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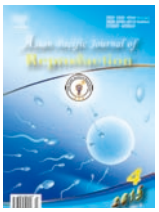

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Epigallocatechin gallate exacerbates fluoride-induced oxidative stress mediated testicular toxicity in rats through the activation of Nrf2 signaling pathway

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ABSTRACT

Objective: To explore the ameliorative potential of epigallocatechin gallate (EGCG) by evaluating markers of oxidative stress, apoptosis, and inflammation and antioxidant competence in Fl intoxicated rats. **Methods:** The animals were divided in to four groups that is control, EGCG alone, NaF, and EGCG with NaF. Group III animal were exposed to Fl as sodium Fluoride (NaF) (25 mg/kg BW) for 4 weeks. After the completion of the treatment, the testis tissues has been removed and used for the experimental observations. **Results:** Pre-administration of EGCG to Fl intoxicated rats showed a significant normalization in the levels of steroidogenic enzymes, testosterone, sperm functions, oxidative stress markers and antioxidant status. The altered levels of proinflammatory cytokines and apoptotic markers were also relapsed in close proximity to control. In addition, EGCG significantly improved antioxidant status and reduced the oxidative stress and pathological changes in testes. The mRNA and protein analysis also substantiated that EGCG pre-treatment markedly enhanced the expression of Nrf2 and its target genes HO-1, NQO1 and γ GCS and suppressed the expression of Keap1 in testis. **Conclusion:** Altogether, our findings supports that EGCG attenuates Fl toxicity in testis through Nrf2 activation.

1. Introduction

Fluoride (Fl) is an essential trace element to the human body, for decades it has been employed for the prevention of dental caries [1]. Meanwhile, as a pervasive natural pollutant, fluoride can normally enter into the human body through drinking water, food, industrial pollution, drugs, cosmetics, *etc*. However, drinking water is the major source of daily intake of fluoride [2]. Accumulating evidences illustrated that in addition to hard tissues like teeth and skeleton, excessive fluoride exposure can also cause injury to the soft tissues such as, brain, heart, liver, kidney and testis [3, 4]. These findings heightened the concerns regarding the multi-exposure routes as well as the multi-organ toxicity elicited by fluoride. The reproductive toxicity is of great concern to the public, as clearly demonstrated by epidemiological studies that environmental exposure to fluoride was associated with male infertility and low birth rates of people living in the area of endemic fluorosis [5, 6]. However, increased evidences demonstrated that fluoride exposure also affected the soft tissues including testes [7]. Epidemiological data have also indicated that fluoride may adversely affect the reproductive systems of men living in endemic areas of fluorosis [8].

A number of mechanisms have been proposed to explain fluoride induced testicular toxicity. Among them, oxidative stress has been observed as the major cause for its toxicity in tissues, liver, kidney, brain, and testes in animals and in people living in areas of endemic fluorosis [9-13]. Fluoride has also been reported to hamper the

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activity of many antioxidant enzymes such as superoxide dismutase, glutathione peroxidase, and catalase. Moreover, fluoride can deplete glutathione levels, often resulting in too much production of reactive oxygen species (ROS) at the mitochondrial level, and directing to the damage of cellular components [14, 15]. Although the fertility of spermatozoa mainly on the maintenance of mitochondrial transmembrane potential ($\Delta\psi$m) which could be altered by Fl, proposing a possible mechanism of Fl induced toxicity in testes[16]. However, the possible mechanism(s) by which Fl damages the testicular function remains ambiguous.

Recently, mounting alertness in finding natural antioxidant phytoconstituents from plants has been drawn more concern. Plant materials and products are rich repository of an array of biologically active compounds such as antioxidants and free radical scavengers. Flavonoids are the polyphenolic entities richly available in plants and served as indispensable components of the human diet. The flavonoids have been suggested to exhibit a powerful antioxidant capacity because of their supremacy to scavenge free radicals, together with the upregulation of antioxidant defences. Catechins are the principal ingredients of green tea and primarily consist of epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG), and epigallocatechin gallate (EGCG) [17]. Epigallocatechin gallate (EGCG) has been well established polyphenol from green tea and is widely used against oxidative damage, because of its antioxidant ability to repress the free radical mediated inflammation[18, 19]. It has already been reported to protect the testes against ischemia/reperfusion-induced apoptosis in rat [20]. EGCG is also been exploited as a nutritional supplement because of its countless health benefits and affirmative property that surpasses any other usual antioxidants.

To the best of our knowledge, this is the first study concerning the effect of EGCG against Fl-induced oxidative testicular injury. Hence, the present study has been designed to evaluate the defensive role of EGCG on Fl-provoked testicular toxicity and provide proof for its molecular mechanism of protection with particular attention to the oxidative stress, inflammatory and apoptotic pathways.

2. Materials and methods

2.1. Chemicals

Sodium fluoride (NaF), Epigallocatechin gallate (EGCG), bovine serum albumin was purchased from Sigma Chemical Co., St. Louis, MO, USA. Nrf2, HO-1, γGCS, NQO1, Keap-1, Bcl-2, Bad, caspase-3, and cytochrome-C, antibodies were purchased from Santa-Cruz Biotechnology, Inc, USA and Goat anti-rabbit, anti-mouse and Rabbit anti-goat secondary antibodies were purchased from Genei, Bangalore, India. All other chemicals and solvents were of certified analytical grade and purchased from S.D. Fine Chemicals, Mumbai or Himedia Laboratories Pvt. Ltd., Mumbai, India. Reagent kits were obtained from span Diagnostics, Mumbai, India.

2.2. Animals

This study used 24 healthy adult male albino Wistar rats weighing 270 ±10 g. The animals were purchased from the Central Animal House, Faculty of Medicine, Annamalai University, Annamalainagar. They were kept in well-ventilated plastic cages under standard laboratory conditions. The animals were housed in a laboratory-controlled environment (25 ℃, 50% humidity) and the lighting schedule was maintained at 12 h of light per day. Feed (Lipton India Ltd, Mumbai, India) and water were provided ad libitum. All animal experiments were conducted in accordance with the Ethical Norms on Animal Care and use approved by the Institutional Animal Ethical Committee (IAEC) of Rajah Muthiah Medical College and Hospital (Reg. No. 952/2012/CPCSEA), Annamalai University, Annamalai nagar, India.

2.3. Experimental design

In this experiment, a total of 24 rats were used. Six rats were used in each group. Two rats from each group were used for histological study. The 4 groups were as follows: group I, normal control rats treated with vehicles alone daily for four weeks. Group II, normal rats treated with EGCG dissolved in 10% tween 80 with a daily dose of 40 mg/kg for 4 weeks [21]. Group III, rats were orally treated with NaF in saline with a daily dose of 25 mg/kg body weight for 4 weeks[22]. Group IV rats orally pre-administered with EGCG at an interval of 90 minutes and then administered with NaF in normal saline with a daily dose of 25 mg/kg body weight for 4 weeks.

At the end of the experimental period, rats were fasted overnight and all the rats were anesthetized and then sacrificed by cervical decapitation. Blood was collected and serum and plasma were separated by centrifugation. The final body weight, organ weight of the rats was recorded. The testes were excised, removed the connective tissues, weighed and homogenized in 100 mM potassium phosphate buffer containing 1mM EDTA, pH 7.4 using a Potter-Elvehjem type homogenizer and centrifuged at 12 000×g for 30 min at 4 ℃. The supernatant was collected and used for various biochemical assays. Testes were collected at 24 h after Fl intoxication. Testes were divided in two parts: left one was kept at -80 ℃ for Western blotting and apoptosis study. The right one was used for the histological and immunohistochemical study. The testes were fixed in Bouin's solution for 1-2days, embedded in paraffin by routine method, serially sectioned at 5 μm thickness and stained in hematoxylin/eosin for light microscopic evaluation.

