Corosolic acid suppresses the expression of inflammatory marker genes in CCL4-induced-hepatotoxic rats

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Abstract: The aim of the study was to asses the anti-inflammatory effects of corosolic acid on the carbon tetrachloride (CCL4) toxicity in rats. Liver toxicity was induced by administered CCL4 (single dose (1:1 in liquid paraffin) orally at 1.25 ml/kg. Rats were pretreated with CRA for 7 days before made CCL4 toxicity at 20 mg/kg BW. The mRNA levels of TNF-α, IL-6, iNOS, COX-2 and NF-κB were assayed by reverse transcriptase PCR analysis. The mRNA levels of proinflammatory cytokines such as TNF-α, IL-6, and the inflammatory markers such as iNOS, COX-2 and NF-κB were significantly up regulated in CCl_4 induced rats and treatment with corosolic acid significantly reduced the expression of the above indicators. Our results suggest that the inhibition of TNF-α, IL-6, iNOS, COX-2 and NF-κB by corosolic acid, a potential candidate could possess anti-inflammatory activity besides its hepatoprotective effect in CCl4 liver toxicity in rats.

Keywords: Carbon tetrachloride, hepatotoxicity, corosolic acid, anti-inflammatory.

INTRODUCTION

Liver diseases are generally raised by various drugs, or viral infection (Lee *et al.,* 2007), and are considered a serious health problem all over the world. The CCl_4 induced hepatotoxicity is a well-proven experimental model for inducing acute liver injury, trichloromethyl-free radical (CCl_3* or CCl_3OO*) is formed by bio-transformation (Weber *et al.,* 2003). These reactive species attacks antioxidant enzymes such as glutathione (GSH), catalase and superoxide dismutase (SOD), lead to lipid peroxidation and liver damage (Jo *et al.,* 2001); Additionally, CCl4 toxicity may generate various endogenous reactive oxygen and nitrogen species, these molecules then stimulates the secretion of inflammatory mediators from hepatic macrophages, so that are assume to induce CCl4-liver injury (Recknagel, 1989). During inflammatory process, macrophages are helpful in maintaining the homeostasis of immunological phenomenon, together with the increased secretion of inflammatory cytokines and mediators such as IL- 1β, IL-6, TNF-α, NF-κB, iNOS, and COX-2 (Zeilhofer and Brune 2006; Jachak, 2007). Severe liver injury and its associated inflammation may possibly lead to the hepatic damage, fibrosis, then end with cirrhosis. The restrain of inflammatory cytokines and mediators could be a new remedial approach against liver inflammation.

Polyphenolics, a class of compounds, antioxidants derived from natural source, have generated extensive attention as possible beneficial mediators for a wide variety of chronic diseases. Corosolic acid (CRA), a triterpenoid, which is derived from *Actinidia valvata* Dunn and it was identified in many Chinese medicinal herbs (Fukushima *et al.*, 2006) and banaba leaves (Yamaguchi *et al.,* 2006). It has been reported that CRA exhibited hepatoprotective (Abdullah, 2013) and antihyperglycemic activity in rat models and clinical trials (Fukushima *et al.,* 2006; Miura *et al.,* 2006). It has been reported that CRA demonstrated cytotoxic activity in *in vitro* studies (Ahn *et al.,* 1998; Yoshida *et al.,* 2005).

Besides its hepatoprotective role, the anti-inflammatory aspect of corosolic acid remains unclear. Consequently, this study validated the protective effect of corosolic acid against CCl4 toxicity and a potential mechanism associated with hepatotoxicity.

MATERIALS AND METHODS

Chemicals

CRA was purchased from Mansite Pharmaceutical Co., Ltd (Chendu, China), CCl4, primary and secondary antibodies for TNF-α, IL-6, iNOS, COX-2 and NF-κ B were purchased from Sigma-Aldrich Co. (St. Louis, Missouri, USA). Other chemicals used in this study were of analytical grade obtained from E. Merck or HIMEDIA, Mumbai, India.

Animals

Male albino Wistar rats (180 to 200g) of Wistar strain, weighing approximately 150 to 180g, were acclimatized for 7 days at room temperature (30±3°C) and relative humidity (55%) in a 12-hour light/dark cycle in a room under hygienic condition. The experiments were carried out as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals and approved by ethics committee of the College of Medicine Research Center at King Saud University, Riyadh, Saudi Arabia (11/3215/IRB). The animals were given access to water and a commercial diet *ad libitum.*

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CCl4-induced hepatotoxicity

Liver toxicity was induced by administered CCL4 (single dose (1:1 in liquid paraffin) orally at 1.25 ml/kg BW at a 6 h interval after given the last dose of CRA on 7th day.

