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Protective Effect of Epigallocatechin Gallate on Fluoride-Induced Oxidative Stress Related Haematotoxicity in Rats.

S Miltonprabu* and S Thangapandiyan

Department of Zoology, Faculty of Science, Annamalai University, Annamalai Nagar - 608002, Tamil Nadu, India.

Research Article

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***For Correspondence**

Department of Zoology, Faculty of Science, Annamalai University, Annamalai Nagar - 608002, Tamil Nadu, India.
Tel: +91 04144 – 238282;
Mobile : +91 9842325222
Fax: +91 04144 – 238080

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ABSTRACT

Fluoride (Fl) is a naturally occurring electro negative compound which is classified as potent toxicant and human carcinogen. Erythrocytes are very expedient model to understand the susceptibility of oxidative stress on membrane induced by different xenobiotics. Fl administration (25mg/kg/BW) significantly increased the percentage of hemolysis, lipid peroxidation markers such as thiobarbituric acid reactive substances (TBARS), conjugated dienes (CD), protein carbonyls contents (PC), and altered haematological parameters, with decreased antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX),glutathione-s-transferase (GST), glutathione reductase (GR) and glucose-6-phosphate dehydrogenase (G6PD). The levels of non-enzymic antioxidants (reduced glutathione, vitamin 'C' and vitamin 'E') and membrane bound ATPases (Na^{+}/K^{+}-ATPase, Mg^{2+}-ATPase and Ca^{2+}-ATPase) were also decreased in F treated rats. Pre oral administration of EGCG (40mg/k/BW) along with Fl for 28 days significantly reduced the levels of TBARS, CD and PC with significantly increased membrane ATPases, membrane integrity, viability, enzymatic and non-enzymatic antioxidants in the RBC's of Fl treated rats. In conclusion, the results clearly indicate that EGCG significantly attenuated the Fl induced haematotoxicity in rats.

INTRODUCTION

Fluoride (Fl) is ubiquitous natural compound and widespread industrial pollutant released into the environment through a combination of natural and anthropogenic processes. In low concentrations Fl has been proven to be beneficial for teeth and bone development, therefore, the prophylactic supplementation of drinking water sources with Fl is widely used in many countries over several decades [1]. In addition, Fl compounds are widely used in industry, agriculture and domestic chemicals such as cleaning products, insecticides, rodenticides. As a result, each year there are thousands of reports related to acute or lethal poisoning due to excessive ingestion of Fl-containing dental products and accidental or suicidal exposure to Fl-containing chemicals at home, industrial workplaces and laboratories. Chronic consumption of high Fl doses results in adverse health effects such as dental and skeletal fluorosis, arthritis, osteoporosis, infertility and mental retardation [2]. Endemic fluorosis is a serious national problem in many counties affecting millions of people using ground waters with high Fl content for their daily needs [3]. Fluoride is often described as a double edged sword because in small doses, it is an essential trace element with remarkable protective effect in preventing dental caries and osteoporosis. On the other hand, excessive exposure to Fl exerts harmful effects on the organism. It may directly or indirectly modulate the enzyme activity by forming complexes with the metal part of enzyme molecules [4]. In this way Fl interferes with the metabolic processes involving carbohydrates, lipids and proteins [5]. Fluoride inhibits enzymes involved in major metabolic pathways for example glycolysis and the Krebs cycle. In addition, Fl inhibits fatty acid oxidation and reduces the activity of pyruvate dehydrogenase, which reduces the amount of acetyl-CoA in the cells. Fluoride negatively regulates the activity of ATPases an enzyme important in the polymerization of amino acids, thus inhibiting the process of bonding the amino acids to peptides and blocking DNA synthesis [6]. Long term exposure to

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Fl compounds induce ROS changes in many cells particularly erythrocytes leading to the damage of cellular function [7].

Reactive oxygen species (ROS) are produced in the erythrocytes by cellular metabolism and other exogenous environmental agents. They are generated by a process known as redox cycling and are catalyzed by transition metals, such as Fe^{2+} and Cu^{2+} [8]. When both humans and animals are exposed to fluoride, they experience an increased formation of ROS/RNS, including peroxyl radicals (ROO·), the superoxide radical, singlet oxygen, hydroxyl radicals (OH·) via the Fenton. Fluoride can inhibit the antioxidant and increase the lipid peroxidation in the RBC via over production of reactive oxygen species [9].

Blood is a major tissue participating in the distribution of fluoride [10]. However, Fl ability to induce red blood cell (RBCs) death, as well as the molecular mechanisms underlying this process, has not been sufficiently investigated. Two studies described the development of anemia in cattle afflicted with fluorosis [11] and in mice exposed to sublethal Fl doses [12] might indicate the premature erythrocyte death. Susheela [13] reported that intoxication of the human body with Fl was associated with severe anemia due to shorter erythrocyte lifespan caused by membrane degeneration that turns them into echinocytes. Recently, exposure of the rat erythrocytes to NaF induced pronounced inhibitory impact on the transport of monovalent cations across plasma membrane associated with the Na^+–K^+-pump inhibition and Ca^{2+}-dependent K^+ loss [14].

