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EFFECT OF CARBOFURAN ON SOME BIOCHEMICAL INDICES OF HUMAN ERYTHROCYTES *IN VITRO*

R. K. SHARMA[1], S. K. JAISWAL[1], N. J. SIDDIQI[2] AND B. SHARMA[1]✉

[1]Department of Biochemistry, Faculty of Science, University of Allahabad; Allahabad, 211 002 India.
[2] Department of Biochemistry, College of Science, PO Box 22452, King Saud University, Riyadh -11495, Saudi Arabia.

Abstract

Pesticides are used in agriculture to protect crops. Its widespread use in agriculture represents a threat not only to the environment but also to human populations exposed to them. Erythrocytes serve as an excellent model system to study the interaction of pro-oxidants. Organocarbamates are known to produce free radical species and to induce toxicity to different body systems resulting into hematological and biochemical perturbations. The information available relating to the effect of organocarbamates on the biochemical indices of human erythrocytes is scanty. Therefore, the present study was carried out to evaluate the impact of carbofuran, a carbamate pesticide, on some key biochemical indices of human erythrocytes' membrane. The oxidative potential of the pesticide was assessed *in vitro* by monitoring the levels of malondialdehyde (MDA) and reduced glutathione (GSH) in human erythrocytes exposed to different sub-acute concentrations (0, 2.5, 5, 10, 25 and 50μM) of carbofuran for different time intervals; maximally up to 120 min. It was observed that the level of MDA was elevated and that of GSH was significantly decreased after treatment of erythrocytes with carbofuran. The results indicated the negative impact of carbofuran in concentration and time dependent manner. Carbofuran was also found to sharply inhibit the activity of membrane bound Na^+K^+-ATPase at higher carbofuran concentrations (10, 25 and 50μM). Further, carbofuran at aforesaid concentrations was also found to cause significant rise in the osmotic fragility of human erythrocytes indicating adverse effect on membrane fluidity. The results of present study suggested that carbofuran was able to alter the oxidative balance and the stability of human erythrocytes membrane.

Key words: Carbofuran, oxidative stress, Na^+K^+-ATPase, Osmotic fragility, Human erythrocytes, GSH, MDA.

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✉ **Corresponding author**
Tel: +91-9415715639
Fax: +91-532-2461157
E-mail: sharmabi@yahoo.com

INTRODUCTION

Carbofuran (2, 3-dihydro-2, 2-dimethyl-7-benzofuranol methyl carbamate), a broad-spectrum pesticide, is commonly used in agricultural practices (7, 12). It is a systemic insecticide, acaricide, and nematicide with a broad spectrum of activity and extensively used for the control of all types of stem borers in rice, sugarcane, fruits, and vegetables (7). It is one of the most toxic carbamate pesticides and known to exert high toxicity to mammalian systems (9)

Erythrocyte is a convenient model to study oxidative damage of cell membranes by various pro-oxidants as well as the pesticides. The erythrocyte membrane has been reported to be highly sensitive to oxidative stress as it contains high amount of polyunsaturated fatty acids (PUFA) as well as higher concentration of oxygen and heme (15, 16). Red blood cells (RBCs) have been reported to act as circulating antioxidant carriers, which protect them from exposure to reactive oxygen species (ROS). Indeed, the erythrocytes have been used for the evaluation of the impact of free-radical induced oxidative stress in humans because of several reasons; for example (i) these cells are continually exposed to high oxygen tensions, (ii) unable to replace damaged components, (iii) their membrane lipid bilayers are rich in PUFA side chains which make them vulnerable to peroxidation and (iv) they have enzymatic and non-enzymatic antioxidant systems (12).

ROS-catalyzed oxidative damage to membrane lipids may impair the stability of erythrocytes and cause oxidative hemolysis or osmotic fragility (26). The osmotic fragility of erythrocytes is frequently used as a measure of erythrocyte tensile strength. It depends on the movement of water into the cell and is related to cellular deformability. The oxidative stress is one of the factors which determine the integrity of erythrocytes (27).

