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PRANLUKAST EFFECT ON THE EARLY STAGES OF LIVER DAMAGE IN RATS TREATED WITH CCl4

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ABSTRACT

Development of liver fibrosis is accompanied by an increased amount of nitric oxide (NO), prostaglandin E2 (PGE2) and 5-lipoxygenase products as leukotrien B4 (LTB4). Carbon tetrachloride (CCL4) induced liver fibrosis in experimental models are often used for investigation of the hepatoprotective effect of drugs. The potential effect of either silymarin and/or pranlukast against CCL4 induced liver damage was examined in a CCL4 model in rats. Sprague Dawley male rats were intraperitoneally injected with CCL4 and received Silymarin and or pranlukast orally once daily for 8 weeks. Both silymarin and pranlukast significantly decreased serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), increased the activities of superoxide dismutase (SOD) and catalase , decreased malondialdehyde (MDA) content in liver and in addition, they decreased (NO) production and transforming growth factor β in CCL4 treated rats compared with CCL4 group.

In conclusion, this study proved that pranlukast protects CCL4 treated rats from liver fibrosis via its ability to decrease oxidative free radicals and transforming growth factor β induced liver fibrosis.

Key Words: *Pranlukast, leukotriens, liver fibrosis*

INTRODUCTION

Carbon tetrachloride induced liver injuries in experimental animal models are often used for the screening of anti-hepatotoxic and hepatoprotective activities of drugs *[1].* CCL4 induced hepatic damage involves increased

lipid per-oxidation, decreased activities of antioxidant enzymes and biotransformation of free radical derivatives *[2].*

CCL4 induced hepatic fibrosis is through 2 steps. The first involves lipid per-oxidation and necrosis of hepatocytes. The second one is the stimulation of Kupffer cell by the free radicals and production of proinflammatory mediators *[3].*

The two mediators potentiate CCL4 induced hepatic lesion are tumor necrosis factor-alpha (TNF-α) and nitric oxide (NO) *[4].*

Silymarin is a standardized extract of the milk-thistle (silymmarianum). Its main active compounds are the flavonoids silibinim, silychristine, silydianin and silibinin *[5].* It is an attractive drug to treat liver disease since it lacks toxic side effects *[6].*

Silymarin prevents or attenuates acute liver injury caused by carbon tetrachloride*[7]* Silymarin prevents fibrosis induced by carbon tetrachloride owing to its antioxidant and radical scavenging properties *[8].*

Pranlukast is a cysteinyl leukotriens receptor antagonist-1, it is similar to montelukast. Arachidonic acid is a poly unsaturated fatty acid that can be metabolized by several enzymes to produce lipid mediators. 5-lipoxygenase metabolizes arachidonic acid to leukotriene B4 and the cysteinyl leukotrienes which possess bronchoconstrictive and proinflammatory effects via action on specific leukotriene receptors *[9].*

Drugs that inhibit 5-lipoxygenase pathway or antagonize the cysteinyl-leukotriene receptors are effective treatment for bronchial asthma and pulmonary fibrosis *[10, 11].* Leukotriens have been exerting potent effects on fibroblast migration, proliferation and production of extracellular matrix proteins in vitro suggesting that they may also be able to stimulating mesenchymal cells to grow and deposit collagen in vivo *[12].*

Transforming growth factor- β is one of the biomarkers of fibrotic lung disease activity *[13].* It is a cytokine that acts upon proliferation, migration, differentiation and apoptosis of cells and accumulation of extracellular matrix components. It is detected in different organ fibrosis as lung, liver, kidney and skin *[14].*

So, this study was aimed to investigate the effect of pranlukast on CCL4 induced rat liver injury, compared it to the standard antifibrotic Silymarin and to examine 5-lipoxygenase pathway which is another strategy for prevention of liver fibrosis.

Materials

Animals

Adult male Sprague Dawley rats (250-300 g) were obtained from the animal house of the research unit at faculty of medicine, Mansoura university.

Chemicals

Drugs were pure materials from Sigma Chemical .CO, St.Louis, MO. USA)

Method:

Establishment of a Rat Model with Hepatic Injury and Fibrogenesis Caused by CCl_4.

The rat model was established using the method originally described by ***Proctor and Chatamra*** *[15]* .

