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EFFICACY OF COROSOLIC ACID ON MITOCHONDRIAL ENZYMES AND DNA DAMAGE AGAINST CCL_4-INDUCED HEPATOTOXIC RATS

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ABSTRACT

The present study is aimed to evaluating the protective effect of corosolic acid (CRA) on mitochondrial enzymes and DNA damage in carbon tetrachloride (CCl_4)-induced hepatotoxic rats. The activities of hepatic mitochondrial enzymes such as isocitrate dehydrogenase (ICH), α-ketoglutarate dehydrogenase (KDH), succinate dehydrogenase (SH), malate dehydrogenase (MDH), NADPH dehydrogenase and cytochrome C oxidase were significantly decreased in CCl_4-hepatotoxic rats, and administration of corosolic acid brought back these parameters towards normal. The lipid peroxidative index in hepatic mitochondrial fractions was increased in CCl_4-treated groups, which were decreased significantly on treatment with CRA. The antioxidants, which were depleted in CCl_4-treated groups, were improved significantly by CRA. An increase in the severity of DNA damage was observed in liver mitochondrial fractions of CCl_4 induced rats. Pretreatment with CRA shows a significant decrease in tail length and tail moment of comet. Thus, the results demonstrate that CRA has potent antioxidant properties, improves mitochondrial enzymes and protective effect against DNA damage in CCl_4 induced liver damage in rats.

Key words: Hepatotoxicity, carbon tetrachloride (CCl_4), corosolic acid, DNA damage.

INTRODUCTION

Liver diseases represent a serious health problem throughout the world, induced by chemicals, alcohol, virus and various toxic intermediates. Oxidative stress plays an important role in the pathogenesis of CCl_4-induced liver injury (Muhtaseb *et al.,* 2008). The CCl_4 induced hepatotoxicity is a well proven experimental model for inducing acute liver injury, is bio-transformed by hepatic microsomal cytochrome P450 to trichloromethyl-free radical (CCl_3* or CCl_3OO*) (Weber *et al.,* 2003). These metabolites react with antioxidant enzymes such as glutathione (GSH), catalase and superoxide dismutase (SOD), lead to lipid peroxidation and liver damage. CCl_4 causes mitochondrial stress and its subsequent dysfunction, can activates signaling cascades involving the activation of caspases, resulting in apoptosis or necrosis (Jo *et al.,* 2001); Kowaltowski and Vercesi, 1999). DNA damage is one of the most sensitive biological markers for evaluating the oxidative stress direct breakage of DNA strands occurs when ROS interacts with DNA. Superoxide radicals can directly or indirectly damage DNA whereas hydrogen peroxide mediates DNA damage by the production of hydroxyl radical via events such as the Fenton reaction (Imlay, 1998). Modern medicine offers few effective treatments apart from natural medicines. Polyphenolic compounds, which are antioxidants of natural origin, have generated considerable interest as potential therapeutic agents for a wide variety of chronic diseases. Corosolic acid (CRA), a triterpenoid, which is derived from *Actinidiavalvata* Dunn and also has been identified in many Chinese medicinal herbs, such as the *Lagerstroemia speciosa*L (Fukushima *et al.,* 2006) and banaba leaves (Yamaguchi *et al.,* 2006). It has been reported that CRA exhibited hepatoprotective (Abdullah, 2013) and anti-diabetic activity in animal models and clinical trials, includes the improvement of glucose metabolism by reducing insulin resistance in a mice model and lowers the post-challenge plasma glucose levels in human (Fukushima *et al.,* 2006; Miura *et al.,* 2006). It was also reported that CRA displayed cytotoxic activity against human cancer cell lines (Ahn *et al.,* 1998; Yoshida *et al.,* 2005). However, no study has been investigated the mitochondrial protective nature of CRA on CCl4 hepatotoxicity. Hence, the present study aims to investigate the effect of CRA on mitochondrial enzymes, antioxidants status and DNA damage in liver tissue from CCl_4 induced hepatotoxicity in rats.

MATERIALS AND METHODS

Chemicals: CCl_4 was purchased from Sigma-Aldrich Co., St. Louis, Missouri, USA. CRA was purchased from Mansite Pharmaceutical Co., Ltd (Chendu, China). All other chemicals used were of analytical grade obtained from E. Merck or HIMEDIA, Mumbai, India.

