

*Full Length Research Paper*

# Combined protective effect of vitamins C and E on cadmium induced oxidative liver injury in rats

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Our study pertains to the potential ability of vitamin C and/or vitamin E, used as nutritional supplements, to alleviate oxidative stress induced by cadmium. Male rats were randomly divided into five groups of eight each. Group I served as the controls; group II received in their drinking water CdCl<sub>2</sub> (200 mg/L); group III received both CdCl<sub>2</sub> and vitamin C (1.5 g/L of water); group IV was treated with CdCl<sub>2</sub> and vitamin E (400 mg/kg diet); and group V received CdCl<sub>2</sub> + vitamin C + vitamin E. The exposure of rats to cadmium chloride for 30 days resulted in a significant decrease in body weight gain. Cadmium treatment also produced oxidative liver injury characterized by increasing serum glucose concentration, glutamate-pyruvate transaminase (GPT), alanine aminotransaminase (GOT) and alkaline phosphatase (ALP) activities. Meanwhile cadmium supplementation decreased serum total protein and albumin in animals. In addition, liver glutathione level, catalase and glutathione peroxidase (GSH-Px) activities were diminished. With vitamin C and vitamin E administration during intoxication of cadmium, corrective effects on Cd-induced oxidative stress in the liver was observed. In conclusion, this study demonstrates that oral exposure to Cd caused reduction in LPO and antioxidant enzyme activities in rat's liver, and vitamin C or vitamin E may have partial ameliorative effects on these disturbances, whereas vitamin C and vitamin E together assured a more efficient protection of the organ against the noticed oxidative stress.

**Key words:** Cadmium, vitamin E, vitamin C, oxidative stress, glutathione, glutathione peroxidase, catalase.

## INTRODUCTION

Cadmium is a very toxic metal, and also an environmental and industrial pollutant which is present in soil, water, air and food (Cinar, 2003; Kaplan et al., 2011). This metal enters surface water from the industrial wastes and found in soil by leaching of sewage sludge through soil (Joshi and Bose, 2002). So, the population can be affected by Cd through food consumption, drinking water and incidental ingestion of soil contaminated by Cd (Hardej and Trombetta, 2004; Valadez-Vega et al., 2011). After absorption, cadmium transported in the plasma is bound to albumin and accumulated mainly in kidney and liver. This metal causes variety of toxic effects on various body tissues of

both human and animals (Kaya et al., 2002). Cadmium is known to cause reproductive disorders, renal and hepatic dysfunction, osteomalacia, neurological impairment, pancreatic activity changes (Hooser, 2007). It also affects various structures and metabolic processes, such as nucleic acids, carbohydrates energy metabolism, protein synthesis and enzyme systems (Cinar, 2003). Chronic cadmium toxicity also causes an oxidative stress through lipid peroxidation and consumption of some antioxidant systems (Cinar et al., 2010). In several reports, administration of antioxidants such as zinc (Uyanik et al., 2001), selenium (Li et al., 2010), diallyl tetrasulfide (Pari and Murugavel, 2005) and quercetin (Morales et al., 2006) have been shown to have protective effect against Cd toxicity. Vitamin C and vitamin E are recognized as essential nutrients for all species of animals. In other words, these vitamins have been shown to have

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protective effect against metal induced toxicity (Rao and Sharma, 2001; Jiraungkoorskul et al., 2007). Therefore, the present study was designed to evaluate the effect of vitamin E and vitamin C separately and in combination against cadmium chloride induced oxidative liver injury in rats.

## MATERIALS AND METHODS

### Chemicals

Vitamin E, vitamin C, cadmium chloride ( $\text{CdCl}_2$ ), 5, 5' dithiobis-2-nitrobenzoic acid (DTNB) and reduced glutathione were purchased from sigma Chemical Co. (St Louis, France), and all other chemicals used in the experiment were of analytical grade.

### Animals

Male albino (Wistar) rats with a body weight of 180 to 210 g were obtained from Pasteur Institute (Algiers, Algeria). Animals were acclimated for two weeks under the same laboratory conditions of photoperiod (12 h light/12 h dark) with a relative humidity of 40% and room temperature of  $22 \pm 2^\circ\text{C}$ . Food and water were available *ad-libitum*.

