research was carried out with a central goal of assessing the molecular mechanism for the hepatoprotective action of telmisartan against CCl4-induced liver fibrosis through investigating its modulatory action on JAK2/STAT3 pathway.

2. MATERIALS AND METHODS

2.1. Chemicals

Telmisartan was kindly supplied by Biopharm for scientific researches and pharmaceutical manufacturing (Giza, Egypt). Carbon tetrachloride was obtained from El-Nasr Pharmaceutical Chemical Company, Egypt. Other used chemicals were of analytical grade.

2.2. Animals

Forty male Sprague-Dawley (180-200 g) were provided from the animal facility of El-Nile for Pharmaceutical and Chemical Industries (Cairo, Egypt). Rats were reserved at precise controlled housing conditions. Animals were left for fourteen days before the start of any experimental processes for acclimatization. Standard rat chow and water were provided to the animals ad libitum. Animal Ethics Committee (Faculty of Pharmacy, Al-Azhar University, Egypt) approved the design of this research (Approval number: 80/2016).

2.3. Treatment protocol

Concisely, animals were distributed to 4 groups (ten rats each) as follow; Group 1 (Control): animals were injected intraperitonially (I.P.) with normal saline for 8 weeks and considered as normal control group.; Group 2 (Corn oil): animals were I.P. injected with corn oil (1ml/kg) two times a week for eight weeks and considered as control vehicle group; Group 3 (CCl4): animals injected I.P. with 1:1 (v/v) CCl4 in corn oil (1 ml/kg), twice a week for 8 weeks[16]; Group 4 (CCl4+Telm): animals injected I.P. with 1:1 (v/v) CCl4 in corn oil (1 ml/kg), two times a week for 8 weeks and orally co-administrated daily telmisartan (10 mg/kg) for 8 week [17]. After 24 hours of last dose administration, blood was collected under mild anesthesia by retro-orbital sinus puncture. Blood was centrifuged, and serum was collected and stored at −80 °C till utilized for biochemical assays. Animals were sacrificed, livers were quickly collected, washed, and their weight were measured. Next, part of the liver tissues was homogenized in phosphate-buffered saline, and the clear homogenate was used for measuring biochemical parameters. Other parts of liver tissue were added in 10% formalin for histological examination, and the last parts were snap frozen to be used for western blot analysis.

2.4. Determination of liver index

Final body weights of the rats as well as their liver weights were recorded. The liver index was determined as it was calculated as liver-to-body weight %.

2.5. Colorimetric and enzyme-linked immunosorbent assays (ELISA)

Markers for hepatic toxicity including serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined utilizing colorimetric assay kits (BioMed Diagnostics, Egypt) depending on the instructions of manufacturer. Hepatic interleukin-6 (IL-6) was assessed using ELISA kit as stated by the recommendation of the manufacturer (MyBioSource, San Diego, USA). Hepatic transforming growth factor-β1 (TGF-β1) level was determined utilizing ELISA kit according to manufacturer's recommendations (MyBioSource, San Diego, USA).

2.6. Assessment of oxidative stress

Malondialdehyde (MDA) concentration, a commonly used biomarker of lipid peroxidation, was assessed by thiobarbituric acid method. It is constructed on the reaction of malondialdehyde with TBA at 98 °C. TBARs were determined calorimetrically using assay kit for lipid peroxidation (MDA) (Biodiagnostic, Giza, Egypt) depending on the instructions of manufacturer. Total antioxidant capacity (TAC) content was measured using commercial kit (Biodiagnostic, Giza, Egypt).

2.7. Western blot analysis

Phosphorylation of hepatic JAK2 and STAT3 proteins were assessed using Western blotting analysis. After blotting, blocking in 5% bovine serum albumin in Tis buffered saline containing 0.05% Tween (TBST) was performed. The membrane was processed with primary antibodies against p-JAK2 and p-STAT3 (1:1,000) (BioBasic Inc., Toronto, Canada) diluted in 1x TBST-buffer for 12 hours at 4 °C, afterwards, membrane was rinsed and incubated for one hour at room temperature with secondary antibody diluted in 1x TBST-buffer (1:10,000) before signal detection using enhanced chemiluminescence (ECL) system.