The existing knowledge about the beneficial role of nutraceuticals represents a great impact on nutritional therapy and stimulated research on favourable properties of bioactive compounds-enriched nutraceuticals. Dietary modifications are one of the key elements in the management of haematological abnormalities. For many years, polyphenolic phytochemicals were thought to protect cells against oxidative damage through scavenging of free radicals. Among all polyphenolics, EGCG has received most attention because of its wide array of biological properties. Epidemiological evidence indicates that consumption of EGCG-rich green tea may be protective against certain chronic diseases in human [15,16,17]

Epigallocatechin gallate (EGCG) is one of the main catechins, extracted from green tea, and was associated with a wide range of physiological effects including antioxidant activities, free radical scavenging, ion chelating and anti-inflammatory properties [18-21]. A recent study from our laboratory showed that EGCG exhibits hepatoprotective activity against Fl induced toxicity. Based on its lipophilic nature and multiple pharmacological actions, we hypothesise that the beneficial effects of EGCG can be extended up to the intracellular fractions of the RBC and prevent the pathological alterations induced by the overproduction of ROS by Fl [22, 23].

Therefore, the present study was designed to evaluate the therapeutic potential of EGCG supplementation against Fl induced haematotoxicity in rats. We also attempt to demonstrate the molecular mechanism of its therapeutic effect by studying the lipid peroxidation, antioxidant enzymes, and other biochemical markers in the erythrocyte fraction of blood.

MATERIALS AND METHODS

Chemicals

EGCG (Fig.1), NaF and calcein-AM, were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals and solvents (glacial acetic acid, heparin, nitro blue tetrazolium chloride, potassium dihydrogen phosphate, reduced glutathione, sodium dihydrogen phosphate, sodium fluoride, trichloro acetic acid, thiobarbituric acid, hydrogen peroxide were purchased) were of certified analytical grade and purchased from S.D. Fine Chemicals, Mumbai or Hi media Laboratories Pvt. Ltd., Mumbai, India. Reagent kits were obtained from span Diagnostics, Mumbai, India.

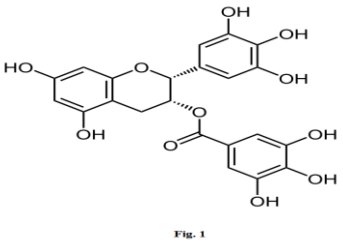

Figure 1: The structure of EGCG

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Animals

Healthy male albino Wistar rats (160-180 g) were obtained from the Central Animal House, Department of Experimental Medicine, Rajah Muthiah Medical College and Hospital, Annamalai University, and maintained in an air-conditioned room (25 ± 2 °C) with a 12 h (light) – 12 h (dark) cycle. Food and water were provided *ad libitum* to all of the animals. The study protocols were approved by the Institutional Animal Ethics Committee of Rajah Muthiah Medical College and Hospital, Annamalainagar (Reg. No. 160/1999/CPCSEA; Proposal No. 952/2012), and the experimental design was performed in accordance with the current ethical norms approved by the Ministry of Social Justices and Empowerment, Government of India and Institutional Animal Ethics Committee, Annamalai University, Annamalai nagar, Chidambaram.

Selection and preparation of the drug

The dose of NaF (25 mg/kg/BW) was selected from the previous reports of Chinoy [24]. NaF dissolved in normal saline and administered daily by intragastric intubation for 4 weeks. EGCG powder was dissolved in 10% tween 80 and given orally, 90 min prior to the administration of NaF, at a dose of 20, 40, and 80 (mg/kg/BW) daily for 4 weeks. A pilot study was conducted with three different doses (Thangapandiyan and Miltonprabu [25]) of EGCG (20, 40 and 80 mg/kg/BW) to determine the dose dependent effect on fluoride treated rats. After 4 weeks of experiment, it was observed that EGCG pre-treatment at the doses of 20, 40 and 80 mg/kg significantly ($p<0.05$) decreased the levels of oxidative markers, and elevate the levels of enzymatic and nonenzymatic antioxidant in Fluoride intoxicated rats. 40 mg/kg of EGCG showed significant effect when compared with the 20 and 80 mg/kg. Hence, we have chosen the effective dose 40 mg/kg of EGCG for our study.

Experimental design

The animals were divided into four groups with 6 animals in each group and were given oral treatments as described below:

- Group I: marked as control, receiving vehicles only
- Group II: marked as positive control receiving a dose of 40 mg/kg/BW of EGCG
- Group III: marked as NaF control receiving a dose of 25mg/kg/BW
- Group IV: marked as pre-treated with EGCG (40 mg/kg/BW) and NaF (25mg/kg/BW)

All the treatments were given orally by intragastric intubation. The total duration of the study was 28 days. Food and water intake were recorded regularly. Forty-eight hours after the administration of the last doses, the rats were anesthetized with an intramuscular injection of ketamine hydrochloride (25 mg/kg) and sacrificed by cervical decapitation. Blood samples were collected into heparinised tubes and processed for biochemical estimation. The obtained blood was centrifuged to separate the plasma and red cells. Packed red cells were washed three times in ice-cold phosphate-buffered saline (PBS-phosphate buffer 0.01 M, pH 7.4, containing 0.15 M NaCl) and used. For oxidative stress parameter determination, the collected blood was centrifuged for 10 min at 5,000 rpm to separate the plasma. While the RBC was washed three times with 0.9% NaCl. Washed-out erythrocytes were lysed with dH2O (1:3, v/v) at 0°C for 30 min. All samples were extracted from lysate. After extraction, samples were stored at -80°C before performing the appropriate analytical method.

Biochemical Analysis

Haematological parameters

Red blood cell count (RBC), white blood cell counts (WBC) were estimated by using Sysmex Automated Haematology Analyzer KX-21N, Sysmex Corporation, Kobe-Japan. Differential WBC counts were done with blood smear stained with Wright's stain. hemoglobin concentration, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet count and Bleeding time was determined by the Duke's method [26], clotting time by Sabrazes's capillary tube method [27].

