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Invasive and Non-Invasive Evaluation of antifibrotic effect of Losartan on Experimentally Induced Liver Fibrosis: A Biochemical and Histological Study

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- Losartan
- Serum TIMP1
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Abstract

Background: Liver fibrosis and cirrhosis are major causes of morbidity and mortality worldwide. Precise determination of liver fibrosis stage is crucial for the choice of optimal therapies. Liver biopsy is the most accurate method used to evaluate progression of chronic liver diseases but it has several complications. Recently many non-invasive markers (NIMs) for assessing liver fibrosis have been developed. **Aim:** This study was done to evaluate invasive method and non-invasive methods in assessment of CCl_4 induced liver fibrosis and the reversibility of liver fibrosis by angiotensin receptor blocker (Losartan). **Methods:** Forty-two adult albino rats were divided into Control group (n =12), CCl4 treated groub (n = 18) and Losartan treated group (n=12). At the assigned times, serum SGPT and SGOT were assayed by the colorimetric method and serum tissue inhibitor metalloproteinase 1 (Timp1) by Elisa. Livers of rats were stained with H & E for histopathological examination, Masson trichrome and Sirius red for liver fibrosis, and Immunostaining for Timp1. **Results & Conclusions:** The results concluded that non-invasive methods can express the difference that occur in liver fibrosis but it cannot estimate its degree so it cannot replace the liver biopsy. Further research could identify other promising non-invasive methods.

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Introduction

Liver fibrosis and cirrhosis are major causes of morbidity and mortality worldwide (1). Fibrosis occurs due to viral hepatitis, metabolic or autoimmune diseases, toxic injury, or congenital abnormalities (2). Liver fibrosis is characterized by an imbalance in the synthesis and degradation of hepatic extracellular matrix (ECM) including collagens (3). Hepatic stellate cells (HSC) are believed to be the main ECM-producing cells in the injured liver (2). Exciting clinical evidence has demonstrated that fibrosis not only undergoes histological reversion (4), but can also be associated with improved clinical outcomes. There are already many existing drugs with antifibrotic mechanism of action (1). For example, renin-angiotensin system (RAS) plays an important role in hepatic fibrosis. Angiotensin II induces contraction and proliferation of human HSCs (3). Losartan is an angiotensin II receptor blocker that has been found to inhibit the progression of hepatic fibrosis (5). Precise determination of liver fibrosis stage is crucial for the choice of optimal therapies (6). Liver biopsy remains the only reliable means to determine prognosis based on the severity of fibrosis. However, liver biopsy is an expensive and invasive procedure associated with a number of complications (7). Thus, non-invasive method to assess severity would be a useful clinical tool (8).

Class II biomarkers for liver fibrosis are indirect methods using serum biochemical and/or hematological tests, based on the detection of common functional alterations in the liver like serum ALT, AST, prothrombin index and macroglobulin. In contrast, class I biomarkers are associated with the process of fibrogenesis, and their presence in the serum is the result of the increased turnover of ECM for example cytokines and chemokines linked to liver fibrosis (1). This study is designed to evaluate invasive method and non-invasive methods in assessment of CCl_4 induced liver fibrosis and the reversibility of liver fibrosis by angiotensin II receptor blocker (Losartan).

Materials and Methods:

Animals:

Forty-two adult albino rats, with average body weight between 180-200 gm, used in this study, were obtained from Medical experimental research center (MERC), Mansoura University, Egypt. They were housed in stainless steel, mesh cages under controlled conditions of temperature (23°C ± 3), and relative humidity. They had free access to standard commercial diet and tap water ad libitum with a normal light-dark cycle throughout acclimatization and experimental periods. All rats were maintained in the animal house, under specific pathogen-free conditions. All the experiments were approved by the ethical committee (code# M170612) and carried out according to the rules and the regulations lay down by the committee on animals' experimentation of Mansoura University.

