Disease Markers 32 (2012) 153–163
DOI 10.3233/DMA-2011-0870
IOS Press

In-vitro carbofuran induced genotoxicity in human lymphocytes and its mitigation by vitamins C and E

Ratnesh Kumar Sharma and Bechan Sharma*
Department of Biochemistry, Faculty of Science, University of Allahabad, Allahabad, Uttar Pradesh, India

Abstract. Various efforts have been made in past in order to predict the underlying mechanism of pesticide-induced toxicity using *in vitro* and animal models, however, these predictions may or may not be directly correlated with humans. The present study was designed to investigate the carbofuran induced genotoxicity and its amelioration by vitamins C and E by treating human peripheral blood lymphocytes (PBLs) with different concentrations (0, 0.5, 1.25, 2.5, 3.75 and 5.0 μM) of this compound. The treatment of PBLs with carbofuran displayed significant DNA damage in concentration dependent manner. The carbofuran induced genotoxicity could be ameliorated to considerable extent by pretreatment of PBLs with equimolar (10 μM) concentration of each of the vitamins C and E; the magnitude of protection by vitamin E being higher than by vitamin C. Also, it was found that the level of protection by these vitamins was higher when PBLs were treated with lower concentrations of pesticide. The significant DNA damage as observed by H_2O_2, a positive control in the present study, and its amelioration by natural antioxidants (vitamins C and E) lend an evidence to suggest that carbofuran would have caused genotoxicity via pesticide induced oxidative stress.

Keywords: Carbofuran, genotoxicity, comet assay, vitamin C, vitamin E, human lymphocytes

1. Introduction

Carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranol methyl carbamate), a broad-spectrum pesticide, is commonly used in agricultural practices (Fig. 1). 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, fruit, and vegetables. It is one of the most toxic carbamate pesticides and known to exert high toxicity to mammalian systems [40]. In the document on "Public Health Goals for Chemicals in Drinking water" prepared by the California Environmental Protection Agency (44), they have reported the detrimental effect of carbofuran on reproductive function in humans through drinking water. Based on their data Office of Environmental Health Hazard Assessment (OEHHA) has a PHG (public health goal) limit of 0.0017 mg/L (1.7ppb) for carbofuran in drinking water. The earlier values for maximum contaminant level (MCL) were 0.01 mg/L (18 ppb) and federal MCL was 0.040 mg/L (40 ppb).

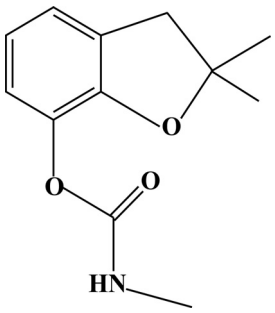

Fig. 1. Chemical structure of carbofuran.

The mechanism involved in the toxicity of carbofuran-induced neuronal damage is linked to free radical-mediated injury. The mechanism of action for carbofuran activity against insects is rapid via reversible inhibition of acetylcholinesterase (AChE), an enzyme

*Corresponding author. Tel.: +91 9415715639; E-mail: sharmabi@yahoo.com; bechansharma@gmail.com.

ISSN 0278-0240/12/$27.50

essential for normal nerve impulse transmission. Also in animals and humans, carbofuran toxicity is characterized by rapid inhibition of activity of neural AChE through carbamoylation of the esteratic site of the enzyme leading to cholinergic signs [38]. This results in accumulation of acetylcholine at nerve synapses and myoneural junctions and causes toxic effects. The carbomoylated enzyme undergoes spontaneous and rapid reactivation. AChE inhibition is a sensitive indicator relative to clinical effects of exposure to carbofuran in both animals and humans (8). Oral administration of carbofuran is reported to cause neuronal injury by excessive generation of reactive oxygen species (ROS) leading to lipid peroxidation (LPO) [1,6]. It has been reported that intra-peritoneal administration of carbofuran at its sub-acute concentrations may induce oxidative stress in Wistar rats [15] and also induced genotoxic effects in epithelial cells across cryptvillus axis in rat intestine [33]. It is also reported that carbofuran induced cytogenetic effects in root meristem cells of *Allium cepa* and *Allium sativum*, a spectroscopic approach for chromosome damage is shown [37]. It has been proven that even increase in intracellular levels of ROS are mediated via carbofuran's inhibition of AChE activity (even at lowest concentrations tested) [15,16].

Blood cells are prone to oxidative stress because they are exposed to high oxygen tension and have polyunsaturated fatty acids in their membrane and hemoglobin-bound iron [27]. However, cellular antioxidant machinery in the form of antioxidative enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione-S-transferase (GST) and antioxidant molecules such as vitamins C and E scavenge active oxygen species to maintain cellular system as native. Several organophosphate pesticides have been reported to cause oxidative damage in erythrocytes [21, 23,32]. ROS-catalyzed oxidative damage of membrane lipids causes the erythrocyte membrane deformability and oxidative hemolysis [9].

The factors, which determine the integrity of blood components, are complex which include oxidative stress [3]. Several insecticides have been reported to bind to human plasma protein fractions [11] and disturb biochemical and physiological functions within blood cells and affect membrane integrity [12]. Carbofuran is lipophilic in nature and its chronic exposure is reported to be responsible for oxidative injury leading to perturbations in membrane structure and functions [6]. Further, Singh et al. [32] have shown *in vitro* induction of oxidative stress in erythrocytes by organophosphate pesticides, which have mechanism of action similar to organocarbamates. However, not much attention has been paid to study the impact of carbofuran on the oxidative stress mediated DNA damage. Further, the protective role of known antioxidants such as vitamin C and E on the pesticide-induced genotoxicity also needs to be explored. Vitamins C and E have been reported to contain antioxidant activity as they can scavenge reactive oxygen species (ROS) and may prevent oxidative damage to important bio-molecules such as DNA, proteins and lipids. Despite acting as an antioxidant, vitamin C regulates the expression of some genes participating in DNA repair processes [14]. Recent studies indicate that vitamin C plays a protective role against free radical mediated carbofuran toxicity in rat [16]. Vitamin C (ascorbic acid) is a known water-soluble antioxidant, which scavenges reactive oxygen and nitrogen species [5]. Besides exerting antioxidant influence directly, vitamin C at higher concentration can promote the removal of oxidative DNA damage from the DNA or nucleotide pool, through the up regulation of repair enzymes, perhaps induced by the vitamin's pro-oxidative properties [2,30,24].

