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Epigallocatechin gallate potentially ameliorates sodium fluoride-induced genotoxicity in rats.

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ABSTRACT
Fluoride compounds are naturally present in soil, water and food. The aim of this study was to investigate the possible ameliorative effect of Epigallocatechin gallate (EGCG) on sodium fluoride induced oxidative stress mediated genotoxicity in the bone marrow cells of rats. Rats were orally (gastric intubation) pretreated with EGCG (40mg/kg BW) followed by Sodium fluoride (NaF) (25mg/kg BW) daily for a period of 28days. NaF intoxicated rats showed a significant increase in the frequency of micronucleus (MN) in polychromatic erythrocytes (PCEs), structural chromosome aberrations (CA) with decreased mitotic index (MI) in bone marrow cells. We also found a significant increased DNA damage in bone marrow cells. Pre- treatment with EGCG (40mg/kg BW) significantly reduced the NaF induced genotoxicity as evidenced with the decreased micronucleus, chromosomal aberration and DNA damage, with increased mitotic index in rat bone marrow cells. In conclusion, the obtained data suggest that EGCG potentially protects bone marrow cells from NaF induced DNA damage and genotoxicity.

Keywords*:* EGCG, NaF, genotoxicity, rat, bone marrow, ros

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1. INTRODUCTION

Sodium fluoride (NaF) has been used ubiquitously for decades, due to its specific and effective caries prophylactic property; as well as used for water fluoridation. Excess fluoride ingestion is the cause of fluorosis in human being. The incidence of fluorosis affecting young and old, men and women is not only confined to India, but occurs in 23 other nations around the globe [1]. Fluoride (FL) enters the body through drinking water, food, toothpaste, mouth rinses, and other dental products; drugs and fluoride dust and fumes from industries using fluoride containing salt and hydrofluoric acid [2]. The fluorosis of human beings is mainly caused by drinking water, burning coal and drinking tea while that of animals is mainly by drinking water and supplementing feed additives such as calcium monohydrogen phosphate containing high levels of fluoride [3]. Intake of high levels of fluoride is known to cause structural changes, altered activities of enzymes, metabolic lesions in the brain and influence the metabolism of lipids [4]. Acute poisoning can terminate in death due to blocking cell metabolism since fluorides inhibit enzymatic processes, particularly metalloenzymes responsible for important vital processes [5].

There are number of Fl induced genotoxic studies done in vitro [6–8] using cell lines or *in vivo* [9, 10] are contradictory to the results that showed lack of genotoxic potential [11–13]. There was a report of increased chromosome aberrations in mice/rat bone marrow and testes *in vivo*, but other studies, using similar protocols and dose ranges, have reported Fl induced chromosome damage [14]. Based on the epidemiological, in vitro and in vivo studies in human, human cell lines and rodents respectively, the National Research Council-US report [15] on fluoride in drinking water noted that the genotoxic effects of fluoride at environmental concentrations are contradictory. Zeiger et al. [16] in their review considered chromosome damage induced by fluoride *in vivo* as an unresolved issue. In humans [1, 17, 18] and in animal models [10], a close association between chronic fluoride toxicity and increased oxidative stress has been reported. But there are ample evidences in favor of the genotoxic potential of fluoride in humans, in vitro as well as *in vivo*, exhibited by increased frequency of chromosomal aberrations, micronuclei induction, sister chromatid exchanges, etc. [19]. The minimal risk level for daily oral fluoride uptake was determined to be 0.05 mg/kg/day, based on a non-observable adverse effect level (NOAEL) of 0.15mg fluoride/kg/day for an increased fracture rate. Estimations of human lethal fluoride doses showed a wide range of values, from 16 to 64 mg/kg in adults and 3 to 16 mg/kg in children [20].