Oral administration of carbofuran is reported to cause neuronal injury by excessive generation of reactive oxygen species (ROS) leading to lipid peroxidation (LPO) (1). It has also been reported that intra-peritoneal administration of carbofuran at its sub-acute concentrations may induce oxidative stress in Wistar rats (20) and genotoxic effects in epithelial cells across cryptvillus axis in rat intestine (7). It has been proven that even the increase in intracellular levels of ROS is mediated via inhibition of AChE activity by carbofuran even at lowest concentrations tested (19, 20).

The studies concerning the *in vitro* effect of carbofuran on biochemical markers from the membrane of human eryhtrocytes have not been systematically carried out. Keeping these facts in view, we envisaged in the present study to explore the *in vitro* impact of carbofuran on the levels of oxidative stress parameters using human erythrocytes.

MATERIALS AND METHODS

Carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranyl N-methylcarbamate) in powder form (99.6%) was generously supplied by Rallis India Limited (Bangalore, India) as a gift. Sodium di-hydrogen phosphate (NaH_2PO_4), di-Sodium-hydrogen phosphate (Na_2HPO_4), potassium chloride (KCl), sodium chloride (NaCl), thiobarbituric acid (TBA) and trichloroacetic acid (TCA) were purchased from E. Merck, Darmstadt, Germany and Dimethylsulfoxide (DMSO) from (Qualigens). Reduced glutathione (GSH) and heparin were purchased from HIMEDIA La-

boratories Pvt. Ltd. India. All other chemicals used in the study were analytical grade and purchased locally.

Collection of blood and isolation of erythrocytes

The protocol of study was in conformity with the guide lines of the Allahabad University Ethical Committee. A volume of 3 ml human blood was collected in heparin containing vials by venipuncture from a group of 28 to 32 years old healthy subjects, not exposed to radiation, drugs or any antioxidant supplementation including vitamins C and E. The blood was centrifuged at 1800×g for 10 min at 4°C. After the removal of plasma, buffy coat and upper 15% of the packed red blood cells (RBCs), the RBCs were washed twice with cold PBS (0.9% NaCl, 10mM Na_2HPO_4, pH 7.4). Erythrocyte 'ghosts' from leucocyte-free RBCs were prepared by an osmotic shock procedure, using the method of Dodge et al. (4). The protein content was determined by the method of Lowry et al. (14) using bovine serum albumin (BSA) as a standard.

In vitro effect of carbofuran on the levels of MDA and GSH contents of erythrocytes

A stock solution of carbofuran was prepared by dissolving it into 10% DMSO and 0.1% DMSO was used as vehicle control. The experimental groups included (I) a vehicle control i.e. the erythrocytes incubated in buffer containing 0.1% DMSO without carbofuran used as the non-treated control cells and (II) the erythrocytes incubated with various concentration (2.5,5.0,10.0,25.0 and 50.0 µM) of carbofuran.

Packed Red blood cells (RBCs) were suspended in 4 volumes of PBS containing 5mmol/L glucose (pH 7.4). *In vitro* effects were evaluated by co-incubating the erythrocytes in the presence of carbofuran at different concentrations at 37°C for 60min. After incubation the suspensions were centrifuged at 1800×g, the RBCs were washed twice with at least 50 volumes of PBS and then the RBCs were treated with equal volume of 0.154 M NaCl followed by the 10% neutralized β-mercaptoethanol and EDTA solution to give the final 1:20 dilution of the heamolysate to assay for MDA level and GSH content.

Time dependent experiments were also done in which the co-incubation time with carbofuran (50µM) was varied between 15 min to 120 min. Parallel control experiments were also performed.

Determination of GSH content of erythrocytes

Erythrocyte GSH was measured following the method of Beutler et al. (3). This method was based on the ability of the –SH group to reduce 5,5′-dithiobis,2- nitrobenzoic acid (DTNB) and form a yellow coloured anionic product whose OD is measured at 412 nm. The concentration of GSH is expressed in mg/mL packed RBCs.

