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ORIGINAL ARTICLE

Epigallocatechin gallate attenuates arsenic induced genotoxicity via regulation of oxidative stress in balb/C mice

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Abstract

Arsenic is well known genotoxicant which causes the excessive generation of reactive oxygen species (ROS) and inhibition of antioxidant enzyme systems leading to cell damage through the activation of oxidative sensitive signaling pathways. Epigallocatechin gallate (EGCG), the main and active polyphenolic catechin present in green tea, has shown potent antioxidant, free radical scavenging and genoprotective activity in vivo. The present study attempted to investigate antioxidant and geno-protective efficacy of EGCG by regulating arsenic induced oxidative stress in mice. Animals received prophylactic and therapeutic treatments at two different doses (25 and 50 mg/kg b.wt.) of EGCG orally for 15 days and administered arsenic intraperitoneally at dose of 1.5 mg/kg b.wt (1/10th of LD_{50}) for 10 days. Arsenic intoxication revealed enhanced ROS production (114%) in lymphocytes; elevated levels of LPO (2–4 fold); reduced levels of hepato-renal antioxidants (approx. 45%) and augmented genomic fragmentation in hepato-renal tissues; increased chromosomal anomalies (78%) and micronucleation (21.93%) in bone marrow cells and comet tailing (25%) in lymphocytes of mice. Both pre and post treatments of EGCG decreased ROS production, restored lipid peroxidation (LPO) and reduced hepato-renal antioxidants levels, reduced the DNA fragmentation, number of chromosomal aberrations (CA), micronucleation (MN), and comet tailing but prophylactic treatment of 50 mg/kg b.wt was the most effective treatment in regulating arsenic induced oxidative stress. The effectiveness of this dose was furthermore validated by calculating the inhibitory index. Thus, results of present work empirically demonstrate free radical scavenging, anti-oxidative and genoprotective efficacy of EGCG against arsenic toxicity.

Keywords Arsenic · Oxidative stress · Genotoxicity · Epigallocatechin gallate

Introduction

Environmental and occupational exposure to arsenic is a pandemic burden. Globally, 150 million individuals are exposed to arsenic only through contaminated groundwater, though humans are also exposed to arsenic by exposure to contaminated food and soil [1]. The major exposure route of inorganic arsenic (As_i) is through contaminated water [2]. Brinkel et al. have reported various cases of arsenic poisoning in many countries including India, Bangladesh, China, USA, and more than 200 million people are at risk of chronic arsenicosis [3]. Chronic exposure to arsenic is associated with multiple diseases such as skin lesions, peripheral neuropathy, gastrointestinal symptoms, diabetes, renal system effects, cardiovascular disease, and cancer [4]. Several epidemiological studies have also documented exposure of different arsenicals to cancers [5].

Arsenic is considered to be a potentially genotoxic metal to humans in a dose–response manner. Although it is not mutagenic but it can produce chromosomal instability that leads to acentric chromosome formation, elevated indices of micronucleation (MN), sister chromatid exchanges (SCE) and chromosomal aberrations(CA) which have been reported from arsenic-exposed human populations [6]. The actual mode of action of arsenic-evoked genotoxicity still remains to be explored; however genotoxic ability of arsenic majorly lies in its ability to generate reactive oxygen species (ROS)

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[7]. The arsenic-evoked reactive oxygen species from different DNA adducts that can also instigate oxidative damage to DNA that eventually culminates into cytogenetic endpoints such as MN and CA as observed in humans [8]. Arsenic exposure has been observed to induce increased production of 8-oxo-2-deoxyguanosine (8OHdG), a form of oxidative damage to the DNA [9]. Previously numerous studies have implicated that arsenic exposure interferes with the DNA repair proteins leading to increased risk of genotoxicity [5]. Arsenic is recognized to inhibit mismatch repair, base excision repair, and nucleotide excision repair [10]. Recent studies have indicated that the generation of reactive oxygen species, free radicals and deregulated DNA repair resulting from arsenic contributes to DNA damage that leads to the genotoxicity in humans [11].

Interestingly, numerous studies documented plants or their components having antioxidant and free radical scavenging ability, demonstrate remarkable protection against oxidative stress and DNA damage-related conditions. In particular, Catechins that are important dietary ingredients of commonly used beverages consumed around the globe indicate strong anti-oxidative and free radical scavenging efficacy in biological systems. Epigallocatechin gallate (EGCG) is the most abundant and an active catechin which accounts for approximately 59% of total catechins found in green tea. Other catechins include epigallocatechin (EGC) (19%), epicatechin gallate (ECG) (13.6%) and epicatechin (EC) (6.4%) [12]. EGCG possesses two triphenolic groups in its chemical structure, which renders its anti-oxidative potency [13] Evidences indicate that EGCG has extensive pharmacological properties such as antioxidant, anti-apoptotic, anti-cancer and anti-inflammatory [14, 15]. EGCG is a strong free radical scavenger as it traps many reactive oxygen species (ROS) such as superoxide radical anion, hydroxyl radicals, singlet oxygen, nitric oxide and peroxy-nitrite, thus break the chain reaction and cease lipid peroxidation as well as oxidative stress. EGCG also helps in attenuating metal induced organ injury and fibrosis [16]. An electron paramagnetic resonance (EPR) study has reported that each molecule of EGCG has ability to trap/scavenge six superoxide anion or hydroxyl radicals [17]. This capability of EGCG makes it a potent antioxidant, which engulfs ROS generated and further regulates the oxidative stress in a biological system.

Herein, the anti-oxidative and other related potential of epigallocatechin gallate (EGCG) are well studied, however, its efficacy in modulating the DNA damage is still poorly explored in vivo. Thus the present study attempts to investigate the protective efficacy of EGCG against arsenic induced oxidative stress-mediated genotoxicity. Present observations clearly suggest that EGCG considerably inhibits the oxidative stress, owing to its pharmacologically active components like C-gallate ring and eight phenolic hydroxyl groups that make it a strong free radical scavenger, oxidative stress regulator and metal chelator. Moreover, EGCG could afford protection against DNA damage that resulted from arsenic exposure in mice. Thus this study for the first time proposes geno-protective efficacy of EGCG against arsenic. Collectively, this data suggests EGCG might be regarded as a potent geno-protective agent and demands further pharmacological studies.