### Experimental design

Animals were randomly divided into five groups of eight animals each. A control group of animals received tap water and four experimental received either Cd (200 mg/L as  $\text{CdCl}_2$ ), Cd + vit E (200 mg/L  $\text{CdCl}_2$  + 400 mg/kg diet vit E), Cd + vit C (200 mg/L  $\text{CdCl}_2$  + 1.5 g/L vit C in drinking water) or Cd + vit E + vit C (200 mg/L  $\text{CdCl}_2$  + 400 mg/kg diet vit E + 1.5 g/L vit C). We chose to administer the three elements by oral route because it is the main mode of exposure to cadmium in human and animals. The doses of  $\text{CdCl}_2$ , vitamin E, vitamin C and the period of treatment were selected on the basis of previous studies (Chowdhury et al., 1987; Kim et al., 2001; Grosicki, 2004). The experimental procedures were carried out according to the National Institute of Health Guidelines for Animal Care and approved by the Ethics Committee of our Institution. The treatments of rats continued for a period of four weeks. At the end of the experiment, total body weight was recorded and animals were sacrificed by decapitation. At the time of the sacrifice, blood was transferred into ice cold centrifuged tubes. Tubes were then centrifuged for 10 min at 3000 rpm and serum was used for glucose, total protein, albumin, GOT, GPT and alkaline phosphatase assays. Liver was removed immediately and one part was processed immediately for assaying glutathione and antioxidant enzymes activities. The other part was used for light microscopic studies.

### Analytical methods

#### Determination of biochemical parameters

Serum glucose level was estimated with a commercial kit (Spinreact, Spain, ref; 41011) and determined by enzymatic colorimetric method using spectrophotometer (Jenway 6505, Jenway Ltd, UK). However, GOT, GPT and alkaline phosphatase activities were determined with commercial kits from Spinreact, Spain, refs; GOT-1001161, GPT-1001171 and ALP- 1001131 respectively. Total protein and albumin concentration were also

measured utilizing commercial kits (Spinreact, Spain, refs; total protein- 1001291 and albumin- 1001020).

### Tissue preparation

About 1 g of liver was homogenized in 2 ml of buffer solution of phosphate buffer saline 1:2 (w/v; 1 g tissue 2 ml TBS, pH = 7.4), homogenates were centrifuged at  $10000 \times g$  for 15 min at  $4^\circ\text{C}$ , and the resultant supernatant was used for the determination of reduced glutathione and protein levels on one hand and the estimation of catalase and GSH-Px activities on the other hand.

### Estimation of reduced glutathione level (GSH)

Liver GSH content was estimated using a colorimetric technique, as mentioned by Ellman (1959) modified by Jollow et al. (1974), based on the development of yellow color when DTNB (5, 5' dithiobis-(2-nitrobenzoic acid) is added to compounds containing sulfhydryl groups. In brief, 0.8 ml of liver supernatant was added to 0.3 ml of 0.25% sulfosalicylic acid, and then tubes were centrifuged at  $2500 \times g$  for 15 min. Supernatant (0.5 ml) was mixed with 0.025 ml of 0.01 M DTNB and 1 ml phosphate buffer (0.1 M, pH = 7.4). The absorbance at 412 nm was recorded. Finally, total GSH content was expressed as nmol GSH/mg protein.

### Determination of glutathione peroxidase (GSH-Px)

Glutathione peroxidase (GSH-Px) (E.C.1.1.1.9) activity was measured by the procedure of Floche and Gunzler (1984). Supernatant obtained after centrifuging 5% liver homogenate at  $15000 \times g$  for 10 min followed by  $10000 \times g$  for 30 min at  $4^\circ\text{C}$  was used for GPx assay. One milliliter (1 ml) of reaction mixture was prepared which contained 0.3 ml of phosphate buffer (0.1 M, pH = 7.4), 0.2 ml of GSH (2 mM), 0.1 ml of sodium azide (10 mM), 0.1  $\text{H}_2\text{O}_2$  (1 mM) and 0.3 ml of liver supernatant. After incubation at  $37^\circ\text{C}$  for 15 min, the reaction was terminated by addition of 0.5 ml 5% TCA. Tubes were centrifuged at  $1500 \times g$  for 5 min and the supernatant was collected. 0.2 ml of phosphate buffer (0.1 M, pH = 7.4) and 0.7 ml of DTNB (0.4 mg/ml) were added to 0.1 ml of reaction supernatant. After mixing, absorbance was recorded at 420 nm.