Chemicals:

Carbon tetrachloride (CCI4) solution was purchased from El-Gomhoria Company, Mansoura, Egypt. It was given by intraperitoneal injection of 1 ml/kg sterile CCI*4* dissolved in corn oil at 1:1 ratio twice weekly as previously

described by Kim et al. (9) . Losartan 100 mg from Amriya pharmaceut co, Mansoura_Egypt, in the form of oral tablets. It was crushed and suspended uniformly in 1% solution of carboxyl methylcellulose (CMC) and given orally by gastric gavage (10).

Experimental design:

The rats were randomly divided into three groups: I. Control group (n =12): Rats received intraperitoneal (i.p.) injections of 1mg/kg corn oil twice daily and were sacrificed after 8,10 and 12 weeks from the start of the experiment. II. CCl4 treated group (n = **18):** Rats received 1ml/kg CCl4 dissolved in corn oil (1:1) twice a week for 8 weeks Then rats were sacrificed either: (CCl4a, 6 rats) immediately,(CCl4.b, 6 rats) two weeks or (CCl4.c, 6 rats) four weeks after last CCl4 injection. III. Losartan treated group (n=12): Rats received CCl4 for 8 weeks followed by oral treatment of Losartan (10mg/kg/day) by gastric gavage for: (Losartan.a, 6 rats) two weeks or (Losartan.b, 6 rats) four weeks then animals were sacrificed.

Samples collection:

At the assigned times, all rats were anaesthetized with diethyl ether; samples of blood were drawn from the eye sockets and collected for assessment of liver enzymes and assay of Timp1 by Elisa. Then, rats were sacrificed. Livers of rats were rapidly removed from the abdominal cavities and were processed for histopathological examination by light microscope.

Biochemical analysis:

Assessment of liver functions*:*

The serum samples were obtained by centrifugation of blood samples for 10 min at 5000 g at 4°C. The serum levels of glutamate-pyruvate-transaminase (GPT) and glutamate-oxalate-transaminase (GOT) were assayed according to the colorimetric method using clinical test kits (Elitech, UK) (11).

Assay of Serum Tissue inhibitor of metalloproteinase-1 (Timp1) by Elisa. Collected blood samples were allowed to coagulate at room temperature for 10-20min. Then they were centrifuged at 2000-3000 rpm and the supernatant was removed. The manufacturer instructions of Timp1 Elisa kit obtained from Glory Science Company, code 30435, USA, were followed.

Histopathological examination:

Immediately after sacrifice, livers specimens were processed for paraffin sectioning. Tissues were stained with Hx& E (12) for histopathological examination. Masson trichrome (13) and Sirius red (14) staining were used for fibrosis detection. The Sirius red stained sections of all groups were subjected to image analysis of the area occupied by collagen fibers using image analyzer computer system using program Image J (version1.48).

Immunostaining for Timp1 (15):

Liver tissues were examined by immunohistochemistry for detection of TIMP-1. Sections were counterstained with hematoxylin. Positive cells showed brown granules in the cytoplasm. The interpretation of immunohistochemistry expression was done in a qualitative and subjective manner according to Seidal et al., (16).

Statistical analysis:

The statistics were done by using computer software SPSS version 16 (Chicago, IL, USA). Data were expressed as the mean ± SD. Multiple comparisons were performed using one-way analysis of variance (ANOVA) followed by the independent sample T test. P value <0.05 was considered as statistically significant.

Results:

Biochemical results:

Liver function tests (SGOT & SGPT): In control group, the serum level of glutamate-pyruvate-transaminase (GPT) and glutamate-oxalate-transaminase (GOT) were in the normal range (25.60 ± 5.41 U/L and 101.34 ± 10.05 U/L respectively) As compared to control group, there was high significant increase in SGOT and SGPT in both CCl4 treated group for 8 weeks (CCl4 a) and animal sacrificed two weeks after last CCl4 injection (CCl4 b). but these values were significantly lower in (CCl4 b) than the values of subgroup (CCl4a). In animal sacrificed four weeks after last CCl4 injection (CCl4 c), SGOT and SGPT values showed high significant increase as compared to the control but these values were significantly less than subgroup CCl4 a and subgroup CCl4 b (Table 1).

