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# Vitamin C acts as a hepatoprotectant in carbofuran treated rat liver slices *in vitro*

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ABSTRACT

Carbamates, most commonly used pesticides in agricultural practices, have been reported to produce free radicals causing deleterious effects in animals. The present study was designed to assess the carbofuran induced oxidative stress in rat liver slices *in vitro* and also to evaluate protective role of vitamin C by incubating them in Krebs-Ringer HEPES Buffer (KRHB) containing incubation media (Williams medium E (WME) supplemented with glucose and antibiotics) with different concentrations of carbofuran. The results demonstrated that carbofuran caused significant increase in lipid peroxidation and inhibition in the activity of hepatic superoxide dismutase (SOD) in concentration dependent manner. The data with incubation medium reflected that carbofuran at lowest concentration caused an increase in SOD activity followed by its inhibition at higher concentration. Carbofuran treatment caused inhibition in the activity of catalase in liver slices and WME incubation medium. Pre-incubation of liver slices and the WME media with vitamin C restored the values of biochemical indices tested. The results indicated that carbofuran might induce oxidative stress in hepatocytes. The pretreatment with vitamin C may offer hepatoprotection from toxicity of pesticide at low concentration only.

## 1. Introduction

The use of pesticides in agriculture remains the most effective method for protection of plants and animals from a large number of pests. The carbamates are chemicals mainly used in agriculture as insecticides, fungicides, herbicides, nematocides, and/or sprout inhibitors. The application of carbamates has been preferred over organophosphates and organochlorines as these compounds were extremely toxic and possessed delayed neurotoxic effects [1,2]. Organocarbamates share with organophosphates in their abilities to inhibit cholinesterases and therefore exhibit similar symptomatology during acute and chronic exposures [3]. Positive correlations have been reported between acetylcholine accumulation and oxidative stress [4,5].

Carbofuran ($C_{12}H_{15}NO_3$; 2,3-dihydro-2,2-dimethyl-7-benzofuranol methylcarbamate), commonly known as Furadan, is a carbamate pesticide and used in the farm practices in order to increase crop productivity. It is also used as an insecticide, nematicide and acaricide due to its short half life in the environment [6]. The adverse effects of carbofuran like myopathy have been reported to be mediated by oxidative stress and the excessive generation of free radicals such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) [2,7,5]. The production of ROS is caused by a mechanism in which xenobiotics, toxicants and pathological conditions generate oxidative stress and induce tissue damage in liver, kidney and brain [8]. The protein oxidation oxidative damage to nucleic acids and peroxidation of lipids mediated by free radicals are considered to be crucial events of the cytotoxic actions of ROS [9]. Studies by Kaur and Sandhir [10] have shown an increased lipid peroxidation and a decrease in glutathione level in the liver of carbofuran exposed rats. In another study, we have reported the increased oxidative stress and its amelioration by vitamin C in the different tissues of carbofuran treated rats [11–14]. Vitamin E [15] and Curcumin [14] have also been reported to be potent ameliorative antioxidants against carbofuran induced toxicity.

In the present study, we have used rat liver slices which have been widely exploited by many researchers as an *in vitro* model of the organ for toxicological assessment. Such tissue slices contain natural and

*Abbreviations:* KRHB, Krebs-Ringer HEPES Buffer; WME, Williams medium E; SOD, superoxide dismutase; ROS, reactive oxygen species; RNS, reactive nitrogen species; KCl, potassium chloride; NADH, nicotinamide adenine dinucleotide; TBA, thiobarbituric acid; EDTA, Ethylenediaminetetraacetic acid; TCA, trichloroacetic acid; DMSO, Dimethylsulfoxide; BSA, Bovine serum albumin; NaCl, sodium chloride; $CaCl_2$, calcium chloride; $MgSO_4$, magnesium sulfate; $CuSO_4$, copper sulphate; NaOH, sodium hydroxide and MDA Malonaldialdehyde

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intact environment in every possible manner including their intercellular and cell-matrix interactions. Previously, the preparation of slices from several mammalian tissues such as liver [16–19], lung [20], kidney [21], intestine [22], spleen [23], brain [24], heart [25], prostate [26] and several tumor types [27,28] and their corresponding applications in many different studies have been demonstrated by several researchers [29]. These workers have suggested the use of these tissues slices for many biochemical and toxicological studies including xenobiotics metabolism and their transport, testing the safety and efficacy of drugs using diseased tissues, ischemia/reperfusion damage [30,31] and specificity of viruses as carriers for gene therapy agents [32,33].

Several other workers have used liver slices for the analysis of the metabolic rates and fates of physiological substrates [34] as well as xenobiotics [17], modulation of hepatic enzymes [35–37], uptake of transporter-mediated drugs [38,39]. In addition, liver slices have also been used to study the biochemical and molecular bases of drug toxicity [40–42] and to investigate the early toxicological markers [43–45]. This system has been preferably used because of it is easy and cheap to study with numerous slices. However, the applications of none of these organ slices have been utilized to demonstrate xenobiotics induced production of oxidative stress, the impact on biochemical parameters of liver slices and amelioration by antioxidants.