Isolation of erythrocytes and erythrocyte ghost membranes

Erythrocytes and their ghost membranes were prepared by Dodge *et al.* [28] and Fairbanks *et al.* [29] with slight modifications. Packed cells were washed with saline. Packed cells were washed with Tris-buffer, 0.31 M, and pH 7.4 and used for biochemical estimations. Another packed cell was used for hemolysis by adding hypotonic 5 mm phosphate buffer (pH 8.0) with the addition of 1 mm EDTA. After 4–6 h, the erythrocyte ghosts sedimented by centrifugation at 12000 rpm for 45 min at 4–6 °C. The hemolysate was used for the antioxidant assay. The erythrocyte membrane pellets were suspended in 0.02 M Tris-buffer (pH 7.2) and used for various biochemical assays. Protein content in the RBC membrane was determined by Lowry *et al.* [30].

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Estimation of erythrocytes membrane lipid peroxidation

Lipid peroxidation in terms of thiobarbituric acid reactive substances was measured according to the method of Esterbauer and Cheeseman [31] and the Protein carbonyl levels were measured according to method described by Reznick and Packer [32]. Measurement of conjugated diens in erythrocytes was according to Konings [33].

Determination of nonenzymatic antioxidants

Reduced glutathione (GSH) content was estimated according to the method of Beutler *et al.* [34] and expressed as µmoles/g Hb. Total sulfhydryl groups (TSH) were measured after reaction with dithionitrobis benzoic acid, using the method of Ellman [35]. Concentrations of vitamins C and E were measured following the methods of Omaye *et al.* [36] and Desai [37], respectively.

Assay of enzymatic antioxidants

Erythrocytes from the second tube were lysed by four-fold dilution with H_2O, followed by repeated freezing–thawing cycles. SOD (U/g Hb) activity was estimated according to the method described by Misra and Fridovich, [38]. CAT (U/g Hb), activity was determined using the method described by Aebi [39], by measuring hydrogen peroxide decomposition at 240 nm. GPx (U/g Hb) activity was assayed using the method described by Flohe and Gunzler [40], by the subsequent oxidation of NADPH at 240 nm with t-butyl-hydroperoxide as substrate. The values were expressed in units per gram of hemoglobin. GR activity in erythrocytes was assayed by the methods of Goldberg and Spooner [41]. The GR activity in erythrocytes has been expressed as nM NADPH oxidized to NADP/ g of Hb/min. Glutathione-S-transferase (GST) measured by Buetler [42]. Glucose-6-phosphate Dehydrogenase (G6PD) activity was measured by using kit (from (R&D) span diagnostics Ltd., India) used for a rapid quantitative measurement of G6PD activity coupled to a simultaneous evaluation of the hemoglobin content in the same sample, expressing results in Units/gram Hemoglobin (U/ g Hb).

Assay of membrane bound enzymes

Na^+/K^+-ATPase was measured was assessed in erythrocyte membrane preparation according to the method of Quigley and Gotterer, [43]. Na^+/K^+-ATPase activity was measured under two conditions; in the presence of Mg^{2+}, Na^+/K^+ (total ATPase) and secondly in the presence of Mg^{2+}, Na^+/K^+ and ouabain. The Na^+/K^+-ATPase activity was measured as the difference between total ATPase activities and ouabain-insensitive ATPase activities. The inorganic phosphate released by the action of ATPase's was estimated by the method of Fiske and Subbarrow, [44]. The Ca^{2+}-ATPase activity was measured according to the method of Desaiah *et al.* [45]. The Mg^{2+}-ATPase activity was determined in the presence of 1mM EGTA (which specifically chelates Ca^{2+} ion) and this was subtracted from the total activity in order to obtain the net Ca^{2+}-ATPase activity.

Determination of erythrocyte viability

The viability of rat erythrocytes was assessed using calcein-AM (calcein acetoxymethyl ester) according to procedure described by Bratosin *et al.* [46] Calcein, non-fluorescent membrane-permeable dye, rapidly enters viable cells where it is converted by the cytosolic esterases into green fluorescent calcein retained in the cells with intact membranes but extruded from dying or damaged cells. Calcein-AM was prepared as a 10 mM stock solution in DMSO, aliquoted and stored at -20ºC. The stock solution was diluted by incubation medium to 100 µM working solution before each experiment. The aliquots of control and NaF-treated RBCs (100 µl, 1% hematocrit) were incubated with 5 µM calcein-AM for 45 min at 37ºC in the dark. Then the samples were diluted in 1 ml of incubation medium for immediate flow cytometry. Flow cytometric analysis was performed on EPICS XL cytometer (Beckman Coulter Inc., Brea, CA, USA) using SYSTEM II (Version 3.0) software for acquisition and analysis. The fluorescence channel FL-1 was set on logarithmic scale. The viability of 3×10^4 cells was analyzed in each experiment.

Determination of erythrocyte membrane integrity

The integrity of erythrocyte plasma membrane was determined in hemolysis assay. After incubation with NaF the samples were sedimented (3000 rpm at 4 ºC for 5 min) and the supernatants were collected. The cell hemolysis was measured photometrically at 405 nm based on the hemoglobin (Hb) content in the supernatant. The absorption of the supernatant of erythrocytes lysed in distilled water was taken as 100% hemolysis.