In animals treated with CCl4 for eight weeks then two weeks Losartan (Losartan a), the serum levels of SGOT and SGPT showed high significant decrease as compared to those of subgroup 2a but these levels were still significantly high as compared to the control group. Comparing these values to those of Losartan b they showed significant decrease. In animals treated with CCl4 for eight weeks then four weeks Losartan (Losartan b). The level of SGOT and SGPT showed high significant decrease as compared to Losartan a but these levels were still significantly high as compared to the control values. Comparing these values to those of CCl4c, they showed significant decrease (Table 1) .

Serum level of tissue inhibitor metalloproteinase 1 (Timp1):

In control group, serum level of Timp1 was 8.88 ± 2.18 pg/ml. In CCl4 treated group for 8 weeks (CCl4 a), serum level of Timp1showed high significant increase as compared to control group. In animal sacrificed two weeks after last CCl4 injection (CCl4 b) Serum Timp1 showed significant increase as compared to control group but these values were significantly less than subgroup CCl4a .In animal sacrificed four weeks after last CCl4 injection (CCl4 c), serum level of Timp1 was significantly increased as compared to control but these values were significantly less than subgroup (CCl4a) and subgroup (CCl4b)

(Table 2).

In animals treated with CCl4 for eight weeks then two weeks Losartan (Losartan a) , serum level of Timp1 showed high significant decrease as compared to group CCl4a and group CCl4b but this level was still higher than those of control group. In animals treated with CCl4 for eight weeks then four weeks Losartan (Losartan b), serum level of Timp1 showed high significant decrease as compared to group CCl4a, group CCl4b and group CCl4c but these levels were still significantly high as compared to the control value (Table 2).

Liver Histopathology:

Haematoxylin and Eosin (Hx & E) stained sections : As shown in Figure 1, The histological appearance of the liver in all control rats was normal with no histological abnormalities or evidence fibrosis. The liver sections were formed of the classical hepatic lobules with central hepatic venules and portal triades at the periphery. In animal received CCl4 injection for 8 weeks (CCl4 a), there was marked distortion of liver architecture. Hepatocytes showed steatosis. Portal area was infiltrated by mononuclear inflammatory cells. There was increase of connective tissue around portal tract and central veins. These signs were relatively reduced in animal sacrificed two weeks after last CCl4 injection (CCl4 b) and showed more decline in animal sacrificed four weeks after last CCl4 injection (CCl4 c) (Figure 1).

In animals treated with CCl4 for eight weeks then two weeks Losartan (Losartan a), hepatocytes appeared normal with vesicular nuclei. Mild mononuclear inflammatory infiltration was seen in portal areas and hepatic sinusoids. Thick fibrous septa were often present and they were short and incomplete radiating from central vein or portal tracts into the surrounding parenchyma . Similar results were observed in animals treated with CCl4 for eight weeks then four weeks Losartan (Losartan b) but with scanty thin fibrous septa (Figure 1).

Masson trichrome and sirus red stained sections:

In the control group, portal tracts were seen at the corner of each lobule surrounded by collagen fibers. Fine collagen fibers also were seen surrounding the central vein, portal tract and in the wall of sinusoid. In subgroup CCl4a, there was marked increase in the amount of collagen fibers in the portal tracts and around central veins. Thick well-developed septa could be seen throughout the sections connecting portal tract and central veins together. There were marked distortion of the liver architecture with pseudolobules formation (Figure 2).In subgroup CCl4b, septal fibrosis was still detected. Septa were less in thickness and pseudo lobules were larger than those of subgroup CCl4 a. In subgroup (CCl4c), thin septal fibrosis was often seen connecting the portal areas. Septa were less in thickness and pseudolobules were larger than those of animals sacrificed immediately and animals sacrificed 2wks after last injection of CCl4(CCl4 b). In subgroup 3a, moderate increase in the amount of collagen fibers in the portal tracts and around central veins. Thick well-developed septa could be seen throughout the sections connecting portal tract and central veins together. Similar signs in subgroup Losartan b with thin septa were seen connecting portal tract and central veins (Figure 2). Area occupied by collagen fibers in sirus red stained sections and their multiple comparisons between all groups are demonstrated in Table 3.