Efforts to prevent and treat fluoride induced genotoxicity by therapeutic measures had only limited success [21]. Consumers all over the world are becoming more conscious of the nutritional value, health benefits and safety of their food and its ingredients. In addition, there is a preference for natural functional food ingredients that are believed to be safer, healthier and less subject to hazards than their artificial counterparts. Evaluation of the functional properties of naturally occurring substances, especially those that are present naturally in human diets, has been of interest in recent years [22]. Green tea (*Camellia sinensis*) is one of the most commonly consumed beverages in the world and is a rich source of polyphenols known as catechins (30 to 36% of dry weight) including EGCG, which constitutes up to 63% of total catechins.The main flavonoids present in green tea is catechins; among them, epigallocatechin 3- gallate (EGCG) has the highest antioxidant capacity [23]. EGCG has many biological functions such as antioxidant [24], antimutagenic [25] and anti carcinogenic effects [26]. EGCG has been shown to be 25 to 100 times more potent than vitamins C and E in terms of antioxidant activity. A cup of green tea typically provides 60 to 125 mg catechins, including EGCG. Scientific reports proved that EGCG possesses many of the structural components that contribute to their antioxidant property. EGCG has a gallate moiety esterified at the 3rd position of the C ring, the catechol group (3,4,5-trihydroxyl groups) on the B ring and the hydroxyl groups at the 5th and 7th positions on the A ring (Fig. 1).

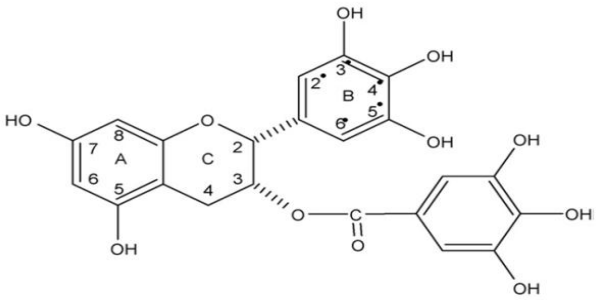

Fig. 1

Fig. 1. The structure of Epigallocatechin gallate

The potent free radical scavenging activity of EGCG was attributed to the presence of the C ring gallate group. The observation was also made that the more hydroxyl groups in the catechins (EGCG) possess, more effective free radical scavenger the catechins becomes [27]. In this work, we have intended to investigate the therapeutic potentials of EGCG by evaluating chromosomal aberrations, micronucleus formations,

mitotic index and DNA strand breaks in bone marrow cells of NaF intoxicated rats.

2. MATERIALS AND METHODS

2.1 Chemicals

Sodium fluoride (NaF), Epigllaocatechin gallate (EGCG), 1,1',3,3'- tetramethoxy propane, bovine serum albumin were purchased from Sigma Chemical Co., St. Louis, MO, USA.

Normal melting point agarose (NMA), low melting point agarose (LMPA), ethidium bromide (EtBr), triton X-100 were purchased from Sigma–Aldrich Co. (USA). 2-nitrobenzioc acid, dithiothreitol, di-sodium salt of ethylenediaminetetra acetic acid (EDTA), were purchased from Hi Media, Mumbai, India. Giemsa, Tris buffer, dimethyl sulfoxide (DMSO), trichloroacetic acid, methanol, hydrogen peroxide, acetic acid, potassium chloride, sodium citrate monohydrate, sodium hydroxide (NaOH), sodium chloride (NaCl) were purchased from Himedia Laboratories Pvt. Ltd., India. All other chemicals and solvents were of certified analytical grade and purchased from S.D. Fine Chemicals, Mumbai, India. Reagent kits were obtained from span Diagnostics, Mumbai, India.

2.2 Animals and diet

Healthy adult male albino rats of Wistar strain, bred and reared in the Central Animal House, Department of Experimental Medicine, Rajah Muthiah Medical College, and Annamalai University were used for the experiment. Males were preferred to avoid complications of the estrous cycle. Animals of equal weight (160–180 g) were selected and housed in polypropylene cages lined with husk and kept in a semi natural light/dark condition (12 h light/12 h dark). The animals had free access to water and were supplied with standard pellet diet (Amrut Laboratory Animal Feed, Pranav Agro Industries Ltd., Bangalore, India), a constituent of protein (22.21%), fat (3.32%), fiber (3.11%), balanced with carbohydrates (> 67%), vitamins and minerals. Animal handling and experimental procedures were approved by the Institutional Animal Ethics Committee, Annamalai University (Registration Number: 952/2012/CPCSEA) and the animals were carried in accordance with the "Guide for the care and use of laboratory animals" and "Committee for the purpose of control and supervision on experimental animals."