Statistical analysis

All the data were analyzed with SPSS/10 student software. Hypothesis testing methods included one way analysis of variance (ANOVA) followed by LSD. The data were expressed as the mean ± SD for three different sets of experiments and results were considered as significantly at $P < 0.05$. Statistically significant variations are

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compared as follows: control erythrocyte vs. erythrocytes + EGCG; erythrocytes + EGCG vs. erythrocytes + NaF; erythrocytes + NaF vs. erythrocytes +40 mg/kg/BW EGCG.

RESULTS

Effect of EGCG on food intake, body mass change, and organ: body mass ratio in control and experimental rats

The effect of FI and EGCG on food and water intake, body mass change, and the organ: Body mass ratios (%) in normal and experimental animals are presented in Table 1. In FI-treated rats, water and pellet consumption were significantly (P < 0.05) decreased and a significant decrease in body mass was observed. Pre oral administration of EGCG showed significant (P < 0.05) increase in the body mass ratio was found FI-treated rats. No significant changes were observed between the control group and rats treated with EGCG alone.

Table 1: Effect of EGCG on food intake, body weight change, and organ: body mass ratio in control and experimental rats

Groups	Body weight			Food intake(g/ 100 g bw/ day	Water intake (mL/rat/day)
	Initial (g)	Final(g)	%Change		
Control	157.00±1.89	172.00±3.52	11.68±0.49[a]	12.15±1.17	19.17±2.08
EGCG	158.00±2.18	174.00±2.74	12.23±0.50[a]	11.85±1.10	20.76±1.76
FI	156.00±2.32	142.00±2.54	6.53±0.40[b]	7.20±0.92	16.44±1.46
FI+EGCG	159.00±1.46	170.00±2.62	8.55±0.58[c]	10.65±1.19	17.57±1.71

Values are given as mean ± SD from six rats in each group. Values not sharing a common superscript letter (a–c) differ significantly at p<0.05 (DMRT)

Effect of EGCG on Haematological parameters

The effect EGCG on hematological parameter studies in the blood of control and experimental rats are presented in Table 2. The hematological parameters such as bleeding time (min), clotting time (s), RBC (Lakhs), Hb (g/dL), MCV(fl), MCH (pg), MCHC (g/dL), (%), WBC (thousands), lymphocyte (%), neutrophils (%), were examined. FI administered rats showed a significant (p< 0.05) reduction in the hematological parameters such as RBC, Hb, Circulating platelets, WBC, lymphocyte, and neutrophils when compared to control. The Pre oral administration of EGCG significantly (p< 0.05) improved these altered parameters, such as, higher counts of RBC, WBC, lymphocyte, and neutrophils compared with FI alone treated groups. EGCG alone treated rats also shows a significant (p< 0.05) increase in the haematological parameters when compared to control.

Table 2: Effect of EGCG on hematological parameter studies in the blood of control and experimental rats.

Parameters studied	Control	EGCG	FI	FI + EGCG
RBC (Lakhs)	80.5 ± 3.17a	80.7 ± 3.13a	58.2 ±7.26b	70.1 ± 8.72c
Hb (g/dL)	19.30 ± 1.0a	19.17 ± 0.9a	15.87 ± 0.75b	18.8 ± 0.72c
MCV(fl)	60.4 ± 2.28a	60.4 ± 2.30a	70.3 ± 3.40b	62.5 ± 2.50c
MCH(pg)	15.05 ± 0.81a	15.07 ±0.83a	17.31 ± 1.39b	15.39 ± 1.02c
MCHC(g/dL)	30.02 ± 1.32a	29.05 ± 1.31a	32.19 ± 2.13b	30.06 ± 1.03c
WBC(Thousands)	10.26 ± 0.29a	10.16 ± 0.28a	8.58 ± 0.25b	9.86 ± 0.34c
Neutrophil (%)	58.3 ± 7.20a	59.6 ± 7.21a	68.8 ± 8.13b	50.11 ± 7.08c
Lymphocyte (%)	50.8 ± 6.13a	49.14 ± 6.14a	68.9 ± 8.13b	53.9 ± 7.09c
Bleeding time (s)	78.01 ± 5.62a	75.02 ± 5.14a	160 ± 20.2b	105 ± 9.80c
Clotting time (min)	8 ± 0.17a	8.2 ± 0.17a	6.8 ± 0.16b	7.7 ± 0.18c

Values are given as mean ± SD from six rats in each group. Values not sharing a common superscript letter (a–c) differ significantly at p<0.05 (DMRT)

Effect of EGCG on RBC membrane lipid peroxidation

Effect of EGCG on the oxidative stress marker indices in the RBC membrane of control and experimental group of animals were shown on Figure. 2. The activities of oxidative stress markers such as TBARS, protein carbonyl (PC) contents and conjugated diens (CD) were found to be significantly increased (P < 0.5) in FI treated animals. Pre oral administration of EGCG was found to be significantly (P < 0.5) reverted the lipid peroxidation

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markers as compared to control animals. There was no significant difference observed between the EGCG control and vehicle control animals.

