Evaluation Of Possible Antifibrotic Effect Of Cholecalciferol, Calcipotriol, Aliskiren, Ramipril And Eprosartan On Carbon Tetrachloride Induced Liver Fibrosis In Albino Rats

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Background

the present study aims to evaluate the possible anti-fibrotic effect of calcipotriol, cholecalciferol, aliskiren, eprosartan and ramipril on carbon tetrachloride (CCl₄) induced liver fibrosis in rats. Methods: Fifty six Spague Dawley rats were randomly allocated into seven groups. All rats were exposed to induction of hepatic fibrosis by intraperitoneal (ip) injection of CCl₄ twice/week for 8 successive weeks except control healthy group. The rats were treated with either calcipotriol (20 ug/Kg), Cholecalciferol (500 IU/kg), aliskiren (10mg/kg), eprosartan (60mg/kg) and ramipril (10mg/kg) simultaneously with CCl₄ treatment. Results: all the tested drugs have significant improvement in liver function tests (liver enzymes, bilirubin and albumin), oxidative stress markers (reduced glutathione, malondialdehyde and nitric oxide), transforming growth factor-b1 (TGF-b1) and fibrotic score. Only calcipotriol and cholecalciferol are significantly decrease renin gene expression in liver and kidney tissues while cholecalciferol alone has significant increase in serum calcium and 25 hydroxy vitamin D [25(OH)D] levels. Conclusion: these results suggest that calcipotriol, cholecalciferol, aliskiren, eprosartan and ramipril have antifibrotic effect. Vitamin D receptor (VDR) agonists (Calcipotriol and cholecalciferol) inhibit renin gene expression and have better antifibrotic effect than renin angiotensin blockers (aliskiren, eprosartan and ramipril). Eprosartan has better antifibrotic effect than aliskiren and ramipri

Introduction

Liver fibrosis is a dynamic reversible wound healing process of chronic liver injury characterized by excessive deposition of extracellular matrix (ECM) in liver tissue. Liver fibrosis is usually caused by viral hepatitis, biliary obstruction, nonalcoholic steatohepatitis and alcohol abuse (1).

Hepatic stellate cell (HSC) in the perisinusoidal space of Disse is the main source of ECM deposited in liver fibrosis. HSC present in resting quiescent state and liver injury leading to HSC activation (2). Activation of HSC composed of two main phases, initiation and perpetuation (3). Initiation result from paracrine stimulation of HSC by all surrounding cells including hepatocytes, sinusoid-

al endothelium, kupffer cell and platelets. During perpetuation phase, there is continuous autocrine and paracrine release of pro-inflammatory, pro-fibrogenic and pro-mitogenic factors which maintain HSC in the active form. There are many cytokines involved in HSC activation but the most important one is TGF- β 1 (4).

Inflammatory cells and cytokines have important role in both hepatic fibrosis and resolution. Cytotoxic T cells have Pro-fibrogenic effect while natural killer cells have anti-fibrogenic effect through induction of HSC apoptosis(5). Some Interleukins (ILs) have pro-fibrogenic effect like IL-1 and IL-17 while others have anti-fibrogenic effect like IL-10, IL-6 and IL-22(6). Damaged hepatocytes release

mediators, such as reactive oxygen species, lipid peroxidation products and acetaldehyde which are strong activator of HSC (7).

Hepatic renin–angiotensin system (RAS) is over expressed in liver fibrosis. Angiotensin-II increase TGF- β 1 and collagen release from HSC through AT1 receptor stimulation (8). Also, angiotensin II activates nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in HSC and increase formation of reactive oxygen species, cell migration and inflammatory cytokines, all of which contribute to liver fibrosis (9). There's high prevalence of vitamin D deficiency has been implicated in the development of liver fibrosis, hepatocellular carcinoma and hepatic osteodystrophy (12-14).

Our study was designed to evaluate the antifibrotic effect of Calcipotriol, Cholecalciferol, Aliskiren, Eprosartan and Ramipril on hepatic fibrosis and answer many questions that still present until now to effectively guide clinical practice. These questions includes, is hepatic fibrosis associated with vitamin D deficiency? Is vitamin D administration can inhibit hepatic fibrosis and what is the mechanism? Is vitamin D administration can inhibit renin gene expression in hepatic fibrosis and if the answer is yes, what is the difference between vitamin D and RAS blockers? Is improvement of hepatic fibrosis can restore normal vitamin D level? What is the preferred to prescribe in hepatic fibrosis, active or inactive vitamin D? What is the best to be used in hepatic fibrosis, direct renin inhibitor, ARBs or ACEI?

Material and Methods

Drugs and chemicals

- Carbon tetrachloride as liquid (purchased from Sigma-Aldrich, UK).
- Calcipotriol 10 mg as powder (purchased from Sigma-Aldrich, UK).
- Cholecalciferol 200000 IU as oily liquid (purchased from Memphis pharmaceutical and

chemical industries, Egypt).