Determination of malondialdehyde (MDA) level in erythrocytes

Lipid peroxidation was measured according to the method of Esterbauer and Cheeseman (5). Packed erythrocytes (0.2 ml) were suspended in phosphate buffer, pH 7.4. The lysate (1 ml) as mentioned in the materials and methods section, was added to 1ml of 10% trichloroacetic acid (TCA) and mixture was centrifuged for 5 min at 3000×g. The supernatant (1 ml) was added to 1 ml of 0.67% thiobarbituric acid (TBA) in 0.05 mol/l NaOH and boiled for 30min at 90ºC, cooled and the absorbance was recorded at 532 nm. The concentration of MDA was determined from a standard plot using malondialdehyde as standard.

Determination of Na^+/K^+-ATPase activity in erythrocytes

The Na^+-K^+-ATPase activity was determined by the method of Shuhail and Rizvi (25). For measurement of Na^+-K^+-ATPase activity in the membrane, erythrocyte ghosts (0.8–1.5 mg of protein) were incubated with the carbofuran at different doses in PBS (pH 7.4) for 1h at 37°C prior to the estimation of Na^+-K^+-ATPase activity. The final assay mixture contained 0.8mg membrane protein per ml, 140mM NaCl, 20mM KCl, 3mM $MgCl_2$, 30mM imidazole (pH 7.25), 5×10^{-4} M Ouabain and 6mM ATP. The reaction was stopped by adding 3.5ml of a solution containing 0.5M H_2SO_4, 0.5% ammonium molybdate and 2% SDS. The amount of liberated inorganic phosphate was estimated by the method of Fiske and Subbarow (6). Parallel control experiments were also performed in which carbofuran was replaced with an equal amount of 0.1% DMSO. Time dependent experiments were done in which the incubation time was varied between 15 min to120 min with fixed concentrations of carbofuran.

Determination of pesticide induced erythrocyte fragility assay

Carbofuran induced hemolysis of erythrocyte membrane was done according to Sharma et al. **(**23). Osmotic fragility test was estimated by adding 50µl of washed erythrocyte suspension of preincubated with various concentrations (2.5,5.0,10.0,25.0 and 50.0µM) of carbofuran at room temperature for 30 min containing 5 ml of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8 and 0.9% saline solutions (pH 7.4). After incubation, the tubes were centrifuged at 5000xg for 10min to pellet the hemolysed cells and the absorbance of the supernatant was measured at 540 nm.

$$\% \text{ EOF} = \text{O.D.x} - \text{O.D.y} / \text{O.D.y} \times 100$$

Where O.D. x is optical density at a given NaCl concentration and O.D. y is O.D. at 0.1% NaCl concentration.

Statistical analysis of data

Statistical analysis was performed using Graph Pad Prism version 5.01 for Windows, Graph Pad Software, San Diego California USA. All values were expressed as mean ± standard deviation of 3 to 5 observations. Data were analyzed using one way analysis of variance (ANOVA) followed by Bonferroni's post-hoc test for multiple pair wise comparisons between the various treated groups. The value were significant at ***, ** and * $p < 0.001$, $p < 0.01$ and $p < 0.05$, respectively, when compared to control.

RESULTS

The results of *in vitro* incubation of the erythrocytes with varying concentrations of carbofuran (2.5, 5, 10, 25 and 50µM) for 1h at 37°C indicated increase in the erythrocyte MDA content as compared to control. The pesticide exerted its effect in the concentration dependent manner (Fig.1A). The rise in the level of erythrocytes' lipid peroxidation product malondialdehyde (MDA) was found to be maximum at 50µM with 204% increase, which was highly