Materials and methods

Experimental animals

Adult balb/C female mice weighing around 25–35 g were procured from the Central Animal House, Panjab University, Chandigarh and used throughout the experimental studies. Animals were kept in temperature- and humidity-controlled conditions on a 12:12-h light–dark cycle and were fed with commercially available rat pellet diet (Ashirwad Industries, Punjab, Hindustan Lever, India), had water and feed ad libitum. The mice were acclimatized for 7 days prior to experimental use. All the animals were housed, cared and used experimentally in accordance with the 'Guide for the Care and Use of Experimental Animals' approved by Institutional Animal Ethics Committee, Panjab University, Chandigarh (Registration Number: 45/GO/ReBi/S/99/CPCSEA). Females have been used for the current study as both the sexes of balb/C mice display similar response against different metal toxicities [18].

Chemicals

Epigallocatechin gallate (EGCG, Mol.Wt. 458.372) used for present study was supplied by Cayman, USA. Sodium meta arsenite ($NaAsO_2$) was obtained from Himedia Pvt. Ltd. DCFH-DA was purchased from Sigma. Chemicals like tri-sodium citrate, acridine orange, fetal bovine serum, EDTA, tris HCl, DMSO, Triton, Low melting Point Agarose (LMPA), Normal melting Point Agarose (NMPA) were purchased from Himedia laboratories Pvt. Ltd. Mumbai and colchicine were obtained from Sisco Research Laboratory Mumbai. Ethidium bromide and proteinase K were acquired from Chromous Biotech Limited, Bangalore. NaCl, HCL and NaCl were procured from Central Drug House (P) Ltd. New Delhi. All chemicals were of analytical grades specifications.

Effective dose estimation of arsenic and epigallocatechin gallate

For obtaining the toxicologically effective dose of arsenic, LD_{50} of the arsenic compound has been calculated by employing probit analysis using SPSS software 21 that came

out to be 15.71 mg/kg b.wt. for calculating LD_{50}, a range the range of toxicity was established by giving different doses (10, 20, 30, 40, 50 mg/kg b.wt.) of arsenic to six different groups and mortality rate of mice in these groups was observed for 96 h [19]. In the present study, the sub-lethal dose (1/10th of LD_{50}) was given intraperitoneally for 10 days to induce observable genotoxicity in the peripheral blood lymphocytes, hepato-renal tissues and bone marrow cells of mice. The sub lethal dose of 1.5 mg/kg b.wt. for a short duration of ten days was given to mimic the environmentally relevant exposure of arsenic in humans.

The different doses of EGCG (25 and 50 mg/kg b.wt.) were selected based on the results obtained in preliminary experiments to evaluate the maximally tolerated dose that did not evoke micronucleated erythrocytes. Two doses of EGCG (25 and 50 mg/kg b.wt.) used in the study were also supported by already existing literature that suggested the effective anti-oxidative efficacy of EGCG against different toxic agents between the ranges 5–100 mg/kg b.wt. in rodents [20, 21]. Oral dosing mimics the most commonly used mode of administration of substances to humans which are being tested for the remedial purpose [22].

Experimental design

The animals were divided into following nine groups of five animals each. EGCG treatment was given as prophylactic and therapeutic treatments.

Group I	Control group was administered normal saline (i.p.)
Group II	Arsenic treated—mice were administered 1.5 mg/kg b.wt. (1/10th of LD_{50} of sodium meta arsenite) of arsenic intraperitoneally for 10 days
Group III	DMSO treated (Carrier group)—mice were administered 0.05% of DMSO for 15 days orally
Group IV	*EGCG* treatments (EGCG-25 mg)—mice were administered 25 mg/kg b.wt. of EGCG for 15 days orally
Group V	Pre-EGCG treatment (Pre-EGCG-25 mg)—mice were given 25 mg/kg b.wt. of EGCG for first 15 days orally, and arsenic was administered (1.5 mg/kg b.wt.) for next 10 days
Group VI	Post EGCG treatment (Post-EGCG-25 mg)—mice were administered 1.5 mg/kg b.wt. of arsenic (i.p.) for first 10 days and then were given 25 mg/kg b.wt. of EGCG orally for next 15 days
Group VII	EGCG treatment (EGCG-50 mg)—mice were given 50 mg/kg b.wt. of EGCG for 15 days orally
Group VIII	Pre-EGCG treatment (Pre-EGCG-50 mg)—mice were given 50 mg/kg b.wt. EGCG for first 15 days orally and 1.5 mg/kg b.wt. of arsenic was administered for next 10 days
Group IX	Post-EGCG treatment (Post-EGCG-50 mg)—1.5 mg/kg b.wt. of arsenic was administered intraperitoneally for first 10 days, then 50 mg/kg b.wt. of EGCG was given orally

Animals were sacrificed after 24 h of last exposure under mild ether anesthesia and blood samples were obtained from jugular vein. After blood sampling, animals were dissected and bone marrow was taken from femurs and liver and kidney tissues were procured. ROS production was carried out in blood lymphocytes, lipid peroxidation (LPO), reduced glutathione (GSH), catalase (CAT), superoxide dismutase (SOD), glutathione-*S*-transferase (GST) and glutathione reductase (GR) from 10% homogenates of hepato-renal tissue, DNA fragmentation from both the tissues, chromosomal aberrations and micronucleation from bone marrow cells and comet tailing from blood lymphocytes.

Measurement of ROS production

Blood lymphocytes were isolated by centrifugation of diluted blood in histopaque density gradient (108, Sigma) according to the manufacturer's guide. Freshly isolated lymphocytes were resuspended in PBS (pH 7.4) to a final concentration 10^6 cell/ml. ROS generation was recorded by flow cytometry using peroxide sensitive fluorescence probe, 2,7-dichlorofluorescein diacetate (DCFH-DA).

Estimation of oxidative stress markers and hepato-renal antioxidants

10% homogenates were prepared in Tris–HCl buffer (pH-7.4) using a homogenizer at 4 °C. The extent of lipid peroxidation was measured according to the method of Buege and Aust [23]. Reduced glutathione was assayed by the method of Beutler et al. [24]. Estimation of various antioxidants was assessed by estimating catalase (CAT) by Luck [25], superoxide dismutase (SOD) by Kono [26], glutathione-*S*-transferase (GST) by Habig et al. [27] and glutathione reductase (GR) by Horn [28] methods spectrophotometrically.

