Protective role of black tea and vitamin C during sub-acute toxicity of carbofuran in rats

Kumar D. [A-F], Sharma B. [A-F] , Rizvi SI [A-F].[*]

Department of Biochemistry, University of Allahabad, Allahabad U. P. 211002, India

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A - Conception and study design, **B** - Data collection, **C –**Data analysis, **D** - Writing the paper,
E – Review article, **F** - Approval of the final version of the article

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ABSTRACT

Purpose: Carbofuran toxicity on rats was studied during sub-acute exposure. This work was undertaken to evaluate the protective effect of aqueous black tea extract and vitamin C against a rat model of oxidative stress induced by treatment with carbofuran, an organocarbamate insecticide.
Materials and methods: The levels of lipid peroxidation, reduced glutathione and ascorbic acid were assessed by determining the extent of oxidative stress in the erythrocytes of rats.
Results: The results clearly demonstrated that the treatment of rats with sub-acute concentration of carbofuran caused significant elevation in the levels of oxidative stress and decrease in the contents of glutathione and ascorbic acid. The introduction of black tea extract and vitamin C augmented the antioxidant defense mechanism in alleviating the carbofuran induced oxidative stress.
Conclusion: The findings that the pretreatment with black tea and vitamin C can mitigate carbofuran induced toxicity lend evidence that supplementation with either black tea extract and/or vitamin C have a therapeutic potential in amelioration of oxidative stress in mammalian systems.
Key words: Carbofuran, erythrocytes, black tea, vitamin C, oxidative stress

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DOI: 10.5604/01.3001.0009.5162

[*]**Corresponding author:**
Syed Ibrahim Rizvi
Department of Biochemistry
University of Allahabad, Allahabad U. P. 211002, India
Tel.:. +919415305910; e-mail. sirizvi@gmail.com

Received: 03.11.2016
Accepted: 16.12.2016
Progress in Health Sciences
Vol. 6(2) 2016 pp 148-154

INTRODUCTION

The increasing use of pesticides in agriculture and domestic activities for controlling pests is polluting the environment [1]. Carbofuran (2, 3-dihydro-2, 2-dimethyl-7-benzofuranol methyl carbamate) is a commonly used carbamate insecticide, pesticide, nematicide and acaricide [2]. Carbofuran is also known to exert high toxicity to mammalian systems [3]. The mechanism involved in the pathogenesis of carbofuran-induced neuronal damage is linked to free radical-mediated injury [4]. Oral administration of carbofuran is reported to cause neuronal injury by excessive generation of reactive oxygen species (ROS) leading to lipid peroxidation (LPO) [5]. 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 [5].

Erythrocytes are prone to oxidative stress because they are exposed to high oxygen tension and have polyunsaturated fatty acids in the membrane and hemoglobin-bound iron [6]. However, the efficient antioxidant machinery involving antioxidative enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione-S-transferase (GST) as well as other non-enzymatic antioxidant molecules such as glutathione, vitamin C and vitamin E scavenge reactive oxygen species act in concert to maintain the integrity of erythrocyte.

Several natural products including certain beverages have been shown to possess antioxidant potential. Tea (Camellia sinensis) is the most popular beverage world-wide. Of the approximately, 2.5 million metric tons of dried tea manufactured annually, about 80 % is consumed as black tea [7]. The components from black tea are known to exert important medicinal properties; they are reported to be antipyretic, anti-inflammatory, antimicrobial and provide protection against peroxidation of lipids [8].

The present study therefore has been undertaken to evaluate the efficacy of black tea and vitamin C to protect erythrocytes from oxidative stress induced by exposure of Wistar rats to a sub-acute dose of carbofuran.

MATERIALS AND METHODS

Chemicals

Reduced glutathione and 5,5'-dithiobis nitro benzoic acid (DTNB) were purchased from Sigma Aldrich, USA. All other chemicals were of highest purity available from Merck, India and HIMEDIA Labs, India. Premium quality CTC (crush, tear, curl) grade black tea was purchased from the local market.

Preparation of black tea infusion

The black tea extract was prepared from CTC (Curl, Tear and Crush) grade tea. Preparation of aqueous extract of black tea was done by following the method as described by Wei *et al.* (1999) [9-11]. Briefly, 1.25 g of black tea leaves was added to 25 ml of boiling water in beaker and was steamed for 15 min. The infusion was cooled to room temperature and then filtered. The tea leaves were extracted a second time with 25 ml of boiling water and filtered, finally both filtrates were combined to obtain a 2.5% aqueous-tea extract. The resulting clear solution was similar to tea brews consumed by human. This black tea infusion (BTI) was fed to animal model by gavage technique at a dose of 1 ml/100 g body weight at a temperature of 37°C [12].