Experimental animals: Male albino Wistar rats (180 to 200 g) (24 rats) were housed in clean cages at a

temperature of 20 to 24°C, with 12-hr light/12-hr dark cycle and 52% relative humidity in the animal house (College of Medicine, King Saud University). Ethical approval was obtained from the ethics committee of the College of Medicine Research Center at King Saud University, Riyadh, Saudi Arabia (11/3215/IRB).

CCl_4-induced hepatotoxicity: Hepatotoxicity was induced by a single oral dose of CCl_4 (1:1 in liquid paraffin) at 1.25 ml/kg BW at an interval of 6 hr after the administration of the last dose of CRA on the 7th day

Experimental design: The animals were 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 1.25 ml/kg BW (Saba *et al*., 2010) was given at an interval of 6 hr 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 hr 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 morning the animals were killed by cervical dislocation. The blood was collected in clean dry test tubes and the serum was separated by centrifugation at 175 g for 10 min. The liver and kidney were immediately removed and washed in ice-cold saline. The tissues were sliced and homogenized in 0.1 M Tris-HCl buffer (pH 7.0). The homogenates were centrifuged at 48 g for 10 min at 0°C in a cold centrifuge. The supernatants were separated and used for the determination of various parameters.

Biochemical estimations

Isolation of liver mitochondria: The liver mitochondria were isolated from cell debris, nuclei, microsomes, soluble components and contaminant RBC using differential centrifugation by the method of Johnson and Lardy (1981). A 20% (w/v) homogenate was prepared in 0.25 M sucrose containing 0.05 M Tris–HCl buffer and 5.0 mM EDTA. To remove cell debris, tissue fragments and cell nuclei (nuclei pellet), the homogenate was centrifuged at 600g for 10 min. The supernatant fraction was centrifuged in a refrigerated centrifuge at10000g for 5 min at 4°C to bring down the mitochondrial pellet and used for the estimation of various parameters.

Tricarboxylic acid cycle enzymes analysis: The enzyme activity of isocitrate dehydrogenase was assayed by the method of Bell and Baron (1960), a calibration curve was established with α-ketoglutarate as standard. The isocitrate dehydrogenase activity was expressed as nmol of α -ketoglutarate formed/h/mg protein. α-ketoglutarate dehydrogenase was assayed by the method of Reed and Mukherjee (1969), a standard containing potassium ferrocyanide was assayed simultaneously. The activity of α-ketoglutarate dehydrogenase was expressed as nmol of ferrocyanide formed/h/mg protein.

Succinate dehydrogenase was assayed according to the method of Slater and Bonner (1952), The succinate dehydrogenase activity was expressed as nmol of succinate oxidised/min/mg protein. Malate dehydrogenase was assayed by the method of Mehler *et al.* (1948). The activity of the enzyme was expressed as nmol of NADH oxidised/min/mg protein.

Cytochrome c oxidase was assayed by the method of Pearl *et al.* (1963). The enzyme activity was expressed as change in absorbance/min/mg protein. NADH dehydrogenase was assayed according to the method of Minakami *et al.* (1962). The activity of NADH dehydrogenase was expressed as nmol of NADH oxidised/min/mg protein

Lipid peroxidative marker and enzymic antioxidant assay: Thiobarbituric acid reactive substance (TBARS) was estimated by the method described by Niehaus and Samuelson (1968). Value was expressed nmol/mg protein. Superoxide dismutase (SOD) was estimated by the method of Kakkar *et al.* (1984). The enzyme concentration required to inhibit the chromogen produced by 50% in one min under standard conditions was taken as one unit. The specific activity of the enzyme was expressed as units/min/mg protein chronological. Glutathione peroxidase (GPx) was estimated by the method of Rotruck *et al.* (1973). The tubes were centrifuged and the supernatants were assayed for GSH by the method of Ellman (1959).The specific activity of GPx was expressed as micromoles of GSH consumed/ min/ mg protein.

Non-enzymic antioxidant assay: GSH was estimated by the method of Ellman (1959). The amount of GSH was expressed as µg/mg protein. Vitamin C was estimated by the method of Roe and Kuether (1943). The results were expressed as µg/mg protein. Vitamin E was estimated by the method of Baker *et al.*(1940). The value was expressed as µg/mg protein.