2.3 Experimental design

In the present study, NaF was administered orally (gastric intubation) at a dose of 25 mg/kg body weight/day for 4 weeks, which was 1/10 of the oral LD_{50} value of NaF in rats [28]. The EGCG (dissolved in 10% of Tween 80) was administrated orally (gastric intubation) at a dose of 40mg/kg body weight/day daily for 4 weeks. In the experiment totally 24 rats were used. The rats were randomly divided into 4 groups of 6 animals in each.

Group 1: (n = 6) Control rats were administrated orally with normal saline (0.5 ml/day) for 4 weeks .

Group 2: (n = 6) Rats were orally administrated with EGCG alone (40 mg/kg BW/day)[29] for 4 weeks

Group 3: (n = 6) Rats were treated with NaF alone (25 mg/kg BW/day) for 4 weeks

Group 4: (n = 6) Rats were pretreated with EGCG (90 min before) followed by NaF (doses as prescribed above)for 4 weeks

After the last treatment, rats were fasted overnight and were anesthetized with pentobarbital sodium (35 mg/kg, IP) and euthanized by cervical decapitation. The femur bones were removed for the collection of bone marrow cells from control and experimental rats and were used for the micronucleus test, chromosomal aberration, mitotic index and comet assay.

2.4. Micronucleus (MN) assay

The micronucleus test was carried out in rat femoral bone marrow cells and frequencies of micro nucleated-polychromatic erythrocytes (MN-PCE) were evaluated according to the method of Schmid with minor modifications [30-32]. The femoral bone marrow cells were aspirated using syringe and needle (21 G) with 3.0 ml of fetal bovine serum and centrifuged at 800 × g for 10 min. The supernatant was discarded; pellet was mixed, smeared on clean glass slides and fixed in methanol for 5 min. The fixed smear was stained with undiluted May–Gruenwald stain for 5 min followed by diluting May–Gruenwald stain (1:1, v/v in distilled water) for 3 min. The slides were washed with distilled water, stained with Giemsa (10%, v/v in Sorenson buffer) for 10 min and observed under a light microscope (Carl, Zeiss, Berlin, Germany). All slides were coded and scored blind. The incidence of micro nucleated (MN) cells per 500 polychromatic erythrocyte (PCE) was determined for each animal and the percentage of PCEs with MN was calculated. Thousand erythrocytes were scored from each animal to calculate the ratio of polychromatic erythrocyte (PCEs) to normochromatic erythrocytes (NCEs) and the toxic effect of NaF to bone marrow cells was evaluated.

2.5. Chromosome Aberration assay

The chromosome aberration test was performed on rat bone marrow cells with slight modifications [33]. The femoral bone marrow cells were aspirated in RPMI 1640 media and centrifuged at 800x g for 10 min, the pellet was incubated in 8.0 ml of KCl (0.075 M) at 37◦C for 30 min, followed by centrifugation at 800x g for 5 min. The

cells were fixed in Carnoy's fixatives (glacial acetic acid/methanol, 1:3, v/v), washed thrice with Carnoy's fixative at intervals of 10 min. The pellet was resuspended in fixative and dropped on chilled slides from the height of 2–2.5 ft, and air dried. The slides were stained with freshly prepared Giemsa stain (8%, v/v in Sorenson buffer) for 10 min followed by washing with distilled water. A total number of 50 metaphases per animal were evaluated for chromosomal aberrations. The types of aberrations were scored and recorded strictly in accordance with the method of Tice and Ivett [34]. The metaphase cells were scored at 1000x magnification, with selection being based on uniform staining quality, lack of overlapping chromosomes and chromosome number (40±2 chromosomes). Each chromosome aberration recorded was of the following types: A*, A**, as chromatid and isochromatid gaps; B*, B**, as chromatid and chromosome breaks, C* as chromatid rearrangement. Responses were evaluated as the percentage of aberrant damaged metaphase cell (%DC) and as the number of aberrations per cell (CA/cell). Chromatid and chromosome gaps were recorded but were not included in calculations. For a count of the number of CA/cell, chromatid and chromosome breaks, and chromatid rearrangements (dicentric, ring, exchanges) were taken as one, regardless of the number of breakage events involved .