2.8. Histopathological examination

Liver tissue were fixed in 10% buffered formalin, dehydrated in ascending dilutions of ethanol, then embeded in xylene-paraffin. Sections (3 µm) were cut and stained with haematoxylin and eosin (H&E) reagent[18], then visualized under a microscope at 400× magnification, scale bar 25µm.

2.9. Statistical analysis

Data analysis was conducted utilizing statistical package for the social sciences (SPSS) program (version 13) statistical software. All results were expressed as mean ± S.D. Multiple comparisons were performed using ANOVA followed by Tukey test as a Post Hoc test. Significant differences between compared groups was established at a P values less than 0.05.

3. RESULTS

3.1. Telmisartan mitigated the alteration in liver index in carbon tetrachloride-intoxicated rats

Rats injected with carbon tetrachloride appeared weaker and exhausted at the end of the experimental protocol. Additionally, CCl4 treatment significantly decreased the body weight of intoxicated animals in comparison with control rats (p<0.05; Table 1). Meanwhile, animals received telmisartan showed negligible percentage of such symptoms and telmisartan markedly increased the body weight in CCl4-intoxicated animals (Table 1). To assess liver damage, liver index was calculated as liver weight/ body weight (LW/ BW) %. CCl4 significantly increased liver index of CCl4 treated rats in compared to normal group (p<0.05; Table 1). Telmisartan treatment reduced the increase in the liver index as compared with CCl4 intoxicated group (p<0.05).

Table 1. Effects of telmisartan treatment on carbon tetrachloride-induced changes on body weight and liver index% in rats.

Groups	Body Weight (g)	liver weight (g)	LW/BW (%)
Control	244.7 ± 18.2	6.27 ± 0.66	2.48 ± 0.16
Corn oil	252.7 ± 28.5	6.57 ± 1.29	2.45 ± 0.09
CCl4	202.3 ± 17.6$^{*\pi}$	6.86 ± 1.36	3.35 ± 0.04$^{*\pi}$
CCl4+Telm	220.1 ± 17.7$^{\pi}$	7.02± 0.67	3.14 ± 0.10$^{*\pi\delta}$

Results are represented as means ±S.D (n=6).$^{*}P$< 0.05, versus control group, $^{\pi}P$< 0.05 versus corn oil treated group, $^{\delta}P$ < 0.05 versus CCl4 treated group respectively, using ANOVA followed by Tukey as a post-ANOVA test.

3.2. Telmisartan treatment reduced hepatotoxic injury markers in carbon tetrachloride-intoxicated rats

In this study, CCl4 treatment induced liver toxicity that was evidenced from the elevation in serum aminotransferases concentration (ALT and AST). Fig. 2A and 2B show that, treatment with CCl4 markedly elevated ALT and AST compared to normal control group. Telmisartan markedly reduced ALT by 47.6 % and AST levels by 48.7%, respectively versus CCl4 intoxicated rats.

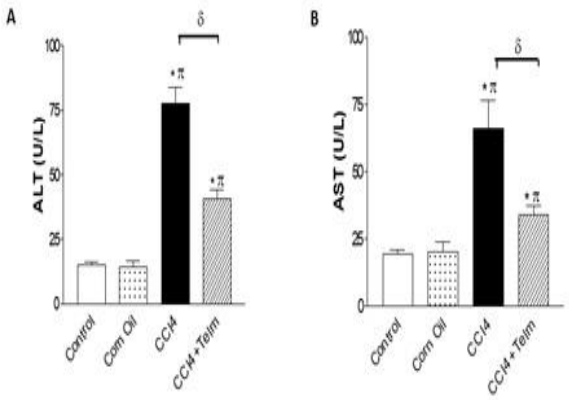

Figure 1. Effect of telmisartan treatment on serum ALT (A) and AST (B) in carbon tetrachloride-treated rats**.** Results are represented as mean ±S.D (n=6). *P < 0.01 versus control group, $^{\pi}P$<0.01 versus corn oil treated group, $^{\delta}P$ < 0.01 versus CCl4 treated group, respectively, using ANOVA followed by Tukey as a post-ANOVA test.