Vitamin E is considered the most effective liposolouble antioxidant found in the human biological system. It interacts with free radicals and prevents lipid peroxidation [17] Vitamin E is known to exist in different molecular forms. It exhibits maximum biological activity as α-tocopherol. It can protect DNA from free radicals attack either by scavenging lipid peroxyl radicals and terminating the lipid peroxidation chain reaction that creates DNA-damaging products or by inactivating reactive oxygen species [41]. Vitamin E has also been reported to have prooxidant properties, depending on the cellular environment [35]. Protective effects of vitamins C and E on the number of micronuclei formation in lymphocytes of smokers have been reported.

The metabolism of carbofuran has been well studied in rats, mice, and lactating cows. Carbofuran is rapidly absorbed, metabolized, and eliminated, primarily via the urine, in the species investigated. Hydroxylation, hydrolysis, and conjugation reactions are the major metabolic steps in carbofuran metabolism. The products of carbofuran metabolism are 3-hydroxycarbofuran and 3-ketocarbofuran. 3-hydroxy-N-hydroxymethylcarbofuran is also produced to some extent in all animals except mouse. The main degradation product of carbofuran occurs at the 3-position of the dihydrobenzofuran structure to give hydroxyl and the resulting ketone derivatives, in addition to N-methyl hydroxylation and hydrolysis of the ester

linkage [39]. All the metabolites undergo conjugation reactions. These degradation products are primarly excreted as conjugates of glucuronides and sulfates .In rat liver homogenates, the main metabolites were 3-hydroxycarbofuran and carbofuran phenol derivatives, indicating that the metabolism *in vivo* follows the same general pattern observed *in vitro*. (Adopted from www.inchem.org/documents/jmpr/jmpmono/v96pr03.htm) [45].

The regulatory agencies in EU and USA have banned the use of carbofuran and carbofuran-related carbamates due to their toxicity to humans. Keeping these facts in view the present study has been therefore undertaken to evaluate pesticide induced DNA damage by different subacute concentrations of carbofuran in human lymphocytes using the alkaline comet assay. Also, endeavours have been made to assess the ameliorative effects of vitamins C and E under these experimental conditions. The results on carbofuran induced genotoxicity could be exploited as a marker which may be useful for potential management of occupational health hazards.

2. Materials and methods

2.1. Chemicals

Carbofuran of 99.5% purity was, kindly gifted by Rallis India. Sodium Ascorbate(vitamin C) and α-tocopherol (vitamin E) were purchased from Qualigens. Other chemicals used in the comet assay were purchased from the following suppliers: Normal melting agarose (NMA) Himedia); sodium chloride (NaCl) and sodium hydroxide (NaOH) from Merck Chemicals (Darmstadt, Germany); dimethylsulfoxide (DMSO) (Qualigens), hydrogen peroxide (H_2O_2), Phosphate buffer saline (PBS), Triton X-100 (HiMedia) and Histopaque1077, ethidium bromide(EtBr), trypan blue and low melting agarose (LMA) from Sigma (St. Louis, MO, USA), ethylenediaminetetraacetic acid(EDTA) disodium salt (HiMedia), Trizma Base (Spectrochem), Coverslip (No.1, 24 $\times$ 60 mm) (Blue Star), Horizontal electrophoresis Unit (GIBCO BRL, Life Technologies).

2.2. Preparation of human peripheral blood lymphocytes (PBLs)

3 ml heparinized whole human blood was collected by venipuncture from a group of 28–32 year old non-smoking healthy male donors, not exposed to radiation, drugs or any antioxidant supplementation including Vitamins C and E. The protocol of study was in conformity with the guidelines of the Allahabad University Ethical Committee. The peripheral blood lymphocyte cells (PBLs) were separated from blood using Histopaque-1077 by the method of Boyum [4]. Briefly, blood was diluted 1:1 with RPMI 1640 medium and layered over Histopaque (3:1) and centrifuged at 800xg for 20 min. The buffy coat was aspirated into 5 ml RPMI 1640 medium and centrifuged at 250xg for 10 min to pellet the lymphocytes. This step is repeated once more. The final pellet was resuspended in about 1 ml of RPMI 1640 medium and counted over a haemocytometer. Nearly 2×10^4 cells per 100 μl of medium are taken for treatment with each concentration of carbofuran.

A stock solution of carbofuran was prepared by dissolving it into 10% DMSO. A stock solution of vitamin E was prepared by dissolving α-tocopherol into 1% DMSO. For supplementation of PBLs, vitamin E (10 μM) final concentration was prepared in RPMI-1640 (R4130; Sigma, St Louis, MO, USA). The final concentration (10 μM) of vitamin C was selected for incubation of PBLs in RPMI-1640. This concentration was chosen on the basis of significant protection offered incase of H_2O_2 induced Oxidative DNA damage. The use of similar concentration has also been reported by Janusz Blasiak et al. [26].

2.3. Viability of human peripheral blood lymphocytes(PBLs)

For the experimentation, the PBLs isolated as above were incubated into RPMI 1640 medium at 37°C. The viability of PBLs was determined by the trypan blue dye exclusion method.

2.4. Human peripheral blood Lymphocytes (PBLs) Treatment

Carbofuran was added to the suspension of the lymphocytes to give final concentrations from the range of 0.5–10 μM. One ml volume of each dose is made in RPMI medium (without FBS) and lymphocytes were added to it. The eppendorff is inverted to mix the cells and test compound. The control cells received only RPMI 1640 medium. The lymphocytes were incubated with carbofuran for 60 min at 37°C. The experiment included a positive control by treating the cells with hydrogen peroxide at 25 μM for 10 min at 37°C [25]. In order to evaluate ameliorative effect of known antioxi-

dants such as vitamins C and E, the cells were preincubated with equimolar concentrations (10 μM) of these vitamins for 30 min at 37°C before addition of different concentrations of carbofuran. Vitamin C was taken from a stock solution in RPMI 1640 and added to the cell suspension to give a final concentration of 10 μM. Vitamin E was derived from stock solutions in DMSO and added to the cells to give a final concentration of 10 μM. The final concentrations of DMSO in the experimental and control sets were 0.1%. The viability of the cells after carbofuran treatment was checked by trypan blue exclusion assay.