Single-cell gel electrophoresis (comet assay)

Lymphocyte preparation: Lymphocytes were isolated using Ficoll–Histopaque (Sigma) using the method of Boyum (1968). Blood was diluted 1:1 with PBS and layered on to histopaque with the ratio of blood and PBS to histopaque maintained at 4:3. The blood was centrifuged at 175g for 35 min. The lymphocyte layer was removed and washed twice in PBS at 140.3 g for 10 min each, and irradiated to an average value of 1.52×10^{-3} mJ/cell. After irradiation, the lymphocytes were kept

at room temperature for 30 min and were then subjected to biochemical assays.

Isolation of hepatocytes: Hepatocytes were isolated from the liver of rats by collagenase perfusion method described by Santhosh (1996). The viability of the cells was tested by trypan blue exclusion and only cell preparations with more than 90% viability were used for experiments and retained their viability in culture. Hepatocytes in minimum essential medium (MEM) were seeded on 35 mm plastic dishes, passively coated with collagen I (50 µg/ ml) and after 4 h unattached cells were removed and the attached cells were incubated with MEM medium. Typically, each culture consisted of an initial density of 1×106 cells in 2 ml culture medium.

Comet assay: DNA damage was estimated by alkaline single-cell gel electrophoresis (comet assay) according to the method of Singh *et al.* (1988). In this method, the cells were first lysed to form nucleoids. During electrophoresis, DNA fragments (from damaged DNA) stream towards anode while the undamaged DNA is trapped within the nucleus. When they are stained with propidium iodide, damaged DNA gives the appearance of a comet tail and undamaged DNA gives as spherical appearance. To prevent additional DNA damage, all steps were conducted under dimmed light or in the dark.

Twenty five images were randomly selected from each sample and were examined at 200 magnification in a fluorescence microscope connected to a personal computer-based image analysis system, Komet v. 5.0 (Kinetics Imaging Ltd., Liverpool, UK). The relative amount of DNA appearing in the tail of the comet (percent tail DNA), tail length and tail moment (% tail DNA x tail length) were linearly related to DNA break frequency.

Statistical analysis: Statistical evaluation was done using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT) using SPSS 10.0 for Windows. The significance level was set at $p< 0.05$.

RESULTS AND DISCUSSION

Carbon tetrachloride has been extensively used for decades to explore the response of liver toxicity, since injury caused by this hepatotoxin replicate those seen in most cases of human liver diseases, which makes it a reliable model to study both signal transduction and cell cycle events *in vivo* (Zoo, 2000; Brennan, 1998). Table 1and 2 represents the restorative effect of CRA on CCl_4 induced mitochondrial tricarboxylic acid (TCA) cycle enzymes and respiratory chain enzymes alterations in control and experimental rats. A significant decrease was observed in the activities of ICH, KDH, SDH and MDH after CCl_4 administration ($p< 0.05$). CRA treatment reversed the altered values of ICH, KDH, SDH, and MDH with a significant improvement towards normal ($p< 0.05$). ICH is also involved in the supply of NADPH needed for GSH production which acts against cytosolic and mitochondrial oxidative damage (Bailey, 2002). Hence, the decline in ICH activity may result in the disturbance of the balance between oxidants and antioxidants and subsequently lead to a pro-oxidant state. Decrease activities of Kreb's cycle enzymes in CCl_4 treated rats might be due to distress by increased levels of free radicals produced by CCl_4 and also deficiency in one or more electron transport chain resulted in decreased activities of these enzymes. However, the intake of CRA increases the activities of TCA cycle enzymes, probably by improving the mitochondrial antioxidant defense system, and overcome the complications associated with the decrease TCA cycle function.