In this research article we have presented the carbofuran induced production of oxidative stress in rat liver slices in terms of alterations in lipid peroxidation and activities of antioxidative enzymes. The hepatoprotective effect of vitamin C from carbofuran toxicity has also been demonstrated upon pretreatment of rat liver slices *in vitro*.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Technically pure (99.6%) carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranyl N-methylcarbamate) in powder form was generously gifted by Rallis India Limited (Bangalore, India) as a gift. Tris HCl, potassium chloride (KCl), sodium-pyruvate, nicotinamide adenine dinucleotide (NADH), Pyrogallol, tris base, EDTA, succinic acid, $H_2O_2$, thiobarbituric acid (TBA) and trichloroacetic acid (TCA) were purchased from E. Merck, Darmstadt, Germany and Dimethylsulfoxide (DMSO) from (Qualigens). Bovine serum albumin (BSA), were purchased from Loba Chemie Pvt. Ltd. India. Diethyl ether [$(CH_3CH_2)_2O$] solution was procured from Sigma-Aldrich, India. β-Hydroxy butyrate, sodium chloride (NaCl), calcium chloride ($CaCl_2$), magnesium sulfate ($MgSO_4$), glucose, Folin-ciocalteu reagent, copper sulphate ($CuSO_4$), vitamin C and sodium hydroxide (NaOH) were purchased from SISCO research laboratory Pvt. Ltd, Mumbai, India.

### 2.2. Animals

Male albino Wistar rats (body weight between 100 and 130 g and 8–10 weeks of age) were selected for all the experiments. Animals obtained from Central Drug Research Institute, Lucknow, India, were housed in polypropylene cages at an ambient temperature of 25 °C-30 °C and 45–55% relative humidity with 12 h each of dark and light cycle. Animals were fed standard rat chow (Golden feed, New Delhi, India) and drinking water ad libitum. The protocols used in the study were according to the guidelines for use and care of laboratory animals and were approved by the Institutional Ethics Committee of the University.

### 2.3. Preparation of KRHB

KRHB is used for slicing and storage of liver slices. This buffer is prepared in 1 liters of a 10 X concentrated KRHB stock solution (10 X KRHB) by dissolving 3.67 g $CaCl_2{\cdot}2H_2O$ in 500 ml of ultrapure water (solution 1). Solution 2 is prepared by dissolving 3.73 g KCl, 69.0 g, NaCl, 2.71 g, $MgSO_4{\cdot}7H_2O$ and 1.63 g $KH_2PO_4$ in ultrapure water to a volume of 500 ml. Then solutions 1 and 2 were mixed and filtered through a 0.45 μm filter. This 10 X KRHB can be stored at 4° C for about 6 months. The KRHB was freshly prepared by dissolving 1.05 g $NaHCO_3$, 2.475 g D-glucose monohydrate and 1.19 g HEPES in about 200 ml of ultrapure water at 4° C. Into this solution, 50 ml of 10 X KHB is mixed followed by addition of 250 ml of ultrapure water at 4° C. This solution may be stored at 4° C. The pH of this solution was maintained to be 7.4 by slow addition of 5 N NaOH solution.

### 2.4. Preparation of WME slice incubation medium

Freshly 500 ml WME slice incubation medium was prepared by adding 1.375 g D-glucose monohydrate and 500 μl gentamicin (50 mg $ml^{-1}$) to WME containing L-gluamine.

### 2.5. Development of liver slice in vitro

Rats were sacrificed by mild anesthesia using Diethyl ether [$(CH_3CH_2)_2O$] solution and followed by the cervical dislocation. The anesthetic used according to the AVMA Guidelines for the Euthanasia of Animals: 2013 Edition. Liver lobes were removed and transferred to pre-warmed Krebs-Ringer HEPES Buffer (KRHB) (Hepes 2.5 mM pH 7.4, NaCl 118 mM, KCl 2.85 mM, $CaCl_2$ 0.5 mM, $KH_2PO_4$ 1.5 mM, $MgSO_4$ 1.18 mM and glucose 4.0 mM) solution. Liver slice culture was maintained following the protocol developed by Wormser et al. [46] and Invittox Protocol No. 42 (1992). A small piece of liver was cut into thin slices. Each culture tube contained 20–22 slices with about 0.5 mm of thickness and the wet weight of each slice was about 10–12 mg. These slices were washed with 10 ml KRHB medium, every 10 min over a period of 1 h. They were then incubated for 60 min in small plugged beakers containing 2 ml KRHB on a shaker water bath at 37 °C. At the end of incubation, the medium was replaced by fresh 2 ml KRHB and incubated for 1 h at 37 °C with ethanol (1372 mM; Invittox Protocol No. 42, 1992). The liver slices were then treated with different final concentrations of carbofuran (0.025, 0.25, 0.5, 1.0, 2.5, 5.0, 10, 25, and 50 μM) for the same time interval i.e. 1 h. The treated liver slice supernatants were taken separately in different centrifuge tubes (2 ml) and used for biochemical estimations. To evaluate the effect of vitamin C on carbofuran treated liver slices, the liver slices were pre-incubated for 10 min in the media with vitamin C at 567.76 μM concentration. For the time dependent study the media was replaced by fresh 2 ml KRHB solution containing 2.5 μM carbofuran in the KRHB solution. The slices were removed at the different time interval (15, 30, 60, 90 and 120 min). The treated livers slices were homogenized (10%, w/v) in 0.25 M sucrose solution and centrifuged at 9000 x g for 30 min at 4 °C. The supernatants were separated by gentle decantation of centrifuged homogenates and used for estimation of lipid peroxidation, activities of superoxide dismutase, catalase and quantification of protein. The control liver slices (untreated) were also processed accordingly parallel to the experimental liver slices.