DNA fragmentation

In order to verify the genomic DNA fragmentation, agarose gel electrophoresis was performed using standard Phenol: Chloroform: Isoamyl alcohol method of Sambrook et al. [29].

Chromosomal aberrations test

Chromosomal preparations from bone marrow cells were carried out according to Das et al. with some modifications [30]. Prior to sacrifice; animals were injected with colchicine (4 mg/kg b.wt.). Femurs were taken out and bone marrow cells were flushed in hypotonic tri-sodium citrate solution, incubated, washed and fixed Carnoy's fixative. Slides are prepared and air dried and stained with Giemsa. 100 well spread metaphasic plate of cells were analyzed per group.

Micronuclei test

Bone marrow cells were flushed using fetal bovine serum. The cells were dispersed by gentle pipetting and centrifugation. The micronuclei formation was analyzed using Acridine orange staining method by Hayashi et al. [31]. The frequencies of micronuclei in polychromatic erythrocytes (PCE) were estimated by scoring 1000 PCE per group.

Comet assay

DNA damage in blood lymphocytes was assessed using single-cell gel electrophoresis according to the method of Singh et al. with minor modifications [32]. Cells were lysed, electrophoresed, neutralized in different buffers and then finally stained with ethidium bromide. A total number of 200 cells were examined for comet scoring and the quantification of DNA strand breaks was performed on obtained images by using Comet Score™ (version 1.5) software to measure the % DNA in the tail, tail moment and olive tail moment.

Inhibitory index

The effective dose of EGCG in attenuating arsenic educed genotoxic disturbances was assessed by inhibitory index formula using method of Madrigal-Bujaidar et al. [33]. This index is determined in chromosomal aberrations, micronuclei formation and comet tailing, as all these are quantitative parameters of genotoxicity.

$$\text{Inhibitory index} = \left[1 - \left(\frac{\text{EGCG plus arsenic treatments (Pre and post - control)}}{\text{Arsenic treatment} - \text{Control}}\right)\right] \times 100$$

Statistical analysis

All the values were expressed as mean ± S.D. and statistical analysis was performed by one way ANOVA (analysis of variance) followed by Tukey's post hoc test. The data analysis was done using SPSS version 21. Value with $p \leq 0.01$ was considered as statistically significant, $p \leq 0.001$ very significant and $p \leq 0.0001$ extremely significant. Symbol (a) denotes the comparison with control group (b) denotes comparison with arsenic treated group and (c) denotes comparison between two doses of EGCG i.e. Pre-EGCG (25 mg/kg b.wt.) and Pre-EGCG (50 mg/kg b.wt.).

Results

ROS measurement

Enhanced ROS generation in arsenic exposed group and its modulation by prophylactic and therapeutic treatments of EGCG are presented in Fig. 1a, b. Ten days arsenic exposure elicited an extremely significant (a*) increase in production of intracellular ROS (114%) in blood lymphocytes (214.92 ± 2.8) as compared to control mice. Pre-treatment with both the doses (25 and 50 mg/kg b.wt.) of EGCG significantly (b*) decreased the elevated ROS levels (49.7% and 60.42%) as compared to arsenic treated mice, suggesting free radical scavenging activity of EGCG (Fig. 1a, b). Whereas, post treatments of EGCG (25 and 50 mg/kg b.wt.) were moderately effective in preventing arsenic induced ROS generation (117.17 ± 1.8 and 132.45 ± 2.7). The pre-treatments of EGCG (25 and 50 mg/kg b.wt.) were more effective in averting enhanced ROS levels (108.05 ± 1.4 and 85.18 ± 2.6). Furthermore, pre-treatment with higher dose (50 mg/kg b.wt.) depicted a noticeable decrease (c*) in ROS levels as compare to lower one in halting arsenic induced increased ROS generation (Fig. 1a, b). No significant effect on the ROS generation was observed in mice treated with EGCG alone treated groups (25 and 50 mg/kg b.wt).

Oxidative stress markers (LPO and GSH) and enzymatic antioxidants (CAT, SOD, GST, GR)

Arsenic exposure for 10 days instigated statistically significant ($p < 0.0001$) intensification in the levels of lipid peroxidation (LPO) in liver and kidney tissues of mice. Arsenic intoxicated group revealed a fourfold increase in LPO (10.31 ± 0.734a*) in the liver (Fig. 2a) and a twofold increase in the kidney (7.63 ± 1.002a*) tissues as compared to control (2.34 ± 0.25 and 3.598 ± 0.224) (Fig. 2a). A significant ($p \leq 0.0001$) decline was observed in reduced glutathione (GSH) levels, revealing 48% (22.58 ± 2.15a*) and 52% (17.724 ± 2.85a*) fall in liver and kidney tissues respectively in comparison to the hepato-renal tissues of the control group (43.55 ± 1.74 and 36.719 ± 2.91) represented in Fig. 2b. The pre-treatment with EGCG at both the doses