The mitochondrial respiratory chain is the major site of ROS production within the cell. Superoxide is thought to be produced continually as a byproduct of normal respiration through the one electron reduction of molecular oxygen. Superoxide itself can react with nitric oxide to form the damaging oxidant peroxynitrite, which is more reactive than superoxide anion (Beckman *et al.,* 1990) CCl_4 is activated by cytochrome P450 (CYP) 2E1, 2B1/B2, and possibly 3A to form trichloromethyl radicals (CCl3*) and trichloromethyl peroxy radicals (CCl3OO*) that cause lipid peroxidation and subsequent tissue damage (Weber *et al.,* 2003). The levels of TBARS increase significantly and the activities of the antioxidant enzymes decrease significantly ($p< 0.05$) in CCl_4 treated group when compared to control. Treatment with CRA, there was a significant decrease in the levels of TBARS and increase in the activities of the enzymatic antioxidants ($p< 0.05$) when compared to CCl_4 treated group. The results are shown in table-3. Table 4 shows the levels of non-enzymatic antioxidants (vitamin C, vitamin E and GSH) in the mitochondrial fraction of liver in control and CCl_4 rats. The levels of non-enzymatic antioxidants were significantly decreased ($p< 0.05$) in CCl_4 rats. Oral administration of CRA improves these parameters towards normal. The increase of lipid mitochondrial peroxidative markers and diminish antioxidant defense as observed in CCL4 treated rats. Treatment with CRA reduces the oxidative damage in mitochondria, which is evidence from the reduce levels of TBARS and enhance antioxidant status. These decrease mitochondrial antioxidants could be due to a feed-back inhibition or oxidative inactivation of enzyme protein caused by excess ROS generation (Ohoto, 2004). It has been suggested that reduction in mitochondrial GSH results in lessening of other antioxidants such as ascorbic acid, α-tocopherol and aggravate the cells to further damage. This condition may exacerbate the overproduction of mitochondrial ROS (Gao *et al.,* 2004). Administration of CRA to CCl_4 rats, decrease lipid

peroxidation, augments antioxidant status and thereby it improves mitochondrial function. This is probably due to antioxidant sparing action of CRA. Previous reports have also supported that CRA possesses antioxidative and free radical scavenging effects (Abdullah, 2013).

The single cell gel electrophoresis or comet assay is a state-of-the-art technique for quantitating DNA damage and repair in vivo and in vitro. Figures 1 and 2 represent the DNA damage in the lymphocytes and hepatocytes of experimental animals. The extent of DNA damage measured as percentage of DNA present in the comet tail (% tail DNA), tail length and tail moment is shown in Tables 5 and 6. CCl_4 rats show about two fold increases in DNA damage compared to that of control rats. Administration of CRA diminishes the DNA damage. CRA treatment to control animals did not show any effect on DNA damage. Determination of DNA damage in this study includes all types of damage detected by comet assay. Treatment with CRA significantly decreases the DNA damage when compared to CCl_4 rats. The genoprotective effect of CRA may be due to the scavenging of free radicals before they damage to DNA, as CRA is a potent antioxidant. However, further studies are needed at the molecular level to find out the exact mechanism of genoprotective effect of CRA.

In conclusion, the results reveal that CRA exhibits beneficial effect on mitochondrial enzymes and DNA damage against CCl_4-induced rats which is mainly due to free radical scavenging and enhances antioxidant properties. These protective effects of CRA characterize a possible development of novel therapeutic agents for liver injury.