- Aliskiren 300 mg/tablet (purchased from Novartis, Espan).
- Eprosartan 600 mg/tablet (purchased from Solvay pharmaceuticals, France).
- Ramipril 5 mg/tablet (purchased from Pharos pharmaceuticals, Egypt).

Animals

This study was carried out on 56 adult male Sprague Dawley rats weighing 250-300 g and freely accessed to water and food. Rats were purchased from the Medical Experimental Research Center, Faculty of Medicine, Mansoura University. All study procedures were performed in accordance with the ethical guidelines and principles for the use and care of laboratory animals adopted by the Scientific Research Ethics Committee, Faculty of Medicine, Mansoura University.

Study design

The studied rats randomly allocated into seven groups (8 rats in each group).

Group 1: Control normal group Rats were received ip olive oil twice/w in a dose equal to CCl₄ for 8 weeks.

Group 2: Control fibrotic group

Rats were received only ip CCl₄ twice/w for 8 weeks. CCl₄ was diluted in olive oil in equal amounts. CCl₄ was administered as 2 ml/kg for two weeks then 1 ml/kg for another two weeks then 0.5 ml/kg in the last four weeks (15).

Group 3: CCl₄ + Calcipotriol (Calcipotriol group)

Rats were received CCl₄ concurrent with ip Calcipotriol at a dose of 20 μ g/Kg/5 days per week for 8 weeks.

Group 4: CCl₄ + Cholecalciferol (Cholecalciferol group) Rats were received CCl₄ concurrent with ip Cholecalciferol at a dose of 500 IU/kg/day for 8 weeks.

Group 5: CCl₄ + Aliskiren (Aliskiren group)

Rats were received CCl₄ concurrent

with oral Aliskiren at a dose of 10 mg/Kg/day for 8 weeks.

Group 6: CCl₄ + Eprosartan (Eprosartan group)
Rats were received CCl₄ concurrent with oral Eprosartan at a dose of 60 mg/Kg/day for 8 weeks.

Group 7: CCl₄ + Ramipril (Ramipril group)
Rats were received CCl₄ concurrent with oral Ramipril at a dose of 10 mg/kg/day for 8 weeks.

Samples collection and storage:

Three days after the last dose of CCl₄ rats were sacrificed using ip thiopental sodium 75 mg/kg (16). Blood samples were collected and centrifuged at 10000 xg for 15 minutes and then the serum is separated and collected in three tubes and stored at - 20°C) for assay of 25(OH)D, liver function tests (AST, ALT, albumin and bilirubin) and serum calcium.

Six pieces of liver tissue were isolated. The first piece sent for histopathological examination. The last five pieces of liver tissue were stored in liquid nitrogen (-80°C) with a piece of renal tissue. Four liver pieces were used to prepare liver homogenate. The fifth piece of liver and the piece of renal tissue were used for renin gene expression.

Preparation of liver tissue homogenate

Liver tissue homogenate was used for asses of oxidative stress markers and TGF-β1. Liver tissue homogenate was prepared by perfusion of liver tissue with PBS (phosphate buffered saline) solution, pH 7.4, containing 0.16 mg/ml heparin to remove any red blood cells and clots, After that by the use of mortar and pestle 1gm of liver tissue was homogenized in 10 ml cold buffer (50 mM potassium phosphate at a pH of 7.5). The homogenized liver tissue was centrifuged for 15 minutes at 12000 xg and the supernatant fluid was used to complete the test according to kits instructions.

I. Biochemical measurements

1. Liver functions tests

- Serum level of alanine amino-transferase (ALT) was measured using colorimetric kits according to manufactures instructions (purchased from Biomerieux, France).
- Serum level of aspartate amino-transferase (AST) was measured using colorimetric kits according to manufactures instructions (purchased from Biomerieux, France).
- Serum total bilirubin was measured using colorimetric kits according to manufactures instructions (purchased from Diamond diagnostics, Egypt).
- Serum albumin level was measured using colorimetric kits according to manufactures instructions (purchased from Biomed diagnostics, Egypt).

2. Oxidative stress markers

- Hepatic malondialdehyde (MDA) was measured in liver tissue homogenate using colorimetric kits according to manufactures instructions (purchased from Bio-diagnostic, Egypt).
- Reduced glutathione was measured in liver tissue homogenate using colorimetric kits according to manufactures instructions (purchased from Bio-diagnostic, Egypt).
- Nitric oxide was measured in liver tissue homogenate using colorimetric kits according to manufactures instructions (purchased from Abnova , United kingdom).

3. Serum calcium

Serum calcium was measured in serum using colorimetric kits according to manufactures instructions (purchased from Sigma-Aldrich, United Kingdom).

4. Serum 25(OH)D

Serum 25(OH)D was analyzed using ELISA kits and measured according to manufactures instructions (purchased from MYBiosource, USA).