### Assay of catalase activity

The activity of catalase was determined according to the method of Aebi (1984). The reaction mixture (1 ml) that contained 0.78 ml of phosphate buffer (0.1 M, pH = 7.4), 0.2 ml of liver supernatant, and 0.02 ml of  $\text{H}_2\text{O}_2$  (0.5 M) was prepared. The reaction was started by adding  $\text{H}_2\text{O}_2$  and the decomposition of  $\text{H}_2\text{O}_2$  was monitored following the decrease in absorbance at 240 nm for 1 min. The enzyme activity was calculated by using an extinction coefficient of  $0.043 \text{ mM}^{-1} \text{ cm}^{-1}$ .

### Protein determination

The protein content of tissues samples were measured by the method of Bradford (1976) using bovine serum albumin as a standard.

### Histological studies

For histological examination, liver was dissected and immediately

**Table 1.** Body weight gain and liver weights of control male rats, treated with Cd, Cd-vit C, Cd-vit E and Cd-vit C-vit E after 4 weeks of treatment.

Parameter	Experimental group				
	Control (n = 8)	Cd (n = 8)	Cd-vit C (n = 8)	Cd-vit E (n = 8)	Cd-vit C-vit E (n = 8)
Initial body weight (g)	206 ± 6.4	209.1 ± 7.6	197.4 ± 8.0	190.9 ± 3.7	183.8 ± 5.6
Final body weight (g)	201 ± 3.2	108 ± 4.8***	159.1 ± 8.1***c <sup>β</sup>	163.1 ± 5.9***c <sup>β</sup>	181.1 ± 4.1*** <sup>c</sup>
Liver weight (g)	9.0 ± 0.3	12.2 ± 0.3***	10.4 ± 0.4 <sup>bμ</sup>	10.2 ± 0.2***c <sup>β</sup>	8.8 ± 0.3 <sup>c</sup>

Statistically significant differences from control: \*p < 0.05, \*\*\*p < 0.001; from Cd: <sup>b</sup>p < 0.01, <sup>c</sup>p < 0.001; from Cd-vit E-vit C: <sup>μ</sup>p < 0.05, <sup>β</sup>p < 0.01. Values are given as mean ± SEM, n = number of animals.

**Table 2.** Changes of biochemical parameters of control male rats, treated with Cd, Cd-vit C, Cd-vit E, and Cd - vit C- vit E after 4 weeks of treatment.

Parameter	Experimental group				
	Control (n = 8)	Cd (n = 8)	Cd-vit C (n = 8)	Cd-vit E (n = 8)	Cd-vit E-vit C (n = 8)
Glucose (mg/100 ml)	107.7 ± 2.4	193 ± 2.4***	160.1 ± 2.6***c <sup>β</sup>	146.8 ± 5.6*** <sup>c</sup>	138.8 ± 5.3*** <sup>c</sup>
Total protein (g/100 ml)	8.7 ± 0.3	6.1 ± 0.3***	7.45 ± 0.3 <sup>aβ</sup>	7.28 ± 0.3 <sup>aβ</sup>	8.5 ± 0.1 <sup>c</sup>
Albumin (g/100 ml)	4.9 ± 0.2	3.2 ± 0.3***	4.2 ± 0.1***b <sup>β</sup>	4.5 ± 0.2*** <sup>c</sup>	4.7 ± 0.1 <sup>c</sup>
GOT (U/L)	62.4 ± 4.6	135.2 ± 3.9***	71 ± 3.6 <sup>cμ</sup>	76.2 ± 3.6 <sup>cβ</sup>	58.3 ± 2.1 <sup>b</sup>
GPT (U/L)	42.9 ± 3.9	78.6 ± 1.5***	46.7 ± 3.7 <sup>c</sup>	58.1 ± 2.7 <sup>ck</sup>	45.6 ± 0.4 <sup>c</sup>
(ALP) (U/L)	109.9 ± 2.5	292.3 ± 2***	245.2 ± 9.5*** <sup>c</sup>	218.5 ± 11.4*** <sup>c</sup>	213.6 ± 10.2*** <sup>c</sup>