Immunohistochemical staining for tissue inhibitor metalloproteinase 1 (Timp1):

In control animals, there was only a mild positive expression of Timp1 in vascular endothelial cells and myofibroblasts existed in the wall of portal tract. The reaction appeared as brown particles in the cytoplasm. No positive expression was found in the nucleus (Figure 3). In animal received CCl4 injection for 8 weeks (subgroup (CCl4a), there was a strong positive expression of Timp1 in vascular

endothelial cells and myofibroblasts existed in the wall of portal tract and fibrous septa. In animal sacrificed two weeks after last CCl4 injection (CCl4 b), moderate decline in Timp1 positive stain. The positive cells were demonstrated in the wall of portal tract and in the fibrous septa (Figure 3). In animals sacrificed four weeks after last CCl4 injection (CCl4 c), marked decline in Timp1 positive stain in all sections.

In animals treated with CCl4 for eight weeks then two weeks Losartan (Losartan a), few Timp1 positive cells were seen compared to CCl4 group. They were observed in fibrous tissue septa and in the wall of portal tract. In animals treated with CCl4 for eight weeks then four weeks Losartan (Losartan b) , Timp1 positive cells were markedly diminished in all sections. Just few cells expressing this reaction appeared in the portal area in this group (Figure 3).

Table (1): Liver function test (SGOT, SGPT) values (mean ± SD) and multiple comparisons of SGOT and SGPT between all groups

		Multiple comparisons P values				
SGPT (U/L)		**CCl4 a**	**CCl4 b**	**CCl4 c**	**Losartan a**	**Losartan b**
Control	25.60 ± 5.41	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*
CCl4 a	125.80 ± 7.36		<0.001*	<0.001*	<0.001*	<0.001*
CCl4 b	94.80 ± 3.27			<0.001*	<0.001*	<0.001*
CCl4 c	70.60 ± 2.41				<0.001*	<0.001*
Losartan a	84.00 ± 2.92					<0.001*
Losartan b	54.60 ± 2.97					
SGOT (U/L)		<0.001*	<0.001*	<0.001*	<0.001*	<0.001*
Control	101.34 ± 10.05					
CCl4 a	328.40 ± 35.31		<0.001*	<0.001*	<0.001*	<0.001*
CCl4 b	237.00 ± 12.04			<0.001*	<0.003*	<0.001*
CCl4 c	177.20 ± 5.17				<0.020*	<0.001*
Losartan a	202.80 ± 5.26					<0.001*
Losartan b	158.00 ± 5.83					

P value <0.05 was considered as statistically significant.

P value <0.00l was considered as statistically highly significant.

Table (2): Serum levels (Mean ± SD) and multiple comparison of Timp1 between all groups.

	TIMP 1 (pg/ml)	Multiple comparisons P values				
		CCl4 a	**CCl4 b**	**CCl4 c**	**Losartan a**	**Losartan b**
Control	8.88 ± 2.18	<0.00*	<0.001*	<0.002*	<0.001*	<0.001*
CCl4 a	22.56 ± 3.69		<0.001*	<0.001*	<0.001*	<0.001*
CCl4 b	18.90 ± 1.10			<0.001*	<0.001*	<0.010
CCl4 c	12.31 ± 2.25				<0.472*	<0.001*
Losartan a	13.23 ± 2.50					<0.001*
Losartan B	9.73 ± 1.26					

P value <0.05 was considered as statistically significant.