5. Hepatic TGF- β 1

TGF- β 1 in liver tissue homogenate was analyzed using ELISA kits and measured according to manufactures instructions (purchased from eBioscience, Vienna, Austria)

II. Renin gene expression (semi-quantitative PCR)

a) RNA extraction:

RNA was extracted from liver and renal tissue by Gene JET RNA purification kit (purchased from Thermo Scientific Com, Lithuania). 30 mg of tissues (liver or kidney) were placed into liquid nitrogen and grinded thoroughly with a mortar and pestle and after that 300 μ L of lysis buffer supplemented with β -mercaptoethanol were added. Lysis Buffer contains guanidine thiocyanate, a chaotropic salt capable of protecting RNA from endogenous RNases. 600 μ L of diluted proteinase K were added to the sample (lysate) followed by vortex and incubated at 25°C for 10 minutes. The lysate was centrifuged for 10 minutes at 13000 xg and supernatant fluid was transferred to new RNase-free microcentrifuge tube. The lysate was mixed with 450 μ L of ethanol 100% and loaded on a purification column. The column was centrifuged for one minute at 13000 xg and the flow-through was discarded. Guanidine thiocyanate and ethanol cause RNA to bind to the silica membrane on a purification column while the lysate was spun through the column during centrifugation. Impurities were effectively removed from the membrane by washing the column with wash buffer in three steps. First step was done by addition of 700 μ L of wash buffer 1 to the column and centrifuged for 1 minute at 13000 xg and the flow-through was discarded. Subsequently, 600 μ L of wash buffer 2 were added and centrifuged for 1 minute at 13000 xg and the flow-through was discarded. The last step was done by addition of 250 μ L of wash buffer 2 to the column and centrifuged for 2 minutes at 13000 xg and the flow-through was discarded. Pure RNA was then eluted by ad-

dition of 100 μ L of nuclease free water and centrifugation for 1 minute at 13000 xg and stored at -20°C to be used for formation of complementary DNA (17).

b) Formation of complementary DNA from RNA (Reverse Transcription):

Extracted RNA transformed to DNA using Revert aid kit (purchased from Thermo Scientific Com, Lithuania). RNA was reverse transcribed by addition of 1 μ L oligo (dT) primer, 2 μ L from 10 mM mixed deoxynucleotide triphosphate (dNTPs), 4 μ L of 5x reaction buffer, 20 unit of RNase inhibitor, 200 unit of reverse transcriptase and 9 μ L nuclease free water. The mixture was incubated at 42°C for 1 hour then the reaction terminated by heating for 5 min at 70°C. The reverse transcription product stored at -20°C to be used for polymerase chain reaction (PCR) (17).

c) Amplification of Renin complementary DNA by PCR:

Amplification of complementary DNA was performed by Dream Tag green PCR kit (purchased from Thermo Scientific Com, Lithuania). PCR was performed in a thermal cycler using 2 μ L of complementary DNA in a total volume of 50 μ L containing 3 μ L from 25 mM MgCl₂, 1.5 μ L of renin sense primer 5'CAGTACTATGGTAGATCGGCT3', 1.5 μ L of renin antisense primer 5'ACTCCATCAACAGCCTGAGC3' (48), 1 μ L from 10 mM dNTP mix, 0.5 μ L Taq DNA polymerase, 5 μ L from 10x PCR buffer and 35.5 μ L nuclease free water. Conditions of the reaction were 94°C for denaturation, 50°C for annealing, 72°C for elongation, 1.0 mM for Mg Cl₂ concentration and product size was 362 base pair (17).

d) Calculation of results:

PCR products were electrophoresed on 3% agarose gel and visualized by ethidium bromide staining under UV light. Result images photographed by digital camera. Images were analyzed

using total lab quant software to obtain band intensity represented as area under the peak (45).

III. Histopathological examination of liver tissue

Liver tissue was fixed in 10% neutral buffered formalin solution then the liver specimens putted in paraffin sections 6 μ m thick for histopathological examination by:

- a) Haematoxylin & eosin (H&E) stain
- b) Masson Trichrome stain (special stain for fibrous tissue). Images were analyzed by image analysis software program and take score according to percentage of collagen content (49).

Results

I. Liver functions

The control fibrotic group showed significant impairment in liver functions in the form of increased serum ALT, AST, total bilirubin and decreased serum albumin as compared to control normal group. Treatment with either Calcipotriol, Cholecalciferol, Aliskiren, Eprosartan or Ramipril caused significant improvement in liver functions in the form of decrease in the mean serum level of ALT, AST enzyme, total bilirubin and increase in the mean serum level of albumin as compared to control fibrotic group except the effect of ramipril on serum albumin which is not significant (table 1).