Statistically significant differences from control: \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001; from Cd: <sup>a</sup>P < 0.05, <sup>b</sup>P < 0.01, <sup>c</sup>P < 0.001; from Cd-vit C-vit E: <sup>μ</sup>P < 0.05, <sup>β</sup>P < 0.01, <sup>k</sup>P < 0.001. Values are given as mean ± SEM, n = number of animals.

fixed in bouin solution for 24 h, processed by using a graded ethanol series, and then embedded in paraffin. The paraffin sections were cut into 5 μm thick slices and stained with hematoxylin and eosin for light microscopic examination (Haoult, 1984). The sections were then viewed and photographed.

### Statistical analysis

Data were given as means ± SEM. Statistical significance of the results obtained for various comparisons was estimated by applying one way analysis of variance (ANOVA) followed by Protected Least Significant Difference Fisher's test (PLSD Fisher) and the level of significance was set at p < 0.05.

## RESULTS

### Effects of treatments on body weight gain, absolute, and relative liver weights

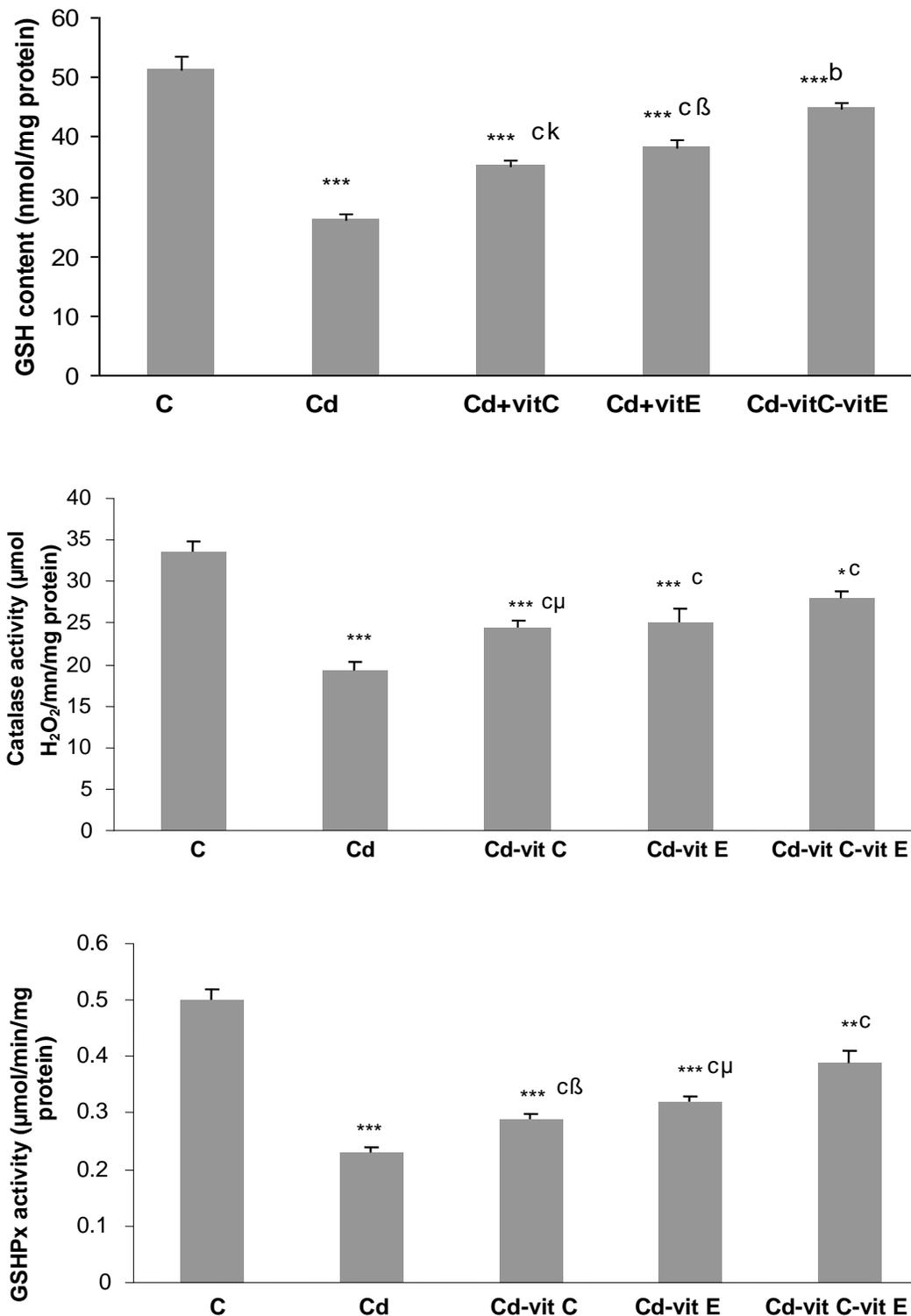
In this investigation, the body and liver weights of rats subjected to different treatments are shown in Table 1. In Cd-treated animals, the results showed obviously significant decrease (p < 0.001) in body weight as compared to the control group. In addition, a significant increase of Cd-treated group in liver weight was noticed (p < 0.001). However, vitamin C, vitamin E and vitamin C + vitamin E supplies, the body weight gain became significantly greater (p < 0.001) than in rats exposed to cadmium and the liver weight decreased p < 0.01 and p < 0.001.