### 2.6. Measurement of MDA level

Lipid peroxidation was measured in the cytosolic fraction of hepatic tissues as well as in the KRHB incubation media by following the method of Niehaus and Samuelsson [47] and the results were expressed as nmol MDA/mg protein using the extinction coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$.

### 2.7. Estimation of the activities of antioxidant enzymes

The activity of superoxide dismutase (SOD, E.C. 1.15.1.1) was measured by following the method of Marklund and Marklund [48]. It is a spectrophotometric measurement of optical density of colored complex involving pyrogallol auto-oxidation at 412 nm for 3 min at the

interval of 30 s with or without the enzyme protein. One unit of the enzyme activity was expressed as 50% inhibition of auto- oxidation of pyrogallol per min.

Catalase (CAT, E.C.1.11.1.6) activity was measured according to the method of Beers and Sizer [49] by measuring the decrease in the absorbance for $H_2O_2$ consumption at 240 nm at the interval of 30 s for 3 min. One unit of CAT activity was defined as micromoles of $H_2O_2$ decomposed per min using molar extinction coefficient of $H_2O_2$ (43.6 $M^{-1}cm^{-1}$).

### 2.8. Estimation of total protein content

The protein content in the sample was measured by the modified Lowry et al. [50] using BSA as a standard. The amount of protein was calculated from the standard curve.

### 2.9. Statistical analysis

Each sample was run in triplicate. Values were expressed as mean ± standard deviation (SD) tissue, for n = 5 to 6 rats. Values between groups were compared using Dunnet's comparison tests. Values were considered significant if $P < 0.05$. Statistical analysis was performed by means of In-Stat package for personal computers version 5 (GraphPad_Software, Inc., San Diego, USA).

## 3. Results

### 3.1. Effect of carbofuran on the activities of antioxidant enzymes in rat liver slices

Superoxide dismutases (SODs) form the first line of defense against reactive oxygen species. Fig. 1(a) and (b) show that carbofuran caused a significant inhibition of SOD in the liver tissue and incubation medium from a concentration of 0.5 and 0.25 μM, respectively. Similarly, Fig. 1(c) and (d) demonstrate that carbofuran exhibited a parallel inhibitory effect on SOD in the liver tissue homogenate and the secretary products present in the incubation medium from 60 min of post incubation which continued up to 120 min of incubation. However, vitamin C reversed the SOD inhibition to near normal levels except at a carbofuran concentration of 2.5, 5 and 5 μM in the liver tissue and medium, respectively (Fig. 1(e) and (f)). Inhibition of SOD by increasing concentration and/or incubation time of carbofuran may be due to inhibitory effect of superoxide anions on SOD (***$p < 0.001$) generated by carbofuran [51].

Catalase forms the second stage defense antioxidant enzyme that dissipates $H_2O_2$ formed from dismutation of superoxide anions by superoxide dismutases or those formed from other sources. Fig. 2(a) and (b) show that carbofuran from a concentration of 0.25 μM and 0.5 μM till 5.0 μM significantly inhibited catalase in the liver tissue and the secretary products present in the incubation medium, respectively. Hepatic catalase was inhibited by carbofuran from 60 min of incubation till 120 min (Fig. 2(c) and (d)). In the incubation medium, however, there was an increase in catalase activity after 30 min followed by significant inhibition of its activity from 60 min till 120 min of incubation. Vitamin C was able to reverse carbofuran induced catalase inhibition up to 2.5 μM of carbofuran concentration in liver (Fig. 2(e)) and 5.0 μM in medium (Fig. 2(f)).

### 3.2. Effect of carbofuran on the level of MDA in rat liver slices

The results presented in Fig. 3(a) show the effect of different concentrations of carbofuran on lipid peroxidation in liver slices. Carbofuran at doses of 0.5, 1.0, 2.5 and 5.0 μM caused significant increase ($P < 0.05$) in lipid peroxidation in the slices. When extent of lipid peroxidation under this condition was monitored in the incubation medium which contained the liver slices, it was found that carbofuran at doses of 0.5, 1.0, 2.5 and 5.0 μM caused significant increases ($P < 0.05$) in lipid peroxidation in the secretary products present in the incubation medium the WME incubation medium. The results are shown in Fig. 3(b).

The time dependent alterations in the levels of MDA in liver slices were also evaluated after incubation with 2.5 μM of carbofuran. The results presented in Fig. 3(c) indicated that carbofuran caused a significant increase ($P < 0.001$) in malonaldehyde formation after 60 min of incubation which continued to increase up to 120 min. Fig. 3(d) shows a parallel increase in MDA liver in the secretary products present in the incubation media after incubation with 2.5 μM of carbofuran. An earlier significant increase ($P < 0.01$) in lipid peroxidation which started at 30 min after incubation was observed which continued up to 120 min. Fig. 3(e) and (f) showed the effects of vitamin C on carbofuran induced lipid peroxidation in the hepatic tissues and the secretary products present in the incubation medium incubation medium, respectively. Vitamin C was able to reverse increased lipid peroxidation induced by all the doses of carbofuran tested except 5 μM carbofuran in the liver and incubation medium to near normal levels. At a higher carbofuran concentration of 5 μM vitamin C failed to neutralize increased lipid peroxidation both in the liver slices as well as in the incubation medium which could be possibly due to saturation of its antioxidant capacity.