2.6. Mitotic index determination

The mitotic index was used to determine the rate of cell division. The slides prepared for the assessment of chromosomal aberrations were also used for calculating the mitotic index. Randomly selected cells on the slides were monitored to determine the number of dividing cells (metaphase stage) and the total number of cells. At least 1000 cells were examined in each preparation.

2.7. Single cell gel electrophoresis (Comet assay)

The alkaline comet assay was performed according to the method of Singh et al. [35] with minor modifications. Animals were sacrificed and femurs (bone marrow cells) were removed and then single cell suspension was prepared as previously described by Sasaki et al. [36]. Briefly, bone marrow cells were minced in 0.075 M NaCl solution containing 0.024 M Na_2EDTA (pH 7.5). Followed by the centrifugation, the cells were resuspended in phosphate buffered saline. The bone marrow cells were washed in 0.075 M NaCl solution containing 0.024 M Na_2EDTA (pH 7.5) and resuspended in phosphate buffered saline. Slides were prepared by mixing the cell suspension with 1% low melting point agarose; layered on the slide base coated with 1% normal melting point agarose and placed in a chilled lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Trizma, 10% DMSO, and 1% Triton X-100, pH 10.0) at 4 ◦C for 1 h. Then the slides were subjected to DNA unwinding in chilled alkaline solution (300 mM NaOH and 1 mM Na_2EDTA, pH >13) for 20 min and subsequently electrophoresis was performed at 0.7 V/cm and 300 mA at 4 ◦C for 25 min in freshly prepared electrophoresis buffer (1 mM EDTA disodium salt and 300 mM NaOH). After electrophoresis the slides were neutralized with Tris buffer (400 mM, pH 7.4). Slides were stained with 20µg/ml Ethidium bromide (EBR) (1:10,000) dilution) and stored at 4 ◦C in a humidified slide box until scoring. Slides were scored at a final magnification of 400 ×using an image analysis system (Komet 5.5, Kinetic Imaging, Andor Technology, Nottingham, UK) attached to a fluorescence microscope (Leica, Germany) equipped with an attachment of a CCD camera. The comet parameters used to measure DNA damage in the cells were tail DNA (%). Images from 150 random cells (per animal) were analyzed as per the guidelines [37].

2.8. Statistical analysis

Statistical analysis (Mean ± SD) was performed by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT) using Statistical Package for the Social Science (SPSS) software package version 16.00. Results were expressed as mean ± standard deviation for six rats in each group. Followed by the post hoc test, least significant difference (LSD). Values were considered statistically significant when $p<0.05$.

3. RESULTS:

3.1. Genotoxicity biomarkers

3.1.2. Effect of EGCG on Micronucleus (MN) test

The effects of NaF on frequency of MN-PCEs and PCE/NCE ratio in rat bone marrow cells of control and experimental rats are presented in Table 1.

Groups	% MN-PCE (Mean ± SD)	% PCE/NCE (Mean ± SD)
Control	0.06 ± 0.002[a]	2.10 ± 0.02[a]
EGCG	0.05 ± 0.001[a]	2.12 ± 0.03[a]
Fl	0.24 ± 0.012[b]	1.50 ± 0.02[b]
EGCG + Fl	0.13 ± 0.007[c]	2.01 ± 0.04[c]

Table 1. Shows the micro nucleated polychromatic erythrocyte (MN-PCEs) in the bone marrow cells of control and experimental rats.

Values are given as mean ± SD from six rats in each group. [a-c] values with different superscript letter (a-c) in the same column differ significantly at P<0.05 (DMRT).

A significantly (P<0.05) increased in number of MN-PCEs with significant (P<0.05) decreased PCE/NCE was observed in rat exposed to NaF treated rats compared with control. The cytotoxic effect of NaF on

bone marrow cell was tested by assessing polychromatic erythrocyte/normo chromatic erythrocyte (PCE/NCE) ratio. Pre administration of EGCG along with NaF intoxicated rats showed a significant (P<0.05) decreased MN-PCEs with significant (P<0.05) increased PCE/NCE when compared with NaF alone treated rats. There was no significant change between the control and EGCG alone treated rats.