### Effect of treatment on serum biochemical parameters

Treatment with Cd caused significant increase of serum glucose, GOT, GPT and ALP (p < 0.001) compared to the control group. Meanwhile the concentration of serum total protein and serum albumin were diminished (p < 0.001). Whereas, the supplementation of vitamin C or vitamin E either alone or together with cadmium produced a recovery in the above mentioned biochemical parameters p < 0.05 (Cd-vit C), p < 0.05 (Cd-vit E) and p < 0.001 (Cd-vit C-vit E) for total protein and p < 0.01 (Cd-vit C), p < 0.001 (Cd- vit E) and p < 0.001 (Cd-vit C-vit E) for albumin and p < 0.001 (Cd-vit C), p < 0.001 (Cd-vit E) and p < 0.001 (Cd-vit C-vit E) for glucose (Table 2). In addition, the activities of serum hepatospecific enzymes serum GOT, GPT and alkaline phosphatase were generally significantly decreased (p < 0.001) in animal groups treated with vitamin C and vitamin E either alone or in combination. Moreover, vitamin C and E together showed more efficacy than vitamin C or vitamin E alone when comparing Cd-vit C and Cd-vit E with Cd-vit C-vit E animals (Table 2).

### Effect of treatment on hepatic oxidative stress parameters

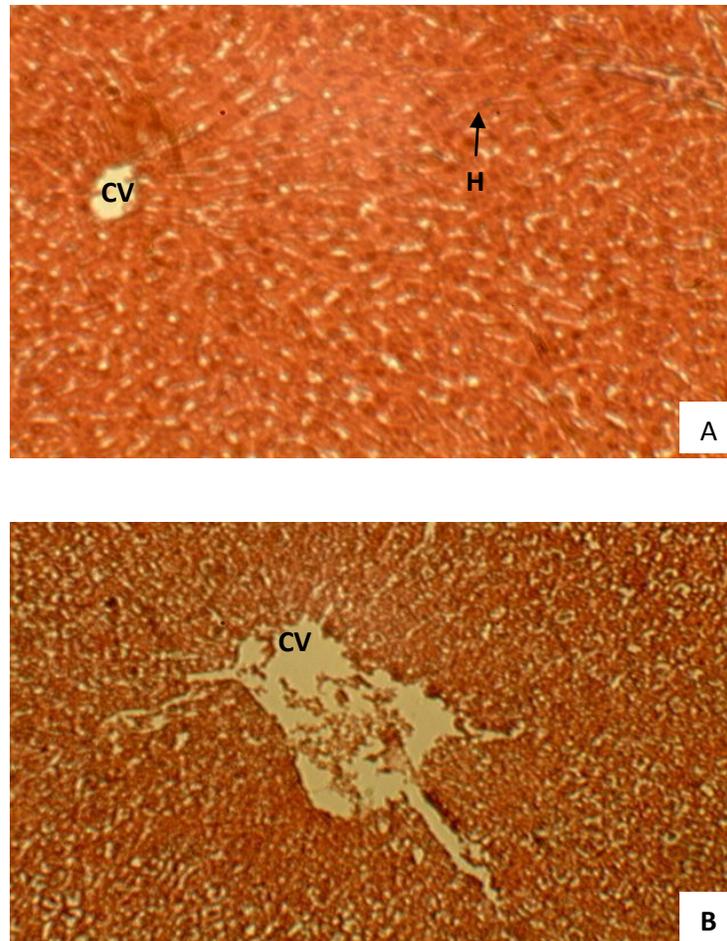
The exposure to Cd produced a significant adverse effect on the liver redox status, which is indicated by a