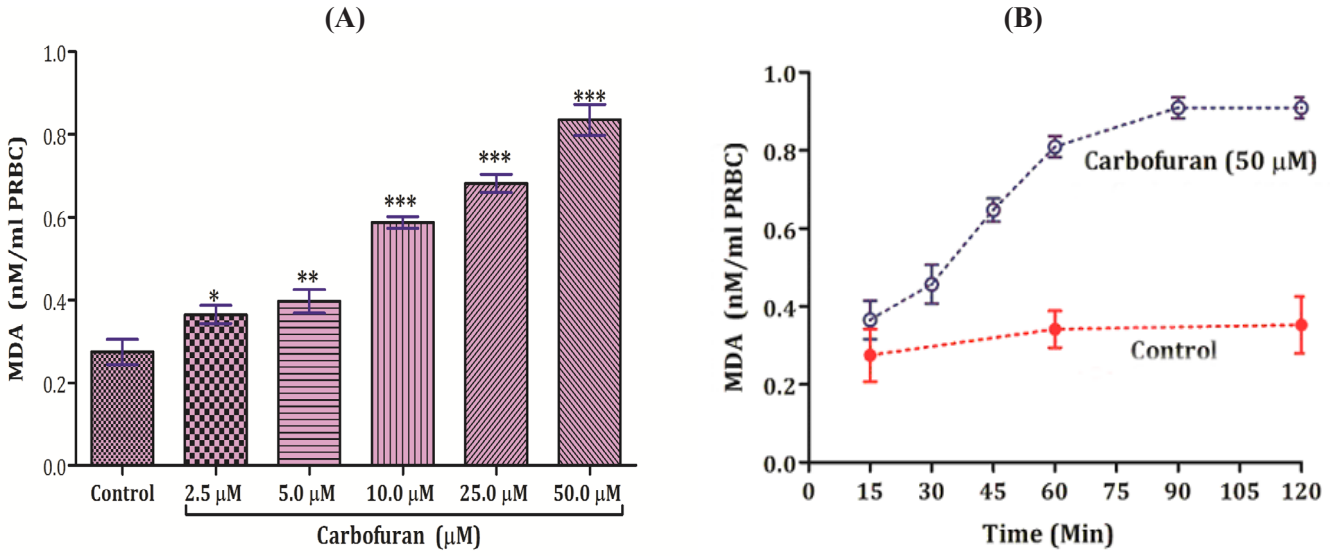

Figure 1. (A): Concentration dependent carbofuran induced lipid peroxidation in human erythrocytes. The human RBCs were treated for 1hr with different concentrations of carbofuran (2.5, 5, 10, 25 and 50µM) as mentioned in Materials and Methods. MDA content was expressed in nM/mL PRBC. PRBC=packed red blood cells. The values are mean ±SD of 5 independent experiments. The signs ***, ** and * represent the significant values at $p < 0.001$, $p < 0.01$ and, $p < 0.05$, respectively, in comparison to that of control. The control experiment was carried out by incubating the RBCs *in vitro* with equal volume of DMSO (0.1%) as mentioned in Materials and Methods. **(B):** Time dependent effect of 50µM carbofuran on membrane lipid peroxidation in erythrocytes treated for varying durations (15, 30, 45, 60, 90 and 120min). The lower panel (●) indicates the control experiment wherein the RBCs were incubated *in vitro* with equal volume of DMSO (0.1%) as mentioned in Materials and Methods. The data represent the mean values of three independent experiments. ○=carbofuran (50µM) treatment.