3.3. Telmisartan treatment reduced oxidative stress and inflammation in carbon tetrachloride-treated rats.

Figure 3A shows that TAC level in liver tissue of CCl4 treated animals was significantly reduced by 55.4 % versus normal group. Telmisartan significantly increased hepatic TAC content in intoxicated animals by 162.3% as compared to CCl4 group. Also, MDA was markedly greater ($p < 0.01$) in liver tissue of CCl4 treated animals (370%) in comparison with control group. Telmisartan treatment markedly mitigated the oxidative damaging effect of CCl4 as it reduced lipid peroxidation by 51.9% versus CCl4-treated rats (Fig. 3B). Additionally, inflammation in the hepatic tissue of CCl4 intoxicated animals was markedly elevated as evidenced from the marked rise in IL-6 level (355%) in comparison with normal group. However, treatment with telmisartan significantly reduced IL-6 level by 42.7%, as compared to CCl4-intoxicated rats (Fig. 3C). These data confirm that telmisartan treatment markedly mitigated oxidative stress and inflammation ($p < 0.01$) in carbon tetrachloride-treated rats.

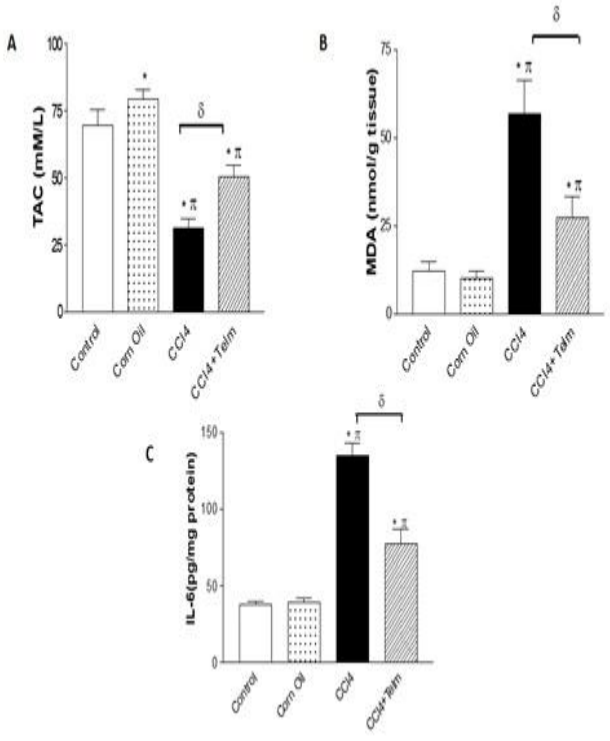

Figure 2. Effect of telmisartan treatment on hepatic TAC (A), MDA (B), and IL-6 (C) in carbon tetrachloride-treated rats**.** Results are represented as mean ±S.D (n=6). $*P < 0.01$ versus control group, $^{\pi}P<0.01$ versus corn oil treated group, $^{\delta}P < 0.01$ versus CCl4 treated group, respectively, using ANOVA followed by Tukey as a post-ANOVA test.

3.4. Telmisartan treatment ameliorated liver fibrosis in carbon tetrachloride-intoxicated rats

TGF-β is one of the main contributors to pathological fibrosis nearly in all organs, particularly the liver. Activated TGF-β signaling in injured liver tissue encourages the activation and proliferation of hepatic stellate cell (HSCs), thereby inducing excessive extracellular matrix (ECM) deposition[19]. For that we assessed the hepatic level of TGF-β1 as a marker for fibrosis. CCl4 injection significantly increased TGF-β1 level by 252.3% as compared to that of control animals ($p < 0.05$). Treatment with telmisartan markedly decreased TGF-β1 levels by 47.6%, versus CCl4 treated rats ($p < 0.01$).