**Figure 1.** Values of glutathione and catalase and GSH-Px in liver of control male rats, treated with (Cd), Cd-vit E, Cd-vit C and Cd-vitE-vit C after 4 weeks of treatment. Statistically significant differences from control: \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001; from Cd, <sup>a</sup>P < 0.05, <sup>b</sup>P < 0.01, <sup>c</sup>P < 0.001; from Cd-vit C+vit E: <sup>μ</sup>P < 0.05; <sup>β</sup>P < 0.01; <sup>k</sup>P < 0.001. Values are given as mean ± SEM, for group of 8 animals each.

significant reduction ( $p < 0.001$ ) in reduced glutathione level, catalase and GSH-Px activities (Figure 1).

Treatments with vitamin E, C and a combination of both vitamins restored and ameliorated these biomarkers.



**Figure 2.** Effect of cadmium (cadmium chloride) on histological damage in the liver. Control (A), treated with Cd (B). Optic microscopy section were stained using the haematoxylin-eosin method (400 $\times$ ). CV: Central vein, H: Hepatocytes.

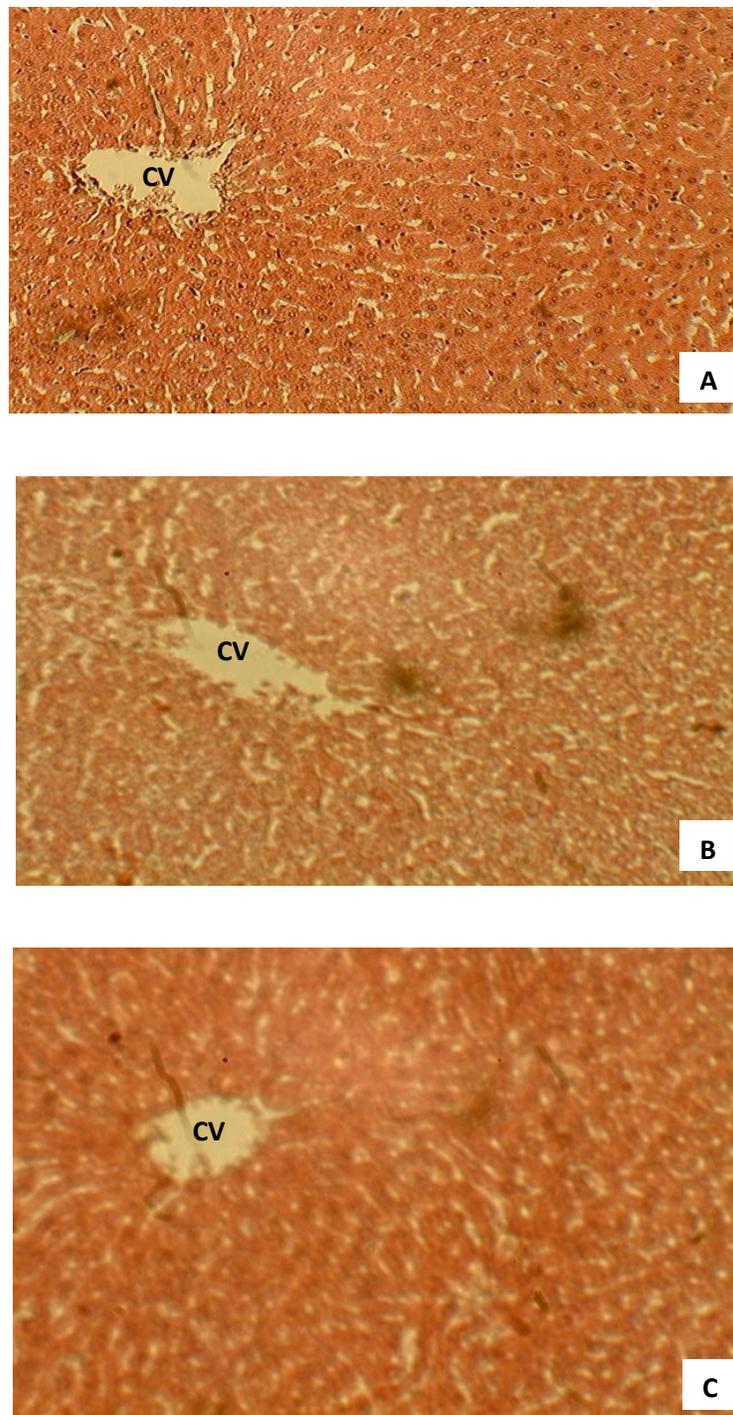
### Histological results

Histopathological investigations showed that the administration of Cd produced severe hepatic damage including extensive degeneration of hepatocytes with necrosis, inflammation, the presence of cellular debris within a central vein and cytological vacuolization (Figure 2B) when compared with control rats (Figure 2A). However, the combination groups of Cd-vit C, Cd-vit E and Cd-vit C-vit E showed prominent recovery in the form of the liver histo-architecture such as the reduced cytoplasmic vacuolization and the normal sinusoidal spaces, but still binucleated cells were seen, though, the lamellar pattern of hepatocytes was restored to almost normal (Figure 3).

### DISCUSSION

The decreased weight gain of rats observed in this study