## 4. Discussion

### 4.1. Perturbations in the activities of antioxidant enzymes due to carbofuran treatment of rat liver slices

Superoxide dismutases (SODs) dissipate superoxide to $H_2O_2$ and $O_2$, which are the most important parts of antioxidant enzyme defense against ROS, particularly superoxide anion radicals [52]. Catalase dissipates $H_2O_2$ formed from dismutation of superoxide anions by superoxide dismutases. The restoration of catalase activity after vitamin C treatment may be attributed to the supporting role of this vitamin in improving the rate of removal of $H_2O_2$ in metabolically normal animals [53].

Vitamin C is a water soluble antioxidant, has been reported to ameliorate the free radical induced damage by carbofuran [54,55]. Since ascorbic acid is water soluble, it can work both inside and outside the cells to combat free radical damage [55]. There are several transport mechanisms to get it inside: Glucose-dependent transporters like GLUT1 and 3 as well as more specialized sodium-dependent transporters for ascorbic acid, SVCT1 and 2 [56]. Vitamin C can "donate electrons to free radicals such as hydroxyl and superoxide radicals and quench their reactivity" [57,58]. Vitamin C reported to be an antioxidant [59], has been shown to mitigate lead induced oxidative stress toxicity [60]. The extract of *C. limon* fruit, which is rich in vitamin C has also been reported to play a protective role against free radical mediated carbofuran induced hepatotoxicity in rats [13]. Therefore, the present study confirms the protective role of vitamin C on carbufuran induced alterations in enzyme activities only up to a certain concentration of carbofuran. At a higher concentration of carbofuran (5 mM), vitamin C was not able to completely restore activities of antioxidant enzymes to normal level. This may be due to fixed concentration of vitamin C used for all concentrations of carbofuran tested. At higher carbofuran concentrations, the antioxidant effect of vitamin C may be limited due to saturation of its antioxidant capacity.

### 4.2. Carbofuran induces alterations in rat liver slices

The lipid peroxidation of biomembranes results in production of several compounds that are routinely used as molecular markers of oxidative stress. Malonaldehyde is one of the most widely used indicators of the cellular redox state [61]. In present study elevated lipid peroxidation caused by increased doses of Carbofuran and increased