3.1.3. Effect of EGCG on Chromosome aberration (CA)

The chromosome aberrations observed in bone marrow metaphase cells of rats are depicted in Table 2.

Table 2. Shows the chromosomal aberration in bone marrow cells of control and experimental rats.

Values are given as mean ± SD from six rats in each group. [a-c] values with different superscript letter (a-c) in the same column differ significantly at P<0.05 (DMRT).

Abbreviations used: Fl: Fluoride, EGCG: Epigallocatechin gallate, A*: Chromatid gaps; A**: Chromosome gaps; B*: Chromatid breaks; B**: Chromosome breaks; C*: Chromatid rearrangement; D*: Chromosome deletion.

Groups	Chromosomal aberrations							No. of aberrations	Average No. of Aberration Mean ± S.D
	A*	A**	B*	B**	C*	D*	Polyploidy		
Control	2	0	1	0	-	2	3	8	1.65 ±1.02[a]
EGCG	1	0	1	2	-	3	7	7	1.58 ±1.01[b]
Fl	80	22	48	56	-	35	23	264	62.32 ±23.27[c]
EGCG + Fl	31	4	21	28	-	10	8	102	15.35 ±8.12[d]

NaF intoxicated rats showed a significant (P<0.05) increased frequency of chromosome aberrations when compared with control. Pre administration with EGCG to NaF intoxicated rats discovered a significant (P<0.05) decreased in the chromosomal aberrations compared with NaF alone treated rats. EGCG alone pre administrated rats did not exhibit any changes compared with control.

3.1.4. Effect of EGCG on mitotic index

The mitotic index was used to determine the rate of cell division in bone marrow cells of exposed to control and experimental rats (Fig 2).

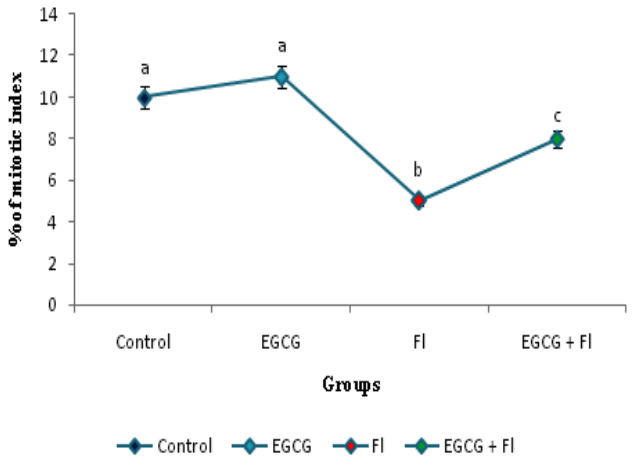

Fig. 2

Fig. 2. Effect of EGCG on mitotic index of bone marrow cells of control and experimental rats. Statistical significance was determined by one way ANOVA followed by post hoc test. Values are given as mean ± SD from six rats in each group. Bars with different superscript letter (a–c) differ significantly at p<0.05 (DMRT).

The mitotic index was significantly decreased in NaF intoxicated rats compared with control rats. Pre administration with EGCG to NaF treated rats showed significant increased mitotic index when compared with NaF alone treated rats. EGCG alone treated rats have also shown similar index of the control.

3.1.5. Effect of EGCG on Comet assay

Figures 3 and 4 display the level of DNA damage in control and experimental rats. A significant (P < 0.05) increase in different comet assay parameters such as % tail length (Fig.4A) and tail moment (Fig.4B) was noted in rats treated with NaF when compared with the control rats. Pre-oral administration of EGCG with NaF significantly (P < 0.05) reduced the NaF-induced DNA damage to near control levels. Control rats and rats treated with EGCG alone showed minimal or no DNA migration.