Experimental design

The animals were randomly divided into four groups of six animals each as given below. CRA was suspended in 0.1% DMSO, and fed to rats via an oral route at 20 mg/kg BW for 7 days. Then a single oral dose of CCl_4 (1:1 in liquid paraffin) at 1.25ml/kg BW (Saba et al., 2010) was given at an interval of 6 h after the administration of last dose of CRA. Groups IV was administered CRA at 20 mg/kg BW and also administered CCl_4 at an interval of 6 h after the administration of last dose of CRA on the 7th day.

Group I: Control rats received 0.1% DMSO only
Group II: Control rats received CRA (20 mg/kg BW) in 0.1%DMSO
Group III: CCl_4 (1:1 in liquid paraffin) at 1.25 ml /kg BW
Group IV: CCl_4+CRA (20 mg/kg BW) in 0.1%DMSO

On 8th day, the animals were anesthetized (ketamine (25 mg/kg BW)) and sacrificed by cervical dislocation. Liver was removed, cleared off blood and immediately transferred to ice-cold containers containing saline and used for the determination of various inflammatory markers.

Quantitative RT-PCR gene expression

All glass-wares were rinsed with diethyl-pyrocarbonate (DEPC) treated water to inhibit RNases. Total RNA was isolated using guanidiumthiocynate-chloroform-phenol method of Chomczynski and Sacchi (1987).

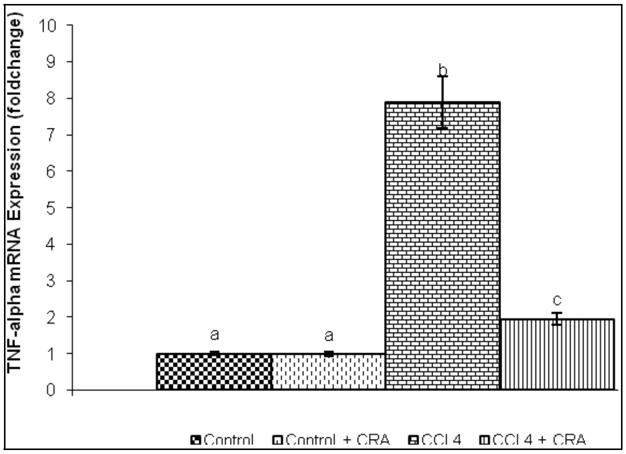

Fig. 1: Effect of corosolic acid on liver TNF-α mRNA expression level in CCL4 induced hepatoxic -rats.

Histogram depicts quantitation of three independent experiments (means ± S.D). The TNF-α mRNA expression was normalized with the expression of the GAPDH mRNA in each sample. Values not sharing a common superscript differ significantly at p ≤ 0.05 (DMRT).

Isolation of total RNA

The 1gm of tissue samples were grinded and homogenised (100mg/1mL) in RNA isolation buffer. Total RNA was isolated using the guanidium thiocynate-chloroform-phenol method of Chomczynski and Sacchi (1987). Total RNA (2μg) was reverse transcribed and 4μl cDNA obtained was used for the PCR amplification to estimate the expression of various genes listed below. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as an internal standard. The amount of RNA was quantified in a UV-spectrophotometer by measuring the absorbance at 260/280 nm against DEPC water as the blank. All samples were stored at -80ºC until required.

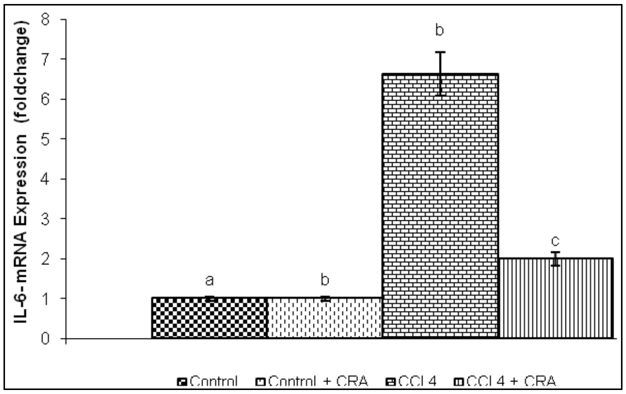

Fig. 2: Effect of corosolic acid on liver IL-6 mRNA expression level in CCL4 induced hepatoxic -rats.

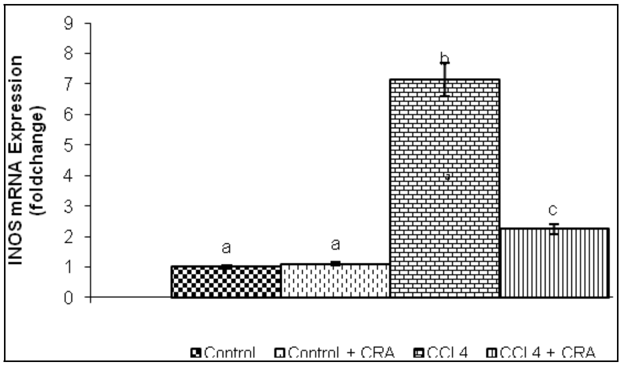

Fig. 3: Effect of corosolic acid on liver INOS mRNA expression level in CCL4 induced hepatoxic-rats.