Animal model and study protocol

The experiment was carried out with 36 male 4 ± 0.5 months old Wistar rats with body weight between 160 ± 15g. They were housed in a temperature controlled room (25±5°C) with 12-h light–dark cycles for at least 1 week. All rats were fed with a normal laboratory diet nutrients rich pellets containing total energy as fat, protein and carbohydrates, and had free access to drinking water. After the stabilization period of one week, the rats were randomly divided into six groups, containing six animals each (n= 6). Group I: Normal control receiving no treatment/supplementation; Group II, animals leveled as (CF) received once oral dose of carbofuran 1.6 mg/kg body weight equivalent to 20% LD_{50} dissolved in 0.5 ml of edible ground nut oil (purchased from Adani Wilmar Limited, Gujarat, India, under the brand name Fortune) [13]. The animals from groups of III and IV received aqueous black tea extract at 1 ml/100 g body weight/day [9] and V and VI group rats were treated with vitamin C at 200 mg/kg body weight/day in 0.5 ml distilled water [12] via gavage technique (oral route). Rats were administered black tea extract (BTE) and vitamin C 10 days before the carbofuran treatment. The pretreatment of black tea extract and vitamin C was provided in order to build antioxidant pool in animal body before pesticide exposure. Carbofuran treatment was given 1.6 mg carbofuran/kg body weight once after 10 days administered of black tea extract and vitamin C, after that animals were monitored for 24h. The biochemical estimations were conducted after the completion of 24 h treatment of carbofuran.

Collection of blood, isolation of red blood cells and plasma

After the treatment periods were over, rats were sacrificed under light anesthesia. Blood samples were collected by cardiac puncture into 10 unit/ml heparin rinsed anticoagulant syringes, and then red blood cells were pelleted by centrifugation at 1800g for 10 min at 4°C. After the removal of plasma (immediately frozen at -80°C until use for biochemical assays), buffy coat, and the upper 15% of packed red blood cells (RBCs), the RBCs were washed twice with cold phosphate buffered saline (PBS) (0.9% NaCl and 10 mmol·L^{-1} Na_2HPO_4; pH 7.4) and then used for experiment. All protocols for experiments were approved by the Animal Care and Ethics Committee of University of Allahabad.

Determination of erythrocyte malondialdehyde (MDA) Content

Erythrocyte MDA was measured according to the method of Esterbauer and Cheeseman (1990) [14] with slight modification. Packed erythrocytes (0.2 ml) were suspended in 3 ml PBS containing 0.5mM glucose, pH 7.4. The lysate (0.2 ml) was added to 1 mL of 10 % trichloroacetic acid (TCA) and 2 ml of 0.67 % thiobarbituric acid (TBA) and boiled for 20 min at 90-100ºC followed by cooling. Then the mixture was centrifuged at 1000 g for 5 min and the absorbance of supernatant was read at 532 nm. The concentration of MDA in erythrocytes was calculated using extinction coefficient ($\varepsilon = 1.56x10^5$ M^{-1} CM^{-1}) and is expressed as nmol·mL^{-1} of packed erythrocytes.

Determination of erythrocyte reduced glutathione (GSH)

Erythrocyte GSH was measured following the method of Beutler (1984) [15]. The methods is based on the ability of the –SH group to reduce 5,5'-dithiobis, 2-nitrobenzoic acid (DTNB) and form a yellow colored anionic product whose optical density is measured at 412 nm. Concentration of GSH is expressed in mg ml^{-1} packed RBCs and was determined from standard plot.

Determination of ascorbic acid content

Ascorbic acid content was estimated in the plasma by using the calorimetric method [16].

Measurement of erythrocyte PMRS activity

The activity of the erythrocyte PMRS was measured by the reduction of ferricyanide the method are described earlier [17]. Briefly, packed RBC (0.2 ml) were suspended in PBS containing 5 mM glucose and 1 mM freshly prepared potassium ferricyanide to a final volume of 2.0 ml. The suspensions were incubated for 30 min at 37°C and then centrifuged at 800 g at 4°C. The supernatant collected was assayed for ferrocyanide content using 4, 7-diphenyl-1, 10-phenanthroline disulfonic acid disodium salt, absorption was recorded at 535 nm (ϵ = 20,500 M^{-1} cm^{-1}). The results are expressed in µmol ferrocyanide/ml PRBC/30 min.

Statistical analysis

All data are presented as means ± SEM and statistical analyses were conducted using the software PRISM version 5.01 Differences among treatments were determined using a *t*-test.