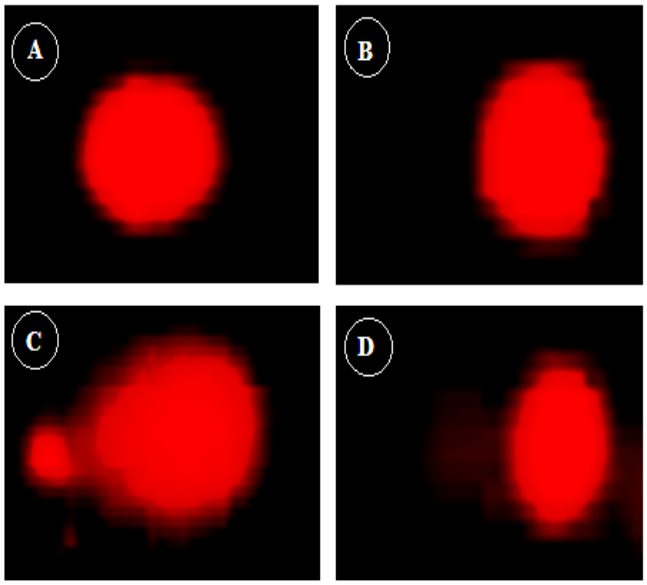

Fig. 3

Fig. 3. Representative photomicrographs of comets stained with ethidium bromide showing the DNA migration pattern in bone marrow cells (A) Control group shows no DNA migration. (B) Epigallocatechin gallate (EGCG)-treated group shows no DNA migration. (C) NaF-treated group shows extensive DNA migration. (D) EGCG-treated + NaF-intoxicated group shows minimal DNA migration. Magnification ×200.

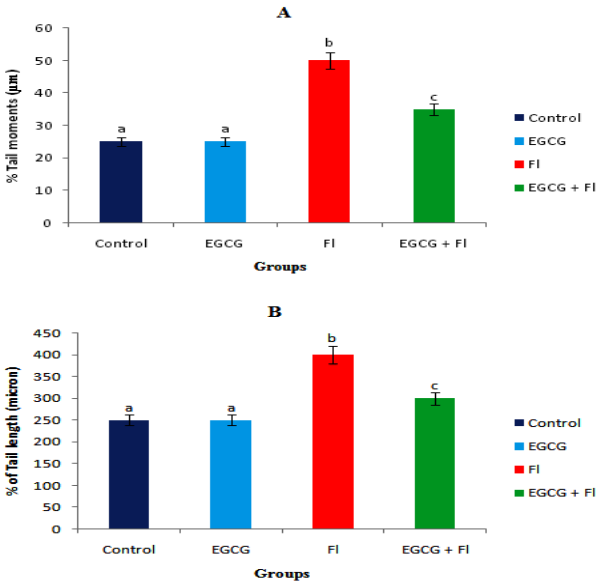

Fig. 4

Fig. 4. Effect of Epigallocatechin gallate (EGCG) on DNA damage (in terms of tail moment (4 A) and % of tail length (4 B)) in the bone marrow cells of control and experimental rats. Values are the mean ± SD for 6 rats in each group. Bars with different superscript letter (a–c) differ significantly at p<0.05 (DMRT).

4. DISCUSSION

The micronucleus (MN) is widely used as a screening method for detecting the clastogenicity of chemicals. MN confirming its sensitivity as biomarkers of genotoxicity [38]. NaF induced ROS have a major role in the mediation of cellular damage and involvement of ROS in genotoxicity has been well documented [39]. The excessive formation of ROS by fluoride leads to oxidative stress, which can trigger cell damage by oxidizing macromolecular structures and modifying their biological functions that ultimately causes cell cycle arrest and cell apoptosis [40]. In our present study, we observed increased levels of MN in the bone marrow cells of NaF treated groups, which was similar to the previous report of Khalil [41]. Similar contradictory finds on the genotoxic potential of NaF in mammalian cells using *invitro* and *invivo* systems have also been reported [42, 43]. However, rats pretreated with EGCG along with NaF showed significantly decreased levels of MN number and ROS generation which could be due to its free radical scavenging nature. These agree well with the existing literature of EGCG as an antioxidant during various oxidative insults [44].