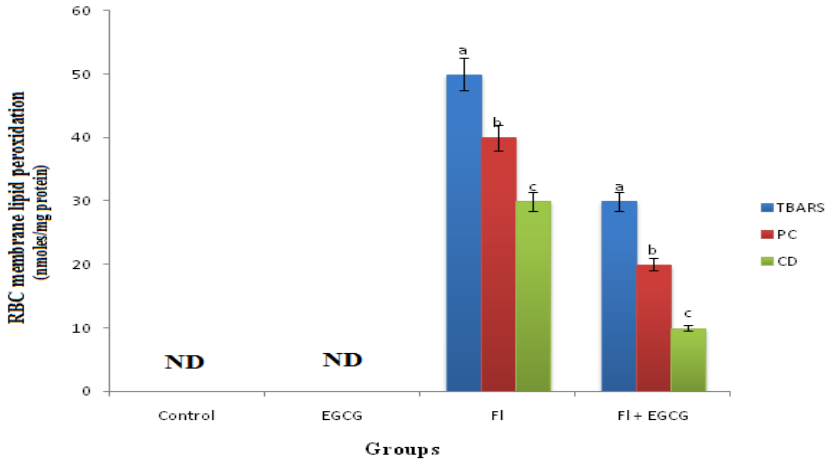

Fig. 2

Fig. 2 Effect of EGCG on RBC membrane lipid peroxidation (TBARS), protein carbonyl content (PC), and Conjugated diens (CD) of control and experimental rats. Values are expressed as mean ± SD for groups of six rats in each. Statistical significance was determined by one way ANOVA followed by post hoc test. Values are given as mean ± SD for six rats in each group. Values not sharing a common superscript letter (a–c) differ significantly at $p<0.05$ (DMRT).

Effect of EGCG on fluoride induced changes in nonenzymatic antioxidant of erythrocytes

Table 3 showed the levels of non-enzymatic antioxidants namely GHS vitamins C and E in control and experimental rats. The levels of non-enzymatic antioxidants namely GSH, vitamins C, and E were significantly ($p < 0.05$) decreased in the rat erythrocytes treated with FI, when compared to control group. The depleted level of GSH, vitamins C, and E was significantly ($p < 0.05$) restored with EGCG pre-administration in FI intoxicated rats. There was no significant improvement found to be EGCG alone treated rats when compared with control.

Table 3: Effect of EGCG on fluoride induced changes in nonenzymatic antioxidant of erythrocytes membrane

Groups	Control	EGCG	FI	FI + EGCG
GSH (µmol/g Hb)	5.73 ± 0.75a	5.78 ± 0.54a	3.50 ± 0.34b	4.98 ± 0.54c
Vlt.C (mg/dL)	1.55 ± 0.07a	1.73 ± 0.08a	0.90 ± 0.06b	1.43 ±0.09
Vlt.E (mg/dL)	1.20 ± 0.02a	1.21 ± 0.01a	0.87 ± 0.03b	1.02 ± 0.05c

Values are given as mean ± SD from six rats in each group. Values not sharing a common superscript letter (a–c) differ significantly at $p<0.05$ (DMRT)

Effect of EGCG on fluoride induced changes in enzymatic antioxidant activity of erythrocytes

The effect of EGCG on erythrocyte enzymatic activity namely SOD, CAT, GPx, GR, GST and G6PD in control and experimental rats showed on table 4. There was a significant ($p<0.05$) decrease in the activities of SOD, CAT, GP_X, GR, GST and G6PD in FI intoxicated rats when compared to control. Pre administration of EGCG along with F showed significant ($p<0.05$) recovery relating to the activities of SOD, CAT, GP_X, GR, GST and G6PD were observed when compared with FI alone treated rats. EGCG alone treated rats showed significantly ($p<0.05$) increased activity of these enzymatic antioxidant compared to control.

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Table 4: Effect of EGCG on fluoride induced changes in enzymatic antioxidant activity of erythrocytes membrane.

Groups	Control	EGCG	Fl	Fl + EGCG
SOD (U/g Hb)	120.1 ± 3.1a	121.3 ± 5.3a	109.6 ± 2.72b	119.7 ± 3.26c
CAT (U/g Hb)	135.8 ± 1.3a	136.6 ± 10.1a	115.4 ± 2.3b	123.2 ±6.03c
GPx (U/g Hb)	80.5 ± 2.4a	83.3 ± 7.9a	70. 1 ±1.5b	80.4 ± 9.2c
GR (U/g Hb)	0.26 ± 0.2a	0.27 ± 0.2a	0.12 ± 0.5b	0.20 ± 0.4c
GST (U/g Hb)	0.46 ± 0.1a	0.45 ± 0.3a	0.27 ± 0.2b	0.36 ± 0.4c
G6PD (U/g Hb)	7.5 ± 1.70a	7.6 ± 1.80a	4.8 ± 1.40b	6.98 ± 0.50c

Values are given as mean ± SD from six rats in each group. Values not sharing a common superscript letter (a–c) differ significantly at $p<0.05$ (DMRT)

Effect of EGCG on erythrocytes membrane bound ATPases

Figure 3 shows the effect of pre-administration of EGCG on Fl intoxicated rat erythrocyte membrane bound Na^+/K^+-ATPase, Mg^{2+}-ATPase and Ca^{2+}-ATPases levels in control and experimental rats. The levels of erythrocyte membrane bound ATPases in Fl treated rat were found to be significantly ($p<0.05$) decreased when compared to control group. Pre-administration of EGCG along with Fl had significantly ($p<0.05$) increased the levels of erythrocyte membrane bound ATPases when compared to Fl alone treated group. EGCG alone treated rats showed no changes of membrane bound ATPase when compared with control rats.