2.4. Determination of reactive oxygen species (ROS) in testes

The amount of reactive oxygen species (ROS) in testes was measured using 2', 7'-dichlorofluorescin diacetate (DCF-DA), which gets converted into highly fluorescent DCF by cellular peroxides (including hydrogen peroxide). The assay was performed as described by us previously. Briefly, the testes was homogenized in 1 mL of ice-cold 40 mM Tris-HCl buffer (pH 7.4) and dilute (0.25%) with the same buffer and placed on ice. The samples were divided in to two equal fractions. In one fraction 40 μL of 1.25 mMDCF-DA in methanol was added for ROS estimation. Another fraction, to which 40 μL of methanol was added, served as a control autofluorescence. All the samples were incubated for 15min in a 37 ℃ water bath. Fluorescence was determined at 488nm excitation and 525 nm emission using fluorescence plate reader.

2.5. Determination of Δ^5 3 β -HSD and Δ17 β -HSD activities

Testicular Δ^5 3 β -HSD and Δ17 β -HSD activities were measured spectrophotometrically following the methods of Talalay [23] and Jarabak *et al* [24] respectively.

2.6. Determination of plasma testosterone level

Plasma testosterone level in all experimental rats was determined by an enzyme immunoassay kit (Assay Designs, Ann Arbor, MI, USA).

2.7. Determination of epididymal sperm count and motility

Epididymis sperm count and sperm progressive motility were evaluated by the method of Linder *et al* [25]. Accordingly; epididymal spermatozoa were obtained by mincing the epididymis with anatomical scissors in 5 mL of physiological saline and incubated at 32 ℃ for 2 min. An aliquot of this solution was placed in Neubauer hemocytometer and motile sperm were counted by using a microscope at 400 × magnification. Non-motile sperm numbers were first determined, followed by counting of total sperm.

Sperm motility was expressed as a percent of motile sperm of the total sperm counted. Percentage of morphologically abnormal spermatozoa was determined by the method described by Evans and Maxwell [26]. According to this method, slides were prepared with Wells and Awa stains for morphological examination and 1% eosin B and 5% nigrosine in 3% sodium citrate dehydrate solution for live-dead ratio. A total of 400 sperm cells were counted on each slide under a light microscope at 100 × magnification.

2.8. Determination of mitochondrial membrane potential (Δ ψ m)

Testes mitochondria were isolated following the method of Hodarnau *et al* [27]. Mitochondrial membrane potential (Δ ψ m) was estimated on the basis of cell retention of the fluorescent cationic probe Rhodamine 123 [28].

2.9. Determination of lipid peroxidation and oxidative stress markers

Lipid peroxidation was estimated spectrophotometrically by measuring thiobarbituric acid reactive substances and lipid hydroperoxides by the method of Niehius and Samuelson, Jiang et al. respectively [29, 30]. Protein carbonyl content was determined by the method of Levine *et al* [31]. The levels of conjugated dienes were assessed by the method of Rao and Recknagel [32].

2.10. Determination of non enzymatic antioxidant levels in testes

Reduced glutathione and Total sulfhydryl groups were determined by the method of Ellman [33]. Vitamin C concentration was measured as previously reported Omaye *et al* [34]. Vitamin E was estimated by the method of Desai [35].

2.11. Determination of enzymatic antioxidant levels in testes

Superoxide dismutase activity was determined by the method of Kakkar *et al* [36]. The activity of catalase was determined by the method of Sinha [37]. Glutathione peroxidase activity was estimated by the method of Rotruck *et al* [38]. Glutathione S-transferase activity was determined by the method of Habig *et al* [39]. Glutathione reductase was assayed by the method of Horn and Burns [40]. The estimation of glucose-6-phosphate dehydrogenase was carried out by the method of Beutler [41]. The total protein content of tissue homogenate was estimated as described previously by Lowry *et al*[42].

2.12. RT-PCR

Total RNA was isolated from the testes using the TRizol reagent (Invitrogen Corp., Carlsbad, CA, USA) according to the manufacturer ' s instructions. The A260/280 ratio was found to be in the range of 1.8–2.0, and total RNA was also electrophoresed on a 1% agarose gel (Sigma, St. Louis, MO) to visually assess RNA quality. The total RNA was converted into cDNA with 3 μ g total RNA using Revert Aid First Strand cDNA Synthesis Kits (Fermentas, Hanover, MD, USA). The gene-specific primers were designed based on the corresponding mRNA sequences with Primer Version 5.0 (Table 1). To obtain the relative quantitative values for gene expression, the housekeeping gene β -actin was used as an internal control. Real-time PCR reactions were performed in an ABI PRISM 7900 HT PCR system (Applied Biosystems, Framingham, MA, USA), and SYBR Green PCR Master Mix reagent kits (Applied Biosystems, Foster City, CA) were used according to the manufacturer ' s instructions. The conditions for real-time PCR were as follows: 95 ℃ for 10 min followed by 40 cycles of 95 ℃ for 15 s, 60 C for 1 min. After PCR, a melting curve analysis was performed to demonstrate the specificity of the PCR product. Every sample was analyzed in triplicate. The fluorescence threshold value was calculated using SDS 2.2.1 system software and the 2^{-Ct} method was applied to the quantitative calculation [43].

Table 1
Effect of EGCG on Fl induced oxidative stress parameters in control and experimental rats.

Groups	TBARS (nmoles/ g tissue)	LOOH (nmoles/g tissue)	PC (nmoles/mg protein)	CD (nmoles/mg protein)
Control	21.29 ± 0.96[a]	0.82 ± 0.10[a]	1.58 ± 0.10[a]	1.80 ± 0.15[a]
EGCG	21.72 ± 1.10[a]	0.84 ± 0.02[a]	1.48 ± 0.12[a]	1.70 ± 0.10[a]
Fl	32.77 ± 1.13[b]	1.90 ± 0.52[b]	3.30 ± 0.28[b]	4.57 ± 0.25[b]
EGCG + Fl	25.38 ± 1.09[c]	0.80 ± 0.45[c]	1.88 ± 0.15[c]	2.37 ± 0.14[c]

Values are given as mean ± SD for six rats in each group. Values with different superscript letter (a-c) in the same row or column differ significantly at $P<0.05$ (DMRT); [a] values not differ significantly from control and EGCG group at ($P<0.05$); [b] values differ significantly from control and drug control group at ($P<0.05$); [c] values differ significantly from Fl group at ($P<0.05$).

2.13. Immunohistochemistry

To examine the protective effects of EGCG on apoptosis in the testes, NF-kB expression was assessed by immunohistochemical staining. Testes sections on polylysine coated slides obtained were fixed in neutral buffered formalin and embedded in paraffin and were treated for NF-kB antibody for immunohistochemical analysis. The procedures were processed according to the manufacturer ' s protocol recommended for the NF-kB immunohistochemistry with slight modifications. For nuclear factor-kB (NF-kB) expressions, 4 μm thick sections were prepared from different animal groups. Sections were deparaffinised, rehydrated and endogenous peroxidase activity was blocked with H_2O_2 in methanol. Sections were pre-treated in citrate buffer (pH 6.0) in a microwave and incubated at room temperature with monoclonal anti-NF-kB antibodies (Thermo Scientific, USA, dilution 1:100). Ultra vision detection System (Thermo Scientific) was used as follows; sections were incubated with biotinylated goat anti-polyvalent, then with streptavidin peroxidase and finally with DAB plus a chromogen. Slides were counterstained with hematoxylin, visualized under a light microscope and the extent of cell immunopositivity was assessed. The number of immunopositive cells was counted in 5 separate microscopic fields in each slide and the mean number for each slide was obtained, then the mean±SD was calculated for each group (10 slides).