Histogram depicts quantitation of three independent experiments (means ± S.D). The TNF-α mRNA expression was normalized with the expression of the GAPDH mRNA in each sample. Values not sharing a common superscript differ significantly at p≤0.05 (DMRT).

PCR primer design

Gene sequence information was obtained from the Entrez nucleotide database, and all primer sequences were designed using the online tool Primer 3-BLAST from the GenBank and the primers were obtained from Sigma Chemical Company, St. Louis, MO, USA. Sense and antisense primers for the gene of interest (table 1) were designed to have a closely matched melting temperature. All primer sets underwent preliminary testing to ensure that they were effective.

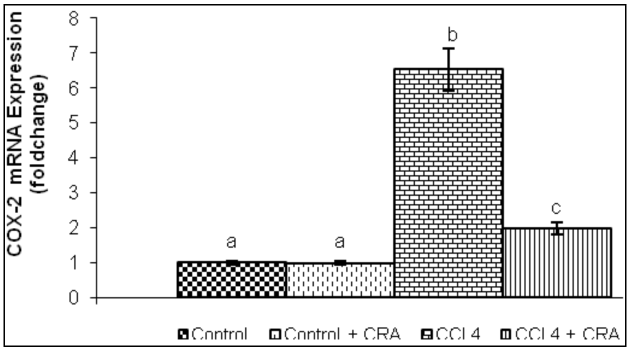

Fig. 4: Effect of corosolic acid on liver COX-2 mRNA expression level in CCL4 induced hepatoxic -rats.

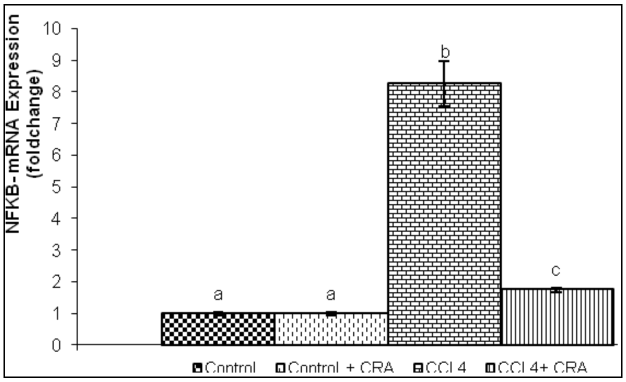

Fig. 5: Effect of corosolic acid on liver NFκB mRNA expression level in CCL4 induced hepatoxic -rats.

Histogram depicts quantitation of three independent experiments (means ± S.D). The TNF-α mRNA expression was normalized with the expression of the GAPDH mRNA in each sample. Values not sharing a common superscript differ significantly at $p \leq 0.05$ (DMRT).

STATISTICAL ANALYSIS

Statistical evaluation was performed using one-way ANOVA followed by Duncan's multiple range test (DMRT) using statistical package of social science (SPSS Inc., Chicago, IL, USA) 16.0 for Windows. Significance level was set at $P<0.05$.

RESULTS

Fig. 1 and 2 show the mRNA expression of TNF-α and IL-6 in control and corosolic acid treated CCl4 treated rats. Treatment with corosolic acid, TNF-α and IL-6 were significantly reduced the mRNA expression implies its significant role in the reduction of CCl4-induced inflammatory events.

The mRNA expressions of iNOS and COX-2 (fig. 3 and 4) were up-regulated significantly in CCl4 induced hepatotoxic rats while treatment with corosolic acid significantly down-regulated the mRNA expressions Fig. 5 shows the effect of corosolic acid on liver NFκB mRNA expression level in CCL4 hepatoxic rats. The mRNA expressions of liver NFκB mRNA (fig. 3 and 4) were up-regulated significantly in CCl4 induced hepatotoxic rats while treatment with corosolic acid significantly down-regulated the mRNA expressions

DISCUSSION

CCl4 toxicity is widely used as an experimental model of severe hepatic cell damage caused by reactive oxygen species (ROS) generation and subsequent activation of immune cells, which results in inflammatory liver disease (Hayden and Ghosh, 2008). CCl4 produces ROS that not only related to tissue damage, but it could also initiate inflammatory process. Oxidant-antioxidant imbalance triggers kupffer cell via the NADPH oxidase pathway or intracellular ROS-dependent kinase activated pathway (McMullan and Brown, 2009). Subsequently, a Kupffer cell produces inflammatory cytokines and also it can activate inflammation in the adjacent cells.