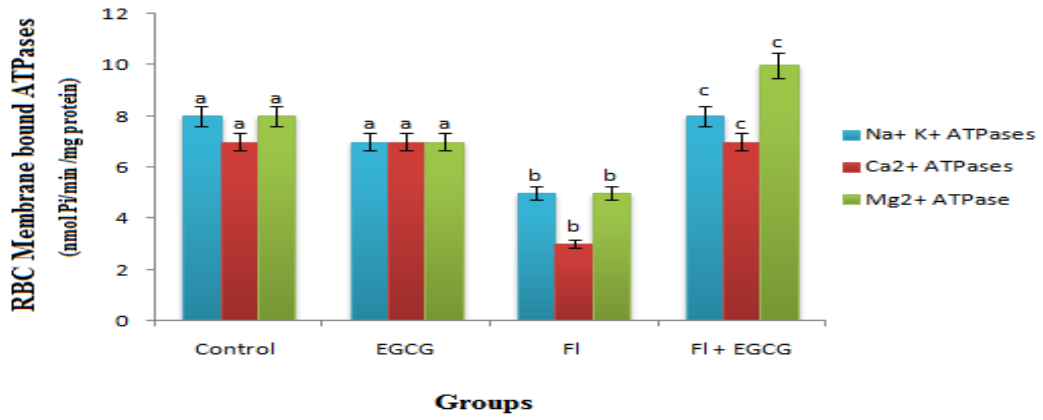

Fig. 3

Fig. 3 Effect of EGCG on RBC membrane bound ATPases (Na^+/K^+-ATPase, Mg^{2+}-ATPase and Ca^{2+}-ATPases) of control and experimental rats. Values are expressed as mean ± SD for groups of six rats in each. Statistical significance was determined by one way ANOVA followed by post hoc test. Values are given as mean ± SD for six rats in each group. Values not sharing a common superscript letter (a–c) differ significantly at $p<0.05$ (DMRT).

Effect of EGCG on fluoride altered rat erythrocytes viability

Figure 4 showed the effect of EGCG on viability of Fl-treated rat erythrocytes in control and experimental animals. Using flow cytometry analysis with calcein-AM, indicate the cell esterase activity was significantly ($p<0.05$) decreased in erythrocytes of Fl intoxicated rats when compared with control. A significant ($p<0.05$) increased levels of erythrocyte esterase activity was observed after EGCG administrated compared to Fl alone treated rat. There was no improved activity of erythrocyte esterase found in EGCG alone treated rats when compared to control.

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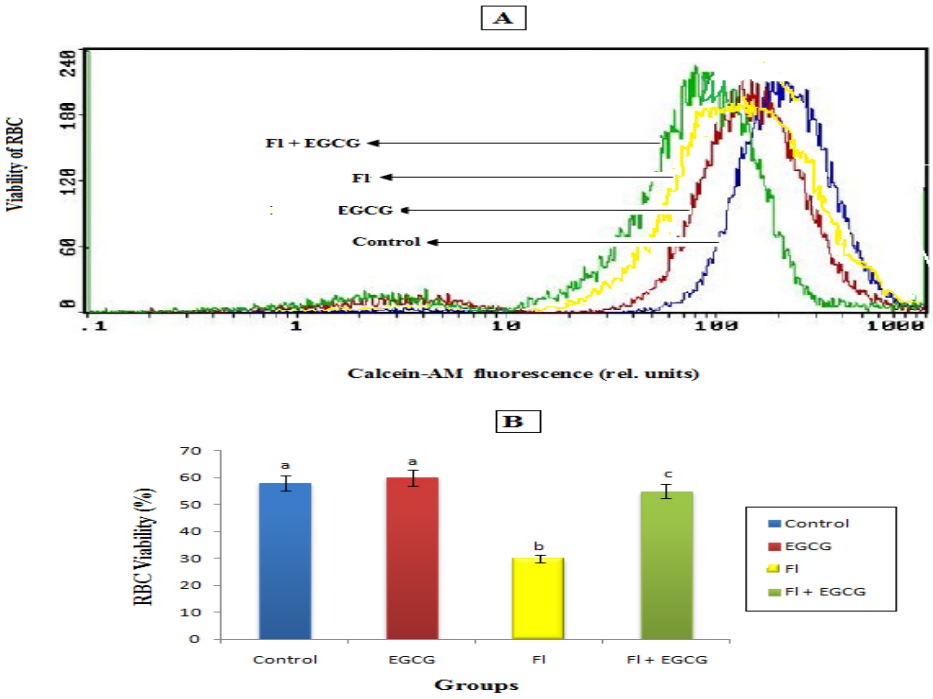

Fig. 4

Fig. 4 Effect of EGCG on rat erythrocyte viability. (a) Overlay of flow cytometric analysis of esterase activity in NaF (25mg/kg/BW) exposed of normal control rats. Abscissa – log scale of green fluorescence intensity of calcein-AM (FL1), ordinate – relative cell number. Mean fluorescent calcein intensity: Control–233, EGCG-235, NaF–190, and NaF+EGCG-230. Shown are the data from one representative experiment of 6 independent experiments giving similar results. Number of counted cells: 30,000. (b) Viability (mean ± SD, n = 6) of rat erythrocytes exposed to NaF (25mg/kg/BW) 4weeks. Values not sharing a common superscript letter (a–c) differ significantly at p<0.05 (DMRT).

Effect of EGCG on fluoride altered erythrocyte membrane integrity

The effect of EGCG on Fl altered membrane integrity of control and experimental rat erythrocytes were shown on Figure 5. A significant (p<0.05) increased hemolysis was decreased level of membrane integrity were observed in the Fl intoxicated rats when compared with control. Administration of EGCG significantly (p<0.05) decreased the hemolysis and increase membrane integrity of erythrocytes when compared to Fl alone treated rats. There was no significant hemolysis observed EGCG alone treated rat erythrocytes.