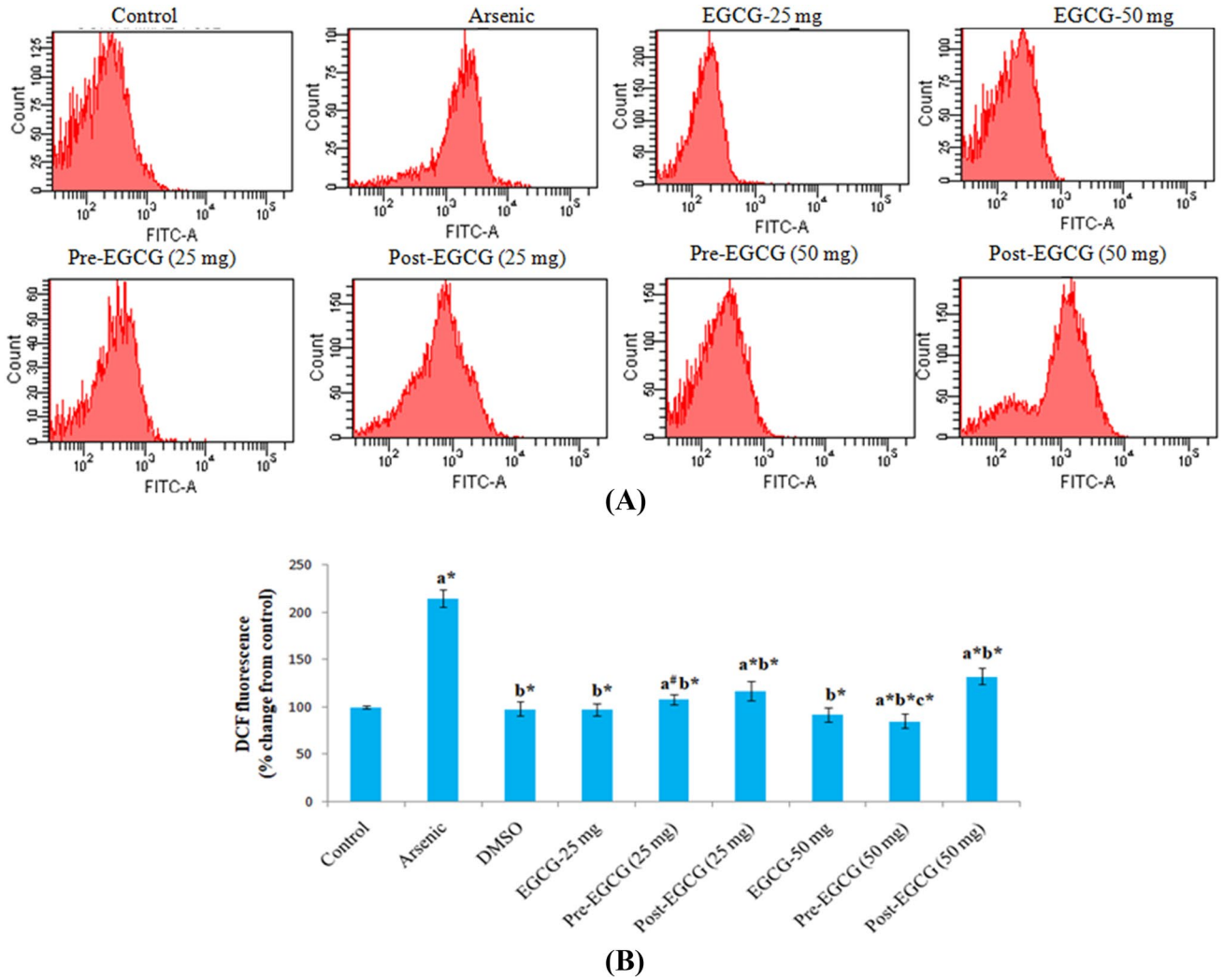


Fig. 1 **a** Representing histograms of ROS production obtained by FACS analysis in different groups. **b** DCF fluorescence (% change from control) in blood lymphocytes of control, arsenic, EGCG-25 mg, Pre-EGCG (25 mg), Post-EGCG (25 mg), EGCG-50 mg, Pre-EGCG (50 mg) and Post-EGCG (50 mg) treated groups. Values are shown as Mean ± S.D. (n = 5). Levels of significance: # ≤ 0.005 (statistically significant); * ≤ 0.0001 (very statistically significant). a = comparison with control; b = comparison with arsenic; c = comparison between Pre-EGCG (25 mg) and Pre-EGCG (50 mg)

(25 and 50 mg/kg b.wt.) significantly ($p < 0.001$) decreased the elevated LPO levels by 25% ($7.75 \pm 0.289a^*$) and 51% ($5.02 \pm 0.687b^{\#}$) in liver and by 19% ($6.179 \pm 0.264a^{\#}$) and 35% ($4.927 \pm 0.358b^{\#}$) in kidney tissues when compared to arsenic treated group (Fig. 2a) The GSH levels were also restored by 44% ($32.52 \pm 1.91a^{\#}b^{\#}$) and 72% ($38.78 \pm 1.98b^*$) in liver and 52% ($26.875 \pm 3.00a^{\#}b^{\#}$) and 80% ($31.857 \pm 1.66b^*$) in kidney tissue respectively in both pre-EGCG treatments (25 and 50 mg/kg b.wt.) when compared with arsenic treated group (Fig. 2b). Post treatments of both the doses were moderately effective in reducing hepato-renal LPO levels and enhancing the GSH levels in mice. Among prophylactic treatments, the higher dose (50 mg/kg b.wt.) was more effective (c^*) in regulating arsenic induced oxidative stress and offering antioxidant potential (Fig. 2a, b).

Arsenic exposure elicited an extremely significant ($p \leq 0.0001$) decline in the activities of antioxidants enzymes. It decreased catalase levels by ~40%, SOD by ~45%, GST by ~50% and GR by ~40–50% in hepato-renal tissues of mice as compared to control group. Pre-treatment of EGCG at both the doses (25 and 50 mg/kg b.wt.) significantly restored the activities of catalase by 46% ($a^{\#}b^{\$}$) and 72% ($b^{\#}$), SOD by 34% ($a^{\#}$) and 73% ($b^{\$}$), GST by 31% ($b^{\#}$) and 67% ($a^{\$}b^*$) and GR by 52% ($a^*b^*$) and 74% ($a^{\$}b^*c^{\#}$) respectively in hepatic tissue as compare to arsenic treated group (Table 1). Both pre-treatments (25 and 50 mg/kg b.wt.) also reinstated the activities of catalase by 20% ($a^{\#}b^{\#}$) and 44% ($b^*c^{\#}$), SOD by 33% ($a^{\#}$) and 65% ($b^{\#}c^{\$}$), GST by