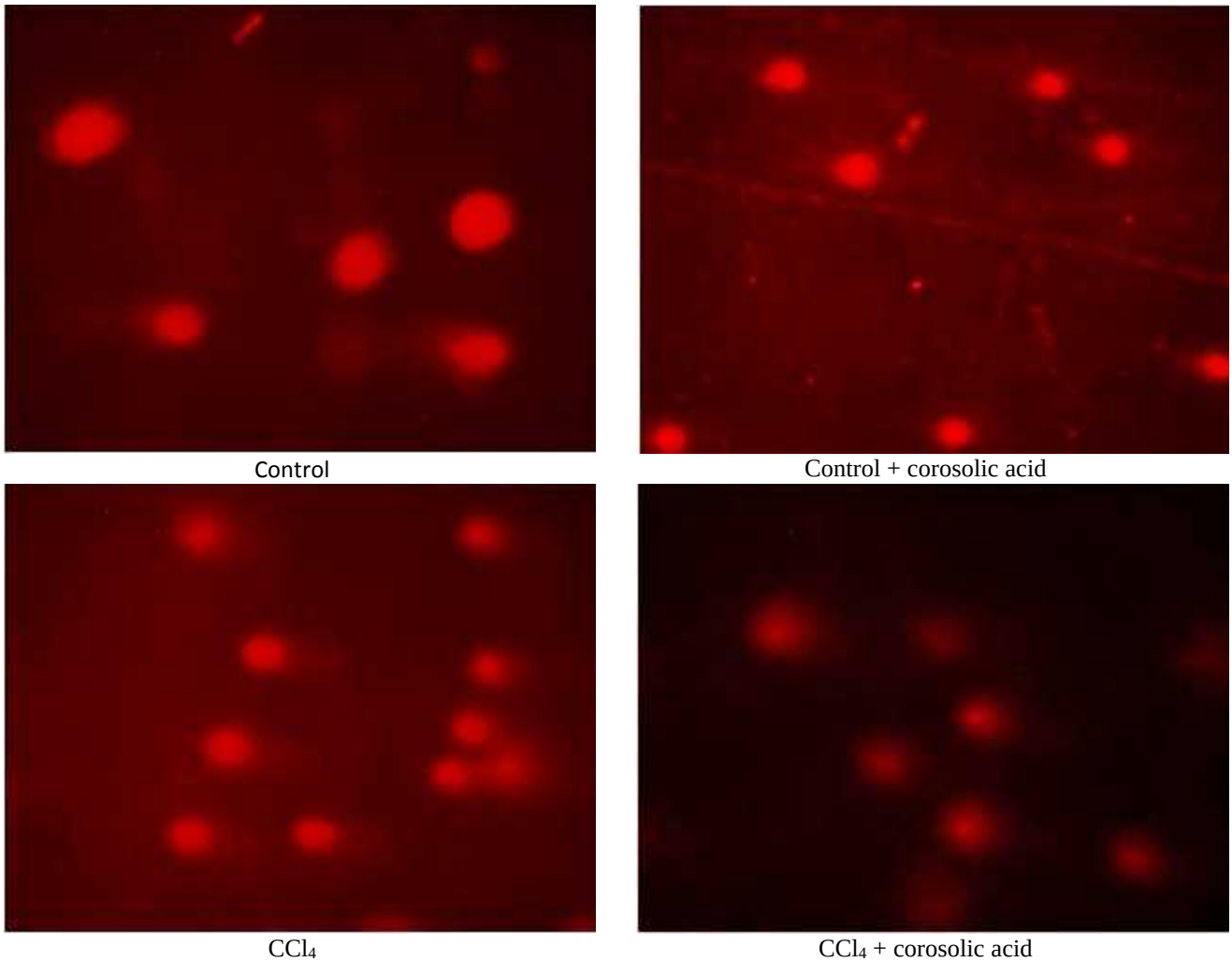

Figure 1. Effect of corosolic acid on DNA damage in the blood lymphocytes of CCl4-hepatotoxic rats