In separate set of experiments to evaluate the effect of vitamins alone on the lymphocytes, the cells were preincubated with 0–10 μM of vitamin concentrations for 30 min at 37°C. In order to evaluate the ameliorative effect of vitamins, the lymphocytes pretreated with different concentrations (0–10 μM) of each of these vitamins were subjected to H_2O_2 (10 μM) treatment for next 30 min at 37°C. In this case, the experimental set containing H_2O_2 (10 μM) with zero concentration of vitamins served as positive control. Simultaneously, the cells incubated in only RPMI medium and RPMI (without FBS) + DMSO (0.1%) served as negative controls.

2.5. Slide preparation

The basic alkaline technique of Singh et al. [34], as further specified by Anderson et al. [13] and Collins et al. [7], was followed for slide preparation. Each of the fully frosted microscope slide was covered with 110 μl 0.5% normal melting agarose (NMA) at about 45°C in Ca^{2+} and Mg^{2+} free PBS. It was immediately covered with a large no.1 cover slip and kept at room temperature for about 5 min to allow the agarose to solidify. This layer was used to promote the attachment of the second layer of 0.5% LMA. Approximately 10,000 cells were mixed with 75 μl of 1.0% LMA to give 0.5% final LMA concentration. After gently removing the cover slip, the cell suspension was rapidly pipetted over the first agarose layer, spread using a cover slip, and maintained on an ice-cold flat tray for 5 min to solidify. The cover slip was removed and a third layer of 0.5% LMA (75 μl) at 37°C was added, spread using a cover slip, and again allowed to solidify on ice for 5 min. After removal of the cover slips, the slides were immersed in cold lysis solution containing 2.5 M NaCl, 100 mM Na_2EDTA and 10 mM Tris-HCl, pH 10. The 1% Triton X-100 (v/v) and 10% DMSO (v/v) were added just before use for a minimum of 1 h at 4°C.

2.6. The alkaline single-cell gel electrophoresis (*SCGE*)

The slides were removed from the lysis solution, drained and placed in horizontal gel electrophoresis tank (GIBCO BRL, Life Technologies) side by side, avoiding spaces and with the agarose ends facing each other, nearest the anode. The buffer tank was filled with fresh and chilled electrophoresis solution (about 4°C) containing 1 mM Na_2EDTA and 300 mM NaOH, pH 13 to a level of about 0.25 cm above the slides. Before electrophoresis, the slides were left in the solution for 20 min to allow the unwinding of DNA and the expression of alkali labile damage. Electrophoresis was conducted at a low temperature (4°C) for 30 min using 24V (0.8V/cm) and adjusting the current to 300 mA by raising or lowering the pre-chilled buffer (about 4°C) level and using a power supply. All of these steps were carried out under dimmed light (the tank was covered with a black cloth) to prevent the occurrence of additional DNA damage due to stray light. After electrophoresis, the slides were taken out of the tank. Neutralizing buffer (0.4 M Tris-HCl, pH 7.5) was added drop wise gently to neutralize the excess alkali and the slides were allowed to stay for 5 min. This neutralization process was repeated three times.

2.7. Staining of gel slides

The slides were rinsed with the chilled distilled water and drained excess water on tissue paper followed by addition of 75 μl of EtBr (20 μg/ml) on slides. After 5 min, the slides were dipped into chilled distilled water. The slides were taken out after 5 min and a coverslip was placed on the each slide and kept in a humidified air-tight container to prevent drying of the gel. The slides were analyzed within 4 hours.

2.8. Slide scoring

Slide scoring was done for visualization of DNA damage. The observations were recorded with EtBr stained DNA on slides using a 40x objective lens in a fluorescent microscope (Leica, DM LB Germany). Measurements were made by a computer-based image analysis system Komet 5 developed by kinetic imaging Ltd. (Liverpool,UK) attached to a fluorescence microscope equipped with filters N2.1, excitation wavelength of 515–560 nm and emission wavelength of 590 nm. Images of 100 randomly selected lymphocytes (50 cells from each of two replicate slides), were analyzed from

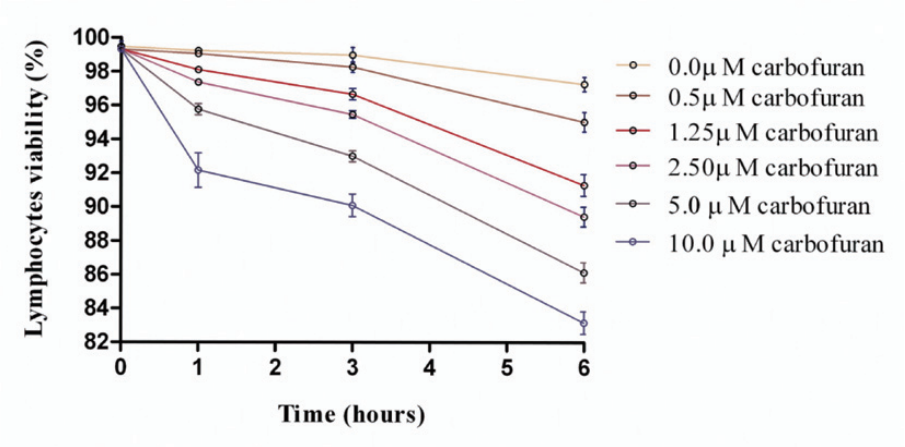

Fig. 2. Time-dose response of various concentration of carbofuran on the viability of human blood lymphocytes as determined by the trypan blue dye exclusion method.

each sample. Out of three comet parameters such as tail length, tail intensity (% DNA in the comet tail) and tail moment as shown by the software (Komet 5), we have used percent tail length to calculate extent of DNA damage.

2.9. Statistical analysis

The statistical analysis of the data has been done using software PRISM 5.1. All values were expressed as mean $\pm$ SD .The results of each experiment were compared using One-way ANOVA followed by Dunnett's test for multiple pair wise comparisons between the various carbofuran concentration treated groups with control. The data obtained for the effect of different concentrations of vitamins C and E (0, 1, 3, 5 and 10 μM) on the basal levels of oxidative DNA damage in the control as well as H_2O_2 treated lymphocytes have been analysed by One-way ANOVA followed by Tukey's Test for multiple pair wise comparisons between the various treated groups . The signs *, ** and *** represent the values significant at $p < 0.05$, $p < 0.01$and $p < 0.001$, respectively.