II. Oxidative stress markers

The control fibrotic group showed significant impairment in oxidative stress markers in the form of decreased reduced glutathione, increased MDA and NO in liver tissue homogenate as compared to control normal group. Treatment with either Calcipotriol, Cholecalciferol, Aliskiren, Eprosartan or Ramipril caused significant improvement in oxidative stress markers the form of increased reduced glutathione, decreased MDA and decreased NO in liver tissue homogen-

ate as compared to control fibrotic group (table 1).

III. Serum calcium and 25(OH)D

The control fibrotic group showed significant decrease in mean serum calcium and 25(OH)D as compared to control normal group. Treatment with either Calcipotriol, Aliskiren, Eprosartan or Ramipril caused non significant increase in mean serum level of calcium and 25(OH)D while treatment with Cholecalciferol caused significant increase in mean serum level of calcium and 25 (OH)D as compared to to control fibrotic group (table 1).

IV. Hepatic TGF- β 1

The control fibrotic group showed significant increase in hepatic TGF- β 1 as compared to control normal group. Administration of either Calcipotriol, Cholecalciferol, Aliskiren, Eprosartan or Ramipril caused significant decrease in the mean level of TGF- β 1 in liver tissue homogenate as compared to control fibrotic group (table 1).

V. Renin gene expression

The control fibrotic group showed significant increase in renin gene expression in liver and kidney tissues as compared to control normal group. Treatment with either Calcipotriol or Cholecalciferol caused significant decrease in renin gene expression in liver and kidney tissues while treatment with either Aliskiren, Eprosartan or Ramipril caused non significant effect on renin gene expression in liver and kidney tissues as compared to control fibrotic group (fig.1&table 1).

VI. Histopathological examination

H&E stain of liver tissue of control fibrotic rats showed multiple anomalies. Hepatocytes especially in pericentral area appeared swollen, apoptotic with foamy degeneration and vacuolated cytoplasm. There was distortion of liver architecture, infiltration with inflammatory cells and congested central and portal veins (fig.3). Masson trichrome

stain of control fibrotic group showed marked hepatic fibrosis in the form of periportal and pericentral fibrosis with fibrous septa between them (fig.10). Treatment with either Calcipotriol, Chole-

calciferol, Aliskiren, Eprosartan or Ramipril caused significant improvement in all pathological changes (fig.4-8) and fibrosis score (fig.11-16) as compared to control fibrotic group.

Table (1): Effect of Calcipotriol, Cholecalciferol, Aliskiren, Eprosartan and Ramipril on Liver Functions, hepatic oxidative stress markers, hepatic TGF- β 1, serum calcium, 25(OH)D and renin gene in CCL4 treated rats (Mean $\pm$ standard deviation, number = 8).