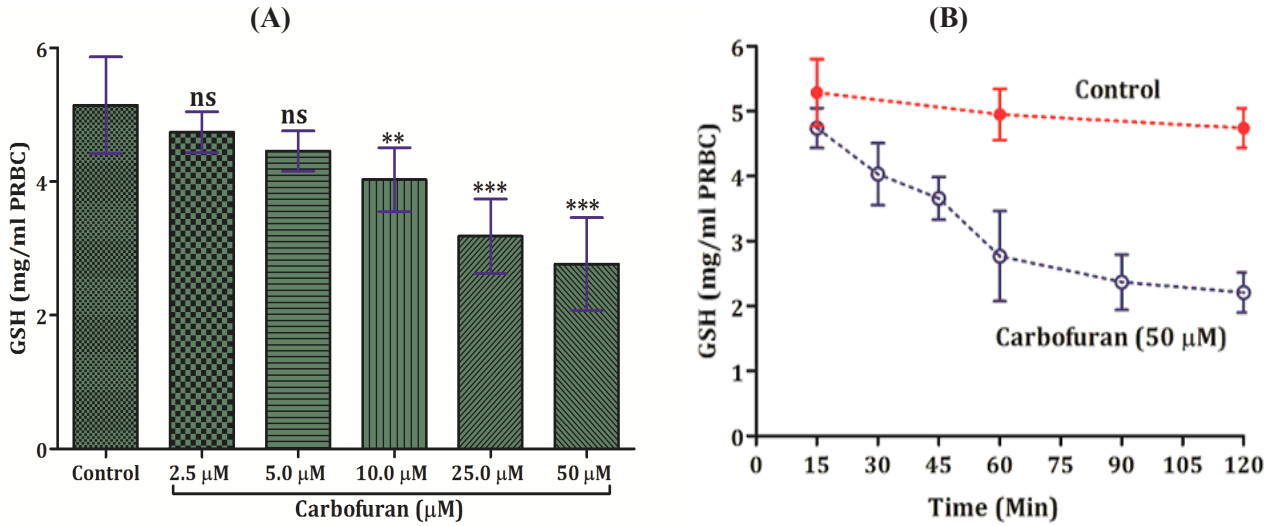

Figure 2. (A): Concentration dependent carbofuran induced changes in intracellular GSH content in human erythrocytes. The treatment of human RBCs with different concentrations of carbofuran (10, 25 and 50µM) was done as described in Materials and Methods. GSH content is expressed in mg/mL PRBC. The values were mean ± SD of three independent experiments. ***, ** and * denote the level of significance at $p<0.001$, $p<0.01$ and $p<0.05$, respectively, as compared to control. **(B): :** Effect of carbofuran (50µM) on the GSH content into the human erythrocytes treated with the pesticide for varying periods of treatment was assessed as described in Materials and Methods. Simultaneously, one control experiment was also carried out with RBCs treated with equal volume of DMSO as described in Materials and Methods. The values represent the mean ± SD of three independent experiments.

significant ($p< 0.001$). The pesticide at relatively lower concentrations such as 5, 10 and 25µM caused increase in levels of MDA by 44, 113 and 148%, respectively. Carbofuran at lowest concentration (2.5µM) tested, resulted into only slight increase ($p<0.05$) in the MDA level as compared to the control (Fig. 1A).

The time dependent effect of carbofuran on the level of MDA content into the erythrocytes was also tested by incubating the cells with the highest concentration of pesticide (50µM) for different treatment periods (15, 30, 45, 60 and 120min). Parallel to it, one control experiment was also run simultaneously and the results were compared at 15 min interval. The results on the effect of carbofuran up to 120 min of incubation as shown in Fig. 1B, displayed significant ($p<0.01$) increase in MDA content in RBCs. As compared to the control, maximum rise in the MDA content was observed after 1h of incubation. The increase in the incubation time beyond 1hr, however, resulted into only slight increase in MDA content in the human RBCs treated *in vitro* with carbofuran (50µM).

The results of *in vitro* incubation of the erythrocytes with varying concentrations of carbofuran (2.5, 5, 10, 25 and 50µM) for 1h at 37°C indicated the depletion in the erythrocytes' GSH content as compared to control. The effect of carbofuran was, however, observed in the concentration dependent manner. The extent of decrease in the

erythrocytes' GSH content was significant and the values being 21, 38 and 46% at 10, 20 and 50μM concentrations, respectively. The lower concentrations (2.5 and 5μM) of carbofuran tested *in vitro* for 1h did not exert any significant effect on the level of GSH in the human RBCs as compared to control (Fig. 2A).

The above experiment with human RBCs was performed using different concentrations of carbofuran for fixed treatment duration (1h). So it was endeavoured to evaluate the effect of maximum concentration of carbofuran tested (50μM) for varying periods of treatment such as 15, 30, 45, 60, 90 and 120min. Simultaneously, a control experiment was also carried out and the data were computed at 15min interval each. The results of the effect of carbofuran on the erythrocytes' GSH content have been presented in Fig. 2B. The maximum depletion in erythrocyte GSH content was observed after 1h of incubation. The increase in the treatment duration further up to 120 min resulted into only slight decrease in GSH content as compared to control.