3. Results

3.1. Viability of human peripheral blood lymphocytes (PBLs)

The effect of different concentrations of carbofuran (0, 0.5, 1.25, 2.5, 3.75, 5.0 and 10 μM) was evaluated on the cell viability of human lymphocytes exposed for 6 h at 37°C. The dependence of survival of human lymphocytes upon treatment with different concentrations of carbofuran up to 6 h, as evaluated by trypan blue exclusion assay, has been demonstrated in Fig. 2. The control cells at these experimental conditions exhibited about 98% cell viability. The results indicated that carbofuran treatment evoked significant decrease in the cell viability. The negative effect of the pesticide on cell viability was recorded in the dose dependent manner. At the highest concentration of carbofuran (10 μM) tested, the viability of human lymphocytes remained up to 83.5% after 6 h of treatment. The results indicated that the gradual decrease in cell viability due to carbofuran treatment was dependent on the duration of exposure. At 1h treatment duration, the range of decrease in cell viability was recorded to be 99–92% which further decreased to 98–90% after 3 h exposure to different concentrations of carbofuran (Fig. 2).

3.2. DNA damage

3.2.1. Effect of vitamin C on the basal levels of oxidative DNA damage in control and H_2O_2 treated lymphocytes

In order to evaluate the effect of vitamin C on the basal level of oxidative DNA damage in the control and H_2O_2 treated lymphocytes, three sets of different experiments were carried out as following: (1) the cells were separately treated by a fixed concentration of H_2O_2 (10 μM) and (2) different concentrations of vitamin C (0, 1, 3, 5 and 10 μM) as well as (3) cells pretreated with these concentrations of vitamins and a fixed concentration of H_2O_2 (10 μM) as described in

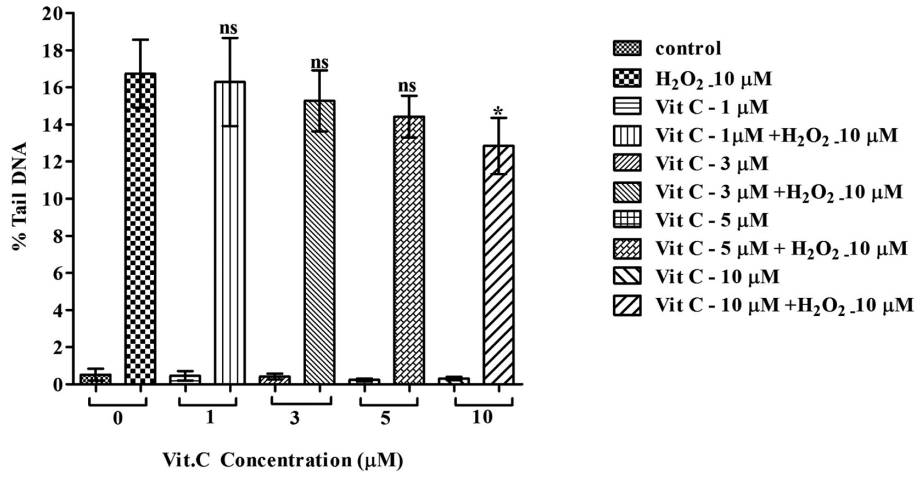

Fig. 3. Effect of different concentrations of vitamin C (0, 1, 3, 5 and 10 μM) on the basal levels of oxidative DNA damage in the control as well as H_2O_2 treated lymphocytes. The results at the each concentration of vitamin treatment have been compared using One-way ANOVA followed by Tukey's Multiple Comparison Test which was applied to compare the difference between multiple paired groups. All values were expressed as mean $\pm$ SD. *, ** and *** represent the values significant at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

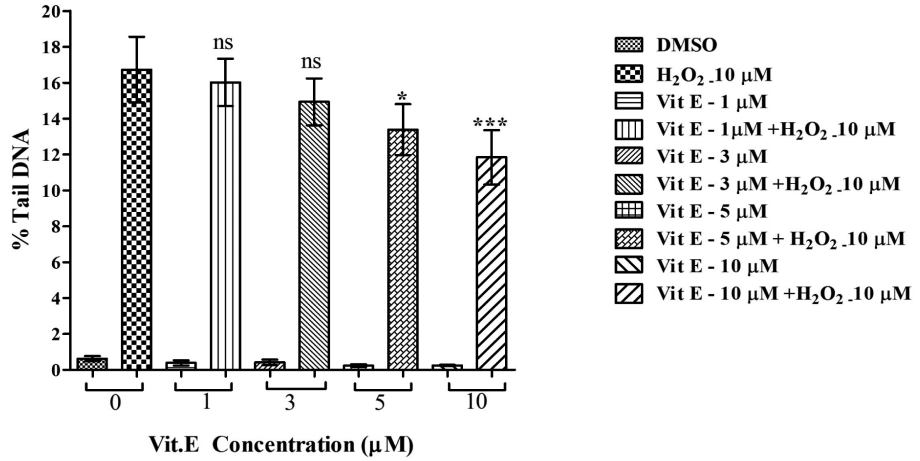

Fig. 4. Effect of different concentrations of vitamin E (0, 1, 3, 5 and 10 μM) on the basal levels of oxidative DNA damage in the control as well as H_2O_2 treated lymphocytes. The results at the each concentration of vitamin treatment have been compared using One-way ANOVA followed by Tukey's Multiple Comparison Test which was applied to compare the difference between multiple paired groups. All values were expressed as mean $\pm$ SD. *, ** and *** represent the values significant at $p < 0.05$, $p < 0.01$and $p < 0.001$, respectively.

Materials and Methods. One set of experiment in which the cells were incubated only in the RPMI medium (without FBS) served as negative control. The results shown in Fig. 3 demonstrated that H_2O_2 was able to cause significant oxidative DNA damage as compared to control. The cells treated with different concentrations of vitamins indicated DNA damage to a very small (about 0.2–0.7%) extent. However, the cells pretreated with different concentrations of vitamin C reflected protection from the oxidative DNA damage induced by H_2O_2 (10 μM). The extent of protection was observed to be in dose dependent manner; the maximum value obtained at 10 μM concentration of vitamin C (Fig. 3).