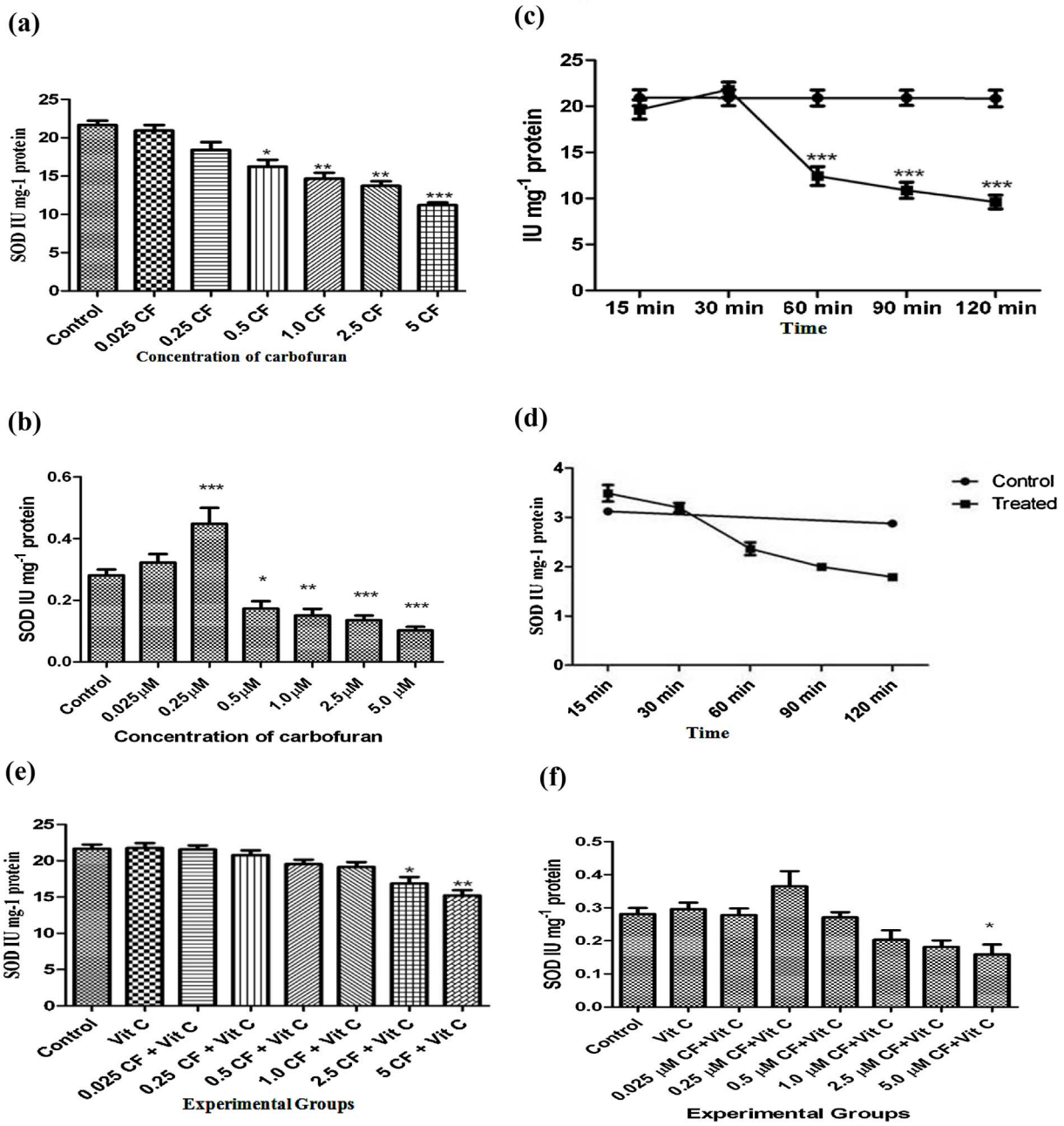


**Fig. 1.** (**a**): Effect of various concentrations of carbofuran (0.025, 0.25, 0.50, 1.00, 2.50, 5.00 (M) on the levels of SOD activity in the supernatant of homogenate (10%, w/v) prepared in 0.25 M sucrose solution rat liver slices in vitro. Control: control group had not treated with any treatment. Level of SOD activity was determined as described in Materials and Methods. The unit of level of SOD activity is IU $mg^{-1}$ Protein. The values were expressed as Mean ± SD. The sign (*), (**), and (***) indicates values significantly decreased the activity from control at ($P < 0.05$). (**b**): Effect of various concentrations of carbofuran (0.025, 0.25, 0.50, 1.00, 2.50, 5.00 µM) on SOD in the supernatant of the incubation medium of rat liver slices in vitro. Control: control group had not treated with any treatment. Level of SOD activity was determined as described in Materials and Methods. The unit of level of SOD activity is IU $mg^{-1}$ Protein. The values were expressed as Mean ± SD. The sign (*), (**), and (***) indicates values significantly decreased the activity from control at ($P < 0.05$). (**c**): Time dependent alterations on SOD activity in the supernatant of homogenate (10%, w/v) prepared in 0.25 M sucrose solution of rat liver slices after incubation with 2.5 µ moles of carbofuran in vitro. Control: control group had not treated with any treatment. Level of SOD activity was determined as described in Materials and Methods. The unit of level of SOD activity is IU $mg^{-1}$ Protein. The values were expressed as Mean ± SD. The sign (***) indicates values significantly increased from control at ($P < 0.05$). (**d**): Time dependent alterations on SOD activity in the supernatant of the incubation medium of rat liver slices after incubation with 2.5 µ moles of carbofuran in vitro. Control: control group had not treated with any treatment. Level of SOD activity was determined as described in Materials and Methods. The unit of level of SOD activity is IU $mg^{-1}$ Protein. The values were expressed as Mean ± SD. (**e**): Effect of vitamin C on carbofuran induced changes in SOD activity level in the supernatant of homogenate (10%, w/v) prepared in 0.25 M sucrose solution of rat liver slices in vitro. Control: control group had not treated with any treatment. The experimental groups of liver slices were treated with different conditions as described in materials and methods. The level of SOD activity was determined as described in Materials and Methods. The unit of level of SOD is IU mg − 1 Protein. The values were expressed as Mean ± SD. The sign (*) and (**) indicates values significant from control at ($P < 0.05$). (**f**): Effect of vitamin C on carbofuran induced changes in SOD activity level in the supernatant of the incubation medium of rat liver slices in vitro. Control: control group had not treated with any treatment. The experimental groups of liver slices were treated with different conditions as described in materials and methods. The level of SOD activity was determined as described in Materials and Methods. The unit of level of SOD is IU mg − 1 Protein. The values were expressed as Mean ± SD. The sign (*) and (**) indicates values significant from control at ($P < 0.05$).