2.14. Western blotting analysis

Western blotting was performed using testicular lysates. In brief, protein extracts from each sample were added to a gel loading buffer (100 mM Tris, pH 6.8, 20% glycerol, 200 mM DTT, 4% SDS, 0.03% bromophenol blue) boiled for 5 min. Proteins (50 μg/sample) in loading buffer were subjected to electrophoresis in 10–15% SDS-polyacrylamide gel for 3 h. The gel was transferred electrophoretically onto a polyvinylidene fluoride membrane (Immobilon-P; Millipore Corp., Bedford, Massachusetts, USA) blocked with 5% nonfat powdered milk in Dulbecco ' s PBS (DPBS) overnight at 4 ℃. The membranes were incubated for 2 h with the following antibodies: Nrf2, HO-1, NQO1, γ GCS, Keap-1, Bcl-2, Bad, Caspase-3, and Cytochrome-C. β -Actin was used as a loading control for total proteins. After washing in DPBS containing 0.05% Tween-20 four times for 10 min each, the membranes were incubated with goat anti-rabbit or goat anti-mouse IgG antibody for 2 h. The membranes were then washed for four times in DPBS containing 0.05% Tween-20 for 10 min each, followed by signal development using an enhanced chemiluminescence (ECL) detection kit from Pierce (Pierce Biotechnology, Rockford, IL).

2.15. Histological observations of testes

For qualitative analysis of testicular histology, the testes samples were fixed for 48 h at 10% formal-saline and dehydrated by passing successfully in different mixture of ethyl alcohol-water, cleaned with xylene and embedded in paraffin. Sections of tissue (5–6 μm thick) were prepared by using a rotary microtome and stained with haematoxylin and eosin and in neutral deparafinated xylene (DPX) medium for microscopic observations.

2.16. Statistical analysis

Results were expressed as mean±SD of a number of experiments (n=6). The statistical significance was evaluated by one-way analysis of variance using SPSS version 13.0 (SPSS, Cary, NC, USA) and the individual comparisons were obtained by Duncan ' s multiple range test (DMRT). A value of P<0.05 was considered to indicate a significant difference between groups. In figures and tables values with different superscript alphabet letters differ significantly at P<0.05.

3. Results

3.1. EGCG effectively scavenges ROS in the testes

The concentration of ROS in the testes of control and experimental rats were shown in Figure 1. A significant (P<0.05) increase in the level of ROS was observed in the testes of Fl intoxicated rats when compared to the control rats. Pre-administration of EGCG to Fl treated rats showed a significant (P<0.05) decrease level of ROS when compared to that of Fl alone intoxicated rats. EGCG alone treated rat also exhibit a significant (P<0.05) decrease in the level of testicular ROS when compared to the control rats.

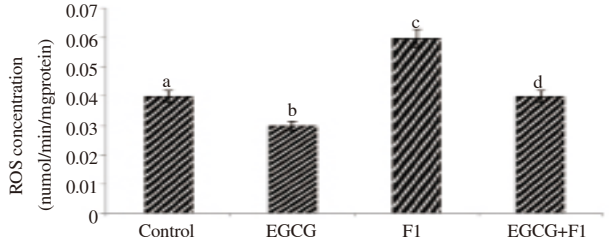

Figure 1. Effect of EGCG on Fl induced ROS concentration in the control and experimental rats.

3.2. EGCG regularizes the body, testicular and epididymal weight in the Fl intoxicated rats

The data in Figure 2 shows the effect of Fl and EGCG on body weight, organ-body weight ratio, testes and epididymal weight in control and experimental rats. Fl treatment caused a significant (P<0.05) decrease in the body weight (3A) with a significant (P<0.05) increase in testicular (3B) and a significant (P<0.05) decrease in epididymal (3C) and cauda epididymal weight (3D) when compared to the control rats. However, pre-administration of EGCG to Fl-exposed rats exerted a significant (P<0.05) abrogation of Fl-induced alterations in testes, epididymal and cauda epididymal weight when compared with Fl alone treated rats. No significant changes were observed in the EGCG alone treated group of rats when compared to that of control group.

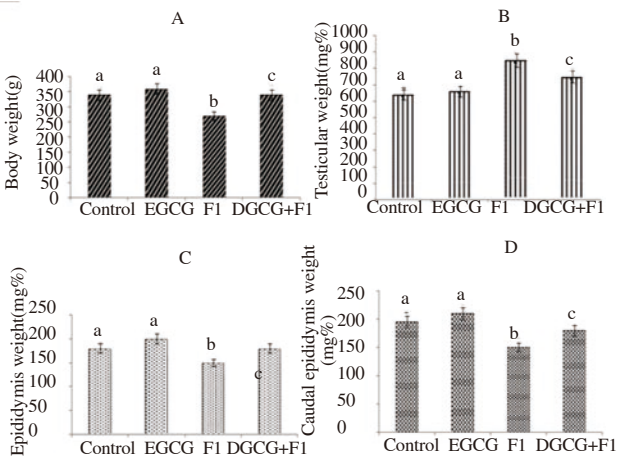

Figure 2. Effect of EGCG and Fl on body weight (A) Testicular weight (B) Epididymal weight (C) and Caudal epididymal weights (D) of control and experimental rats.

3.3. EGCG improves steroidogenic enzymes and testosterone level in the Fl intoxicated rats

Testicular steroidogenic enzymes, Δ^5 3 β -HSD and Δ17 β -HSD as well as the hormone, testosterone play a critical role in male reproduction. In the present study, we observed a significant (P<0.05) decrease in the activity of these enzymes in Fl-treated rats when compared with control rats (Figure 3A). In addition, plasma testosterone level was also decreased significantly (P<0.05) in Fl intoxicated rats (Figure 3B). Pre-treatment with EGCG was found to be more effective in preventing the Fl-induced variation in the activity of testicular enzymes as well as testosterone levels. EGCG alone treated groups did not exemplify any changes in testicular steroidogenic enzymes and testosterone levels.

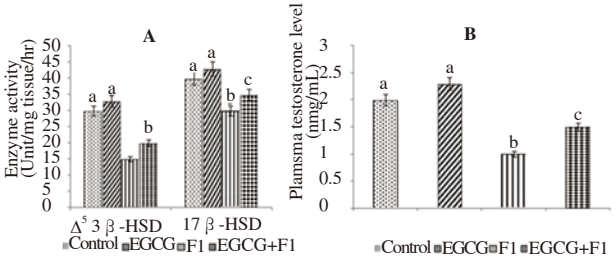

Figure 3. Effect of EGCG on Fl induced changes in the steroidogenic enzyme activity and plasma testosterone level of control and experimental rats.

(Panal A) Testicular steroidogenic enzyme activities namely Δ^5 3 β -HSD and Δ17 β -HSD. (Panal B) Plasma testosterone level.

3.4. EGCG abridges the sperm abnormalities in the Fl intoxicated rat testes

Epididymal sperm concentration, sperm motility, abnormal sperm rate, and live/dead count of sperms were shown in Figure 4. Fl intoxication showed a significant (P<0.05) decrease in epididymal sperm concentration (Figure 4A), sperm progress motility (Figure 4B), dead sperm count (Figure 4C) and increased abnormal sperm rate (Figure 4D). Pre-treatment of EGCG in Fl intoxicated rats brought a significant (P<0.05) improvement in epididymal sperm concentration, sperm motility and live/dead count as well as a significant (P<0.05) reduction in the abnormal sperm rate. No significant change was observed in EGCG alone treated rats in the level of live sperm count, epididymal sperm concentration and sperm motility when compared with control rats.