TNF-α is secreted by local macrophages following CCl4 intoxication and induces phagocytic oxidative metabolism (Morio *et al.,* 2001).H owever, TNF-α appears to be trigger inflammation and fibrosis, but it is not directly involved in hepatocyte necrosis in CCl4 toxicity (Simeonova *et al.,* 2001). Therefore, proinflammatory TNF-α, and IL-6 are key molecules involved in inflammatory liver disease. It has been reported that IL-6 stimulates hepatic inflammation and collagen synthesis in vivo (Choi *et al.,* 1994). In this study, CCl4 toxicity causes significantly upregulated the mRNA expressions of hepatic TNF-α and IL-6. The change in TNF-α and IL-6 expression by corosolic acid treatment implies its significant role in the reduction of CCl4-induced inflammatory events.

Treatment with corosolic acid was significantly down-regulates the mRNA expressions of iNOS and COX-2 against CCl4 treated rats. iNOS is readily upregulated in the liver in conditions such as end toxemia, sepsis and hepatitis (Kane *et al.,* 1997; Wray *et al.,* 1998). iNOS is found principally in hepatocytes (Geller *et al.,* 1993) and kupffer cells. The agents such as endotoxin and inflammatory cytokines are known to up regulate iNOS in liver cells. Contradictory reports concerning the contribution of NO to the pathogenesis of acute toxin-induced liver injury were reported. A beneficial role is based on observations that NO inhibits migration of neutrophils into the liver (Pinzani *et al.,* 1994; Zhang *et al.,* 1994). But the induction of iNOS can also plays a harmful role by inducing tissue damage through the

Table 1: Primers used in this study

Name	Primer sequence
TNF-α	5'- GACAAATCCCAGGATGCAAT-3'- Forward
	5'- CTCCGCTGTGACTCTTGCTT-3'- Reverse
IL-6	5'- AGTTGCCTTCTTGGGACTGA-3' - Forward
	5'- TCCAAGATCTCCCTGAGAACA-3' - Reverse
iNOS	5'- CATCATGGACCACCACACAG-3'- Forward
	5'- TTCAACATCTCCTGGTGGAA-3'- Reverse
COX-2	5'- AGATCCTCCTGTGGGTGCTT-3'- Forward
	5'- TACGGGGCCTTCCAAATGTC-3'- Reverse
NF-κB	5'- TCACCAAGCAGGAAGATGTG-3'- Forward
	5'- GATAAGGAGTGCTGCCTTGC-3'- Reverse
GAPDH	5'-TGCCACTCAGAAGACTGTGG-3'- Forward
	5'- CAACGGATACATTGGGGGTA-3'- Reverse

formation of peroxynitrite (McKim *et al.,* 2003). The toxic effect of iNOS in liver has been reported earlier (Thiemermann *et al.,* 1995; Isobe *et al.,* 1999). Studies have shown that the COX-2 inhibition, one of the pathway to possess the hepatoprotective activity in CCl4-toxicity (Vadiraja *et al.,* 1998). It has been reported that COX-2, seems to be induced in macrophages by various proinflammatory stimuli, such as cytokines and growth factors, leading to increase COX-2 expression and the consequent release of prostaglandins (Planaguma *et al.,* 2005). In addition to cytokines, increased expressions of iNOS and COX-2 mRNA in the CCL4 treated rats were significantly restored by corosolic acid. Thus, corosolic acid effectively suppresses the expressions of proinflammatory cytokines such as TNF-α and IL-6, consequently inhibiting its downstream molecules such as iNOS and COX-2.

Fig. 5 shows the effect of corosolic acid on NF-κB mRNA expression in CCl4 induced hepatotoxic rats. The NF-κB mRNA expressions were up-regulated significantly in CCl4 induced hepatotoxic rats while treatment with corosolic acid significantly down-regulated the expressions. The only transcription factor, which links oxidative stress and tissue injury is NF-κB (that also includes liver injury). NF-κB is an early response transcription factor and the nuclear translocation of NF-κB leads to gene expression of proinflamatory cytokines. NF-κB normally located in cytosol, bound to IκB. In response to activating signals, IκB kinase (IKK) complex is activated and phosphorylates IκB, leading to nuclear translocation of NF-κB (Sun and Karin, 2008) including those involved in toxin-induced liver injury. The expression of iNOS and COX 2 has been shown to dependent on NF-κB activation (Kang *et al.,* 2011). Increased mRNA expression of NF-κB by CCl4 toxicity is one of the reasons for aggravating inflammation. However, these increased expressions were significantly attenuated by corosolic acid.

CONCLUSION

The present study provide evidence that corosolic acid exhibits hepatoprotective effect in CCL4 induced hepatoxicity through improvement of antioxidant defense, suppressing the NF-κB expression and the subsequent inflammatory cascade.

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