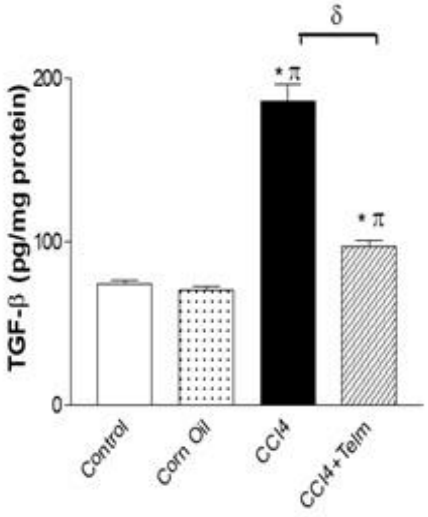

Figure 3. Effect of telmisartan on hepatic TGF-β1 level in carbon tetrachloride-treated rats**.** Results are represented as mean ±S.D (n=6). $*P < 0.01$ versus control group, $^{\pi}P<0.01$ versus corn oil treated group, $^{\delta}P < 0.01$ versus CCl4 treated group, respectively, using ANOVA followed by Tukey as a post-ANOVA test.

3.5. Effect of telmisartan treatment on p-JAK2 and p-STAT3 expression in carbon tetrachloride-intoxicated rats

Figure 5A shows western blotting of p-JAK2 and p-STAT3 protein. Treatment with CCl4 significantly increased p-JAK2 (6.9 fold) and p-STAT3 (5 fold) in hepatic tissue in comparison with normal group. Telmisartan treatment markedly decreased JAK2 phosphorylation by 60.4% and STAT3 phosphorylation by 55.8%, respectively versus CCl4-treated animals.

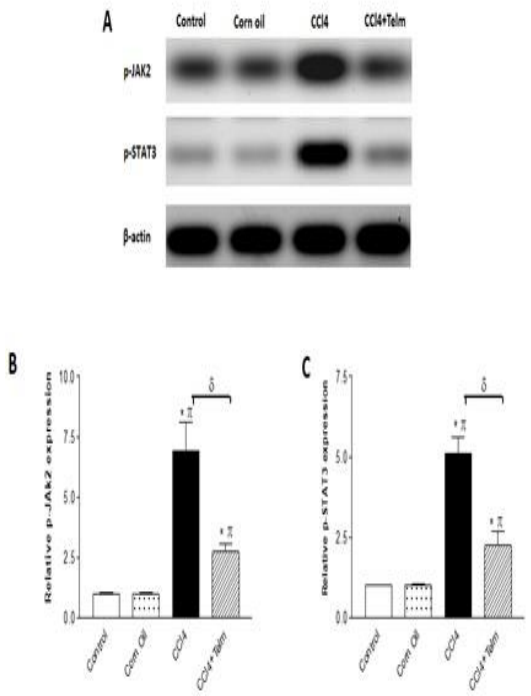

Figure 4. Effect of telmisartan on p-JAK2 and p-STAT3 expression in liver tissue of carbon tetrachloride -intoxicated rats. A: Western blotting of p-JAK2 and p-STAT3 proteins. B and C: Histogram shows the relative level of p-JAK2 and p-STAT3 proteins**.** Data are presented as mean ± S.D. (n = 6). * $p <$ 0.01 versus control group, $^{\pi}P<0.01$ versus corn oil treated group, $^{\delta}P <$ 0.01 versus CCl4 treated group, respectively, using ANOVA followed by Tukey as a post-ANOVA test.

3.6. Telmisartan treatment mitigates the ultrastructural changes in carbon tetrachloride-intoxicated rats

Histological evaluation of liver tissue was done utilizing H&E staining to identify the effect of telmisartan treatment on histological architecture of the liver after CCl4 intoxication in rats (Fig. 1). Carbon tetrachloride treated group showed portal fibrosis with bridging of fibroblasts between the hepatic lobules, mononuclear inflammatory cells infiltration and microvesicular steatosis of hepatocytes, oval cells proliferation as well as karyomegally of hepatocytic nuclei. Treatment with telmisartan reduced all of the previously altered histological features, as liver tissue of the telmisartan treated group showed fine strands of fibroblasts in the portal triad and in between the hepatocytes, mild necrosis of sporadic hepatocytes with slight congestion of hepatic sinusoids and trivial vacuolar degeneration of hepatocytes.