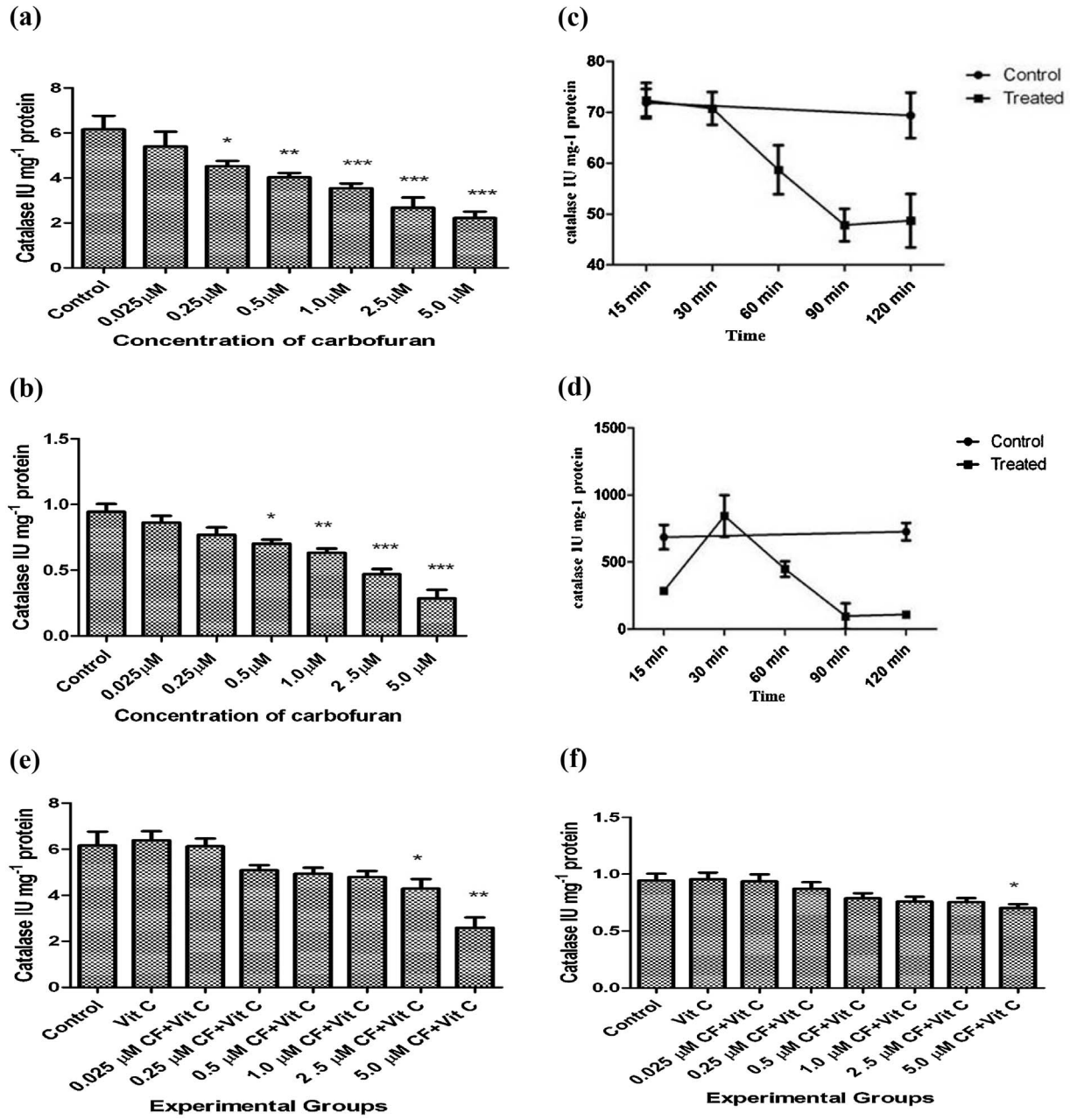


**Fig. 2.** (**a**): Effect of various concentrations of carbofuran (0.025, 0.25, 0.50, 1.00, 2.50, 5.00 µM) on the levels of activity of catalase in the supernatant of homogenate (10%, w/v) in 0.25 M sucrose solution of rat liver slices in vitro. Control: control group had not treated with any treatment. Level of catalase activity was determined as described in Materials and Methods. The unit of level of catalase activity is IU $mg^{-1}$ Protein. The values were expressed as Mean ± SD. The sign (*), (**), and (***) indicates values significantly decreased from control at ($P < 0.05$). (**b**): Effect of various concentrations of carbofuran (0.025, 0.25, 0.50, 1.00, 2.50, 5.00 µM) on the levels of catalase activity in the supernatant of the incubation medium of rat liver slices in vitro. Control: control group had not treated with any treatment. Level of catalase activity was determined as described in Materials and Methods. The unit of level of catalase actvuity is IU $mg^{-1}$ Protein. The values were expressed as Mean ± SD. The sign (*), (**), and (***) indicates values significantly decreased from control at ($P < 0.05$). (**c**): Time dependent alterations on the levels of catalase activity form the supernatant of homogenate (10%, w/v) prepared in 0.25 M sucrose solution of rat liver slices after incubation with 2.5 µmoles of carbofuran in vitro. Control: control group had not treated with any treatment. Level of catalase was determined as described in Materials and Methods. The unit of level of catalase activity is IU $mg^{-1}$ Protein. The values were expressed as Mean ± SD. The sign (■) and (●) indicates values of Experimental and control. (**d**): Time dependent alterations on the levels of catalase activity from the supernatant of the incubation medium of rat liver slices after incubation with 2.5 µmoles of carbofuran in vitro. Control: control group had not treated with any treatment. Level of Catalase was determined as described in Materials and Methods. The unit of level of catalase activity is IU $mg^{-1}$ Protein. The values were expressed as Mean ± SD. The sign (■) and (●) indicates values of Experimental and control, respectively. (**e**): Effect of vitamin C on carbofuran induced changes on the levels of catalase activity from the supernatant of homogenate (10%, w/v) prepared in 0.25 M sucrose solution of rat liver slices in vitro. Control: control group had not treated with any treatment. The experimental groups of liver slices were treated with different conditions as described in materials and methods. The level of catalase activity was determined as described in Materials and Methods. The unit of level of catalase activity is IU $mg^{-1}$ Protein. The values were expressed as Mean ± SD. The sign (*) and (**) indicates values are significant from control at ($P < 0.05$). (**f**): Effect of vitamin C on carbofuran induced changes on the levels of catalase activity from the supernatant of incubation medium of rat liver slices in vitro. Control: control group had not treated with any treatment. The experimental groups of liver slices were treated with different conditions as described in materials and methods. The level of catalase activity was determined as described in Materials and Methods. The unit of level of Catalase activity is IU $mg^{-1}$ Protein. The values were expressed as Mean ± SD. The sign (*) indicates values are significant from control at ($P < 0.05$).