	<i>Normal control</i>	<i>Fibrotic control</i>	<i>Calcipotriol</i> 20 μ g/kg	<i>Cholecalciferol</i> 500 IU/kg	<i>Aliskiren</i> 10 mg/kg	<i>Eprosartan</i> 60 mg/kg	<i>Ramipril</i> 10 mg/kg
<i>AST IU/L</i>	38.9 $\pm$ 16.6	217.8 $\pm$ 32.4 a***	84.7 $\pm$ 18.8 a**b***	98.3 $\pm$ 21.3 a***b***	136.7 $\pm$ 26.7 a***b***c**d*	123.6 $\pm$ 21.8 a***b***c*	152.8 $\pm$ 27.3 a***b***c***d***
<i>ALT IU/L</i>	34.8 $\pm$ 12.7	173.9 $\pm$ 28.2 a***	67.6 $\pm$ 14.6 a**b***	81.2 $\pm$ 16.5 a***b***	112.6 $\pm$ 21.7 a***b***c***d*	102.7 $\pm$ 17.3 a***b***c*	127.5 $\pm$ 23.4 a***b***c***d***
<i>Bilirubin mg/dl</i>	0.7 $\pm$ 0.1	3.1 $\pm$ 0.7 a***	1.0 $\pm$ 0.29 b***	1.3 $\pm$ 0.38 b***	2.1 $\pm$ 0.61 a***b***c***d*	1.7 $\pm$ 0.52 a***b***	2.36 $\pm$ 0.4 a***b***c***d***
<i>Albumin gm/dl</i>	4.1 $\pm$ 0.76	1.8 $\pm$ 0.3 a***	3.7 $\pm$ 0.64 b***	3.3 $\pm$ 0.49 a**b***	2.6 $\pm$ 0.33 a***b***c**	2.9 $\pm$ 0.45 a***b***c*	2.21 $\pm$ 0.37 a***c***d**
<i>Calcium mg/dl</i>	10.7 $\pm$ 1.3	7.4 $\pm$ 1.2 a***	8.6 $\pm$ 1.15	10.13 $\pm$ 1.4 b**	7.8 $\pm$ 1.3 a**d*	8.2 $\pm$ 1.7 a**	7.65 $\pm$ 1.6 a**d*
<i>25(OH)D ng/ml</i>	43.8 $\pm$ 3.5	22.7 $\pm$ 4.45 a***	29.8 $\pm$ 5.6 a***	39.6 $\pm$ 4.7 b***c**	27.3 $\pm$ 4.9 a***d***	28.6 $\pm$ 5.3 a***d**	26.2 $\pm$ 4.2 a***d***
<i>TGF-β1 pg/mg</i>	211.6 $\pm$ 24.6	517.4 $\pm$ 42.6 a***	268.3 $\pm$ 19.9 a**b***	320.8 $\pm$ 21.4 a**b***c*	378.3 $\pm$ 31.7 a**b***c***d*	327.4 $\pm$ 37.6 a**b***c**e*	386.4 $\pm$ 38.3 a**b***c***d***f**
<i>GSH</i>	251.2 $\pm$ 10.3	82.6 $\pm$ 27.9 a***	211 $\pm$ 27.6 a**b***	196.4 $\pm$ 29.7 a**b***	146.3 $\pm$ 28.2 a***b***c***d**	187.1 $\pm$ 24.9 a**b***e*	132.8 $\pm$ 21.8 a**b***c***d***f**
<i>MDA nmol/g</i>	45.2 $\pm$ 10.3	178.3 $\pm$ 27.90 a***	76.4 $\pm$ 11.2 a**b***	87.3 $\pm$ 16.7 a**b***	129.4 $\pm$ 23.6 a***b***c***d**	98.4 $\pm$ 16.2 a***b***e*	136.8 $\pm$ 23.3 a***b***c***d***f**
<i>NO μmol/g</i>	66.7 $\pm$ 11.6	212.1 $\pm$ 32.4 a***	91.1 $\pm$ 25.6 b***	112 $\pm$ 26.4 a**b***	162.7 $\pm$ 24.4 a***b***c***d**	122.6 $\pm$ 27.2 a**b***e*	171.4 $\pm$ 27.3 a***b***c***d***f**
<i>Liver renin</i>	0.38 $\pm$ 0.08	1.85 $\pm$ 0.38 a***	0.81 $\pm$ 0.17 a**b***	0.93 $\pm$ 0.21 a**b***	1.53 $\pm$ 0.3 a***c***d***	1.46 $\pm$ 0.27 a***c***d**	1.64 $\pm$ 0.31 a***c***d***
<i>Renal renin</i>	0.63 $\pm$ 0.13	1.67 $\pm$ 0.42 a***	0.99 $\pm$ 0.12 a**b***	1.09 $\pm$ 0.14 a**b***	1.52 $\pm$ 0.22 a***c***d**	1.47 $\pm$ 0.2 a***c***d**	1.59 $\pm$ 0.25 a***c***d**

Test used: One way ANOVA followed by post-hoc tukey

Significance: * Mild ($p < 0.05$) **Moderate ($p < 0.01$)

a:significance to Normal control group

c: significance to Calcipotriol group

e: significance to Aliskirin group

b: significance to Fibrotic control group

d: significance to Cholecalciferol group

f: significance to Eprosartan group

P:Probability

*** High ($p < 0.001$)

Fig.(1):Renin and control gene (G3PD) expression in liver and renal tissue. A (molecular weight band), B (Normal control band), C (Fibrotic control band), D (Calcipotriol band) E (Cholecalciferol band), F (Aliskirin band) G (Eprosartan band), H (Ramipril band).

Fig.(2): Control healthy rats showing normal hepatocytes, hepatic sinusoids and central vein (H&E).

Fig.(3): Control fibrotic rats showing hepatocyte vacuolation, swelling, necrosis with loss of hepatic architecture and congested central vein (H&E).

Fig.(4): Calcipotriol treated rats showing marked improvement in all pathological changes(H&E).

Fig.(5): Cholecalciferol treated rats showing moderate improvement in all pathological changes(H&E).

Fig.(6): Aliskirin treated rats showing mild improvement in all pathological changes(H&E).

Fig.(7): Eprosartan treated rats showing moderate improvement in all pathological changes(H&E).

Fig.(8): Ramipril treated rats showing mild improvement in all pathological changes (H&E).

Fig.(9): Control healthy rats showing absence of fibrous tissue (Masson Trichrome)

Fig.(10): Control fibrotic rats showing marked fibrosis(Masson Trichrome)

Fig.(11): Calcipotriol treated rats showing marked improvement in fibrosis (Masson Trichrome)

Fig.(12): Cholecalciferol treated rats showing moderate improvement in fibrosis (Masson Trichrome)

Fig.(13): Aliskirin treated rats showing mild improvement in fibrosis (Masson Trichrome)

Fig.(14): Eprosartan treated rats showing moderate improvement in fibrosis (Masson Trichrome)

Fig.(15): Ramipril treated rats showing mild improvement in fibrosis (Masson Trichrome)

Fig.(16): Statistical analysis of liver fibrosis score.