The *in vitro* effect of carbofuran on the activity of Na^+/K^+-ATPase is not well established though pyrethroids are reported to cause perturbations in the level of this enzyme in different animal tissues. In order to assess the effect of carbofuran on the activity of Na^+/K^+-ATPase in the erythrocytes membrane, the enzyme preparation was incubated with different concentrations (2.5, 5.0, 10, 25 and 50μM) of carbofuran for 1h at 37°C and the enzyme activity was measured at the end of treatment duration. The data presented in Fig. 3A shows that carbofuran was able to exert inhibitory effect on the activity of Na^+/K^+-ATPase from erythrocyte membrane as compared to control. The effect of carbofuran was observed to be concentration dependent i.e. maximum (63.3%) decrease in the activity was observed at maximum pesticide concentration (50μM) tested. Carbofuran at lower concentrations (10 and 25μM) caused significant decrease in the enzyme activity up to

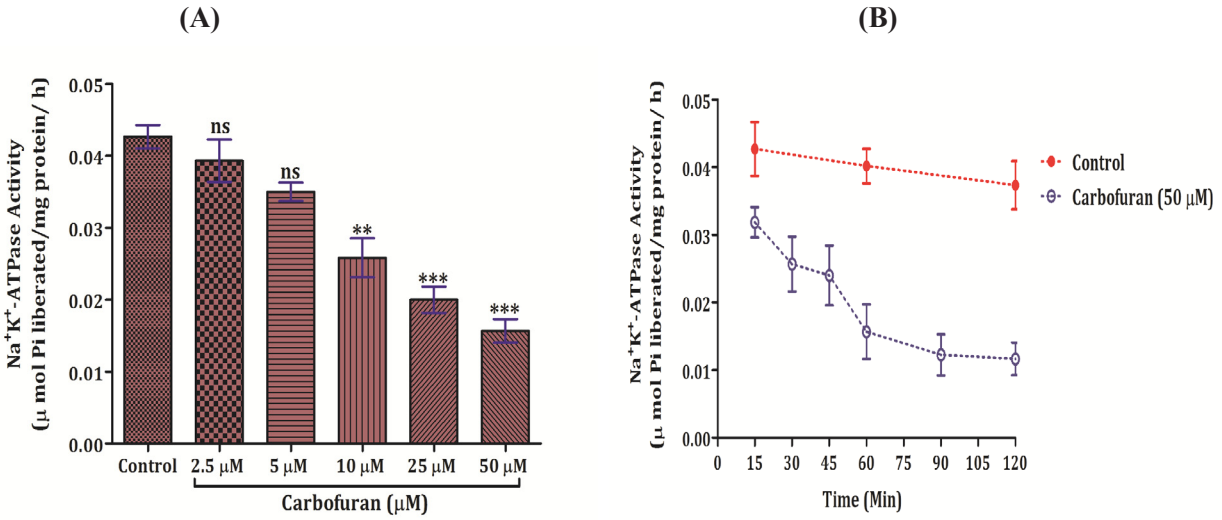

Figure 3. (A): *In vitro* effect of different concentrations of carbofuran (2.5, 5.0, 10 μM, 25 μM and 50 μM) on the activity of Na^+/K^+ ATPase isolated from human erythrocytes membrane. Na^+/K^+ ATPase activity is expressed in μmol Pi liberated/mg protein/h. Values are mean ± SD of 5 independent experiments. ***, ** and * denote the level of significance at $p<0.001$, $p<0.01$ and $p<0.05$, respectively. **(B):** Time dependent effect of 50μM carbofuran on the membrane Na^+/K^+-ATPase activity in the human erythrocytes membrane exposed to the pesticide for 15, 30, 45, 60, 90 and 120 min. The activity of enzyme has been assayed as described in Materials and Methods section. The data are presented as mean ± SD of three different experiments.