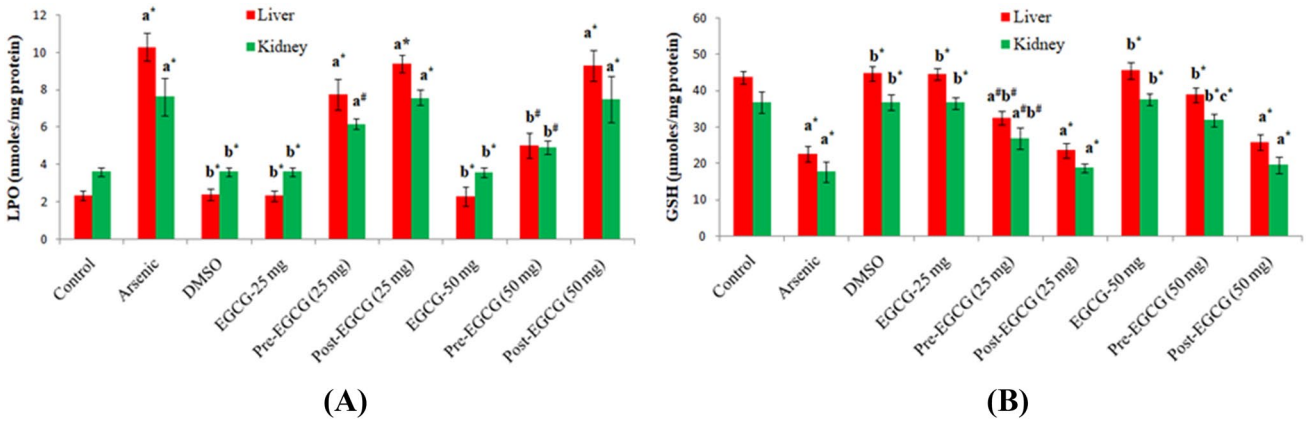


Fig. 2 **a** Lipid peroxidation (LPO) (n moles/mg protein) in liver and kidney tissues of control, arsenic, EGCG-25 mg, Pre-EGCG (25 mg), Post-EGCG (25 mg), EGCG-50 mg, Pre-EGCG (50 mg) and Post-EGCG (50 mg) treated groups. **b** Reduced glutathione (GSH)—µ moles/mg protein in liver and kidney tissues of control, arsenic, EGCG-25 mg, Pre-EGCG (25 mg), Post-EGCG (25 mg), EGCG-50 mg, Pre-EGCG (50 mg) and Post-EGCG (50 mg) treated groups. Values are shown as Mean ± S.D. (n = 5). Levels of significance: Levels of significance: $ ≤ 0.01; @ ≤ 0.05; $ ≤ 0.01; # ≤ 0.005; * ≤ 0.0001. a = comparison with control; b = comparison with arsenic; c = comparison between Pre-EGCG (25 mg) and Pre-EGCG (50 mg)

60% ($a^{\#}b^{\#}$) and 92% ($a^{\$}b^{*}c^{\$}$) and GR by 28% ($a*b^{\#}$) and 57% ($a^{\$}b*c*$) respectively in renal tissue as compared to arsenic treated mice (Table 2). Among both doses, the higher dose (50 mg/kg b.wt.) was found to be more effective ($c^{\#}$) in normalizing the altered antioxidant levels, indicating a dose dependant positive efficacy of EGCG whereas, post treatments were effective in average range.

DNA fragmentation

Agarose gel electrophoretograms represent the genomic DNA damage in liver and kidney tissues of control and different treatment groups. Control and EGCG (25 and 50 mg/kg b.wt.) treated groups revealed intact DNA bands in hepato-renal tissues of mice (lane 1, 2, 5, 6 of Fig. 3a, b). Arsenic intoxication for ten days revealed a significant increase in the level of DNA fragmentation in mice liver (lane 3, 4 of Fig. 3a) and kidney (lane 3,4 of Fig. 3b) as compared to control. Pre-treatment (25 and 50 mg/kg b.wt.) of EGCG to arsenic intoxicated mice considerably lessened the arsenic educed genomic DNA damage. Prior treatment with higher dose (50 mg/kg b.wt.) was more effective in attenuating the genomic damage in mice tissues as shown in electrophoretograms of lane 7 and 8 of Fig. 3a, b revealing geno-protective efficacy of EGCG. Moderate preventive efficacy of EGCG (25 and 50 mg/kg b.wt.) was seen in post treatment groups (lane 9 and 10 of Fig. 3a, b).

Table 1 Protective effect of EGCG against arsenic induced inhibition on antioxidant defense system in liver tissue of mice

Groups	CAT	SOD	GST	GR
Control	57.074 ± 1.232	19.083 ± 0.901	3.163 ± 0.097	129.051 ± 1.272
Arsenic	31.958 ± 1.009a*	10.627 ± 0.568a*	1.693 ± 0.072a*	60.995 ± 1.265a*
DMSO	57.690 ± 1.213b*	19.036 ± 0.688b*	3.156 ± 0.093b*	129.094 ± 1.311b*
EGCG-25 mg	58.671 ± 1.342b*	19.537 ± 1.732b*	3.213 ± 0.481b*	129.431 ± 2.0054b*
Pre-EGCG (25 mg)	46.572 ± 2.917$a^{\#}b^{\$}$	14.281 ± 1.094	2.216 ± 0.736$a*b^{\#}$	92.447 ± 4.925a*b*
Post-EGCG (25 mg)	35.781 ± 3.194a*	10.996 ± 1.457a*	1.754 ± 0.855a*	63.486 ± 2.948a*
EGCG-50 mg	59.121 ± 2.774b*	20.018 ± 0.738b^{*}	3.261 ± 0.118b*	131.811 ± 1.639b*
Pre-EGCG (50 mg)	54.914 ± 1.082$b^{\#}c^{\$}$	18.361 ± 0.681$b^{\$}$	2.832 ± 0.092$a^{\$}b*$	106.575 ± 1.119$a^{\$}b*c^{\#}$
Post-EGCG (50 mg)	39.428 ± 3.441a*	11.058 ± 1.926a*	1.904 ± 0.562a*	68.337 ± 4.013a*

Values are shown as Mean ± S.D. (n = 5). Levels of significance: $^{\$} ≤ 0.05$; $^{\#} ≤ 0.005$; * ≤ 0.0001

a = Comparison with control; b = comparison with arsenic; c = comparison between Pre-EGCG (25 mg) and Pre-EGCG (50 mg)

Table 2 Protective effect of EGCG against arsenic induced inhibition on antioxidant defense system in kidney tissue of mice