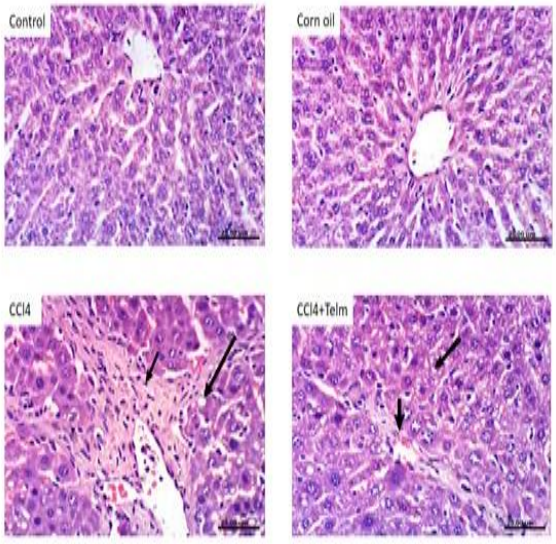

Figure 5. Light photomicrographs of liver sections stained with H&E for histological examination.

Image for section of liver tissue from normal control group shows normal histological structure of the hepatic lobule from the central vein and the hepatocytes arranged in hepatic cords; Image for section of liver tissue from corn oil group shows no histopathological alterations; Image for section of liver tissue from CCl4-treated group shows portal fibrosis with bridging of fibroblasts between the hepatic lobules (short arrow) and microvesicular steatosis of hepatocytes (long arrow); Image for section of liver tissue from CCl4+Temisartan treated group shows fine strands of fibroblasts in between the hepatocytes (short arrow) and vacuolar degeneration of hepatocytes (long arrow) (magnification, ×400; n=6/group).

4. DISCUSSION

One of main leading reasons for human sufferings and mortality worldwide and particularly in Egypt is chronic liver disease. The most significant pathological feature in chronic liver disease is liver fibrosis[20]. However, liver fibrosis is a reversible lesion[21] thus targeting fibrosis regression is a main therapeutic goal nowadays. In the current study telmisartan mitigated all the biochemical and histopathological disturbances caused by CCl4 intoxication. Whereas, telmisartan increased total antioxidant level, reduced disturbed serum hepatic enzyme markers, hepatic inflammatory reaction, and oxidative damage induced by CCl4 intoxication. Interestingly, this study for the first time has shown that, the hepatoprotective action of telmisartan against

CCl4-induced liver fibrosis is partly facilitated through inhibition of JAK2/STAT3 pathway.

The hepatotoxin CCl4 is commonly used to mimic human liver fibrosis in experimental animals[22]. One of the executive mechanisms that participate in progression of CCl4-induced liver fibrosis is oxidative stress. CCl4 and its hepatic metabolism mediate the formation of free radicals and reactive oxygen species with consequent depletion in cellular antioxidant molecules[23,24]. In this study, an increase in liver index and hepatic MDA level accompanied by a reduction in TAC was shown in CCl4-intoxicated animals. These data are in accordance with the increase in serum hepatic injury markers (ALT and AST) that can be explained by the elevated oxidative stress and the consequent lipid peroxidation and liver injury that may alter transport and permeability function of cellular membrane in injured hepatic cells, resulting in outflow of the enzymes from hepatic cells into the blood flow[25]. Conversely telmisartan treatment showed antioxidant and hepatoprotective effects as evidenced from the elevated TAC, decreased liver index, MDA, and serum liver injury markers in CCl4 intoxicated animals[26,28]. The hepatoprotective effects of telmisartan may be in part based on drug's antioxidative action[29,30]. Additionally, telmisartan as a partial peroxisome proliferator-activated receptors-gamma (PPAR-γ), agonist, may modulate the expression of PPAR-γ target genes responsible for inflammation and oxidative stress[31,32]. Additionally, telmisartan as an ARB, inhibits the action of Ang II, which was shown to increase oxidative stress in different experimental models [33]. These results confirm the antioxidant properties as well as the suppressive action of telmisartan on ROS production.