Significance: * Mild ($p < 0.05$)

**Moderate ($p < 0.01$)

*** High ($p < 0.001$)

P:Probability

a: significance to control Fibrotic group

b: significance to Calcipotriol group

c: significance to Cholecalciferol group

d: significance to Aliskirin group

e: significance to Eprosartan group

Discussion

CCl₄ is one of the oldest and most widely used toxin for induction of hepatic fibrosis. CCl₄ is activated by cytochrome p450 oxidase to yield toxic metabolites, including trichloromethyl and trichloromethyl peroxy. The former is responsible for the covalent binding to cell components like glutathione, resulting in its depletion, whereas the latter initiates lipid peroxidation process with consequent formation of by products such as MDA resulting in loss of membrane integrity and cell death (18 & 19).

In the present study, CCl₄ intoxicated rats showed significant disturbance in liver function in the form of increased liver enzymes (AST & ALT), total bilirubin and decreased serum albumin concentration as compared with control normal rats. Increased liver enzymes result from damage and loss of structural integrity of hepatic cells which release AST enzyme (present in cytoplasm & mitochondria) and ALT enzyme (present in cytoplasm). The effect of CCl₄ administration on liver function has been proven previously in many studies (20-22).

In this study, CCl₄ intoxicated rats showed significant increase in hepatic TGF- β 1 which considered one of the most powerful profibrogenic cytokine (23). TGF- β 1 mediates HSC activation, hepatocyte apoptosis, collagen deposition and increase expression of other profibrogenic mediators such as tissue inhibitor metalloprotease 1 (TIMP-1) and connective tissue growth factor. TGF- β 1 was found to play a pivotal role in epithelial to mesenchymal transition (EMT) of hepatocytes. EMT occurs in response to injury, to act as additional source of myofibroblast, which is essential for repair of injured tissue (24).

CCl₄ intoxicated rats showed significant increase in oxidative stress markers manifested by

increase in MDA, NO and decrease in reduced glutathione (GSH) levels in liver tissue homogenate in control fibrotic group. The effect of CCl₄ administration on oxidative stress markers has been proven previously in other studies that found that CCl₄ administration caused oxidative stress in rats by stimulation of liver NADPH oxidase enzyme which produces excess free radicals (superoxide and peroxynitrite) and liver injury (25).

CCl₄ intoxicated rats showed significant increase in local and systemic renin gene expression in liver and kidney tissues respectively as compared to control normal group. Strong evidence supports the presence of functional local RAS in several organs such as pancreas, heart and liver beside systemic RAS (26). Activated HSC and Kupffer cells contain all components of RAS and secrete angiotensin II which activates NADPH oxidase enzyme in HSC and increase formation of reactive oxygen species (9).

The present study showed that CCl₄ intoxicated rats have significant decrease in serum calcium and 25(OH)D. Vitamin D deficiency and insufficiency are considered to be very common problem among patients with CLD. Several studies reported that patients with CLD from various etiologies have been associated with low serum vitamin D level. It has been also reported that the incidence of vitamin D deficiency increase as the liver disease progress (10). Recent meta-analysis study by Abbasi et al. (2016) who collect and analyze data from 641 published articles showed that about 80% of patients with CLD, had severe vitamin D deficiency while the remaining 20% of patients don't have significant deficiency (11).

Many clinical studies showed that low vitamin D level aggravates hepatic fibrosis and vitamin D deficiency can be considered strong independent predictor for hepatic fibrosis. Also, they reported that vitamin D administration can inhibit liver fibro-

sis by activation of hepatic vitamin D receptor (VDR). These results encourage experimental studies to detect molecular mechanisms underlying this antifibrotic effect of VDR. Experimental studies reported that VDR activation can inhibit gene expression of key molecules in hepatic fibrogenesis like $\beta 1$ collagen, TGF- $\beta 1$, α smooth muscle actin (α SMA) and TIMP-1. Also, VDR activation can decrease inflammatory markers and inhibit TGF- $\beta 1$ signaling (12&27-29). On the other hand some studies reported that serum vitamin D level do not correlate with the progression of hepatic fibrosis. El-Maouche et al. (2013) reported that Vitamin D deficiency was highly prevalent in most African American population co-infected with HCV and HIV, but severity of Vitamin D deficiency was not related to bone mineral density or severity of hepatic fibrosis (30). Kitson et al. (2013) and Esmat et al. (2015) reported that Vitamin D level does not predict stage of hepatic fibrosis or sustained virological response in chronic HCV infection (31&32).