Groups	CAT	SOD	GST	GR
Control	43.538 ± 0.984	22.844 ± 0.835	2.661 ± 0.081	78.458 ± 1.265
Arsenic	28.670 ± 1.064a*	12.629 ± 0.763a*	1.231 ± 0.062a*	45.185 ± 1.035a*
DMSO	43.414 ± 1.009b*	22.009 ± 0.699b*	2.657 ± 0.084b*	77.908 ± 1.301b*
EGCG-25 mg	43.665 ± 2.53b*	22.961 ± 1.859b*	2.676 ± 0.183b*	79.441 ± 2.01b*
Pre-EGCG (25 mg)	34.047 ± 3.625a$^{\#}$b$^{\#}$	16.774 ± 2.005a$^{\#}$	1.971 ± 0.261a$^{\#}$b$^{\#}$	58.475 ± 2.27a*b$^{\#}$
Post-EGCG (25 mg)	29.535 ± 2.003a*	13.056 ± 1.472a*	1.373 ± 0.163a*	42.469 ± 2.04a*
EGCG-50 mg	44.135 ± 1.589b*	23.009 ± 0.563b*	2.771 ± 0.086b*	79.895 ± o.609^{b*}b*
Pre-EGCG (50 mg)	41.258 ± 1.004b*c$^{\#}$	20.802 ± 0.742b$^{\#}$c$^{\$}$	2.374 ± 0.092b*c$^{\$}$	71.115 ± 0.901a$^{\$}$b*c*
Post-EGCG (50 mg)	30.114 ± 2.851a*	13.817 ± 1.931a*	1.482 ± 0.149a*	42.742 ± 2.60a*

Values are shown as Mean ± S.D. (n = 5). Levels of significance: $^{\$}$≤0.05; $^{\#}$≤0.005; *≤0.0001

a = Comparison with control; b = comparison with arsenic; c = comparison between Pre-EGCG (25 mg) and Pre-EGCG (50 mg)

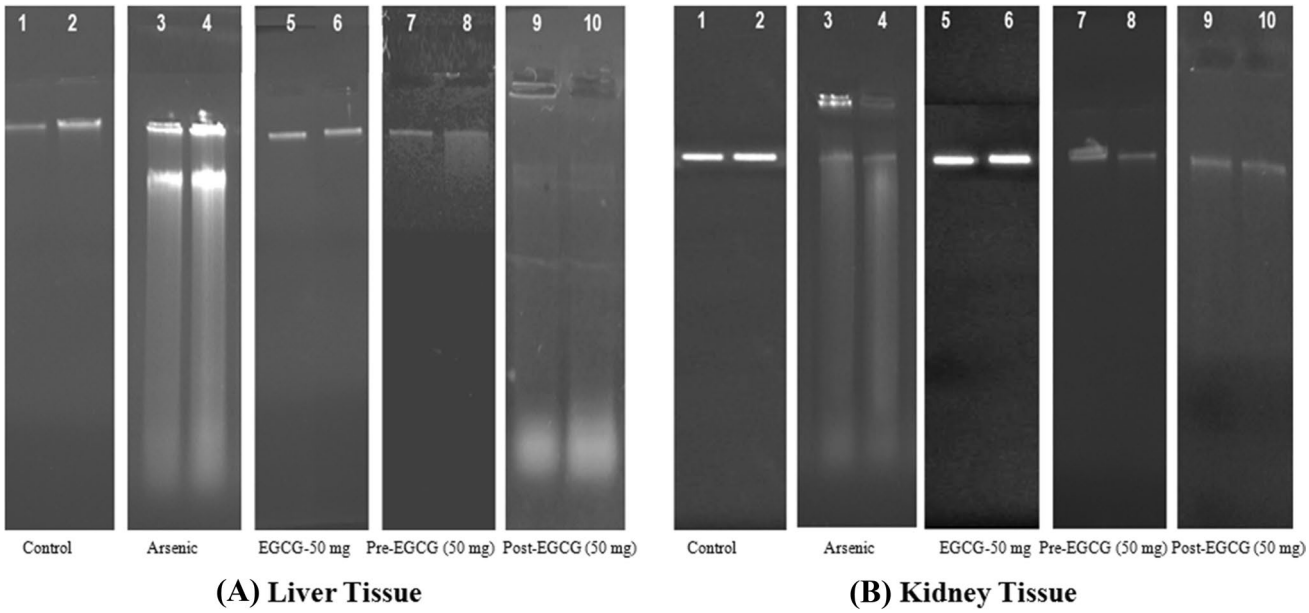


Fig. 3 **a** DNA fragmentation in liver tissue of balb/C mice. Lane 1 and 2—Control, Lane 3 and 4—Arsenic, Lane 5 and 6—EGCG-50 mg, Lane 7 and 8—Pre-EGCG (50 mg), Lane 9 and 10—Post-EGCG (50 mg). **b** DNA fragmentation in kidney tissue of balb/C mice. Lane 1 and 2—Control, Lane 3 and 4—Arsenic, Lane 5 and 6—EGCG-50 mg, Lane 7 and 8—Pre-EGCG (50 mg), Lane 9 and 10—Post-EGCG (50 mg)

Chromosomal aberrations test

Analysis of 100 well spread metaphasic plates of the control groups revealed forty acrocentric chromosomes with well defined contour, typical position of centromere and well spread chromatid arms (97%) (Fig. 4a, b). Ten days arsenic exposure caused various structural and numerical aberrations (Table 3a), physiological (Table 3b), and exchange aberrations (Table 3c) in bone marrow cells of mice. Arsenic exposed group exhibited the highest percentage of exchange aberrations like) ring formation (8%) due to the fusion of telomeric ends of the same chromosome (Fig. 4c). Whereas, 8% cells revealed the formation of bridge i.e. formed by the fusion of the telomeric ends of different chromosomes (Fig. 4d) and centromeric fusion (14%), formed by the fusion of centromeres of two chromosomes (Fig. 4e). 20% of cells revealed physiological aberrations like wooly contour, sticky, and condensed nature. Structural anomalies like one or multiple chromatid breaks (14%) were observed in multiple cells (Fig. 4f). Aneuploidy was observed in 6% of the cells that signifies genotoxic impact of arsenic.