P value <0.00l was considered as statistically highly significant

Table (3): Area occupied by collagen fibers (%) (Mean ± SD)P values of multiple comparisons of area occupied by collagen fibers between all groups

	Collagen fibers area (%) (Mean ± SD)	Multiple comparisons P values				
		CCl4 a	CCl4 b	CCl4 c	Losartan a	Losartan b
Control	1.77 ± .85	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*
CCl4 a	20.81 ± 2.54		<0.001*	<0.001*	<0.001*	<0.001*
CCl4 b	15.35 ± 1.07			<0.001*	<0.002*	<0.001*
CCl4 c	9.84 ± .72				<0.006*	<0.001*
Losartan a	12.37 ± .96					<0.001*
Losartan b	7.39 ± .80					

P value <0.05 was considered as statistically significant.

P value <0.00l was considered as statistically highly significant

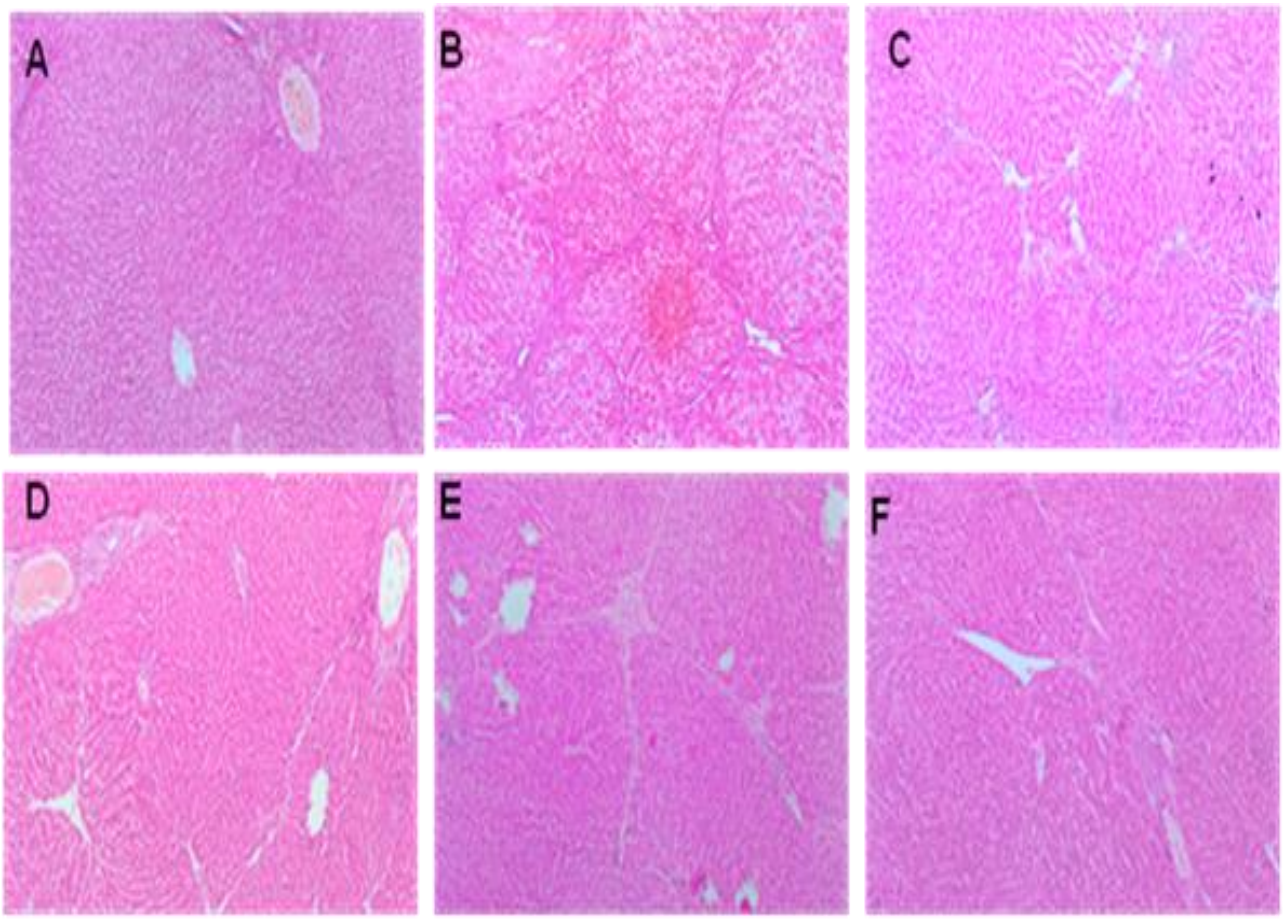

Figure (1): A photomicrograph of Hematoxylin & Eosin stains; ×100 of liver sections in: A. Control rat. B. Animals received CCl4 for eight weeks then sacrificed immediately (CCl4 a). C. Animals sacrificed two weeks after last CCl4 injection (CCl4 b). D. Animals sacrificed four weeks after last CCl4 injection (CCl4 c). E. Animals received Losartan for two weeks after last CCl4 injection (Losartan a). F. Animals received Losartan for four weeks after last CCl4 injection (Losartan b).