The consistent oxidative liver damage caused by CCl4 intoxication induces inflammation that ultimately causes fibrosis of the liver[34]. In accordance with previous reports, accumulating oxidative stress by CCl4 in this study excites a clear inflammatory reaction with inflammatory cells infiltration as well as elevation in inflammatory cytokine level (IL-6) in liver tissue[17,26]. Meanwhile, telmisartan treatment significantly lowered hepatic IL-6 level and inflammatory cell infiltration in liver tissue of CCl4 intoxicated rats. Our data is in line with the evident antioxidant activity seen in telmisartan treated group. These data are also in accordance with other reports in which telmisartan has been shown to significantly reduce lipid peroxidation, inflammatory cytokine secretion and expression, henceforth reduces the progression of tissue injury [26,28,35,36]. Telmisartan has been also documented to significantly reduce inflammatory cell infiltration in liver tissue [26, 37].

HSCs activation due to liver injury goes along with TGF-β1 release from activated Kupffer cells that participate in hepatic fibrosis [38]. Many reports documented that blockage of RAS mitigates liver fibrosis [39]. These findings are in line with our results in which CCl4-intoxication result in marked elevation in hepatic TGF-β1level. Meanwhile, telmisartan treatment significantly reduced TGF-β1level and hepatic fibrosis in intoxicated rats. This data is in line with the ameliorative effect of telmisartan on liver fibrosis that was shown in bile duct-ligated rats via decreasing TGF-β1expression [40] as well as via its partial PPAR-γ agonist effect [32].

Promotion of fibrosis regression by reduction of inflammatory response and inhibition of signaling pathways that initiate/aid in HSCs activation is one of the therapeutic goals in treatment of chronic liver disease [41]. Many reports have shown the involvement of JAK2/STAT3 molecular pathways in HSCs activation[42]. Additionally, many research data documented the contributing role of STAT3 in fibrosis [43]. For that, JAK2/STAT3 pathway was considered as a possible anti-fibrosis target in medical therapy. On such basis, and to explain the molecular mechanisms for the protective action of telmisartan on CCl4-induced liver fibrosis, JAK2/STAT3 signaling was investigated as a putative mechanism.

Our findings indicated that p-JAK2 and p-STAT3 expressions were markedly elevated in CCl4-intoxicated animals, with concomitant elevation in hepatic IL-6 and TGF-β1 levels and subsequent liver fibrosis. These data are in accordance with several previous reports [12,44]. The list of ligands that enhance JAK2/STAT3 pathway include IL-6, TGF-β in addition to Ang II [7,10,45]. Moreover, JAK2/STAT3 proteins are crucial participants in Ang II/AT1R signal transduction[4,46]. Thus, the significant rise in p-JAK2 and p-STAT3 level could be due to increased IL-6 and TGF- β level and most importantly to the exacerbated RAS due to CCl4 injection. Meanwhile, telmisartan has been documented to inhibit JAK/STAT signaling in different experimental models [47,48]. Also, the anti-inflammatory activity of telmisartan [35,36], in addition to its suppressive action on Ang II action assured the positive correlation between the decreased p-JAK2, p-STAT3 expression, reduced TGF-β1 level as well as ameliorated liver fibrosis. Thus, our data corroborate to literature, evidencing that, inhibition of IL-6/JAK2/STAT3 pathway might be a crucial mechanism for the mitigating action of telmisartan against CCl4-induced hepatic fibrosis.

5. CONCLUSION

Conclusively, the current study documented that one of the contributing hepatoprotective mechanisms of telmisartan against CCl4-induced liver injury might be mediated via suppressing oxidative stress, repressing inflammation, and downregulating IL-6/JAK2/STAT3 pathway. These findings are supportive for utilizing telmisartan as a protective and therapeutic agent for liver fibrosis.

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Author Contributions: **LF;** Performed the experiments, collected the data, analyzed the data, performed the graphical and statistical analysis; **AA;** Supervised the experiment execution, supervised the data analysis and revised the manuscript; **KA;** performance of histopathological examination of the liver tissue; **NA;** Developed the research idea, designed the experiments, supervised the experiment execution, supervised the data analysis, and wrote and revised the manuscript; All authors read and approved the manuscript, and all data were generated in-house and that no paper mill was used.

Ethical approval: Animal experiments were approved by Animal Ethics Committee of the Faculty of Pharmacy, Al-Azhar University, Egypt. (Approval number: 80/2016).

Author Consent to Publication: All authors give the Publisher the permission of the Authors to publish the Work.

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