3.2.2. Effect of vitamin E on the basal levels of oxidative DNA damage in control and H_2O_2 treated lymphocytes

Further, In order to assess the effect of vitamin E on the basal level of oxidative DNA damage in the control and H_2O_2 treated lymphocytes, similar experiments were conducted using same concentrations of vitamin E and H_2O_2 under identical experimental conditions. The only difference was that in place of RPMI medi-

Table 1
Protective effect of vitamins on the oxidative DNA damage induced by H_2O_2 (10 μM) in human lymphocytes

Vitamins	Protection (%)				
Concentration (μM)	0	1	3	5	10
C	0	2.68	8.72	13.80	23.25
E	0	4.30	10.75	20.02	29.22

The lymphocytes were preincubated with 0–10 μM of vitamins concentrations for 30 min, then they were subjected to expose to 10 μM H_2O_2 treatment for next 30 min at 37° C. Then DNA damage analysis was done using COMET assay.

um, RPMI containing DMSO (0.1%, v/v) medium was used as a negative control. The results shown in Figure 4 demonstrated that H_2O_2 caused significant oxidative DNA damage as compared to control. The cells treated with different concentrations of vitamins indicated very low basal level of DNA damage (about 0.1–0.7%) extent. However, the cells pretreated with different concentrations of vitamin E displayed protection from H_2O_2 induced oxidative DNA damage. The extent of protection was found to be in dose dependent manner; the maximum value obtained at 10 μM concentration of vitamin E (Fig. 4). The results of protection from H_2O_2 mediated oxidative DNA damage in lymphocytes by vitamins C and E has been summarized in Table 1.

3.2.3. *Carbofuran induced DNA damage in lymphocytes*

As a prerequisite, 85–95% cell viability was used for studying DNA damage in human PBLs due to carbofuran exposure. The results obtained after exposing the human lymphocytes with different concentrations of carbofuran from the experiments shown in Section 3.1 (Fig. 2) suggested that the pesticide treatment was able to influence the cell viability significantly. This could be due to oxidative effect of the pesticide at nuclear level. To test this hypothesis, the experiments were carried out to monitor the tail length of the nucleus of the treated PBLs. Percentage of DNA in the tail of comets is the function of genotoxicity which is originating from the lymphocytes exposed to carbofuran. In present investigation, cells treated with H_2O_2 (25 μM for 10 min) has been used as a positive control. This reagent is known to induce production of free radical species and cause oxidative stress.

The results obtained from measurements of comet assay parameters indicated that control samples displayed low level (0.5–0.6 %) of primary DNA damage (Fig. 5) in lymphocytes. It was observed that after 60 min of incubation of lymphocytes with varying concentrations of carbofuran (1.25, 2.5, 3.75 and 5.0 μM) at 37°C, there was significant increase in DNA damage

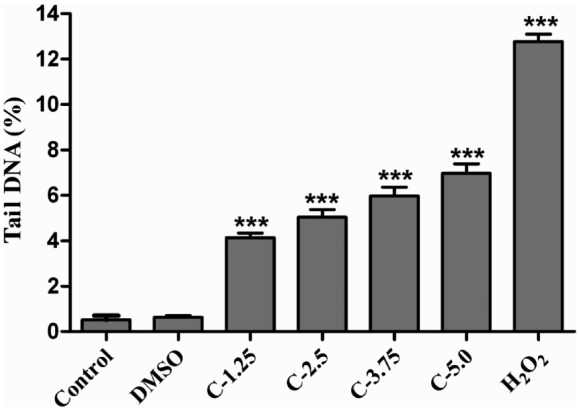

Fig. 5. Genotoxicity caused by control, vehicle DMSO control, carbofuran at 1.25 μM, 2.50 μM. 3.75 μM, 5.0 μM and H_2O_2 in human blood lymphocytes were compared using One-way ANOVA followed by Dunnett's Multiple Comparison Test which was applied to compare the difference between control and treated groups. All values were expressed as mean $\pm$ SD. *, ** and *** represent the values significant at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

($p < 0.001$), the values of DNA damage in terms of percent tail DNA as recorded were found to be 4.13, 5.03, 5.96 and 6.96, respectively. The extent of DNA damage was more at higher concentrations, thereby suggesting its dependence on the pesticide concentration (Fig. 5).

3.2.4. *Effects of vitamins C and E on carbofuran induced DNA damage in lymphocytes*

It was evident from the previous experiments that carbofuran was able to cause significant reduction in cell viability (Fig. 2) and DNA damage in lymphocytes in dose dependent manner (Fig. 5). Some workers have reported that DNA damage by xenobiotics can be minimized or overcome by application of certain antioxidants such as vitamins C and E. To test this notion, the human lymphocyte cells were preincubated with equimolar concentrations of these vitamins (10 μM each) for 30 min at 37°C and then they were exposed to one fixed concentration of carbofuran (1.25 μM) and further incubated for 60 min at same temperature. The results presented in Fig. 4 indicated that the vitamins C and E could protect the DNA from damage by 40.4 and 58%, respectively. The ameliorative effect of Vitamin E was found to be about 18% more than that of vitamin C in this context (Fig. 6).

Similar experiments were carried out by exposing the lymphocytes with increasing concentrations of carbofuran under identical experimental conditions. Figure 6 displays the protective effect of vitamins C and E on the status of DNA in lymphocytes treated with 2.5 μM carbofuran. The results demonstrated that vitamins C and

Table 2
The *in vitro* protective effect of vitamins C and E on carbofuran treated human blood lymphocytes from DNA damage

Carbofuran (μM)	Percent protection from DNA damage by pretreatment of Lymphocytes with vitamins	
	Vitamin C (10 μM)	Vitamin E (10 μM)
1.25	40.43	58.14
2.50	36.38	56.26
3.75	34.56	50.33
5.00	24.85	45.97

The percentage protection of human blood lymphocytes DNA with vitamins C and E was evaluated by preincubating the cells with vitamins C (10 μM) and E (10 μM) and challenged with varying concentrations of carbofuran as mentioned in Materials and Methods. The oxidative DNA damage was measured according to the procedure described in Materials and Methods.