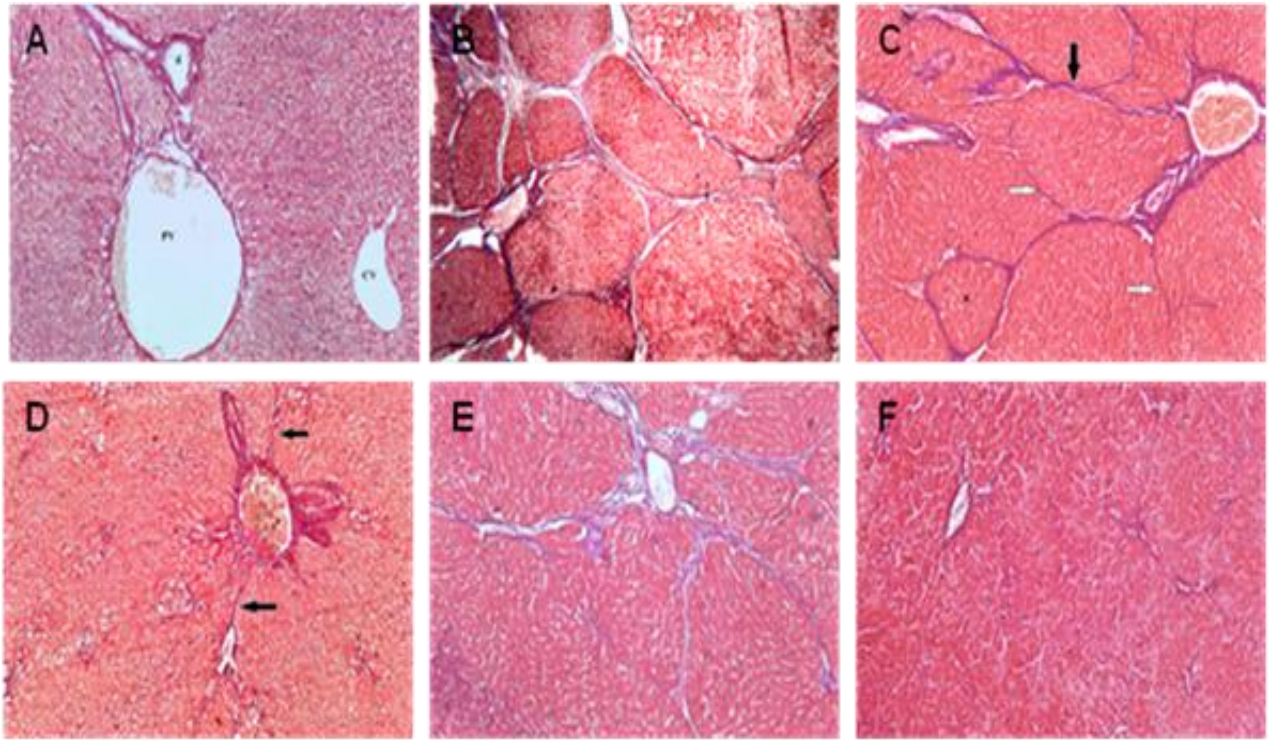

Figure (2): A photomicrograph of Masson's trichrome stain; ×100 of liver sections in: A. Control rat. B. Animals received CCl4 for eight weeks then sacrificed immediately (CCl4 a). C. Animals sacrificed two weeks after last CCl4 injection (CCl4 b). D. Animals sacrificed four weeks after last CCl4 injection (CCl4 c). E. Animals received Losartan for two weeks after last CCl4 injection (Losartan a). F. Animals received Losartan for four weeks after last CCl4 injection (Losartan b).