However, the mechanisms underlying this high prevalence of vitamin D deficiency among patients with CLD are still incompletely understood. There are many probable causes for the observed high prevalence of vitamin D deficiency among patients with CLD. The underlying mechanisms are multifactorial and appear to vary among different liver pathologies. Important possible mechanisms to consider are: reduced exogenous exposure of CLD patients to vitamin D sources from diet and limited exposure to sunlight especially in severe liver disease; decreased intestinal absorption of dietary vitamin D in presence of cholestasis since vitamin D is a fat soluble vitamin and its absorption depends on bile salts; decreased production of albumin and vitamin D binding protein in presence of liver cirrhosis; impaired hepatic hydroxylation of vitamin D to 25(OH)D and finally increased catabolic removal of 25(OH)D (33). High prevalence of vitamin D deficiency in chronic hep-

atitis C may result from decreased vitamin D level and/or activity by HCV. It has been hypothesized that HCV decrease serum vitamin D level through disruption of lipid metabolism decreasing 7-dehydrocholesterol (precursor of vitamin D3) and inhibition of 25 hydroxylation in liver. Inhibition of vitamin D hydroxylation in liver may result from direct effect of HCV or indirect through increase oxidative stress and induction of cytokine as IL-17 and IL-23. It has been suggested that HCV decrease vitamin D activity through inhibition of VDR expression leading to vitamin D resistance and aggravation of HCV associated necrosis, inflammation and fibrosis (34).

In our study treatment with either calcipotriol or cholecalciferol for 8 weeks simultaneously with CCl₄ resulted in marked decrease in hepatic fibrosis score and all pathological features as compared to control fibrotic group. This improvement in histopathological changes is associated with improvement in liver function tests (decrease in liver enzymes, total bilirubin and increase in serum albumin). Moreover, both calcipotriol and cholecalciferol groups showed marked decrease in TGF- $\beta 1$ level and improvement in oxidative stress markers (decreases in MDA, NO and increase in GSH in liver tissue).

Calcipotriol don't have significant effect on serum 25(OH)D and serum calcium levels as compared to control fibrotic group. Calcipotriol has 200 times less effect to cause hypercalcaemia than calcitriol which may explain non significant effect of calcipotriol on serum calcium (12&35). Calcipotriol also don't has direct effect on vitamin D hydroxylation in liver which may explain non significant effect of calcipotriol on serum 25(OH)D. Cholecalciferol showed significant increase in serum levels of 25(OH)D and calcium as compared to control fibrotic group. The effect of Cholecalciferol on 25(OH)D level appears to be result from normal metabolic pathway of Cholecalciferol through direct hydroxy-

lation at site 25 in liver which subsequently activated by hydroxylation at site 1 in the kidney to calcitriol which has potent hypercalcemic effect.

In our study Calcipotriol and Cholecalciferol caused marked decrease in renin gene expression in liver and kidney as compared to control fibrotic group. However, our study is the first study to examine the effect of vitamin D and its active analogue (calcipotriol) on renin gene expression in hepatic fibrosis. Several studies showed that different active vitamin D analogues have protective effect on chronic kidney and cardiac diseases through inhibition of renin gene expression (36&37). The beneficial effects of calcipotriol and cholecalciferol in prevention of hepatic fibrosis are mostly due to activation of VDR. Calcipotriol can directly stimulate VDR while cholecalciferol must be activated by hydroxylation in liver and kidney to calcitriol which is potent activator to VDR (38&39).

In the present study, treatment with aliskiren, eprosartan and ramipril for 8 weeks simultaneously with CCl₄ resulted in a significant reduction in CCl₄ induced hepatic fibrosis and other histopathological changes. This improvement in histopathological changes is associated with improvement in liver function tests as compared to control fibrotic group. However, ramipril don't have significant effect on serum albumin. Moreover, they produced significant improvement of hepatic TGF- β 1 level and oxidative stress markers. Additionally, they don't have significant effect on serum 25(OH)D, serum calcium level or renin gene expressions in liver and kidney tissues as compared to control fibrotic group.

These results are in agreement with those obtained by other studies which reported that Aliskiren and ramipril attenuate liver fibrosis through reduction of angiotensin II formation leading to prevention of liver fibrosis through inhibition of

HSC, inhibition of Kupffer cell, anti-oxidant effect and reducing TGF- β 1 level (40-42). We didn't found any studies evaluating the effect of Eprosartan on liver fibrosis but there are many studies evaluating other angiotensin receptor blocker (ARBs) on liver fibrosis. These studies reported that ARBs attenuate liver fibrosis and improve liver function through reduction of HSC activation, collagen secretion, α -SMA, TGF- β 1, and PDGF (43-44). On the other hand experimental study by Paizis et al. (2001) reported that, Irbesartan doesn't have significant change in liver histology or hydroxyproline content despite of the reduction of the over expression of TGF- β 1 and the matrix protein and type 1 collagen in the liver(46).

Beneficial effect of aliskiren in prevention of hepatic fibrosis is mostly due to direct inhibition of renin enzyme preventing formation of angiotensin-1. Ramipril prevents progression of liver fibrosis through inhibition of ACE preventing formation of angiotensin-II. Eprosartan inhibit hepatic fibrosis through blocking of angiotensin II type 1 receptor (AT1) which expressed on the surfaces of activated HSC. Additionally, blockage of AT1 receptors by eprosartan leads to increase in local angiotensin II concentration that stimulates the unblocked AT2 receptor which decreases hepatic fibrosis and oxidative stress (47).