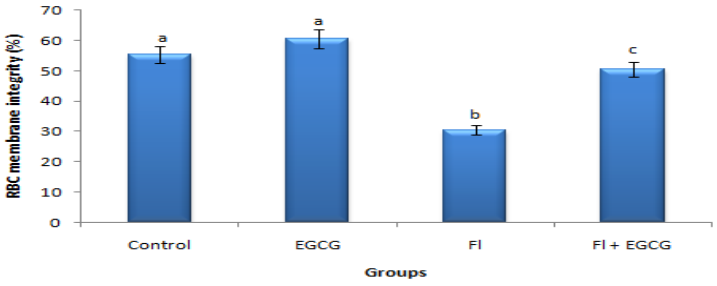

Fig. 5

Fig. 5 Effect of EGCG on RBC membrane integrity of control and experimental rats. Values are expressed as mean ± SD for groups of six rats in each. Statistical significance was determined by one way ANOVA followed by post hoc test. Values are given as mean ± SD for six rats in each group. Values not sharing a common superscript letter (a–c) differ significantly at p<0.05 (DMRT).

DISCUSSION

Oxidative stress describes a state of uncontrolled overproduction of free radicals beyond a threshold for proper antioxidant neutralization causing damage to normal cell. Fluoride consumption is associated with the production of free radicals which can react with polyunsaturated fatty acids to yield lipid hydroperoxides which in turn initiates a lipid-radical chain reaction leading to oxidative damage to cell membrane [47]. Erythrocytes, role and their propensity to generate radical species, may be considered as sensitive and intermediate cells in oxidative reactions [48]. The presence of iron a powerful transitional metal catalyst renders erythrocytes highly susceptible to

peroxidative damage [48]. The membrane of erythrocytes is rich in polyunsaturated fatty acid, a primary target for reactions involving free radicals and may allow the erythrocytes vulnerable to oxidative damage [49]. Among the different indicator used for identifying general health status, the body weight is one of the visible indicators of rats. In addition to the body weight we assessed the food and water changes in the relative control and experimental rats during 4 weeks, experimental period. Reduction in body weight gain and decreased water and food intake in Fl treated rats were found in our study. It has also been reported that Fl exposed rats showed decreased intake of water and food with retardation in growth rate and alterations in organ-body weights [50]. Morphological changes observed in Fl intoxicated rats were significantly attenuated by treatment with EGCG (40mg/kg BW) showed significant effective restoration of the morphological changes when compared to Fl treated rats. EGCG has already been reported to exhibit powerful hydrogen donating, antioxidant, and free radical scavenging properties, in a number of in-vitro systems and in-vivo models [51].

The hematological parameters are probably the more rapid and detectable variations under stress and are fuel in assessing different health conditions [52]. Hence, the haematological parameters in clinical and experimental studies in life sciences cannot be overemphasized. Particularly, literature reports have proved that the alterations in the haematological parameters, from normal state, may be used as valuable indicators of disease, or stress in different animal species [53]. In the present study we observed that F intoxicated rats showed significant altered hematological parameters due to over production of ROS which is in accordance with the reports of Marković *et al.* [54]. Other data from Sharma *et al.* [55] also reported that Fl altered the haematological parameters in female albino rats. Interestingly, EGCG-treated Fl-intoxicated rats showed significant renewal of these hematological parameters by bringing them back to near normal levels. This restoration was mainly due to the strong antioxidant property of EGCG and the presence of its vicinal trihydroxy structure, in which oxygen atoms act as electron donors to form bonds with electrophilic ions that thereby help in the recoupment of the antioxidant defense system [56]. The role of ROS mediated oxidative stress in Fl induced cell death via lipid peroxidation, thus causing erythrocyte membrane dysfunction was well established [57]. MDA, an end product of lipid peroxidation induced by free radical, and its content could reflect the level of lipid peroxidation in the erythrocytes promotes the degradation of membrane integrity and cell viability [58]. In addition to cellular lipids, studies have shown that cellular proteins may also be affected by free radical accumulation. The formation of carbonyl derivatives of proteins is suggested to be a useful measure of oxidative damage to proteins [59]. The carbonyl derivatives of proteins may result from oxidative modification of amino acid side chains and ROS-mediated peptide cleavage. In our study, we observed the increased levels of lipid peroxidation, protein carbonyl content and conjugated diens in the erythrocytes membrane of Fl treated rats. These results are similar with the previous report of shivarajashankara and shivashankara [60]. The administration of EGCG significantly reduced the levels of lipid peroxidation, conjugated diens and protein carbonylation in NaF-intoxicated rats, revealing the free radical scavenging ability of EGCG. This may be due to the presence of 8 hydroxyl groups in a 4 ring structure that is readily dissolved in water and modified into multifactorial components such as stearic, eicosapentaenoic, and docosahexaenoic acids during EGCG metabolism, which have been reported to exhibit enhanced ROS scavenging activity and thereby reduce the Fl-induced oxidative stress [61].