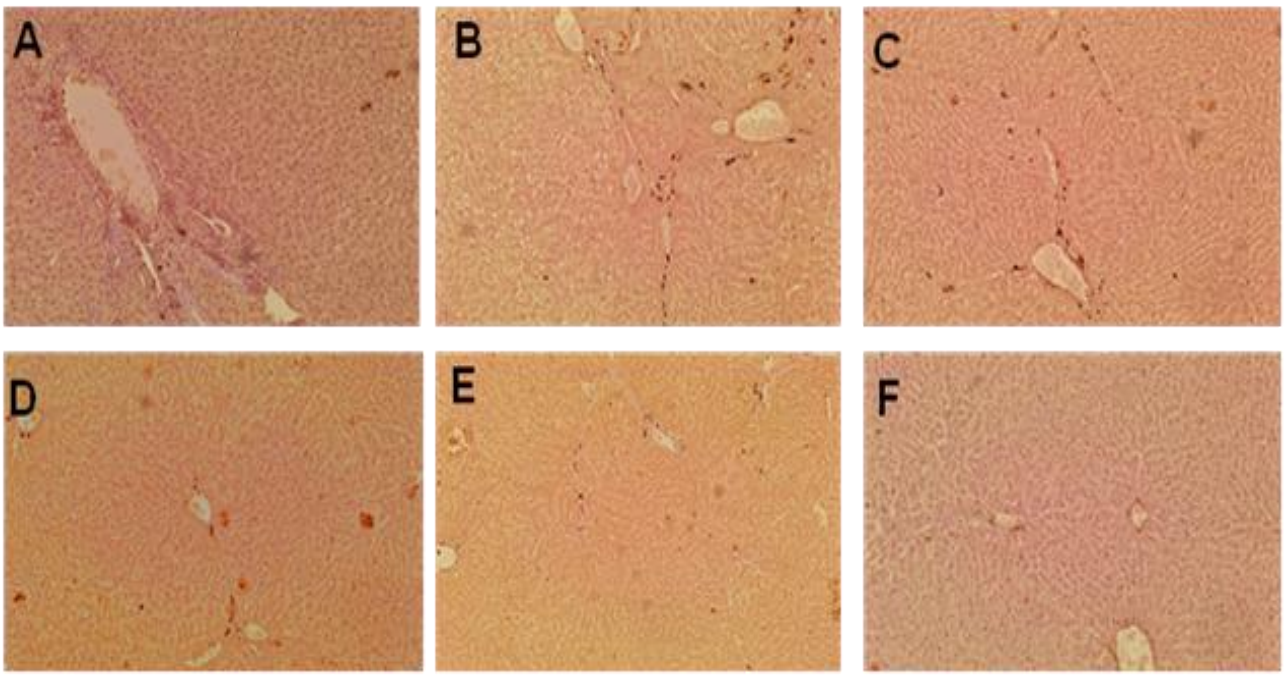

Figure (3): A photomicrograph of immunohistochemistry stain for Timp1 (×100) in liver sections of: A. Control rat . B. Animals received CCl4 for eight weeks then sacrificed immediately (CCl4 a). C. Animals sacrificed two weeks after last CCl4 injection (CCl4 b). D. Animals sacrificed four weeks after last CCl4 injection (CCl4 c) . E. Animals received Losartan for two weeks after last CCl4 injection (Losartan a). F. Animals received Losartan for four weeks after last CCl4 injection (Losartan b).