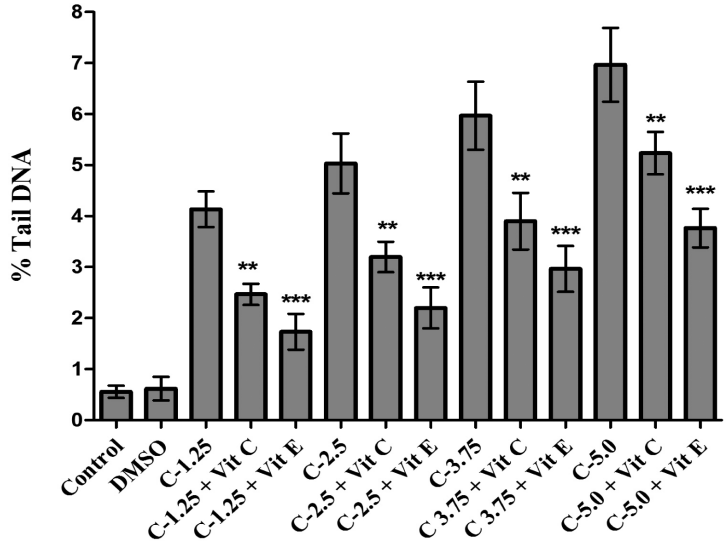

Fig. 6. Genotoxicity caused by control, vehicle DMSO control, varying concentrations of carbofuran (1.25, 2.50, 3.75 and 5.0 μM) in pre incubated human blood lymphocytes with Vitamins C and E were compared using One-way ANOVA followed by Dunnett's Multiple Comparison Test which was applied to compare the difference between control and treated groups . All values were expressed as mean $\pm$ SD. *, ** and *** represent the values significant at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

E were able to protect the DNA damage in human lymphocytes by 36.4 and 56.3% respectively, when Lymphocytes were pretreated with these vitamins and challenged with next higher concentration of carbofuran (3.75 μM), the levels of protection recorded were 34.5 and 50.3%, respectively (Fig. 6). In order to evaluate the extent of protection offered by these vitamins when Lymphocytes were further challenged with the highest concentration of carbofuran tested (5.0 μM), similar experiment was performed. The results shown in Fig. 6 indicated that vitamins C and E could protect the cells from DNA damage by 24.8 and 45.9%, respectively.

The data presented in Fig. 6 reflected an interesting trend. Vitamin E proved to be stronger in imparting protection compared to that of vitamin C at equimolar concentration. The data further suggested that the level of protection provided by these vitamins decreased when the cells were challenged with higher concentrations of carbofuran ($>$ 1.25 μM); the maximum protection (40.43–58%) being obtained at lowest concentration of the pesticide (1.25 μM) which decreased to the tune of 24.8–45.9% at its highest concentration (5.0 μM). These results have been summarized in Table 2.

4. Discussion

Carbofuran is a pesticide often used in agriculture practices in the country with an unintended human exposure. There are some recently published reports

showing carbofuran's genotoxicity [20,43]. A case report shows genotoxic effect of acute carbofuran intoxication in human as evidenced by Comet assay and micronucleus assay in the human blood samples showing significant DNA damage [20]. The mechanism of genotoxicity of this compound is not fully known and the results of some studies remain often inconclusive. Carbofuran treatment has also been reported to induce mitotic inhibition, chromosomal aberrations, micronucleus formation, and sperm abnormality in a dose dependent manner in rats [28,33]. The results of the present investigation showing changes in mean % DNA in tail in human Lymphocytes due to carbofuran exposure corroborates earlier reports.

In normal condition, DNA is localized in condensed manner in the nucleus of the cells. The amount of DNA that migrates in the tail thus making a comet due to treatment with any xenobiotics may be correlated with the DNA damage [7,13,19,20]. The *in vitro* treatment of human lymphocytes with varying concentrations (1.25–5.0 μM) of carbofuran in present investigation displayed DNA damage in the cells forming a comet tail, thereby showing genotoxicity induced by the pesticide. The measurement of mean % tail DNA as a direct estimate of DNA damage followed the similar pattern under these conditions. These results suggest that carbofuran induced alkali-labile sites and/or strand breaks. This could be due to the propensity of carbofuran to free radical-mediated DNA damage as has already been reported by some workers [1].

The results of the present work that a significant DNA damage as observed by different concentrations of pesticide in terms of single strand breaks in DNA as assessed by comet assay caused an increase in tail length in human lymphocytes indicate that carbofuran might have induced oxidative damage at all concentrations of pesticides tested. These findings are in close agreement with the reports from other workers [33]. Kamboj et al. [6] and Rai et al. [16] have reported increased oxidative stress following carbofuran exposure in different regions of rat brain. Milatovic et al. [18] have also shown that carbofuran treatment produces oxidative stress due to marked increase in reactive oxygen species. Damage to cellular DNA by lipid peroxidation plays a major role in cell injury and altered cell functions leading to apoptosis [20]. Thus the role of oxidative stress in DNA damage observed by alkaline Comet assay or single cell gel electrophorasis (SCGE) herein cannot be ruled out.

Another possible mechanism responsible for the observed genotoxic effects of carbofuran may involvement the metabolites of carbofuran, which have been commonly detected in plasma [42]. 3-hydroxycarbofuran and 3-ketocarbofuran have been reported to be potentially genotoxic [36] which may greatly increase the cytotoxic and mutagenic activities of N-methyl carbamate pesticides during carbofuran metabolism in human lymphocytes. However, while studying the carbofuran induced genotoxic effects in epithelial cells across cryptvillus axis in rat intestine have suggested another possible mechanism responsible for the observed genotoxic effects of carbofuran. It may involve conversion of this compound to nitrosoamides in the stomach, because of the presence of nitrite in human gastric juice. N-nitrosation greatly increases the cytotoxic and mutagenic activities of N-methyl carbamate pesticides [33].