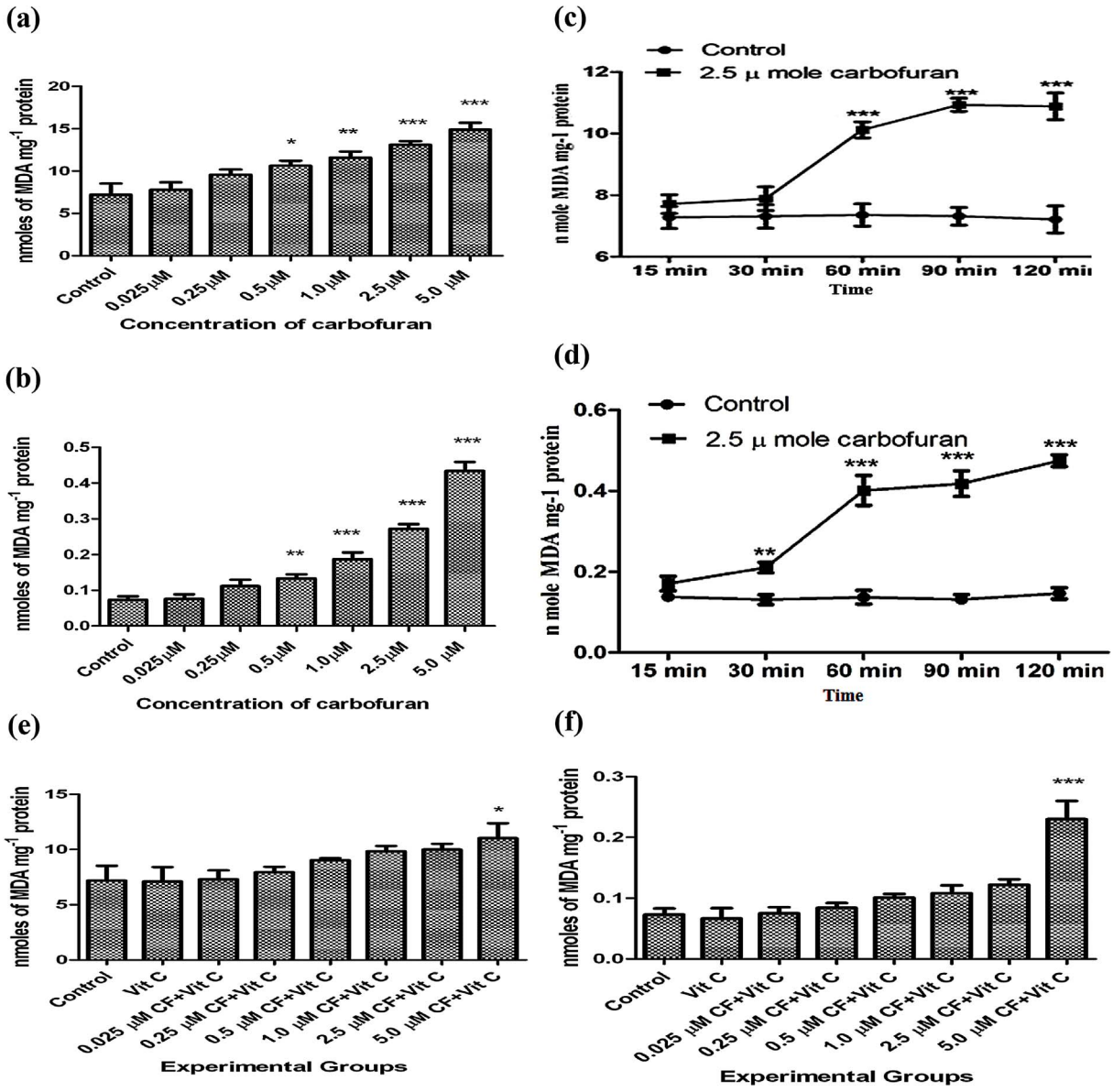


**Fig. 3.** (**a**): Effect of various concentrations of carbofuran (0.025, 0.25, 0.50, 1.00, 2.50, 5.00 µM) on lipid peroxidation in the supernatant of homogenate (10%, w/v) prepared in 0.25 M sucrose solution from rat liver slices in vitro. Control: control group had not treated with any treatment. Level of MDA was determined as described in Materials and Methods. The unit of level of MDA is $mg^{-1}$ Protein. The values were expressed as Mean ± SD. The sign (*), (**), and (***) indicates values significantly increased from control at ($P < 0.05$). Carbofuran at doses of 0.5, 1.0, 2.5 and 5.0 µM has been observed to be significantly increased. (**b**): Effect of various concentrations of carbofuran (0.025, 0.25, 0.50, 1.00, 2.50, 5.00 µM) on lipid peroxidation in the supernatant of incubation medium of rat liver slices in vitro. Control: control group had not treated with any treatment. Level of MDA was determined as described in Materials and Methods. The unit of level of MDA is $mg^{-1}$ Protein. The values were expressed as Mean ± SD. The sign (*), (**), and (***) indicates values significantly increased from control at ($P < 0.05$). Carbofuran at doses of 0.5, 1.0, 2.5 and 5.0 µM has been observed to be significantly increased. (**c**): Time dependent alterations in MDA levels in the supernatant of homogenate (10%, w/v) prepared in 0.25 M sucrose solution from rat liver slices after incubation with 2.5 µ moles of carbofuran in vitro. Control: control group had not treated with any treatment. Level of MDA was determined as described in Materials and Methods. The unit of level of MDA is $mg^{-1}$ Protein. The values were expressed as Mean ± SD. The sign (***) indicates values significantly increased from control at ($P < 0.05$). (**d**): Time dependent alterations in MDA levels in the supernatant of homogenate (10%, w/v) in 0.25 M sucrose solution of the supernatant from the incubation medium of rat liver slices after incubation with 2.5 µ moles of carbofuran in vitro. Control: control group had not treated with any treatment. Level of MDA was determined as described in Materials and Methods. The unit of level of MDA is $mg^{-1}$ Protein. The values were expressed as Mean ± SD. The sign (***) indicates values significantly increased from control at ($P < 0.05$). (**e**): Effect of vitamin C on carbofuran induced changes on the level of lipid peroxidation from the supernatant of homogenate (10%, w/v) prepared in 0.25 M sucrose solution of rat liver slices in vitro. Control: control group had not treated with any treatment. The experimental groups of liver slices were treated with different conditions as described in materials and methods. The level of MDA was determined as described in Materials and Methods. The unit of level of MDA is $mg^{-1}$ Protein. The values were expressed as Mean ± SD. The sign (*) indicates values significantly increased from control at ($P < 0.05$). (**f**): Effect of vitamin C on carbofuran induced changes on the levels of lipid peroxidation in the supernatant of the incubation medium in vitro. Control: control group had not treated with any treatment. The experimental groups of liver slices were treated with different conditions as described in materials and methods. The level of MDA was determined as described in Materials and Methods. The unit of level of MDA is $mg^{-1}$ Protein. The values were expressed as Mean ± SD. The sign (*) indicates values significantly increased from control at ($P < 0.05$).