Discussion:

This study is performed to correlate between invasive method (liver biopsy) and non-invasive methods (SGOT, SGPT and serum TIMP 1) in evaluation of CCl_4 induced liver fibrosis and the reversibility of liver fibrosis by angiotensin receptor blocker (Losartan).

Comparing values of animals that received CCl4 for 8 weeks and sacrificed immediately and values of control animals, SGOT increased by 4 folds, SGPT increased 2.5 fold and serum Timp 1 increased 10 folds. The values of SGOT and SGPT were in agreement with Mihailovic et al. (17) who stated that the liver function tests increase of CCl4 treated group. Nei et al. (15) noted that serum Timp1 increased significantly in CCl4 induced liver fibrosis as compared to control group but it couldn't reflect the grade of fibrosis . Histopathological examination showed the same observation reported by Al Gamdi, (18) who stated that CCl_4 injection cause fatty changes, ballooning degeneration, cell necrosis and centrilobular inflammatory infiltrate. Masson's trichrome and Sirius red stained slides in rats treated with CCl4 for eight weeks showed abundant septa radiating from central veins and portal tracts and marked bridging fibrosis. These findings are in parallel to the results reported by kisseleva et al., (2012) (19). Image analysis revealed that slides of animals treated with CCl4 for eight weeks showed area occupied by collagen fibers increased by 10 folds as compared to control animals.

In the present study, we evaluated the process of spontaneous resolution of fibrosis. Two groups of rats received CCl4 for eight weeks then were left without any form of treatment for either two weeks or four weeks then were sacrificed. The results of animals that were sacrificed two and four weeks after last injection of CCl4 proved the reversibility of liver fibrosis even without treatment and ensured the spontaneous resolution capacity of the liver tissue. As values of animals sacrificed four weeks after CCl4 treatment were less than animals sacrificed two weeks after CCl4 treatment, this prove that resolution of liver fibrosis is a continuous process. These results are similar to data reported by Iredale, (20).

Losartan has been found to inhibit the progression of hepatic fibrosis (21). So, it is a major candidate in clinical studies as an anti-fibrotic drug (22). Losartan is an angiotensin II receptor blocker that acts upon AT1 receptors (23). AT1 receptors blockade can reduce the activated HSCs accumulation and attenuates liver fibrosis (9). In this present study we used Losartan as antifibrotic drug. Most of studies perform simultaneous administration of CCl4 and Losartan to evaluate its protective effect but in the present study we administered CCL4 for eight weeks then losartan 10 mg/kg/day orally for either two weeks or four weeks to evaluate its curative effect and compare this effect with spontaneous resolution. Values estimating fibrosis in Losartan treated groups were less than spontaneous resolution groups. So, losartan attenuated liver fibrosis by a degree more than spontaneous resolution.

The results of non-invasive methods (SGOT, SGPT and serum Timp1) and the results of invasive method (image analysis of area occupied by collagen fibers) observed in animals that

received CCl4 for eight weeks and control animals showed increase of the values than control group but the increase was not by the same degree. However in spontaneous resolution groups and losartan treated groups, the results of two methods were more close. These results in agreement with Sebastiani , (24) stated that combination of non-invasive methods and liver biopsy greatly improves the diagnostic performance and the need for liver biopsy is reduced by 50%-80% but cannot be completely avoided. Also Patel et al. (25) who stated that combination of more than one serum markers may reliably differentiate moderate/severe fibrosis from those with no/mild fibrosis but accurate delineation between stages was not possible. Thus, our observations denote that non-invasive methods can express the difference that occur in liver fibrosis but it cannot estimate its degree so it cannot replace the liver biopsy.

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