120 male Sprague-Dawley rats were randomly divided into four groups (thirty rats/group). Group 1 was the vehicle control in which rats were intraperitoneally (IP) injected with the vehicle olive oil. Group 2 was the CCl_4 group in which rats were IP injected with CCl_4, Group 3 was a silymarine treated group in which rats were injected with CCl_4 and received silymarine at 50mg/kg *[8]*. Group 4 was pranlukast treated group in which rats were injected with CCl_4 and received pranlukast hemihydrate at 10 mg/kg *[16]*. All rats were fed with chow diet and kept at 21-25°C under a 12-h dark/light cycle. All protocols were approved by our local committee of Animal Care and Use Committee. Rats in groups 2, 3, and 4 were IP injected with a mixture of CCl_4 (0.1 ml/100 g body weight) and olive oil [1:1 (v/v)] every other day for 8 weeks. Silymarine and Pranlukast were suspended in sterile PBS and given once daily by gavages. The control animals in group 1 was similarly handled, including IP injection with the same volume of olive oil and oral administration of the same volume of PBS only. Rats in group 2 received oral administration of the same volume of PBS. Forty-eight hours after the last CCl_4 injection, rats were sacrificed after being anesthetized by IP pentobarbital (50 mg/kg). The liver from each rat was cut in pieces and rapidly frozen at -70 for measurement of the following parameters.

Analyses of the Pathological Indexes for Hepatocytic Death and Hepatic Injury.

- **Liver function tests**

Blood was collected from each rat by heart puncture when sacrificed. After coagulation, sera were collected and stored at -20°C for further analyses. Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) [17] and total bilirubin *[18]* were determined spectrophoto-

metrically using commercial kits (BIO diagnostic Co., Egypt).

- **Determination of the hepatic Content of Hydroxyproline.**

  This experiment was performed using a colorimetric method described by Bergman and Loxley *[19]*. In brief, three small pieces of liver tissues randomly excised from the liver of every rat in the rat model were hydrolyzed in 6 N HCl at 110°C for 24 h, and subsequently they were neutralized with NaOH. Isopropanol in citrate acetate-buffered chloramine T (*Sigma-Aldrich St. Louis, MO*) was added to aliquots of the hydrolysate, followed by the addition of Ehrlich reagent (*Sigma*). The chemical reaction occurred in dark for 25 min at 60°C. After centrifugation, the absorbance of the supernatant of each sample was read at 558 NM using a 96-well plate spectrometer (SpectraMax 190). *Trans*-hydroxyproline was used as the standard for quantification.

- **Determination of hepatic TGF-** β**.**

  Levels of hepatic TGF-β in rats were determined by using a corresponding ELISA kit purchased from BD Biosciences (San Jose, CA) according to the protocol provided by the manufacturer. In brief, Microplates were coated with 100 µl/well of capture antibody, and then they were incubated overnight at 4°C. After washing, the plates were blocked with Assay Diluent (BD Biosciences) at room temperature (RT) for 1 h. One hundred microliters liver extract in PBS supplemented with protease inhibitors, was added to each well of the plate, followed by incubation for 2 h at RT. Working Detector (100 µl; BD Biosciences) was loaded into each well, and the plate was incubated for an additional 1h at RT before the addition of Substrate Solution (100 µl; BD Biosciences). The reaction was stopped by adding Stop Solution (50 µl; BD Biosciences). The absorbance was read at 450 NM, with reference wavelength at 570 NM using a 96-well plate spectrometer (SpectraMax 190; Molecular Devices, Sunnyvale, CA). Calculation of the concentrations of the cytokine was performed in a log-log linear regression according to the instructions in the protocol.

- **Determination of oxidative stress biomarkers**

  Superoxide dismutase (SOD) *[20]* catalase (CAT) *[21]* and malondialdehyde (MDA) *[22]* were estimated in liver tissues as indicators of oxidative stress.

- **Determination of hepatic nitric oxide levels:**

Hepatic nitric oxide levels were measured by a colorimetric method as described by **Montgomery** and ***Dymock*** *[23].*

- **Determination of total protein:**

Total protein is needed for tissue parameters calculation was determined by the method described by ***Lowry, et al***. *[24].*

Statistical analysis

All data were expressed as mean ± standard deviation (SD). ANOVA with Tukey's post-hoc test was used for repeated measures comparison. P value ≤ 0.05 was considered as statistically significant. All analyses were carried out using the SPSS computer program version 11.0 for windows.