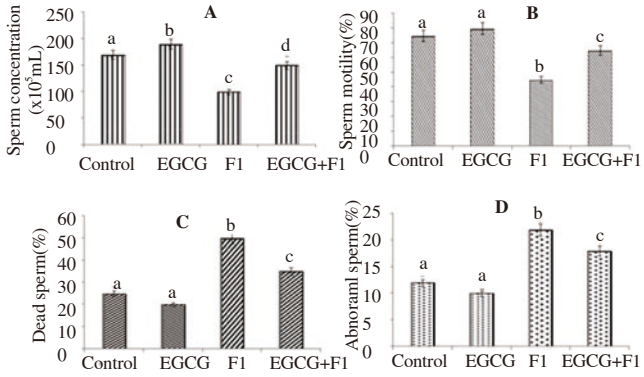

Figure 4. Effect of EGCG on Fl induced changes in the sperm concentration (A) sperm motility (B) dead sperm (C) and abnormal sperm (E) of control and experimental rats.

3.5. EGCG restrains the level of mitochondrial membrane potential in the Fl intoxicated testes

Mitochondrial membrane potential (Δ ψ m) is an important indicator of cell viability. The effect of EGCG on Fl induced testicular mitochondrial membrane potential was analyzed in control and experimental rats. As shown in Figure 5, the level of testicular mitochondrial membrane potential was significantly (P<0.05) decreased in Fl treated rats when compared with control rats. Pre-treatment with EGCG significantly (P<0.05) increased the mitochondrial membrane potential when compared with Fl-treated group. EGCG alone administrated rat revealed a significant increase (P<0.05) mitochondrial membrane potential when compared to that of control.

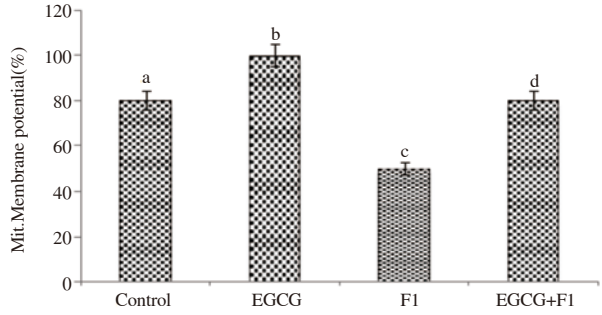

Figure 5. Effect of EGCG on Fl induced changes in the level of mitochondria membrane potential of control and experimental rats.

3.6. EGCG suppresses the level of oxidative stress markers in the Fl intoxicated testes

Table 1 shows the level of oxidative stress markers in control and experimental testes. Oxidative stress markers, such as TBARS, LOOH, PC, and CD were significantly (P<0.05) increased in Fl intoxicated rats when compared with control rats. However, pre-treatment with EGCG in Fl-treated rats significantly (P<0.05) decreased the level of oxidative stress markers when compared with Fl-treated rats. However, no significant statistical changes were observed in rats treated with EGCG alone in comparison with that of the control rats.

3.7. EGCG augments the non-enzymatic antioxidant levels in the Fl intoxicated testes

Table 2 depicts the effect of EGCG on Fl induced changes of non-enzymatic antioxidant levels in control and experimental rats. There was a significant (P<0.05) decreased levels of non-enzymatic antioxidant was observed in Fl intoxicated rats compared with control. Surprisingly, Pre-administration of EGCG significantly (P<0.05) increased the levels of non-enzymatic antioxidants near to normal control. No significant changes in the non-enzymatic antioxidant levels of EGCG alone treated group when compared to the control rats.

Table 2

Effect of EGCG on Fl induced changes in the non-enzymatic antioxidant levels of control and experimental rats.

Groups	GSH (nmoles/ mg protein)	SH (nmoles/ mg protein)	Vit. C (µmol/ mg tissue)	Vit.E(µmol/ mg tissue)
Control	6.56 ± 0.41[a]	13.62 ± 0.78[a]	0.58 ± 0.13[a]	0.69 ± 0.09[a]
EGCG	6.88 ± 0.55[a]	12.31 ± 0.62[a]	0.67 ± 0.10[a]	0.75 ± 0.07[a]
Fl	4.70 ± 0.30[b]	6.35 ± 0.35[b]	0.47 ± 0.09[b]	0.52 ± 0.03[b]
EGCG + Fl	6.50 ± 0.35[c]	10.65 ± 0.48[c]	0.52 ± 0.10[c]	0.70 ± 0.05[c]

Values are given as mean ± SD for six rats in each group. Values with different superscript letter (a-c) in the same row or column differ significantly at P<0.05 (DMRT); [a] values not differ significantly from control and EGCG group at (P<0.05); [b] values differ significantly from control and drug control group at (P<0.05);[c] values differ significantly from Fl group at (P<0.05).

3.8. EGCG enhances the enzymatic antioxidant levels in the Fl intoxicated testes

Table 3 illustrates the activities of enzymatic antioxidants status in the testes of control and experimental rats. Fl treated rats showed a significant (P<0.05) decrease in the activities of SOD, CAT, GPx, GST, GR and G6PD when compared to control rats. Pre-treatment of EGCG to Fl intoxicated rats significantly (P<0.05) increased the activities of these enzymatic antioxidants when compared to Fl-treated rats. EGCG alone treated rats didn't exhibit any changes in the antioxidant enzyme level when compared with control.

Table 3

Effect of EGCG on Fl induced changes in the antioxidant levels of control and the experimental rats.

Groups	SOD	CAT	GPx	GR	GST	G6PD
Control	86.25±3.18[a]	60.22±2.83[a]	156.20±5.58[a]	100.79±2.22[a]	10.02±0.43[a]	7.05±0.13[a]
EGCG	85.15±3.12[a]	61.08±2.52[a]	157.10±4.37[a]	102.41±4.35[a]	10.03±0.33[b]	8.06±0.15[a]
Fl	48.67±8.08[b]	30.62±2.17[b]	86.84±7.85[b]	64.56±4.27[b]	5.23±0.13[b]	2.31±0.10[b]
EGCG + F1	70.27±3.35[c]	46.82±2.38[c]	130.34±6.05[c]	90.98±4.78[c]	8.78±0.31[c]	5.65±0.39[c]

Values are given as mean ± SD for six rats in each group. Values with different superscript letter (a-c) in the same row or column differ significantly at P<0.05 (DMRT); [a]values not differ significantly from control and EGCG group at (P<0.05); [b] values differ significantly from control and drug control group at (P<0.05); [c] values differ significantly from Fl group at (P<0.05).

SOD - One unit of enzyme activity was taken as the enzyme reaction, which gave 50% inhibition of NBT reduction in one minute/mg protein; CAT- µ moL of H_2O_2 utilized/min/mg protein; GPx- µ g of GSH consumed/min/mg protein; GST- µ moL of CDNB-GSH conjugate formed/ min/mg protein; GR-nmol of NADPH oxidized/min/mg protein; G6PD were expressed as nmol of NADPH formed/min/mg protein.