The water soluble vitamin C and fat soluble vitamin E are known for their antioxidation potentials in addition to their several other biochemical functions. The novel repair action of vitamin C upon *in vivo* oxidative DNA damage has been documented [30]. The protective effects of vitamins C and E on the number of micronuclei in lymphocytes in smokers and their role in ascorbate free radical formation in plasma have been demonstrated [31]. The protective effect of vitamin C on the levels of 8-hydroxy-2'-deoxyguanosine in peripheral blood lymphocytes of chronic hemodialysis patients have been reported [14]. In another such study the effect of vitamin E therapy on oxidative stress and erythrocyte osmotic fragility in patients on peritoneal dialysis and hemodialysis has been demonstrated [3]. Vitamins C and E are known to react with free radicals of oxygen including others such as hydroxyl, superoxide, pentadienyl and the oxygen-derived peroxyl free radicals [24]. Very recently carbofuran induced oxidative stress in rat brain and its amelioration by vitamin C as well as some medicinal plant extracts have been reported [15,16]. It has also been shown that carbofurane may induce chromatin instability in mammalian cells [29]. The results of the present study indicating the protection of DNA from damage by vitamins C and E (10 μM each) when pre-incubated with human lymphocytes followed by challenge with different concentrations of carbofuran are in consonance with earlier reports [30,31]. Further, our observations that the vitamin E offered more protection to Lymphocytes from the carbofuran induced oxidative stress mediated DNA damage than that of vitamin C; could be due to lipophilic nature of vitamin E which enables it to reach into the lymphocytes membrane more readily than vitamin C thereby quenching the free radical species.

In conclusion, the results of the present study suggest that carbofuran may induce production of free radicals which may generate oxidative stress thereby causing DNA damage in human PBMCs. Though we have not studied but it is quite possible that this compound could also be inhibiting the DNA repair processes. The significant DNA damage as observed by H_2O_2, a positive control in the present study, and its amelioration by natural antioxidants (vitamins C and E) lends an evidence to suggest that carbofuran would have caused genotoxicity via pesticide induced oxidative stress. The supplementation of these vitamins may help minimize the genotoxic potential of carbofuran and also the risk of pesticide mediated occupational health hazards to farmers using such pesticides.

Acknowledgement

RKS is grateful to the University Grants Commission (UGC)-New Delhi for providing research scholarship for this work at the Department of Biochemistry, University of Allahabad-Allahabad, India. RKS is also thankful to Dr. K.C. Gupta, Director, and Dr. Alok Dhawan, Senior Scientist, IITR-Lucknow, India for providing facilities for COMET assay.

References

[1] A. Agrawal and B. Sharma, Pesticide induced oxidative stress in mammalian systems, *Int J Biol Med Res* **1**(3) (2010), 90–104.

[2] A. Carr and B. Frei, Does vitamin C act as a pro-oxidant under physiological conditions? *FASEB J* **13**(1999), 1007–1024.

[3] A. Uzum, O. Topark, M.K. Gumustak, S. Ciftci and S. Sen, Effect of vitamin E therapy on oxidative stress and erythrocyte osmotic fragility in patients on peritoneal dialysis and hemodialysis, *J Nephrol* **19** (2006), 739–745.

[4] A. Boyum, Isolation of lymphocytes, granulocytes, and macrophages, *Scand J Immunol* **5** (1976), 9–15.

[5] A.C. Carr and B. Frei, Human neutrophils oxidize low-density lipoprotein by a hypochlorous acid-dependent mechanism: the role of vitamin C, *Biol Chem* **383** (2002), 627–636.

[6] A. Kamboj, R. Kiran and R. Sandhir, N-acetylcysteine ameliorates carbofuran induced alterations in lipid composition and activity of membrane bound enzymes, *Mol Cell Biochem* **286** (2006), 107–114.

[7] A.R. Collins, M. Dusinska, M. Franklin et al., Comet assay in human biomonitoring studies: reliability, validation and applications, *Environ Mol Mutagen* **30** (1997), 139–146.

[8] B.W. Wilson, J.R. Sanborn, M.A. O'Malley, J.D. Henderson and J.R. Billitti, Monitoring the pesticide exposed worker, *Occup Med State of the Art Rev* **12** (1997), 347–363.

[9] B. Tavazzi, D. Pierro and A.M. Amorini, Energy metabolism and lipid peroxidation of human erythrocytes as a function of increased oxidative stress, *Eur J Biochem* **267** (2000), 684–689.

[10] C.M. Menzie, Metabolism of Pesticides, U.S. Department of the Interior,Bureau of Sport Fisheries and Wildlife, *Publication* **127** (1969), Washington, DC: U.S. Government Printing, p. 216.

[11] C. Datta, J. Gupta, A. Sarkar and D. Sengupta, Effect of organophosphorus insecticide phosphomidon on antioxidant defence components of human erythrocyte and plasma, *Indian J Exp Biol* **30** (1992), 65–67.

[12] D. Agarwal, P. Sultana and G.S.D. Gupta, Oxidative stress and glutathione redox system in erythrocytes from rats treated with hexa chloro hexane, *Food Chem Toxicol* **29** (1991), 459–462.

[13] D. Anderson, T.W. Yu, B.J. Philips and P. Schmezer, The effect of various antioxidants and other modifying agents on oxygen radical generated DNA damage in human lymphocytes in the comet assay, *Mutat Res* **307** (1994), 261–271.

[14] D.C. Tarng, T.Y. Liu, and T.P. Huang, Protective effect of vitamin C on 8-hydroxy-2'-deoxyguanosine level in peripheral blood lymphocytes of chronic hemodialysis patients, *Kidney Intern* **66** (2004), 820–831.

[15] D.K. Rai, R.K. Sharma, P.K. Rai, G. Watal and B. Sharma, Role of aqueous extract of *Cynodon dactylon* in prevention of carbofuran induced Oxidative stress and acetyl cholinesterase inhibition in rat brain, *Cell Mol Biol* **57**(1) (2011), 135–142.

[16] D.K. Rai, P.K. Rai, S.I. Rizvi, G. Watal and B. Sharma, Carbofuran-induced toxicity in rats:Protective role of vitamin C, *Experi and Toxicol Pathol* **61** (2009), 531–535.

[17] D.M. Aabdallah and N.I. Eid, Possible neuroprotective effects of lecithin and alpha tocopherol alone or in combination against ischemia/reperfusion insult in rat brain, *J Biochem Mol Toxicol* **18** (2004), 273–278.