is consistent with some previously published reports (Horiguchi et al., 1996). Weight gain depends on availability of nutrients. Therefore, the observed reduction in weight gain could have been due to the decrease in food intake, or due to the overall increased degeneration of lipids and proteins as a result of cadmium toxicity (Erdogan et al., 2005). The findings from this investigation also indicate an increase of liver weight. Thus, the administration of cadmium chloride might have led to selective accumulation of cadmium in certain organs especially liver. However, the co-administration of vitamin C and/or vitamin E to the cadmium treated animals improved body and liver weights. Cadmium chloride group animals also showed a high level of glucose. The elevation in serum glucose is a common result of heavy metal toxicity and is usually linked with inhibition of insulin release from Langerhans' islets (Dormer et al., 1973; Kechrid et al., 2006) or with a block of glucose utilization by cells even in the presence of



**Figure 3.** Effect of vitamin E and vitamin C coadministered with cadmium on histological damage in the liver. Cd-vit C (A), Cd-vit E (B), and Cd-vit C-vit E (C). Optic microscopy section were stained using the haematoxylin-eosin method (400×). Vitamin E and vitamin C coadministered with Cd maintained granular cytoplasm and normal histoarchitectural pattern of hepatic parenchyma.

elevated concentrations of insulin (Sunderman et al., 1976) or due to disruption in glucagon secretion result in

high glycogen breakdown and new supply of glucose production from other non carbohydrate sources such as

proteins (Massanyi et al., 1995). However, there is an amelioration of blood glucose concentration in cadmium animals treated either with vitamin E or vitamin C alone or in combination.