3.9. EGCG abates the pro-inflammatory cytokines in the Fl intoxicated testes

The effect of EGCG on the mRNA expression of pro-inflammatory cytokines in control and experimental rats are depicted in Figure 6. The mRNA expression of TNF- , IL-1β, iNOS and COX-2 was significantly (P<0.05) increased in Fl-treated rats when compared with control rats (Figure 6 A-D). Immunohistochemical examination of testes section also revealed that Fl administration caused significant increase in the immunoreactivity of NF-kB p65 in the seminiferous tubules (Figure 7C) as compared to the control rats (Figure 7A). On the other hand pre-treatment with EGCG showed a significant reduction in the Fl-induced over expression of pro-inflammatory cytokines and the immunoreactivity of NF-kB p65 in the testicular tissue as compared to the Fl intoxicated rats (Figure 7D). EGCG alone treated group didn't show any significant changes in these inflammatory markers when compared to control rats (Figure 7B).

3.10. EGCG stabilizes the mRNA expression of Nrf2, Keap1γ-GCS, NQO1 and HO-1in the Fl intoxicated testes

The effect of EGCG on the mRNA expression of Nrf2, Keap1, γ-GCS, NQO1 and HO-1 in the testes tissues of control and

experimental groups of rats are depicted in Figure 8. The mRNA expression of Nrf2 and its down regulatory genes such as γ-GCS, NQO1 and HO-1 were significantly (P<0.05) declined with simultaneous elevation of Keap1 gene in the testes tissues of Fl treated group of rats. However, these altered mRNA expressions were significantly (P<0.05) normalized in the Fl treated group of rats with the pre-administration of EGCG. On the contrary, EGCG pre-treatment to control group of rats demonstrate a significant statistical difference in comparison to control group of rats.

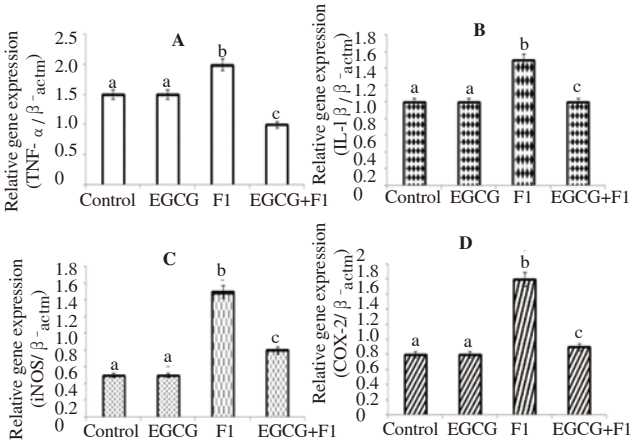

Figure 6. Effect of EGCG on Fl induced changes in the levels of inflammatory markers of control and experimental rats.

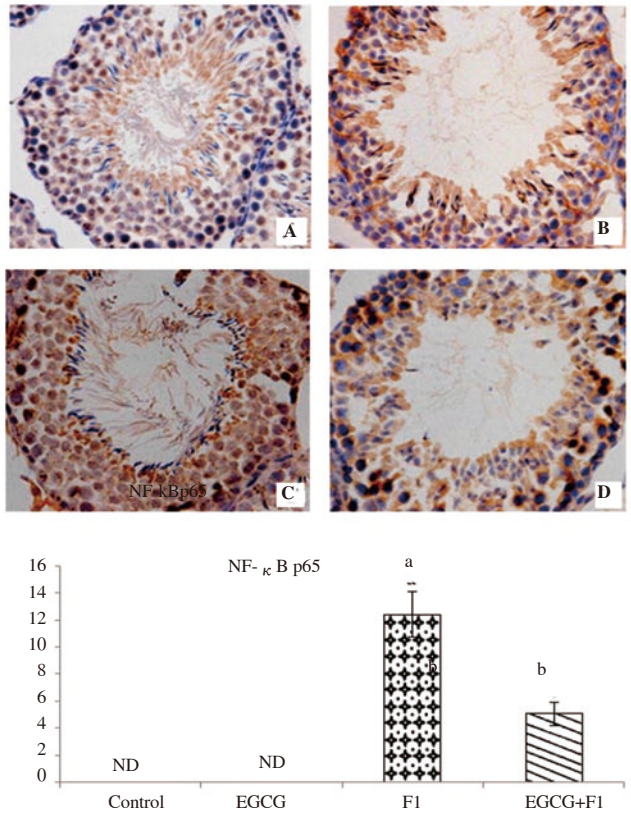

Figure 7. Representative photomicrographs of NF- κ B p65 (×400) immunohistochemistry of control and experimental rats.

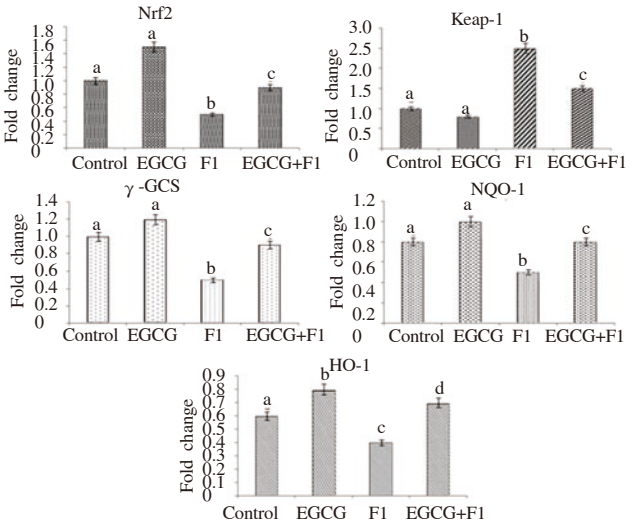

Figure 8. Effect of EGCG on Nrf2 Keap1 γ -GCS NQO1 and HO-1 mRNA expressions in the testes of control and experimental rats.

3.11. EGCG normalizes the protein levels of Nrf2, Keap1, γ-GCS, NQO1 and HO-1in the Fl intoxicated testes

The effect of EGCG on the protein levels of Nrf2, Keap1, γ-GCS, NQO1 and HO-1 in the testes tissues of control and experimental groups of rats are depicted in Figure 9. The protein levels of Nrf2, γ-GCS, NQO1and HO-1 were significantly (P<0.05) declined with a simultaneous elevation of Keap1 protein in the testes of Fl treated group of rats. However, these altered protein levels were significantly (P<0.05) normalized in the Fl treated group of rats with the pre-administration of EGCG. On the other hand, EGCG alone treated group also exhibit a significant differences on the protein levels of Nrf2, Keap1, γ-GCS NQO1 and HO-1 in comparison to the control group of rats.

3.12. EGCG stabilizes the protein expression of Bcl-2, Bad, Cyt-c and Cas-3 in the Fl intoxicated testes

The effect of pre- treatment of EGCG on the levels of Bcl-2, Bad, Cyt-c and Cas-3 in testes tissues of control and experimental groups of rats are demonstrated in Figure 10. The levels of Bcl-2, Bad, Cyt-c and Cas-3 in control rats administered with EGCG did not reveal any statistical difference when compared with that of control group of rats. Conversely, there was a significant (P<0.05) decrease in the level of Bcl-2 with a significant (P<0.05) increase in the levels of Bad, Cas-3 and Cyt-C in the testes of Fl treated rats in comparison with control group of rats. Moreover, pre- treatment with EGCG to Fl group of rats significantly (P<0.05) restored the altered levels to near normalcy when compared with Fl treated group of rats.