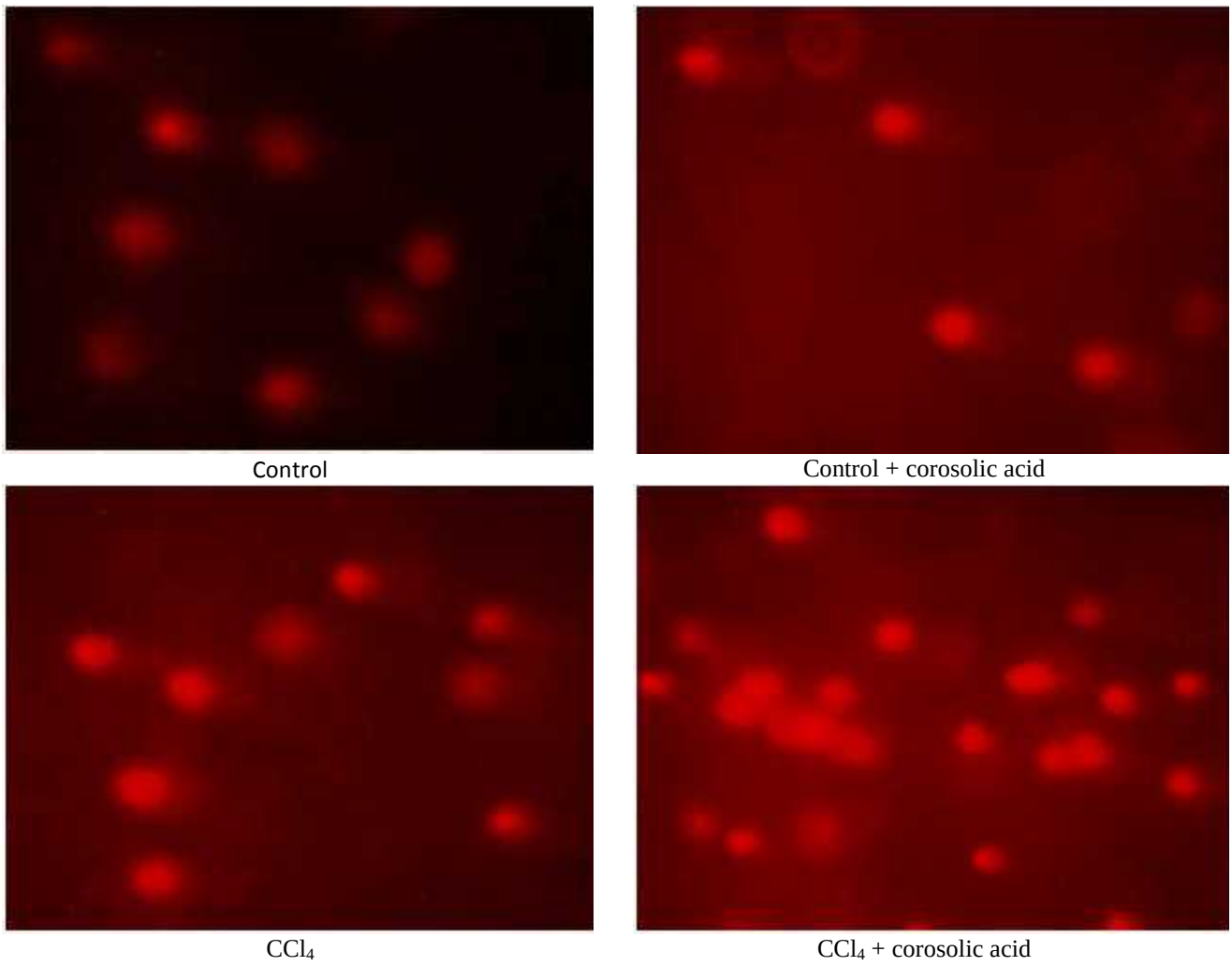

Figure 2. Effect of corosolic acid on DNA damage in the liver splenocytes of CCl4-hepatotoxic rats

Table 1. Effect of corosolic acid on the activities of mitochondrial tricarboxylic acid cycle enzymes in CCl4-hepatotoxic rats

Groups	Isocitrate dehydrogenase (U^a/mg protein)	α-Ketoglutarate dehydrogenase (U^b/mg protein)	Succinate dehydrogenase (U^c/mg protein)	Malate dehydrogenase (U^d/mg protein)
Control	780.33 ± 58.66^a	176.81 ± 13.39^a	41.13 ± 3.12^a	364.41 ± 27.14^a
Control + corosolic acid (20 mg/kg body wt.)	775.11 ± 53.55^a	181.44 ± 14.41^a	39.17 ± 2.54^a	360.25 ± 27.01^a
CCl4-hepatotoxicity (1.25 mL/kg body wt.)	511.55 ± 39.85^b	105.18 ± 7.40^b	15.11 ± 1.19^b	214.15 ± 19.22^b
CCl4 + corosolic acid (20 mg/kg body wt.)	$752.61 \pm 51.55^{a,c}$	$157.14 \pm 12.01^{a,c}$	33.10 ± 2.19^c	333.39 ± 26.14^c

U^a – nmol of α-ketoglutarate formed/h; U^b – nmol of ferrocyanide formed/h; U^c – nmol of succinate oxidized/min; U^d – nmol of NADH oxidized/min

Values are expressed as means ± S.D. for six rats in each group. Values not sharing a common superscript differ significantly at $p < 0.05$ (DMRT).