RESULTS

Figure 1 shows the lipid peroxidative damage of erythrocytes measured in terms of MDA subsequent to carbofuran treatment and supplementation with black tea extract and vitamin C.

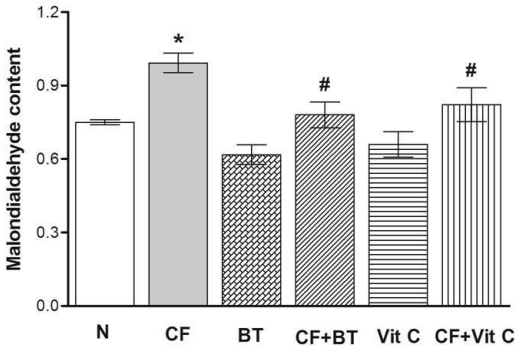

Figure 1. Effect of the carbofuran induced toxicity and preventive action of aqueous black tea extract and vitamin C on malondialdehyde (MDA) content from erythrocytes of rats. The content of MDA was determined in the hemolysates prepared from erythrocytes of nut oil-treated (N) normal rats, CF carbofuran treated, BT black tea treated, vitamin C treated, CF+BT carbofuran induced toxicity with black tea extract treated and CF+vitamin C carbofuran induced toxicity with vitamin C treated rats. Concentration of MDA is expressed as nmol·L^{-1} of packed erythrocytes. Treatment with carbofuran caused an significantly increase in MDA content (*$p<0.01$) compared with control. Treatment with black tea extract and vitamin C showed significant protection against carbofuran induced toxicity ($^{\#}p<0.01$) compared with negative control.

Subjecting rats to carbofuran-induced oxidative stress caused a significant ($p<0.01$) increase

in MDA level (75.52%) above the basal values. Supplementation with aqueous black tea extract significantly decreased (96.11%) lipid peroxidation after carbofuran treatment. Vitamin C also protected erythrocytes from carbofuran-induced oxidative stress, as evidenced by decrease in MDA level (91.11%). Rats when supplemented alone with black tea extract and vitamin C caused a mild reduction in lipid peroxide level compared to normal rats.

We observe a significant ($p<0.01$) decrease in erythrocyte reduced glutathione (GSH) content in carbofuran-treated rats (Figure 2). However, pretreatment with black tea to CF-treated rats significantly protected the decrease in GSH content due to carbofuran. vitamin C was more effective than black tea in improving intracellular GSH level in post carbofuran treatment conditions. When given alone with either black tea or vitamin C, there was an increment of GSH level in erythrocyte (Figure 2).

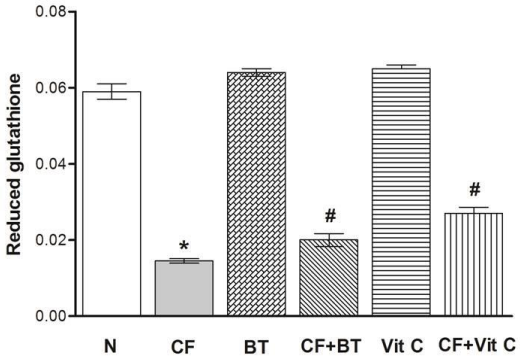

Figure 2. Effect of the carbofuran induced toxicity and preventive action of aqueous black tea extract and vitamin C on reduced glutathione (GSH) activity from erythrocytes of rats. The activity of GSH was determined in the hemolysates prepared from erythrocytes of nut oil-treated (N) normal rats, CF carbofuran treated, BT black tea treated, vitamin C treated, CF+BT carbofuran induced toxicity with black tea extract treated and CF+vitamin C carbofuran induced toxicity with vitamin C treated rats. Concentration of GSH is expressed in milligram per milliliter packed RBCs. Treatment with carbofuran caused an significantly decrease in GSH content ($^{*}p<0.001$) compared with control. Treatment with black tea extract and vitamin C showed significant protection against carbofuran induced toxicity ($^{\#}p < 0.01$) compared with negative control.

The plasma ascorbic acid content was found to be significantly ($p<0.01$) decreased in carbofuran-treated rats. However, the aqueous black tea extracts supplementation caused significant (87.35%) increment of ascorbic acid content in carbofuran-treated rats (Figure 3).