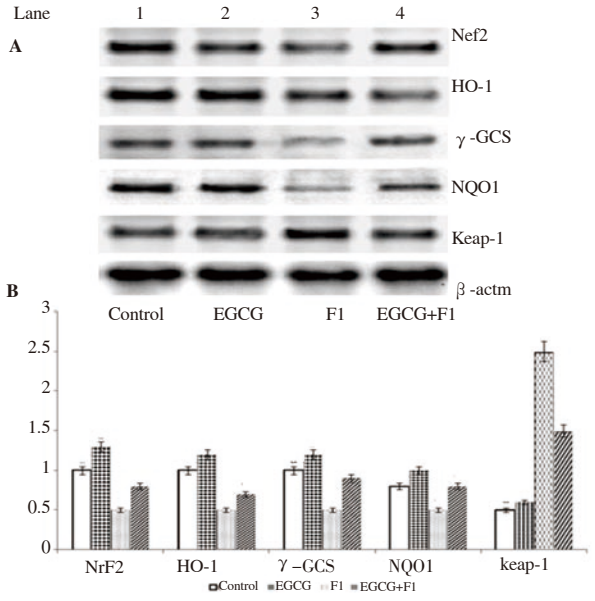

Figure 9. Effect of EGCG on Nrf2 Keap1 γ -GCS NQO1 and HO-1 protein expressions in the testes of control and experimental rats by western blot. Lane 1. Control Lane 2. EGCG Lane 3. Fl-control Lane 4. EGCG + Fl. B. Effect of EGCG on the mRNA expression level of Nrf2, Keap1, γ -GCS, NQO1 and HO-1 in control and experimental rats by RT-PCR.

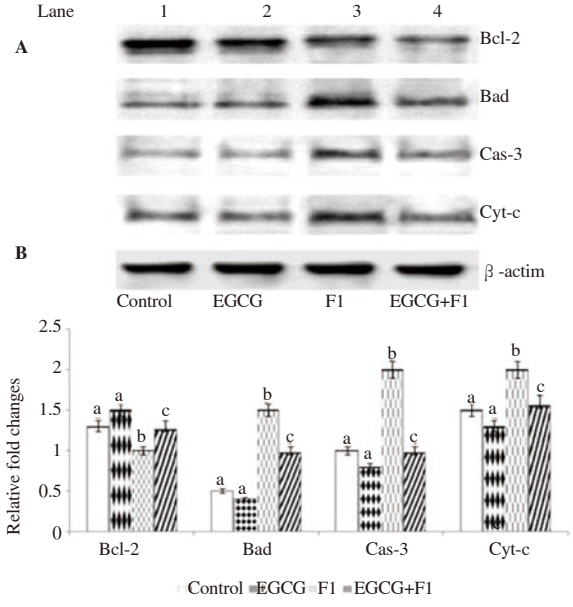

Figure 10. Effect of EGCG on Bcl-2 Bad Cas-3 and Cas-9 protein expressions in the testes tissue of Control and experimental rats by western blot.

Lane 1. Control Lane 2. EGCG Lane 3. Fl-control Lane 4. EGCG + Fl. (B) Effect of EGCG on Bcl-2, Bad, Cas-3 and Cas-9 on protein fold change in control and experimental rats.

3.13. EGCG preserves the histological architecture of the Fl treated testes

The biochemical and molecular findings on the protective efficacy of EGCG were further substantiated with the histological examination of the testes tissue. The control testes showed normal histological features, characterized with the well-organized distribution of cells in the seminiferous epithelium, including the outermost layer of spermatogonia and sertoli cells, the middle layer of spermatocytes, and the innermost layer of sperm cells, suggestive of normal spermatogenesis (Figure 11A). EGCG alone administered group displayed the normal active seminiferous tubules with complete spermatogenic series (Figure 11B). Fl intoxicated rats showed more pronounced alterations in the testes such as atrophy of the seminiferous tubule, injury of spermatogonia and decrease of spermatocytes, as well as the absence of elongated spermatids in the severely damaged seminiferous tubules, indicative of impaired spermatogenesis and loss of germ cells (Figure 11C). Pre-administration of EGCG to Fl treated rats showed the significant abrogation of degenerative changes induced by Fl and helps to rejuvenate the testicular tissue into its normal histoarchitectural pattern (Figure 11D).

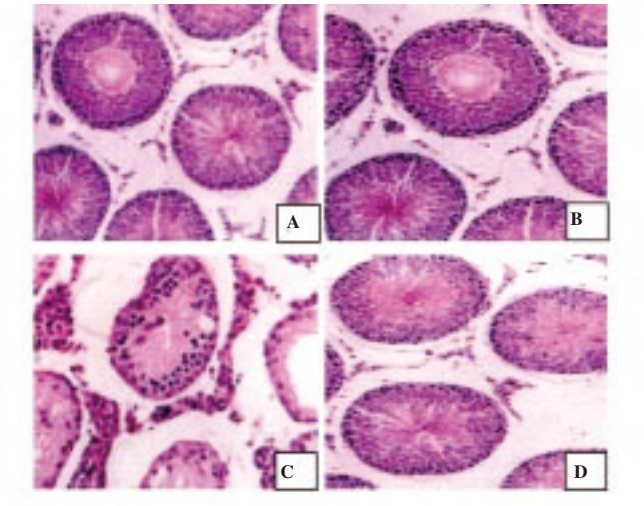

Figure 11. Effect of EGCG on Fl induced changes in the testes of control and experimental rats.

Testes cross section was stained with H&E from all the groups of rats at × 200 magnification.

4. Discussion

Fluoride compounds have been recognized as a persistent global contaminants and their exposure may cause oxidative stress mediated sperm dysfunction and male infertility[44]. Reports on the oxidative stress responses of organisms exposed to Fl can help us to understand the mechanism through which fluoride exerts its toxicity on organisms and hence the outcomes of the results can be used to explain the impact of Fl on organisms in the environment. However, the information is currently inadequate on the toxic effect of Fl on the testis. In fact, the mammalian testes afford a suitable and indispensable model for studying the undesirable effect of pollutants on spermatogenesis and maturation of spermatozoa. The oxidative

stress induced by Fl compounds appears to be of great significance in understanding the impact of Fl with reference to the male reproductive function.

A progressive decrease in the body weight, testicular and epididymal weight of Fl challenged rats clearly demonstrated the Fl induced oxidative stress related testicular damage. This is in line with the reports of Ghosh *et al*. [45] who reported the enhanced oxidative testicular injury with decreased body weight in Fl intoxicated rats. Pre-administration of EGCG significantly reduces the testicular damage with a significant restoration of body weight, testicular and epididymal weight. This might be due to the presence of gallate and catechol moieties along with 8 hydroxyl groups in the EGCG and have been easily dissociated during EGCG metabolism and effectively scavenges free radicals and protect the rat testes from Fl induced oxidative stress.

Oxidative stress is a recognized mode of action of Fl exposure in animal and humans living in areas of endemic fluorosis. Fl is thought to inhibit several antioxidant enzymes and alter glutathione level, often resulting in the excessive production of ROS at the mitochondrial level, leading to the cell death and testicular dysfunction. In the present study, we observed a significant increase level of testes ROS in Fl intoxicated rats which clearly suggested that increased free radicals can impair the function of mitochondria in the rat testes. Pre-treatment with EGCG, effectively suppressed the levels of ROS in testes and enhanced the mitochondrial transmembrane potential through its strong free radical scavenging and antioxidant activity[18].

In the present investigation, the activity of hydroxysteroidogenic enzymes ($\Delta^5 3\beta$-HSD and $\Delta 17\beta$-HSD) and plasma testosterone levels were significantly decreased in Fl intoxicated rats which were in corroborate with the previous report of Gosh *et al* [45]. The mechanism of inhibition of these enzymes was principally due to the over production of ROS by Fl which leads to the degradation of plasma FSH and LH. These two gonadotrophins are the prime regulators for hydroxysteroidogenic enzymes and testosterone synthesis in testes[46]. An increase in Fl induced oxidative stress causes ROS-induced damage to macromolecules such as DNA, protein, and key enzymes involved in testicular steroidogenesis and spermatogenesis in germ cells. EGCG pre-treatment repealed all these undesirable effects and oxidative stress-associated changes induced by Fl. This protective efficacy of EGCG as an antioxidant in organ pathophysiology has been attributed to its ability by stabilizing biological membrane and scavenging reactive oxygen species[18].