Conclusion

Calcipotriol, Colecalciferol, Aliskirin, Eprosartan and Ramipril have antifibrotic effect and may be tried in strategy for treatment of chronic liver diseases. Further clinical studies are recommended to test for the safety and efficacy of these drugs in human.

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الملخص العربي

تقييم التأثير المحتمل المضاد للتليف للكوليكالسيفيرون، كالسيبوترايول،

ألسكرين، رامبيريل و ابروسارتان على تليف الكبد الناجم عن رباعي

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استاذ الفارماكولوجيا الاكلينيكية بكلية طب المنصورة****

تليف الكبد عبارة عن عملية ديناميكية لالتئام الجرح الناتج عن امراض الكبد المزمنة. هناك أسباب متعددة تؤدي إلى تليف الكبد منها: الفيروسات الكبدية ،ادمان شرب الكحوليات ، الركود الصفراوي و ترسب الدهون فى الكبد. الخلية الكبدية النجمية هى المصدر الرئيسى للاليف المترسبة فى الكبد. الى الان لا يوجد علاج دوائى قائم على الدليل لعلاج تليف الكبد لذلك كان هدف هذه الرسالة وهو اختبار قدرة بعض الادوية الواعدة مثل كوليكالسيفيرون و كالسيبوترايول و الادوية المثبطة لإنزيم الرنين (اليسكرين) و المثبطة لانزيم تحويل أنجيوتنسين (رامبيريل) و قافلات مستقبلات AT1 (ابروسارتان) والتي يحتمل ان يكون لها تأثير وقائي ضد تليف الكبد. وقد تم استخدام ٥٦ جرذا من الجرذان البيضاء تتراوح اوزانهم بين ٢٥٠ الى ٣٠٠ جرام وتم تقسيمهم الى سبع مجموعات كل مجموعة بها ٨ من الجرذان. المجموعة الاولى (الضابطة الطبيعية) تم اعطائها زيت زيتون فقط فى التجويف البريتونى للجرذان. المجموعة الثانية (الضابطة المصابة بتليف الكبد) تم اعطائها مادة رباعي كلوريد الكربون فى التجويف البريتونى للجرذان. وأما باقى الخمس مجموعات المتبقية فقد تم إحداث التليف الكبدى بهم بمادة رباعي كلوريد الكربون لمدة ٨ أسابيع متتالية وتم إعطاؤهم أدوية كالسيبوترايول و كوليكالسيفيرون و اليسكرين و ابروسارتان و رامبيريل بالترتيب ولمدة ٨ أسابيع متتالية متزامنا مع إعطاء مادة رباعي كلوريد الكربون. وقد اظهرت الدراسة النتائج التالية. فى المجموعة الضابطة الطبيعية: كان كلا من مستوى تحاليل الدم و الأنسجة و صورة نسيج الكبد فى الحدود الطبيعية. فى المجموعة الضابطة المصابة بتليف الكبد بمادة رباعي كلوريد الكربون فقط لمدة ٨ أسابيع متتالية كان نسيج الكبد مليء بتليف حول الأوردة المركزية و الوريد البابى الكبدى وتليف متصل بينهما وقد حدث تشوه كامل للترتيب البنائى بالمقارنة بالجرذان القياسية الطبيعية. وأما باقى الخمس مجموعات المتبقية فقد تبين ان استخدام أدوية كالسيبوترايول و كوليكالسيفيرون و اليسكرين و ابروسارتان و رامبيريل مع مادة رباعي كلوريد الكربون ادى الى عرقلة عملية تقدم التليف الكبدى وذلك من خلال حدوث نقص كبير فى كمية الألياف حول الأوردة المركزية و الوريد البابى الكبدى وقد تم تأكيد هذه النتائج التى جاءت فى تحليل أنسجة الكبد أيضا عن طريق إختبار تحليل الصورة وتقدير المساحة التى تحتلها الألياف. وقد صاحب هذا التحسن فى شكل

انسجة الكبد تحسن ملحوظ في وظائف الكبد ودلالات التليف ودلالات الأكسدة في أنسجة الكبد و أيضا نقص التعبير الجيني لإنزيم الرينين في نسيج الكبد والكلي بالمقارنة بالمجموعة الضابطة المصابة.و من الجدير بالذكر هنا أن الادوية التي تنشط مستقبلات فيتامين د (كالسيوترايول و كوليالكاليفيرول) كان لها التأثير الأقوى في عرقلة تليف الكبد وتقليل التعبير الجيني لإنزيم الرينين وتحسين وظائف الكبد ومضادات الاكسدة بالمقارنة بالأدوية الأخرى التي تعمل على تثبيط نظام رينين انجيوتنسن الدوستيرون.