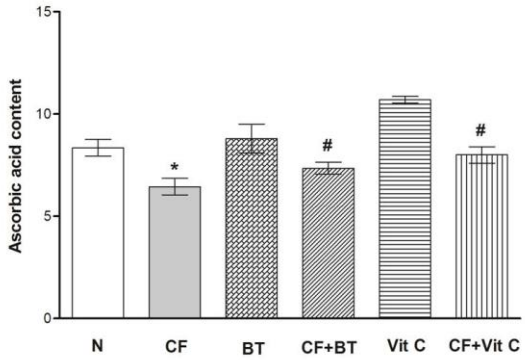

Figure 3. Effect of the carbofuran induced toxicity and preventive action of aqueous black tea extract and vitamin C on ascorbic acid content from plasma of rats. The content of ascorbic acid was determined in the plasma of nut oil-treated (N) normal rats, carbofuran (CF) treated, BT black tea treated, vitamin C treated, CF+vitamin C carbofuran induced toxicity with black tea extract treated and CF+vitamin C carbofuran induced toxicity with vitamin C treated rats. Concentration of ascorbic acid is expressed in milligram per milliliter plasma. Treatment with carbofuran caused an significantly decrease in ascorbic acid content ($^{*}p<0.01$) compared with control. Treatment with black tea extract and vitamin C showed significant protection against carbofuran induced toxicity ($^{\#}p < 0.01$) compared with negative control.

The results reflected that treatment of rats with vitamin C also augmented vitamin C content in erythrocytes near to normal level (96.58 %). Treatment of rats with vitamin C alone caused significantly higher (124.06 %) content of ascorbic acid as compared to normal ones. Black tea supplementation caused about 86.0 % increment in the level of ascorbic acid.

We observe significant ($p<0.01$) activation of erythrocyte PMRS activity (142%) in rats subjected to carbofuran treatment (Figure 4). The higher activity of erythrocyte PMRS in carbofuran treated rats is the result of generation of oxidative stress concomitantly with toxicity. However, aqueous black tea extract (BTE) and vitamin C supplementation significantly ($p<0.01$) decreased the PMRS activity (87% and 85% respectively) with respect to normal control animals. The activity of PMRS comes to near basal level in carbofuran treated

rats supplemented with aqueous black tea extract and vitamin C justifying that rats receiving treatment

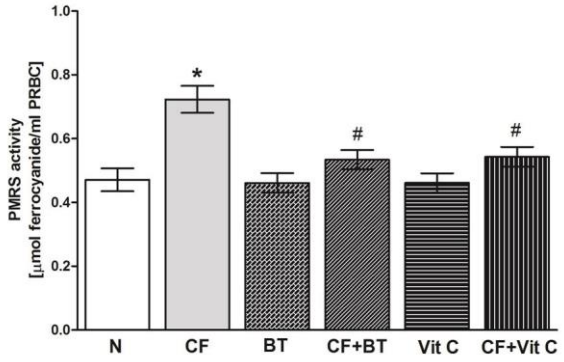

Figure 4. Effect of the carbofuran induced toxicity and preventive action of aqueous black tea extract and vitamin C on plasma membrane redox system (PMRS) content from erythrocytes of rats. The content of PMRS was determined in the hemolysates prepared from erythrocytes of nut oil-treated (N) normal rats, CF carbofuran treated, BT black tea treated, vitamin C treated, CF+BT carbofuran induced toxicity with black tea extract treated and CF+vitamin C carbofuran induced toxicity with vitamin C treated rats. Activity of PMRS is expressed as micromole ferrocyanide/ml PRBC/30 min. Treatment with carbofuran caused an significantly increase in PMRS activity (*$p<0.01$) compared with the control group. Treatment with black tea extract and vitamin C showed significant protection against carbofuran induced toxicity ($^{\#}p<0.01$) compared with negative control.

have an augmented antioxidant defense and can cope easily the oxidative stress generated due to chronic toxicity in carbofuran treated rats.

DISCUSSION

We have provided evidence that a sub-acute single dose of carbofuran can efficiently induce oxidative stress in the erythrocytes of the experimental rats. Oxidative stress is reported to play an active role in development/progression of several diseases [18]. A certain amount of oxidative damage to biomolecules takes place even under normal physiological conditions. However, the rate of this damage increases during aging and some other degenerative conditions such as during environmental toxicant induction [19,20]. In the present study we have observed significant increment of lipid peroxidation in rat erythrocytes after carbofuran treatment. Our results are in agreement with other workers who have reported an altered membrane lipid composition as well as increased lipid peroxidation in synaptic membranes under chronic exposure to carbofuran, underlining the possible interaction of carbofuran with cell membranes [5]. Pretreatment of rats with black tea and vitamin C significantly protected the erythrocytes from CF-induced lipid peroxidation. This effect of black tea and vitamin C can be explained on the basis of their antioxidant properties in the aqueous medium [8,21]. In addition, the green tea and vit E has also been reported to protect erythrocytes against oxidative stress-induced lipid peroxidation [22].