Mitochondrial damage, as evaluated by the loss of mitochondrial membrane potential was observed in the mitochondria of Fl intoxicated rats. Mitochondrial membrane potential ($\Delta \psi m$) of spermatozoa is widely used to characterize the functional status of spermatozoa. In our study, a significant decrease in mitochondrial membrane potential ($\Delta \psi m$) in Fl treated rat strongly suggested the mitochondrial dysfunction which leads to the abnormal structure and non motility of sperms[47]. Fl ions can bind with the functional groups of mitochondrial enzymes to cause an inhibitory effect on glycolytic and Krebs cycle pathway, which are sensitive to the inhibition of Na+/K+-ATPase leading to ATP depletion and a disturbance in cell membrane potential. The decreased ATP production and increased cellular levels of ADP, AMP, GDP and Pi leads to the dysfunction of spermatozoa because it needs large amounts of energy to fertilize the Oocyte [48]. Fl induced oxidative stress impose an energy deprivation in both structural and physiological defects and inhibition of energy producing enzymes in the sperm mitochondria. Pre administration of EGCG in Fl intoxicated rats significantly restored the mitochondrial transmembrane potential and sperm functions through its strong antioxidant action and maintains the normal functional status of the testes.

Earlier studies suggested that oxidative stress is a chief causative factor in understanding the male reproductive dysfunction. Because of high concentration of polyunsaturated fatty acids and low antioxidant capacity, male germ cells could be vulnerable to oxidative stress[45]. Fl is known to produce oxidative damage in the testes by enhancing peroxidation of membrane lipids, a destructive process exclusively carried out by free radicals. Present study demonstrated that exposure to Fl significantly increased the ROS mediated lipid peroxidation, which may mess up a variety of intra and extra mitochondrial membrane transport systems thus contributing to apoptosis[49]. Furthermore, lipid peroxidation is the process of oxidative degradation of polyunsaturated fatty acids (PUFAs) and its incident in biological membrane causes impaired membrane function, structural integrity, decrease in membrane fluidity and inactivation of a several membrane bound enzymes. Oxidative stress induced by Fl can be confirmed by measuring lipid peroxidative products such as TBARS, hydroperoxides, and other lipid oxidative markers[50, 51]. In addition to lipid peroxidation the formation of carbonyl derivatives of proteins are also suggested to be a valuable marker for protein oxidation. The carbonyl derivatives of proteins may result from oxidative modification of amino acid side chains and ROS-mediated peptide cleavage. In our study, Fl intoxication significantly enhanced the levels of these oxidative stress markers in testes. Pre administration of EGCG significantly reduced the levels of lipid peroxidation and protein carbonylation in Fl intoxicated rats, and thus exhibits the strong free radical scavenging capability of EGCG. This might be mainly due to the presence of tri-phenolic groups in a 4 ring structure that is easily 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 reduces the Fl induced oxidative stress[52].

Reduced glutathione is an imperative cellular thiol antioxidant present in the tissues. Depleted GSH levels in Fl intoxicated rats may be due to its enhanced utilization for augmenting the activities of GPx and GST. Depleted level of GSH by Fl was restored

significantly in the EGCG pre-administered rats. Hydrophilic antioxidant, Vitamin C appears to be the prime contour of antioxidant defense against reperfusion damage during the return of bloodflow, while lipophilic antioxidant vitamin E participate a key role in protecting the integrity of cellular membranes from oxidative damage[9, 10]. Decreased level of vitamin C and E by Fl could be due to its enhanced utilization by ROS or could be due to the depletion of cellular GSH level. The decreased level of these non-enzymatic antioxidants upon Fl intoxication is in accordance with the results in which testicular lipid peroxides were increased along with the decreased antioxidants in rats intoxicated with Fl[9]. The depleted levels of GSH, vitamin C and E were significantly restored in EGCG pre-administered rats undoubtfully revealed the antioxidant nature of EGCG. Based on results of our earlier investigations, EGCG could be proposed to play its protective role by its direct as well as indirect antioxidant activities. As a direct antioxidant, it could quench and detoxify several reactive intermediates, like nitric oxide, hydrogen peroxide, hydroxyl radical etc. As an indirect antioxidant, it could prevent the changes in oxidative stress-induced membrane permeability and stabilize it.

SOD activity decreased significantly in the Fl intoxicated rats, which might be due to an excessive formation of superoxide anions. A decrease in SOD activity can result in the decreased removal of superoxide anions which can be harmful to the testes. The activities of H_2O_2 scavenging enzymes CAT and GPx were also decreased significantly in the testes of Fl intoxicated rats. The turn down activity of these enzyme levels may be explained by the fact that excessive superoxide anions may inactivate SOD thus, resulting in an inactivation of the H_2O_2 scavenging enzymes [46]. EGCG was reported to scavenge superoxide radicals and hydrogen peroxide[21]. In view of this, it was expected that EGCG could decrease the workload of SOD, CAT/GPx and reduce the free radical mediated inactivation of enzymes and thereby uphold the activities of enzymatic antioxidants. Diminished levels of GPx, GST and GR may be due to loss of enzymes by the events of increased lipid peroxidation and protein oxidation. GPx, GST and GR are 'SH' dependent enzymes, and their 'SH' groups can react with Fl induced ROS, leading to their inactivation. The diminution of G6PD activity in Fl intoxicated rats showed an impaired generation of NADPH, which is required for the reduction of GSSG to GSH[53]. The inhibition of these enzymes not only reflect oxidative stress, but also render the cells to further oxidative damage, since GPx, GST and GR are the indispensable enzymes in cytoprotection against ROS. Pre-administration of EGCG restores the activities of these enzymes by restoring the glutathione level and counteracting the free radicals produced by Fl.

In harmony with the previous reports, the present results also confirmed that the oxidative stress and increased production of proinflammatory cytokines are implicated in the pathogenesis of testicular injury upon Fl intoxication. It has been reported that during testes reperfusion, neutrophils and macrophages released the free radicals and pro-inflammatory cytokines that can induce apoptosis and thus act as a mediators of inflammation in testes[54]. Even though the testis is an immune privileged organ, inflammatory condition in this site is one of the most compelling etiological features of male infertility[55]. Under normal physiological conditions, inflammatory mediators including TNF- , IL-1β, iNOS and COX-2, as well as transcription factor NF-κB participate in an important regulatory function in testicular steroidogenesis, spermatogenesis and maturation of the sperm[56–58]. In our present study, augmented production of ROS upon Fl intoxication can stimulate the proinflammatory mediators in the testes, and thereby disrupts the normal spermatogenesis. It has been reported that both TNF- and iNOS over-expression can stimulate germ cell apoptosis and disrupt sertoli tight junction integrity and germ cell attachment[54,58]. An increase in IL-1β expression also cause an inhibition of testosterone production by leydig cells and reduce the intensity of spermatogenesis via apoptosis, whereas COX-2 is the major source of inflammation-associated prostaglandin synthesis that induces hypertrophy and loss of contractility in testicular peritubular cells, resulting in a diminished sperm output[59]. EGCG pre-administration could effectively suppress the Fl-induced expression of these pro-inflammatory mediators through its strong antioxidant ability and inhibit the activation of NF-kB signaling pathway, which promotes the transcription of other testicular inflammation in rats[18].