Table (1): Biochemical Parameters in the tested groups

	CONTROL	CCL4	CCL4+S	CCL4+P
SERUM ALT (units/L)				
Mean ±SD	23.4±1.9	67.65±5.3*	35.8±3.6#	33.38±2.82#
SERUM AST (units/L)				
Mean ±SD	64.6±4.2	163.3±7.9*	92.49±4.3#	81.9±3.1#
TOTAL BILIRUBIN (mg/dl)				
Mean ±SD	0.47±.07	14.2±2.28*	5.6±1.07 *#	3.3±.84 *#

Significance when p<0.05

* comparison between Control group and CCl4 group

comparison between CCl4 group and CCl4+Silymarin & CCl4+Pranlukast groups

Table (2): NOx concentrations in hepatic tissues.

	CONTROL	CCL4	CCL4+S	CCL4+P
HEPATIC NOx (umol/mg protein) **Mean ±SD**	16.95±2.07	83.28±8.91*	54.75±3.55*#	40.4±1.39*#

Significance when p<0.05

* comparison between Control group and CCl4 group

comparison between CCl4 group and CCl4+Silymarin & CCl4+Pranlukast groups

Table (3): The indexes of lipoperoxidation and activity of antioxidant protection enzymes (SOD, catalase) MDA, transforming growth factor- β and hydroxyproline in the tested groups.

	CONTROL	CCL4	CCL4+S	CCL4+P
SOD (u/mg protein) **Mean ±SD**	4.2±.71	1.59±.35*	3.43±.39#	4.1±.68#
CATALASE (u/mg protein) **Mean ±SD**	8.86±.77	3.66±1.00*	8.49±.79#	8.34±1.23#
MDA (nmol/mg protein) **Mean ±SD**	26.94±2.21	241.11±56.25*	51.27±1.9#	45.29±5.87#
HYDROXYPROLINE (umol /mg protein) **Mean ±SD**	3.88±.80	6.4±.9*	4±.76#	2.98±.8#
TGF -β (pg /mg protein) **Mean ±SD**	479.62±23.7	2101.4±100.02*	1394.94±325.75*#	518.7±45.21#

Significance when p<0.05

* comparison between Control group and CCl4 group

comparison between CCl4 group and CCl4+Silymarin & CCl4+Pranlukast groups

RESULTS:

Liver function tests:

Table 1 shows that CCl4 significantly increased serum activities of ALT, AST and total bilirubin as compared to control ($p < 0.05$). Pranlukast and silymarin each alone caused significant ($p < 0.05$) decrease in the elevated serum of ALT, AST and total bilirubin when compared to CCl4 treated rats.

Hepatic nitric oxide:

CCL4 increased NO production ($p < 0.05$) compared to control group. Both silymarin and pranlukast inhibited CCL4-induced NO production (Tables 2).

Oxidative stress markers:

Table 3 also shows that CCL4 caused depletion of liver catalase and SOD content as compared to control rats.

While catalase and SOD content was significantly increased in both silymarin and pranlukast treated rats ($p < 0.05$).Hepatic MDA content was increased significantly in CCL4 treated rats ($p < 0.05$) compared to control. Reduction of MDA content in liver was observed in silymarin and pranlukast treated rats ($p < 0.05$) compared to CCL4 treated group (Table 3).

Hepatic TGF – β: CCL4 caused a significant increase in hepatic TGF-β ($p < 0.05$) compared to control group (Table 3). This increase was inhibited by administration of either pranlukast and or silymarin ($p < 0.05$).

The hepatic content of hydroxyproline:

Compared to the control, The content of hepatic hydroxyproline was significantly higher in rats injected with CCl_4. The level of hepatic hydroxyproline was significantly reduced in the rats treated with silymarine and or pranlukast ($p<0.05$) (table3).

Discussion:

Carbon tetrachloride has been used as a model for studying the pathogenesis of hepatic necrosis which produces free radicals triggering a cascade of events leading to hepatic fibrosis *[25]*.

This research was done to investigate the effect of silymarin and pranlukast on hepatic fibrosis induced by carbon tetrachloride in rats. In this study the rats were injected every other day for 8 weeks intraperitoneally with carbon tetrachloride which is a hepatotoxic drug causing hepatic injury in the form of ballooning degeneration

of hepatocytes, hepatocellular necrosis, inflammation, fibrosis and the formation of nodules surrounded by scar tissue termed cirrhosis*[26]*.