Table 2. Effect of corosolic acid on the activities of mitochondrial respiratory chain enzymes in CCl4-hepatotoxic rats

Groups	NADH dehydrogenase (U^a/mg protein)	Cytochrome c-oxidase (U^b/mg protein)
Control	42.44 ± 3.05^a	7.89 ± 0.64^a
Control + corosolic acid (20 mg/kg body wt.)	39.09 ± 2.86^a	7.84 ± 0.57^a
CCl4-hepatotoxicity (1.25 mL/kg body wt.)	11.16 ± 1.02^b	2.95 ± 0.18^b
CCl4 + corosolic acid (20 mg/kg body wt.)	30.11 ± 2.11^c	6.31 ± 0.39^c

U^a – nmol of NADH oxidized/min; U^b – change in absorbance x 10^{-2}/min
Values are expressed as means ± S.D. for six rats in each group.
Values not sharing a common superscript differ significantly at $p < 0.05$ (DMRT).

Table 3. Effect of corosolic acid on the levels of TBARS and activities of enzymatic antioxidants in the mitochondrial fraction of the liver of CCl4-hepatotoxic rats

Groups	TBARS (nmol/mg protein)	SOD (U^a/mg protein)	GPx (U^b/mg protein)
Control	1.47 ± 0.11^a	2.34 ± 0.13^a	7.68 ± 0.59^a
Control + corosolic acid (20 mg/kg body wt.)	1.43 ± 0.09^a	2.35 ± 0.12^a	7.61 ± 0.54^a
CCl4-hepatotoxicity (1.25 mL/kg body wt.)	4.15 ± 0.32^b	1.03 ± 0.06^b	4.67 ± 0.39^b
CCl4 + corosolic acid (20 mg/kg body wt.)	1.95 ± 0.14^c	$2.15 \pm 0.17^{a,c}$	6.99 ± 0.53^c

U^a-Enzyme concentration required for 50% inhibition of NBT reduction/min;
U^b-µmol of reduced glutathione consumed/min
Values are expressed as means ± S.D. for six rats in each group.
Values not sharing a common superscript differ significantly at $p < 0.05$ (DMRT).

Table 4: Effect of corosolic acid on the levels of non-enzymatic antioxidants in the mitochondrial fraction of the liver of CCl4-hepatotoxic rats

Groups	GSH (µg/mg protein)	Vitamin C (µg/mg protein)	Vitamin E (µg/mg protein)
Control	13.36 ± 0.98^a	11.03 ± 0.84^a	6.02 ± 0.41^a
Control + corosolic acid (20 mg/kg body wt.)	13.27 ± 0.94^a	11.11 ± 0.88^a	5.97 ± 0.37^a
CCl4-hepatotoxicity (1.25 mL/kg body wt.)	5.89 ± 0.49^b	5.65 ± 0.49^b	2.16 ± 0.12^b
CCl4 + corosolic acid (20 mg/kg body wt.)	$12.89 \pm 0.73^{a,c}$	9.75 ± 0.69^c	5.83 ± 0.36^a

Values are expressed as means ± S.D. for six rats in each group.
Values not sharing a common superscript differ significantly at $p < 0.05$ (DMRT).

Table 5: Effect of corosolic acid on DNA damage in the blood lymphocytes of CCl4-hepatotoxic rats

Values are expressed as means ± S.D. for six rats in each group.
Values not sharing a common superscript differ significantly at $p<0.05$ (DMRT).

Table 6: Effect of corosolic acid on DNA damage in the liver of CCl4-hepatotoxic rats

Groups	% Head DNA	% Tail DNA	Tail length	Tail moment
Control	98.56 ± 4.22^a	1.44 ± 0.13^a	3 ± 0.29^a	0.11 ± 0.02^a
Control + corosolic acid (20 mg/kg body wt.)	96.87 ± 5.14^a	3.13 ± 0.25^a	3 ± 0.17^a	0.24 ± 0.02^a
CCl4-hepatotoxicity (1.25 mL/kg body wt.)	76.68 ± 5.41^b	24.32 ± 1.38^b	24.86 ± 1.77^b	2.58 ± 0.20^b
CCl4 + corosolic acid (20 mg/kg body wt.)	91.73 ± 5.57^c	8.27 ± 0.34^c	6 ± 0.40^c	0.68 ± 0.05^c

Values are expressed as means ± S.D. for six rats in each group.
Values not sharing a common superscript differ significantly at $p < 0.05$ (DMRT).

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