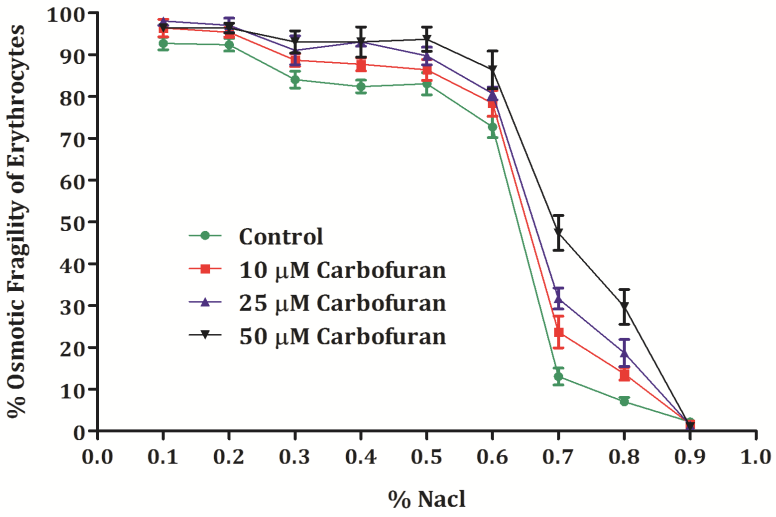

Figure 4. Effect of different concentrations (10, 25 and 50 μM) of carbofuran on the erythrocytes stability. The erythrocytes membrane fragility was tested in absence (control) and presence of carbofuran as described in Materials and Methods. The mean erythrocyte osmotic fragility (MEOF) has been extrapolated from the erythrocytes osmotic fragility curve for a range of NaCl concentrations varying from 0.1% to 0.9%. The percent hemolysis at different NaCl concentrations was calculated using following formula: % EOF = O.D.x - O.D.y / O.D.y × 100; EOF=Erythrocytes osmotic fragility, O.D.x= optical density at a given NaCl concentration; O.D.y = O.D. at 0.1% NaCl concentration.

39.45 and 53%, respectively. However, the lower concentrations of carbofuran such as 2.5 and 5μM caused no significant alteration in the enzyme activity as compared to that of control (Fig. 3A).

The exposure of living systems with any xenobiotics leads to its effect in dose and duration dependent manner. In present work, the attempts were made to assess the effect of treatment duration by exposing the erythrocytes membrane Na^+/K^+-ATPase with a fixed concentration of carbofuran (50μM) for different time periods such as 15, 30, 45, 60, 90 and 120min. The enzyme activity was measured as described In Materials and Methods section. The results presented in the Fig. 3B showed that carbofuran exerted its maximum inhibitory effect on enzyme activity after 1h. However, the increase in the treatment duration beyond 1h resulted in only mild decrease in the enzyme activity as compared to the control.

There are reports suggesting that the exposure of erythrocytes from different animals to the pesticides or any other xenobiotics results into the altered homeostasis of the membrane of RBCs. In order to observe the effect of carbofuran on the osmotic fragility of human erythrocytes, the present experiment was carried out by incubating the cells with different concentrations (10, 25 and 50μM) of the pesticide for 1h. To assess the effect of pesticide treatment on erythrocytes' membrane stability, the concentrations of NaCl corresponding to 50% hemolysis of human erythrocytes in presence of varying concentrations of carbofuran were calculated by extrapolating the results shown in Fig. 4. The mean NaCl concentration corresponding to 50% hemolysis of erythrocytes is expressed as mean erythrocyte osmotic fragility (MEOF).The values of MEOF of erythrocytes in the control set and after treatment with carbofuran (10, 25 and 50μM) were found to be 0.656, 0.675, 0.688 and 0.724 % of NaCl concentration, respectively. The data indicate that exposure to carbofuran may induce 2.8, 5.6 and 10.3% more haemolysis of RBCs as compared to controls at 10, 25 and 50μM carbofuran concentration, respectively.

DISCUSSION

Pesticides are known to cause free radical mediated toxicity in organisms via production of reactive oxygen species (ROS). Pesticide induced oxidative stress mediated toxicity is gradually gaining importance as many classes of pesticides including pyrethroids, organophosphates and organocarbamates have been reported to cause oxidative stress in experimental animals and occupational poisoning cases (2,8,21). Red blood cells can be regarded as circulating antioxidant carriers, reflecting exposure to ROS. Indeed, they have been used as a model for the investigation of free-radical induced oxidative stress because of several reasons; they are continually exposed to high oxygen tensions, they are unable to replace damaged components, their membrane lipids are composed partly of polyunsaturated fatty acid side chains which are vulnerable to peroxidation, and they have antioxidant enzyme systems (12). While ascertaining the carbofuran induced lipid peroxidation in the erythrocytes *in vitro*, the treatment of fresh human RBCs with increasing concentrations of carbofuran indicated significant rise in the MDA levels. These results verify the observations recorded from the blood of the pesticide sprayers. One such report in the erythrocytes of rat exposed to another pesticide, atrazine, suggests induced level of lipid peroxidation causing membrane deformity in the treated cells, which was due to significant decrease in the phospholipid and cholesterol contents of the erythrocytes a membrane (24). They observed the morphological changes in erythrocytes using scanning electron microscopy (SEM) technique which correlates the changes in the biochemical parameters including that of oxidative stress.