There are several possible explanations for the serum glucose reduction. Supplementation of vitamin E or vitamin C might alter insulin receptors in muscle or adipose tissue by increasing membrane motility, secondary to enhance glucose uptake by the diaphragm (Bierenbaum et al., 1985). In this investigation it was also found as a significant diminution in serum total protein and albumin. The decrease in serum total protein and albumin of Cd-treated mice might be due to changes in protein synthesis and/or metabolism (Dostal et al., 1989; Das and Dasgupta, 2000). This result is in agreement with other findings (Yousuf, 2002). Cadmium is one of the heavy metals which induce membrane damage (Uyanik et al., 2001). In the present study, the activities of serum GOT, GPT and alkaline phosphatase were significantly increased, compared to their normal levels. It could be attributed to the hepatic damage resulting in increased release and leakage out of these enzymes from the liver cytosol into the blood stream which gives an indication on the hepatotoxic effect of this metal (Pari and Murugavel, 2005). Consequently, the biochemical perturbations seem to be correlated with the liver histological alteration such as the presence of cellular debris within a central vein and a cytoplasmic vacuolization.

Previous histological studies on liver have documented that Cd-induced changes are characterized by cellular hypertrophy and enlarged nuclei, hepatocyte necrosis, hepatocyte vacuolization and hepatocytes with dilated central vein (Brzóška et al., 2002). The combination treatment of vitamin E or vitamin C with nickel separately or joined, improved the histological alteration induced by nickel, which could be attributed to the antiradicals/antioxidant. The protective action of ascorbic acid on heavy metal toxicity is well documented via its free radical scavenging mechanism and detoxification effect (Suzuki, 1990). However, vitamin E due to its solubility in lipids, plays an important role in protecting lipid-rich structure like liver from free radicals damage and an effective inhibitor of autocatalytic process of lipid peroxidation (Sodhi et al., 2008). It has been shown to be effective in reducing exercise-induced oxidative stress in rats (Metin et al., 2002). In addition, these findings are in good agreement with those obtained by other studies which postulated the beneficial role of vitamin E and vitamin C on histological and enzymatic changes of rats (Das et al., 2006; Ben Amara et al., 2010). Therefore, the supplementation of vitamin E or vitamin C had protected liver function from cadmium intoxication as indicated by the significant restoration of serum total protein, albumin, serum glucose, GOT, GPT and alkaline phosphatase. The diminution of glutathione level in cadmium treated animals may be as a result of oxidative stress, which has occurred in cadmium toxicity. In other words the reduced

antioxidant production due to the increased oxygen metabolites and the elevated free radicals, which caused a decrease in the activity of the anti-oxidant defense system (Gstraunthaler et al., 1983).

Several pathways have been proposed to show the depletion of GSH level in heavy metals toxicity. Firstly, the sulfhydryl group of cysteine moiety of glutathione has a high affinity of metals, forming thermo-dynamically stable mercaptide complexes with several metals. These complexes are inert which can be excreted via the bile (Aposhian, 1989). Secondly, GSH may be oxidized, due to the interaction with the free radicals, induced by nickel. Therefore, GSH level could be consumed during nickel detoxification (Manna et al., 2008). In addition, the decreased activity of hepatic catalase and glutathione peroxidase in cadmium treated animals suggests that either there is an interaction between the accumulated free radicals and the active amino acids of this enzymes (Das et al., 2001) or there is a direct binding of the metal to the active sites of the enzyme (Misra et al., 1990). In group III (cadmium- vit C), group IV (cadmium-vit E) and group V (cadmium-vit C-vit E), the significant improvement of the glutathione level was noticed when compared with that of Group II (cadmium). Thus, the observed normalization of GSH levels, GSH-Px, and catalase activities following vitamin C or vitamin E treatment could be because these vitamins caused a decline in LPO accompanied by an increase in the activities/ level of catalase, GSH-Px and GSH in liver. In other words these vitamins played an action in reducing the levels and accumulation of oxygen reactive species. However, vit C plus vit E proved more effective as compared to individual vitamins alone.

## Conclusion

In conclusion, this study demonstrates exposure to cadmium provoked oxidative liver injury by inducing lipid peroxidation, which led to depletion of liver reduced glutathione, reduction in antioxidant enzyme activities and biochemical parameters variations of rats. However, vitamin E or vitamin C treatment may have partial ameliorative effects on these disturbances caused by cadmium toxicity by increasing GSH level and the activities of antioxidant enzymes ameliorated some biochemical parameters, but vitamin E and vitamin C together exercise a more synergistic effect against the observed oxidative stress.

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