The reduced glutathione (GSH) is a major intracellular tripeptide (Glu-Cys-Gly). It is a non-protein -SH compound accepted as the most important intracellular hydrophilic antioxidant. GSH exists in most tissues of the animal body and is particularity abundant in erythrocytes and the lens. The GSH system is the most important endogenous defense system against oxidative stress in body [23]. Induction of oxidative stress in rats by carbofuran in present study caused depletion of erythrocytes' intracellular level of reduced glutathione. Similar observations have also been recently reported [11]. The decrease in intracellular GSH level could be both due to oxidative stress and the binding of carbofuran to glutathione followed by subsequent elimination of intracellular GSH [24]. The protective effect of black tea and vitamin C supplementation provides evidence that the antioxidant effect of both is effective inside the cell. The reduced GSH content in response to CF may be attributed to the oxidative stress caused due to CF intoxication. The efficient recovery in GSH level highlights the therapeutic efficacy of black tea extract and vitamin C in ameliorating the CF-induced oxidative stress.

The ascorbic acid (ASC) is the primary antioxidant present in plasma. However, the rats, humans, higher primates and guinea pigs cannot make ASC and thus require it through the diet. In the presence of an oxidant, ASC is oxidized first to monodehydro-ascorbate or ascorbate free radical (AFR) and then to dehydroascorbate (DHA), which is unstable and undergoes irreversible hydrolysis to 2,3-diketo-L-gulonic acid, resulting in decreased level of the vitamin. Two molecules of AFR can react with each other to form one each of ASC and DHA. In recent years, there has been much interest in investigating the mechanisms by which ASC level is maintained in blood, The erythrocytes, being the most abundant cells in the blood, have been reported to play a crucial role in recycling ASC in human plasma [25]. Most eukaryotic cells including RBCs have a plasma membrane redox system (PMRS) that

transfers electrons from intracellular substrates to extracellular electron acceptors [26]. This system is activated to maintain a balanced NAD+/ NADH or ASC ratio which is essential for normal energy metabolism, maintenance of homeostasis and neutralization of oxidative stressors outside the cells [27], it also provides a survival mechanism for the cells under stress conditions and during calorie restriction [20]. The importance of red cell PMRS during conditions which results in oxidative stress has recently been highlighted [8,10]. There is evidence that the intracellular ASC donates electrons to extracellular AFR via the PMRS, which incorporates an AFR reductase [28]. Such a redox system enables the cells to counteract oxidative processes effectively and thereby prevent depletion of extracellular ASC. This provides an efficient mechanism for ASC recycling between the intra and extracellular compartments. Since carbofuron induced stress in rats is associated with depletion of ascorbic acid content, these findings support the hypothesis that ascorbic acid deficiency is involved in the pathogenesis of free radical induced oxidative stress in rats. The treatment with vitamin C in present work has shown a rise in content of ascorbic acid, compared to black tea alone treated group. The ASC is known as a strong antioxidant compound. Young *et al.* [29] have shown that ASC supplementation in diabetic rats effectively reduced oxidative stress [29].

Viewed in conjunction with the report of Kamboj *et al.* [5] data from present investigation reflect that oxidative stress is a common feature of carbofuran toxicity whether in prolonged or single exposures. In consonance with our study, Devi et al. [30] have reported that supplementation of aqueous black tea and vitamin C to rats resulted in decreased oxidative stress in erythrocytes. The aqueous black tea and vitamin C supplementation indicates their protective role against carbofuran induced toxicity. To the best of our knowledge this is the first evidence of a black tea antioxidant activity, ameliorating the carbofuran pesticide induced toxicity with reducing the oxidative stress in rats.

CONCLUSION

In the present study, we have provided evidence that a sub acute single dose of carbofuran can efficiently induce oxidative stress in the erythrocyte membrane of the experimental rats. We have shown that vitamin C and the polyphenolic contents present in the aqueous extract of black tea can provide efficient defense against carbofuran induced oxidative damage to the rat erythrocytes.

Acknowledgement

Dileep Kumar was the recipient of Senior Research Fellowship form Council of Scientific & Industrial Research (CSIR), New Delhi, India, currently he is doing research in University of Szeged, Hungary. SIR wishes to acknowledge the support of University Grants Commission, New Delhi for financial support in the form of grant F 37-392/2009 to SIR.

Conflicts of interest

The authors declare no conflicts of interest.

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