From the present observation clearly showed a significant increase in the frequency of structural chromosomal aberration (CA) in bone marrow cells were exposed to NaF. It is well predictable that NaF can damage the DNA and cause chromosomal aberrations from previous findings [45, 46]. It has been suggested that oxidative stress can be a possible mechanism through which fluoride induce damage to DNA molecules in various tissues. Fluoride appears to be a mutagenic agents and induce chromosome aberrations, sister chromatid exchanges, and cytotoxic effects in cultured mammalian cells [47]. In addition, Tiwari et al [48] who reported that low fluoride concentration caused increased chromosomal aberration and DNA damage in human peripheral blood culture. The EGCG pre administration significantly decreased the level of chromosomal aberrations in NaF intoxicated rats due to its strong antioxidant and upregulation of transcription Nrf2 factors, which is involved in the cytoprotective gene regulation by EGCG. This corroborates with previous reports where cytoprotective effects of EGCG have been observed by induction of ARE driven transcription pathway [49].

Mitotic index is a measure for the proliferation status of a cell population. It is defined as the ratio between the number of cells in mitosis and the total number of cells [50].]. In eukaryotic cells, the cell cycle is divided into two brief periods: Interphase and mitosis. The interface has three phases: G1 (cells increase their size), S (DNA replication occurs) and G2 (significant protein synthesis occurs, mainly for the production of

microtubules). The mitosis (M) phase consists of karyokinesis (nuclear division) and finally cytokinesis (cytoplasm is divided into two cells). It has been suggested that fluoride has differential effects on mitosis depending on the cell type [51]. However, some studies provided the evidence that, fluoride exposure on cell cycle progression are closely related to the fluoride concentration. The activation of mitogen activated protein kinases (MAPK), such as p38 and JNK, has been proposed as a possible mechanism for the downstream of G-protein activation by which fluoride exerts its toxic effects on the cell cycle [52]. In the present study, clearly demonstrates that administration of NaF significantly decreased the mitotic index due to arresting of cell cycle by ROS during fluoride induced toxicity which is in line with existing literature of Manivannan et al [53]. However, pre-treatment with EGCG significantly elevated the mitotic index level in NaF treated rats. This may be due to the antigenotoxicity and anticlastogenic activity of EGCG which might be due to the presence of more hydroxyl groups, possess more effective free radical scavenger and it has a potential to bind with the DNA thereby preventing the reaction of free radicals with DNA [54].

The alkaline single-cell gel electrophoresis (SCGE or comet assay) is a rapid and sensitive procedure for quantitating DNA lesions in mammalian cells. This assay enables the assessment of genetic damage in vivo and in vitro in a great variety of cells [55]. The levels of % tail length, and tail moment significantly increased in the bone marrow cells of NaF-treated rats when compared with the control group. Previous reports have shown that lipid peroxidation products of polyunsaturated fatty acids play a major role in the genotoxicity of cells . Several mutagenic and genotoxic lipid peroxidation products, in particular malondialdehyde and 4-hydroxy-2-nonenal, have been shown to bind to DNA and to damage it . A loss of DNA integrity in the form of single strand breaks has been recorded during exposure to NaF. . NaF has a dense negative charge and is biochemically very active, and thus directly affects DNA because of its strong affinity for uracil and amide bonds by the interaction of –NH and F– [56]. In the present study, we observed that NaF intoxicated rats showed significant increase in DNA damage which was in line with the reports of Manivannan et al. [53] who reported that NaF can induce the production of free radicals, which can damage DNA strands directly or by lipid peroxidation initiated by free radicals in bone marrow cells. EGCG pre-administration to NaF treated rats showed decreased DNA damage with increased antioxidant status thereby protecting DNA damage in bone marrow cells. Probably, scientific reports showed that catechin (EGCG) possess many of the structural components that contribute to their antioxidant property. Catechins have a gallate moiety esterified at the 3rd position of the C ring, which is efficiently scavenging free radicals and thereby preventing the ROS induced DNA damage in cells.

In conclusion, the data from the present study suggest that pre-administration of EGCG in NaF intoxicated rats significantly ameliorate genotoxicity via its antioxidant and free radical scavenging capacity. The natural polyphenolic antioxidants like EGCG have excellent scope to counteract against NaF-induced oxidative-stress mediated genotoxicity in exposed subjects via their occupational and environmental settings.

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