incubation time may be due to the damage of membrane lipids by free radicals causing the membranes to lose their fluidity and release of some of their altered macromolecules into the incubation medium. These molecules released into the medium could contribute to a parallel increase in lipid peroxidation in the medium. Earlier studies have described increased lipid peroxidation in the brain and liver of carbofuran treated rats [62]. Malathion, β-cypermethrin and avermectin along with phoxim (one of the organophosphorus pesticides) and Cypermethrin have been reported to induce oxidative stress in various insects [63,64]. Due to high concentration of polyunsaturated fatty

acids in cells, lipid peroxidation is a major outcome of free radical-mediated injury [65,66]. A critically important aspect of lipid peroxidation is that it proceeds until oxidizable substrate is consumed or termination occurs, making this fundamentally different from many other forms of free radical injury in that the self-sustaining nature of the process may cause extensive tissue damage [67]. Two broad outcomes of lipid peroxidation are structural damage to cellular membranes and generation of oxidized products, some of which are chemically reactive and may covalently modify cellular macromolecules [68]. Carbofuran has been reported to cause injury by inhibiting acetylcholinesterase which coincides with increased lipid peroxidation [7]. The lipophilic nature of carbofuran has been reported to cause oxidative injury resulting alterations in membrane structure and function [62,54].

Ladurner et al. [69] have studied the uptake of ascorbate into cultured endothelial cells. They observed that the uptake of ascorbate increases with increasing time. The ascorbate (100 μM) has been reported to exhibit time dependent effect up to 24 h on total eNOS activity level and epithelial BH4 levels. Some workers have reported that vitamin C may exhibit dose- and time-dependent protective effect on NaF mediated alterations in the levels of 3β-HSD and 17β-HSD, SOD, CAT, GPx, GST, γ-GT, and GSH, as well as the activities of sertoli cells *in vitro*. They observed that the enzymes when incubated for 24 and 48 h, maximum protection of them were achieved after longer duration of incubation i.e. 48 h [70].

Our study bears some important limitations such as (i) along with the experimental results with Vitamin C there should have been a parallel result obtained with carbofuran treated group. This data was essential to conclude that Vit C actually decreased the effect of Carbofuran; (ii) in Fig. 2F and G, as well as in Fig. 3E and F, to have conditions without vitamin C seems to be important; (iii) There are two main variables that influence the oxidative stress indicators in our experiments: time and dose response. The best experimental design would have beeen to observe if the dose response and time course curves move to the right or not in the presence of constant amount of Vit C. This design would clearly show how Vit C acts as antioxidant to neutralize some of the oxidative molecules and shift the dose and time response curves to the right; (iv) the data for Control, Vit C, and Carbofuran ± Vit C for both time and dose response curves would have been beneficial. However, in separate experiments we have shown that carbofuran alone can produce serious adverse effects at lower doses. The Fig. 2F and G as well As Fig. 3E and F lack the data without vitamin C but it could be indirectly correlated with the results of other experiments done separately as shown in Figures.

## 5. Conclusions

The use of rat liver slices cultures offers the unique opportunity to investigate the direct toxicological responses in a system exposed *in vitro* (A combined system that more closely mimics the in vivo situation). Therefore it allows more realistic and accurate data and their conclusions to be obtained. The results indicated that rat liver slices when treated *in vitro* with different concentrations of carbofuran displayed significant alterations at the levels of antioxidative indices in both the tissues as well as the secretary products present in the incubation medium, thereby indicating its effects being mediated via generation of oxidative stress, which may be significantly ameliorated by application of vitamin C. Vitamin C being a potential antioxidant was useful in amelioration of pesticide toxicity but failed to offer protection at higher concentration of the pesticide.

## Conflict of interest

The authors declare that they do not have any competing interests.

## Author contributions

All the authors have contributed equally to this work.

## Transparency document

The Transparency document associated with this article can be found in the online version.

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