Epigallocatechin gallate was tested for its geno-protective potential against arsenic induced genotoxicity. Treatment of EGCG (25 mg and 50 mg/kg.b.wt.) for 15 days effectively

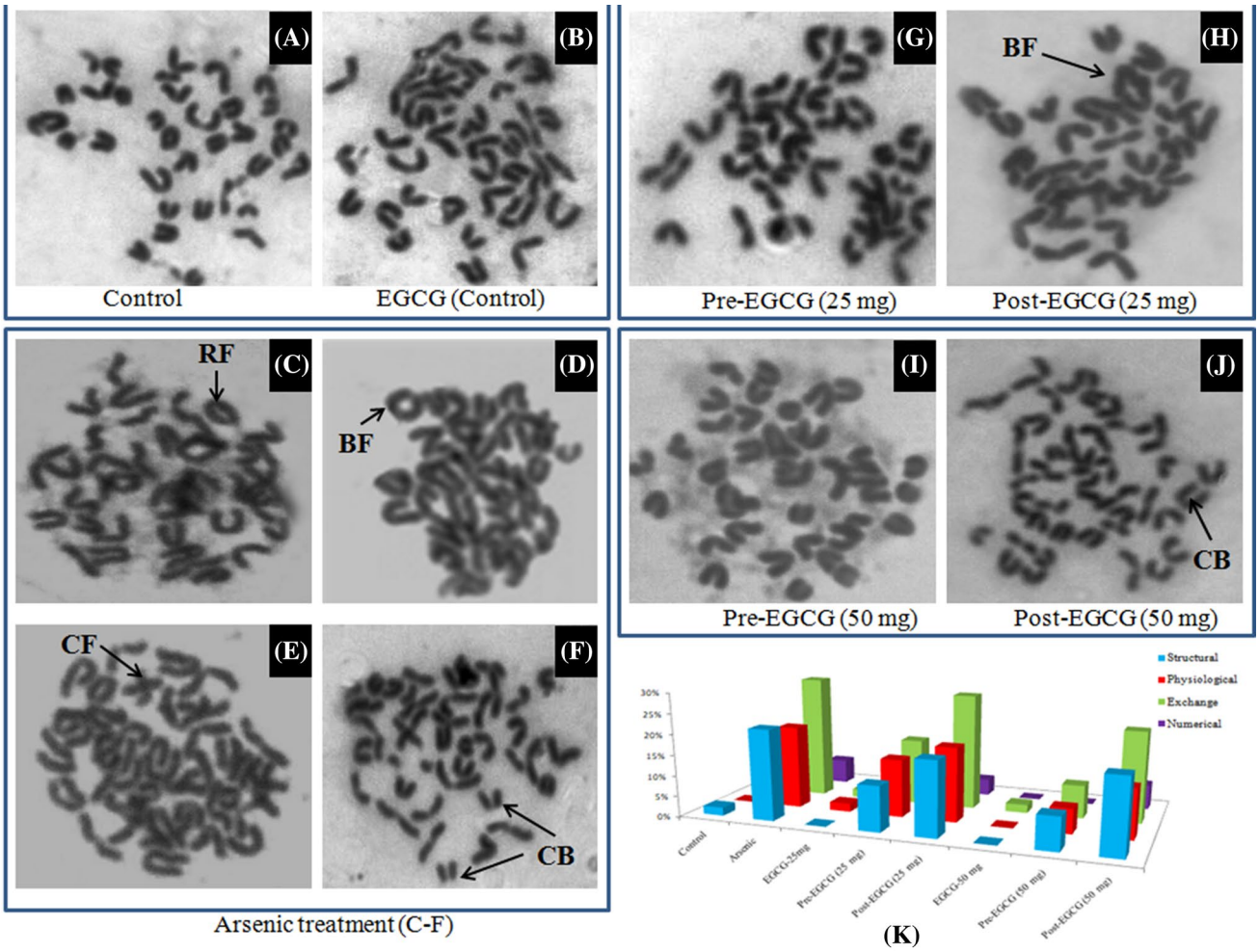


Fig. 4 **a**, **b** Metaphasic spreads of the bone marrow cells of control, EGCG-25 mg and EGCG-50 mg treated groups. **c**–**f** Metaphasic spreads of the bone marrow cells of arsenic treated group. **c**, **d** showing centromeric fusion (CF) and bridge formation (BF); **e, f** showing ring formation (RF) and chromatid breaks (CB). **g**, **h** Metaphasic spreads of the bone marrow cells of Pre-EGCG- 25 mg showing normal extended 40 acrocentric chromosomes and Post-EGCG-25 mg treated groups revealing Y-chromatid break (Y). **i**, **j** Metaphasic spreads of the bone marrow of Pre-EGCG-50 mg representing normal well extended 40 acrocentric chromosomes and Post-EGCG-50 mg treated groups showing ring formation. **k** 3-D Graph showing various types of chromosomal aberrations in control, arsenic, EGCG-25 mg, Pre-EGCG (25 mg), Post-EGCG(25 mg), EGCG-50 mg, Pre-EGCG (50 mg) and Post-EGCG (50 mg) treated groups

attenuated the clastogenic impact of arsenic (Table 3a–c). The percentage of cells with aberrations and deformed chromosomal structure were markedly decreased after the administration of EGCG. Pre-EGCG treatment at lower dose (25 mg/kg b.wt.) effectively reduced the arsenic mediated genotoxic changes by decreasing the exchange aberrations by 50%, physiological aberrations by 40% and structural by approx. 60–70% (Fig. 4g) as compared to arsenic group. Whereas, pre-treatment group with higher dose (50 mg/kg b.wt.) demonstrated highest protective efficacy among different groups by showing 80–90% reduction in genetic perturbations (Fig. 4i). On the other hand, post treatments of both the doses (25 mg and 50 mg/kg b.wt.) were found to be moderately effective in attenuating arsenic induced genotoxicity as these groups still revealed some chromosomal anomalies like bridge formation, ring formation and multiple chromatid breaks (Fig. 4h, j; Table 3a–c). The genotoxic effect of arsenic on chromosomal aberrations and preventive efficacy of EGCG at both doses in pre and post treatments were clearly shown in 3-D graphical representation in Fig. 4k.