Glutathione, an efficient antioxidant present in almost all living cells, is also considered as a biomarker of redox imbalance at cellular level. Carbofuran induced toxicity mediated by oxidative free radicals tend to disturb cellular membrane integrity and enzyme activities in red blood cells (19). The decrease in GSH levels might diminish the overall antioxidant potential of the erythrocyte resulting in increased LPO following carbofuran treatment**.** The evaluation of the impact of carbofuran on the level of GSH in the healthy human erythrocyte by treating the cells *in vitro* with different concentrations of the pesticide exhibited significant reduction in the GSH content; the effect being manifested in the pesticide's concentration and duration dependent manner. In contrast to this finding, Santi et al. (22) have recently reported no significant change in the level of GSH in human erythrocytes when subjected to treatment with another chemical, clomazone, a herbicide. The mechanism involved in GSH depletion after carbamate exposure involves carbomylation of –SH groups as suggested by Ningaraj et al. (17).

Sodium Potassium ATPase (Na^+-K^+-ATPase, EC 3.6.3.9) is a membrane bound sulhydryl containing enzyme whose function is critical for the maintenance of cell viability. This enzyme carries out the transport of sodium and potassium ions against concentration gradient, resulting in the translocation of net charge. The enzyme acts as a current generator and contributes to the membrane potential of nerve cells. This enzyme is known to be an early target for ROS induced damage to intact cells (10,11). In the present study, the significant decrease in Na^+-K^+-ATPase activity in the human RBCs due to carbofuran exposure was found to be in concentration and duration dependent manner. Earlier report from our laboratory in the erythrocytes of rat exposed to carbofuran have suggested that these changes were mediated by carbofuran induced oxidative free radicals which might tend to disturb cellular membrane integrity and enzyme activities (20). Also, in the different organs of some aquatic organisms pesticides induced alterations in the activity of this enzyme have been reported (13,18) suggesting thereby the increased lipid peroxide formation could disturb the anatomical integrity of the biomembrane and diminish its fluidity leading to inhibition of several membrane bound enzymes including Na^+-K^+-ATPase.

The *in vitro* effect of carbofuran on the osmotic fragility of human erythrocytes in the present study indicated decrease in the stability of erythrocyte membrane and increase in the extent of hemolysis, the effect however was in dose dependent manner. There are reports suggesting that due to the exposure of erythrocytes from different animals to the pesticides or any other xenobiotics, the membrane of RBCs exhibits alterations in the biochemical constituents. Previously, it is reported by Rai *et al.* (19) that carbofurane induces free radical mediated membrane damage in the erythrocytes of rat.

Pesticides used in agriculture practices are being reported to cause adverse effects on to the environment as well

as human health. Recently it has been reported that the pesticides including organocarbamates may generate free radical species and cause oxidative stress leading to biochemical perturbations and lipid peroxidation of membranes. The results of the present study indicated that carbofuran was able to cause significant alterations in the levels of malondialdehyde (MDA) and GSH in human erythrocytes exposed to different sub-acute concentrations of carbofuran for different time intervals maximally up to 2h. The level of the MDA was significantly increased and that of GSH got significantly decreased when the human erythrocytes were incubated with carbofuran, the effects being exerted in concentration and time dependent manner. Carbofuran also caused significant inhibition in the activity of Na^+K^+-ATPase as well as sharp increase in the osmotic fragility of human erythrocytes at higher concentrations of carbofuran. Thus, carbofuran was able to induce oxidative stress and adversely influence certain biochemical constituents of the human erythrocytes.

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Other articles in this theme issue include references (28-55).

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