The present study revealed the alterations in the levels of nonenzymatic antioxidants in RBC membrane in response to Fl intoxication. Levels of reduced glutathione (GSH), Vitamins C and Vitamins E were significantly decreased with Fl treatment. These results are corroborates with the findings of Shanthakumari *et al*. [57] who found the decreased levels of non enzymatic antioxidants in Fl treated erythrocytes. Administration of EGCG to Fl intoxicated rat significantly increased the nonenzymatic antioxidant levels in to normal levels compared to control. This is may be due to presence of hydroxyl groups in EGCG which enhances the phase II antioxidant enzyme levels and offering protection against Fl induced oxidative stress.

Antioxidant enzymes, such as SOD, CAT, GPx, GR, GST and G6PD, are considered to be the first line of cellular defense against oxidative stress mediated injury. Assessment of these antioxidant enzymes is an appropriate indirect way to assess the prooxidant–antioxidant status in Fl-induced toxicity. Among them, SOD and CAT mutually function as important enzymes in the elimination of ROS and reactive nitrogen species. SOD is an enzyme responsible for the conversion of superoxide radicals into less harmful products like hydrogen peroxide, while CAT brings about the reduction of hydrogen peroxide and protects tissues from the highly reactive hydroxyl radicals [62]. NaF intoxication significantly reduced the activity of SOD and CAT in our study, which is in line with the finding of Montalvo *et al.* [63] in Fl-treated rats. Glutathione-related enzymes such as GPx, GR, and GST function either directly or indirectly as antioxidants. GPx is a selenium containing enzyme that uses glutathione in decomposing hydrogen peroxides to nontoxic products. In the present study, Fl administration lowered the activities of GPx, GR, and GST in membrane of RBC. Bruce *et al.* [64] reported that the decreased levels of GPx and GST activity and depleted levels of GSH in NaF-treated rats were mainly due to the overproduction of ROS, which is in accordance with the results of this study. GPX, GST, and GR are SH-dependent enzymes, and their SH groups are inactivated by Fl-induced ROS generation, leading to enzyme inactivation. EGCG administration significantly upregulated the levels of GPx, GST, and GR by restoring GSH levels and counteracting the free radicals produced by Fl intoxication. The reduction of G6PD activity in Fl-intoxicated rats showed impaired generation of NADPH, which is required for the reduction of GSSG to GSH [60]. EGCG administration to Fl-intoxicated rats showed significant

rejuvenation of these antioxidant systems back to near normal levels due to the antioxidant boosting nature of EGCG [65,66,67,68].

The diminished activity of membrane-associated enzymes like ATPases has been reported in many pathological conditions [69]. In the present study, a significant decrease in the activities of membrane-bound ATPases in the erythrocyte was observed in Fl-treated rats. Decreased activity of Na^+/K^+ ATPase could be due to enhanced lipid peroxidation by free radicals on F induction, since Na^+/K^+ ATPase is a "SH" group containing enzyme and is lipid dependent. Decreased activity of Na^+/K^+ ATPase can lead to a decrease in sodium efflux, thereby altering membrane permeability [70]. The disruption of membrane permeability or fragmentation of the membrane leads to the leakage of Ca^{2+} ions into cells thereby potentiating irreversible cell destruction. The Ca^{2+} overload medicated Fl also decreased the Ca^{2+} ATPase activity in cell membrane. It is generally accepted that due to high affinity for SH groups, Fl binds avidly to various enzyme proteins and inactivates them. Mg^{2+} ATPase activity is involved in other energy requiring process in the cell and its activity is sensitive to lipid peroxidation. Administration of EGCG in Fl intoxicated rats significantly reduced the level of lipid peroxidation in erythrocytes and sustained the activities of membrane bound enzymes. This may be due to the ability of EGCG to protect the SH groups from the oxidative damage through the inhibition of peroxidation of membrane lipids and stabilizes the membrane. The suppressed erythrocyte viability might be associated with the plasma membrane disruption leading to release of the cellular proteins including hemoglobin. It is indirectly inhibit the erythrocyte membrane integrity [71]. Fl-induced apoptosis was proved many reports that shown to be linked with activation of membrane G-proteins and subsequent stimulation of PKA-, PKC-, tyrosine kinase-, PI-3-kinase-dependent signaling pathways possibly converging at MAP kinases [72]. Moreover, Fl induces a pronounced oxidative stress, leading to the generation of reactive oxygen species (ROS), excessive lipid peroxidation (LPO) and alterations in activities of intracellular antioxidant enzymes such as catalase, superoxide dismutase and glutathione peroxidase [73]. In the present exploration, the viability and membrane integrity of erythrocytes was significantly decreased in Fl intoxicated rats due to over production of ROS which is in agreement with the previous reports of Anuradha *et al.* [74] and Tsai *et al*. [75]. Number of other reports were also attributed that Fl disrupt the outer mitochondria membrane which triggers the release of cytochrome C into cytosol and activates an intrinsic (caspases-9 and -3-dependent) apoptotic pathway and damage the erythrocytes [76]. Administration of EGCG significantly restored the membrane integrity and enhances the viability of erythrocytes in Fl intoxicated rats due to its strong hydrogen donating antioxidant nature which stabilizes the membrane and maintains the integrity and viability of erythrocyte membrane.

CONCLUSION

In conclusion, Fl exposure induces oxidative stress in rat erythrocytes by augmenting hemolysis, lipid peroxidation, protein oxidation and diminishing the activities of membrane bound ATPases, enzymatic and non-enzymatic antioxidants. Oral administration of EGCG counteracted the Fl induced oxidative stress in rat erythrocyte membrane probably by reducing the level of lipid peroxidation, protein oxidation and enhancing the activities of enzymatic and non- enzymatic antioxidants in the RBC membrane.

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