[18] D. Milatovic, R.C. Gupta and M. Aschner, Anticholinesterase toxicity and oxidative stress, *Sci World J* **28** (2006), 295–310.

[19] D.W. Fairbairn, P.L. Olive and K.L. O'Neill, The COMET assay: a comprehensive review, *Mutat Res* **339** (1995),37–59.

[20] D. Zeljezic, A.L. Vrdoljak, N. Kopjar, B. Radic and S.M. Kraus, Cholinesterase inhibiting and genotoxic effect of Acute Carbofuran intoxication in Man: A case report, *Basic and Clin Pharmacol and Toxicol* **103** (2008), 329-335.

[21] F. Gultekin, N. Delibas, S. Yasar and I. Kilinc, *In vivo* changes in antioxidant systems and protective role of melatonin and a combination of vitamin C and vitamin E on oxidative damage in erythrocytes induced by chlorpyrifosethyl in rats, *Arch Toxicol* **75** (2001), 88–96.

[22] G. Speit and A. Hartmann, The comet assay (single-cell gel test). A sensitive genotoxicity for the detection of DNA damage and repair, *Methods Mol Biol* **113** (1999), 203–212.

[23] I. Altuntas, N. Delibas and R. Sutcu, The effects of organophosphate insecticide methidathion on lipid peroxidation and antioxidant enzymes in rat erythrocytes: role of vitamins E and C, *Hum Exp Toxicol* **21** (2002), 681–685.

[24] I.D. Podmore, H.R. Griffiths, K.E. Herbert et al., Vitamin C exhibits pro-oxidant properties, *Nature* **392** (1998), 559.

[25] J. Blasiak, P. Jałoszynski, A. Trzeciak and K. Szyfter, *In vitro* studies on the genotoxicity of the organophosphorusinsecticide malathion and its two analogues, *Mutation Research* **445** (1999), 275–283.

[26] J. Blasiak, E. Gloc, W. Katarzyna and C. Agnieszka, Genotoxicity of acrylamide in human lymphocytes, *Chem Biol Inter* **149** (2004), 137–149.

[27] J. Eritsland, Safety considerations of polyunsaturated fatty acids, *Am J Clin Nutr* **71** (2000), 197–201.

[28] L.K. Chauhan, N. Pant, S.K. Gupta and S.P. Srivastava, Induction of chromosome aberrations, micronucleus formation and

sperm abnormalities in mouse following carbofuran exposure, *Mutat Res* **465** (2000), 123–129.

[29] M. Mladinic, P. Perkovic and D. Zeljezic, Characterization of chromatin instabilities induced by glyphosate, terbuthylazine and carbofuran using FISH assay, *Toxicol Lett* **189** (2009), 130–137.

[30] M.S. Cooke, M.D. Evans, I.D. Podmore et al., Novel repair action of vitamin C upon in vivo oxidative DNA damage, *FEBS Lett* **363** (1998), 363–367.

[31] M. Schneider, K. Diemer, K. Engelhart et al., Protective effects of vitamins C and E on the number of micronuclei in lymphocytes in smokers and their role in ascorbate free radical formation in plasma, *Free Radical Res* **34** (2001), 209–219.

[32] M. Singh, R. Kiran and A. Kamboj, Erythrocyte antioxidant enzymes in toxicological evaluation of commonly used organophosphate pesticides, *Indian J Exp Biol* **44** (2006), 580–603.

[33] N. Gera, R. Kiran and A. Mehmood, Carbofuran adminstration induced genotoxic effects in epithelial cells across cryptvillus axis in rat intestine, *Pest Biochem. and Physiol* (2011), in press.

[34] N.P. Singh, M.T. McCoy, R.R. Tice and E.L. Schneider, A simple technique for quantitation of low levels of DNA damage in individual cells, *Exp Cell Res* **175** (1988), 184–191.

[35] N. Yamashita, M. Murata, S. Inoue, M.J. Burkitt, L. Milne and S. Kawanishi, Alpha tocopherol induces oxidative damage to DNA in the presence of copper(II) ions, *Chem Res Toxicol* **11** (1988), 855–862.

[36] P. Zhou, B. Liu and Y. Lu, DNA damaging effects of carbofuran and its main metabolites on mice by micronucleus test and single cell gel electrophoresis, *Sci China C Life Sci* **48**(Suppl 1) (2005), 40–47.

[37] P.N. Saxena, S.K. Gupta and R.C. Murthy, Carbofuran induced cytogenetic effects in root meristem cells of *Allium cepa* and *Allium sativum*: A spectroscopic approach for chromosome damage, *Pest Biochem and Physiol* **96** (2010), 93–100.

[38] R.E. Gosselin, R.P. Smith and H.C. Hodge, *Clinical Toxicology of Commercial Products*, (5th ed.), Baltimore: Williams and Wilkins, p. II–305. (1984). Philadelphia, PA, USA.

[39] R.J. Kuhr and H.W. Dorough, Carbamate Insecticides: Chemistry, Biochemistryand Toxicology, (1976). Cleveland, OH: CRC Press, Submitted to WHO by FMC Corp.

[40] R.C. Gupta, Carbofuran toxicity, *J Toxicol Environ Health* **43** (1994), 383–418.

[41] R.H. Bisby, S.A. Johnson and A.W. Parker, Quenching of reactive oxidative species by probucol and comparison with other antioxidants, *Free Radical Biol Med* **20** (1996), 411–420.

[42] S.S.E. Petropoulou, A. Tsarbopoulos and P.A. Siskos, Determination of carbofuran, carbaryl and their main metabolites in plasma samples of agricultural populations using gas chromatography tandem mass spectrometry, *Anal Bioanal Chem* **385** (2006), 1444–1456.

[43] S. Soloneski, M.A. Reigosa, G. Molinari, N.V. González and M.L. Larramendy, Genotoxic and cytotoxic effects of carbofuran and furadan on Chinese hamster ovary (CHO_{K1}) cells, *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* **656** (2008), 68–73.

[44] W.H. Hickox, Public health goals for chemicals in drinking water, (2000), in: Carbofuran. California Environmental Protection Agency.

[45] www.inchem.org/documents/jmpr/jmpmono/v96pr03.htm.