Pranlukast (an anti-asthmatic drug similar to Montelukast) administration decreased the serum ALT, AST and bilirubin to their Normal values. This is an indication of hepatic tissue repair in comparison to hepatic injury and loss of cell membranes leading to cellular leakage of the previous parameters in rats that were received CCL4 alone*[27]* .

Pranlukast could produce an antioxidative effect against CCL4 induced hepatic fibrosis through increasing the activities of SOD, catalase, GSH in hepatic tissue and at the same time it decreased MDA and NO levels in hepatic tissue which explains its antioxidative and anti-inflammatory effect. Previous studies have shown that the antioxidant system protects hepatic tissue from damage by oxidative stress and free radical formation. SOD, catalase and GSH are decreased by free radicals produced by lipid peroxidation process in response to CCL4 administration *[27]*.

On the other hand the proinflammatory cytokine Tumor necrosis factor-α and the oxidative NO are produced under the hepatotoxic effect of CCL4. The liver is an inflamed organ as their Kupffer cells release proinflammatory mediators due to the direct action of CCL4 and/or other hepatotoxins *[28, 4]*.

Tumor necrosis factor-α in turn leads to NO production in the hepatic tissue. NO is a highly reactive oxidant can increase oxidative stress peroxynitrite. Pranlukast administration leads to a decrease in hepatic NO, which indicates attenuation in the expression level of inducible, NO synthase and cycloxygenase-2 enzymes *[29]*.

Silymarin produced the same effects as pranlukast on serum hepatic enzyme, antioxidant system and the oxidant hepatic NO. These results are supported by many studies proved the protective hepatic effect of silymarin in CCL4 induced liver injuries through its antioxidant effect *[30]*.

Fibrosis affects many organs as liver and lung and is a cause of significant morbidity and there is no therapy for hepatic fibrosis [31]. Significant molecular insights into

the signaling underlying hepatic fibrosis have been made and showed that in addition to lipid peroxidation, disruption of Ca homeostasis, transforming growth factor-beta (TGF-β) signaling is a major contributor to the fibrogenesis*[31, 1]*.

Pranlukast treated rats produced antifibrotic effect on liver tissue as compared with CCL4 treated rats. Many studies provide evidences that drugs that directly target the formation of prostaglandins and leukotriens or their binding to specific receptors provide opportunities in inflammatory therapy *[32]*.

Evidence indicates that cycloxygenase-2 and 5-lipoxygenase pathways have involved in liver inflammation, tissue remodeling and fibrosis *[33]*. Indeed cycloxygenase-2 expression is upregulated in rats with carbon tetrachloride induced liver injury and in alcoholic liver and steatohepatitis experimental models [33]. Similar to cycloxygenase-2, the upregulation of 5-lipooxygenase has been reported in chronic liver disease and experimental models of liver injury *[34]*.

5-lipoxygenase derived products have been shown to activate hepatic stellate cells and inhibition of their formation induces apoptosis in Kupffer cells the major inflammatory cell type in the liver *[34]*.

Furthermore, blockade of 5-lipoxygenase pathway with a 5-lipoxygenase activating protein inhibitor protects the liver from experimental necroinflammatory damage and fibrosis *[35]*.

On the other hand, cycloxygenase-2 and 5-lipoxygenase play opposite roles in the regulation of expression of interleukin-6 which is a primary proinflammatory cytokine involved in hepatic-inflammatory process *[36]*. Cycloxygenase-2 inhibitor amplified interleukin-6 expression in macrophages whereas 5-lipoxygenase inhibition down-regulated IL-6 expression in these cells *[32]*.

Sipe et al. *[37]* and Marcouiller *[38]* found that among the different eicosanoids, 5-lipoxygenase products and in particular leukotriene B4 is important positive signals for cytokine expression and synthesis of inflammatory cells.

Finally, in the related animal models to the present study, silymarin has antioxidant radical scavenging properties that are leading

to alteration of hepatic Kupffer cell function, lipid peroxidation, collagen production and anti-fibrogenic effect in the liver *[39, 40]*.

CONCLUSION:

Pranlukast has antioxidant anti-inflammatory and antifibrotic action in hepatic tissue damage similar to the reference hepatoprotective drug Silymarin. Pranlukast (the antiasthmatic drug) has antifibrotic effect via inhibition of leukotriens which is a new pathway to prevent fibrosis in hepatic tissue.

AUTHOR CONTRIBUTIONS:

Both authors shared in creating the hypothesis, writing, doing the experimental design and the statistics of this study

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