Recent studies demonstrated that Fl intoxication caused nuclear factor-κB activation with subsequent cascade of events responsible for testicular inflammation[60]. The positive immunohistochemical localization of NF-kB in the present study also confirmed that Fl induced NF-κB-mediated testicular inflammation along with the disruption of sertoli-germ cell junctions and germ cell apoptosis. Upon activation, the free NF-kB in cytosol translocate into the nucleus and activates the expression of a set of NF-kB responsive genes directly or indirectly during generalized oxidative stress that leads to inflammation in the testes. Zhang *et al*. [61] reported that spermatogenesis inhibition was associated with the up-regulation of pro-inflammatory mediators by Fl intoxication. However EGCG pre treatment significantly suppressed the over production of pro-inflammatory mediators and reduced the expression of NF-kB and iNOS in the testes of Fl intoxicated rats. The protective effect of EGCG might be attributed to its ability to inhibit the activation of NF-kB signaling pathway which promotes the transcription of NADPH oxidase, TNF- and iNOS genes. Further we propose that the clutch of NF-kB signaling by EGCG can be simply interpreted by the inhibition of IKK-catalyzed phosphorylation of I-kB in the cytoplasm which leads to the amelioration of inflammation in testes [62].

Several studies on Fl, illustrate the definite role of ROS in executing its detrimental effects. Apoptosis is an active cellular process of gene-directed self-destruction in which cells die in a proscribed fashion either spontaneously or in response to various environmental stimuli or toxicants. Fl induced oxidative stress and ROS have been associated with apoptosis in many cell types

including spermatogenic cells[49]. Mitochondria have been described as the sensor of oxidative stress and loss of its membrane potential can lead to cell death by the release of cytochrome C and other pro-apoptotic factors into cytosol. Translocation of cytochrome C into cytosol is the crucial event in mitochondrial pathway that leads to the formation of apoptosomes and activation of caspase cascade. Increased mitochondrial permeability through the PTPC (permeability transition pore complex) or through selective outer membrane permeabilization is controlled by the Bcl-2 family of proteins. Bcl-2 and Bcl-X are anti-apoptotic members of Bcl-2 family and they could impede cytochrome C release. Bid, Bad, Bik, etc. are the pro-apoptotic members of Bcl-2 family and counteract the cytoprotective effect of Bcl-2 and Bcl-X by promoting cytochrome C release[63–66]. We found that Fl treatment upregulated the expression of Bad as well as down regulated the expression of Bcl-2, consequently reduced the mitochondrial membrane potential, and enhanced the release of cytochrome C and activated caspase-3 in rat testes. EGCG pre-treatment, however, effectively suppressed the activation of caspase-3 as well as the release of cytochrome C and enhanced mitochondrial transmembrane potential by the reciprocal regulation of Bcl-2/Bad thus suggesting the inhibitory effect of EGCG on cell death during Fl mediated oxidative stress mediated apoptosis.

Stimulation of Nrf2-mediated antioxidant system has already been identified as the key molecular target against various oxidative and electrophilic stressors via accumulating in nucleus and inducing expression of cytoprotective enzymes and its related proteins[67]. Under unstimulated state, Nrf2 is sequestered in the cytoplasm by an actin binding repressor protein-Keap1. Upon exposure of cells to oxidative stress or electrophillic compounds with the capacity to oxidize or covalently transform thiol groups, Nrf2 easily dissociates from its repressor protein- Keap1 and translocates into the nucleus, binds to antioxidant response elements and transactivates the genes of detoxifying and antioxidant enzymes such as HO-1, γ-GGL, and NQO1[68]. During Fl induced oxidative stress, the expression of Nrf2, HO-1, γ-GGL and NQO1were significantly decreased along with the increase of Keap1in the testes of rats. However, pre-administration of EGCG significantly inhibits of Keap1 protein and modulates the expression of Nrf2, γ-GGL and NQO1 in Fl mediated oxidative stress in the testes through the up regulation of HO-1 which apparently reveals the antioxidant nature of EGCG.

Previously, it has been reported that polyphenols like EGCG may have a potent ability to modify the SH- residue in Keap1 through ROS production. However, we didn't detect the ROS levels in the testes during EGCG treatment. This result implies that a ROS-independent pathway is involved in the activation of Nrf2 by EGCG. Scientific reports also confirmed that the catechins acquire many of the structural components that contribute to their antioxidant property. Catechins like EGCG have 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. The potent free radical scavenging activity of EGCG was mainly attributed to the presence of the C ring gallate group[69]. The study was also confirmed that the more of hydroxyl groups present in the catechin (EGCG), could exhibit more free radical scavenging ability. EGCG itself is an electrophile (-C=O-) and therefore it could act as a Micheal-reaction acceptor and are able to modify the cysteine and thiol groups in Keap1 suggesting that a possible structure-activity relationship exists between the chemical backbone of the polyphenols and the degree of Nrf2 activation[70]. Therefore, we assume that EGCG may down regulate Keap1 activity, which leads to Nrf2 translocation into the nucleus where it can activate the PhaseII antioxidant genes. Our results also demonstrate that EGCG significantly down regulates the Fl-induced Keap1 activity in the testes tissue. We consider that, the Keap1 inhibition may be mainly due to the electrophilic nature of EGCG or its metabolites. Further, to confirm activation of the Nrf2-ARE system in the present study, we measured the mRNA expression of Nrf2 gene and its three antioxidant genes containing ARE at their promoter regions (Nrf2, contains two ARE sequences), of HO-1, a well-characterized Nrf2 target gene, NQO1, which reduces quinones to hydroquinones to protect against oxidative stress, GCLC, combines Glu and Cys as the first step in GSH production. Previous studies have been reported that the activation of Nrf2 induced the increased expression of HO-1, NQO1 and GCLC[67]. In consistent with this, our findings also showed an increase in the mRNA expression of Nrf2, HO1, NQO1 and GCLC in EGCG pre-treated Fl intoxicated rat testes clearly disclosed the capability of EGCG in the expression of phase-II antioxidant genes via activation of Nrf2 and inhibition of Keap1 signaling pathway. However, a further detailed study is needed to elucidate and confirm this mechanism.

The testicular toxicity induced by Fl and the ameliorative efficacy of EGCG was further confirmed by the histological findings. Histological examination of the experimental testes revealed the atrophy of seminiferous tubules with complete loss of spermatogenic cell layers along with the absence of centrally located spermatozoa in Fl intoxicated animals. Fl-treated rats also exhibited hyalinised seminiferous tubules with marked disruption of spermatogenesis. Additionally, some of the seminiferous tubules showed central debris with damaged sertoli cells. Pretreatment with EGCG could, however, exert a more pronounced effect in ameliorating the histological abnormities caused by Fl intoxication in rat testes. These histological findings further confirmed the cytoprotective nature of EGCG on rat testes intoxicated with Fl.

To summarize, the results of the present study suggest that EGCG afford protection against Fl-induced testicular toxicity through its strong ROS scavenging and anti-oxidative properties without any known side effects. Moreover, EGCG strives as an anti-inflammatory agent by down regulating the NF-kB mediated release of proinflammatory mediators and also prevents the apoptotic events via up-regulating the expression of Bcl-2. Taken together, outcome of the present study evidently proved that EGCG attenuates Fl-induced

oxidative stress mediated testicular toxicity by activating Nrf2/ ARE mediated pathway and thereby reduced the oxidative testicular toxicity elicited by Fl. Further in-depth studies are warranted on the ameliorative role and the molecular mechanism of protection by this dietary supplement on Fl-induced oxidative damage to the testes.

Conflict of interest statement

We declare that we have no conflict of interest.

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