The effective dose of EGCG was determined by evaluating inhibitory index of all treatments of EGCG. Prophylactic EGCG treatment at lower dose (25 mg/kg b.wt.) exhibited 38.5% inhibition on structural and numerical aberrations, 25% on physiological aberrations and 50% on exchange aberrations, whereas, higher dose (50 mg/kg b.wt.) revealed maximum inhibitory index of 73.1% on structural and numerical aberrations, 70% on physiological and 78.6% on exchange aberrations respectively. Post treatments of both the doses of EGCG showed moderate inhibitory indices.

Table 3 Arsenic induced various types of aberrations in bone marrow cells and its modulation by EGCG (Total number of cells observed = 100/treated animal; n = 5)

	Control (%)	Arsenic (%)	EGCG-25 mg (%)	Pre-EGCG (25 mg) (%)	Post-EGCG (25 mg) (%)	EGCG-50 mg	Pre-EGCG (50 mg) (%)	Post-EGCG (50 mg) (%)
(a) Structural and numerical aberrations								
Chromosomal breaks	–	8	–	6	8	–	4	6
Multiple chromatid break	–	6	–	4	6	–	1	5
Aneuploidy	–	6	–	4	4	–	2	6
(b) Physiological aberrations								
Condensed chromosome	–	8	–	6	8	–	2	7
Wooly chromosome	–	6	–	4	6	–	2	5
Sticky chromosome	1	6	2	5	6	–	2	5
(c) Exchange aberrations								
Centromeric fusion	–	14	–	8	12	–	4	12
Ring formation	2	8	1	5	8	1	2	7
Bridge formation	–	8	–	4	8	–	2	7

Table 4 Inhibitory indices of different treatments of EGCG (25 and 50 mg/kg b.wt.) in chromosomal aberrations

Groups	Structural and numerical aberrations (%)	Physiological aberrations (%)	Exchange aberrations (%)
Pre-EGCG (25 mg)	38.5	25	50
Post-EGCG (25 mg)	15.4	0	7
Pre-EGCG (50 mg)	73.1	70	78.6
Post-EGCG (50 mg)	15.4	15	11

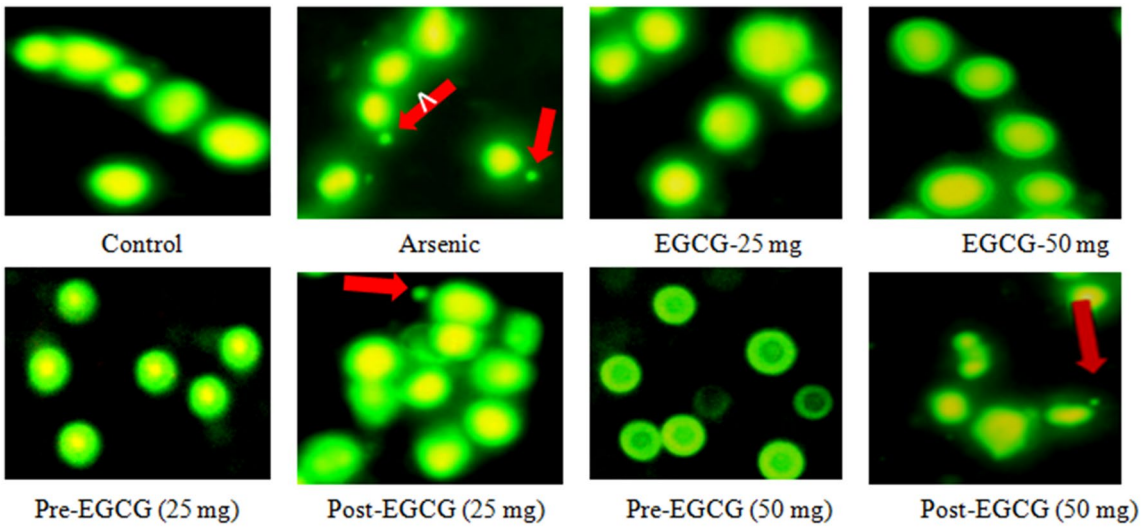
Pre-treatment at higher dose (50 mg/kg b.wt.) was found to be most effective than all other treatments due to its maximum inhibitory index (Table 4).

Micronuclei assay

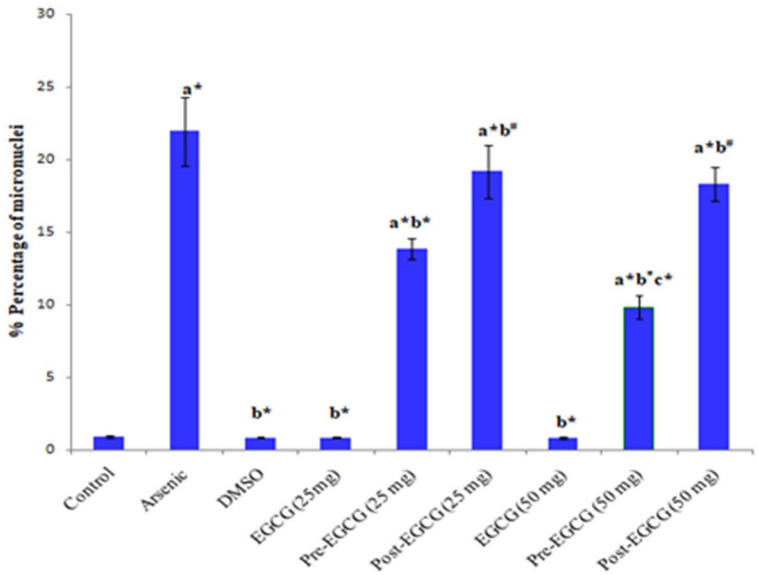
The frequency of micronuclei formation in control and EGCG alone treated groups remained at the basal levels (0.83 ± 0.059 and 0.787 ± 0.094). Sub chronic arsenic exposure for 10 days significantly ($p < 0.0001$,) enhanced the percentage frequency of micronuclei formation (21.93 ± 2.364) in bone marrow cells as compared to control group revealing its clastogenic potential (a*). Pre-treatment of EGCG (25 mg and 50 mg/kg b.wt.) significantly ($p \leq 0.0001$, b*) decreased frequency of micronucleation by 37% and 55.23% in bone marrow cells respectively when compared to arsenic treated group, exhibiting a dose dependant preventive efficacy of EGCG (Fig. 5a, b). The preventive efficacy of EGCG was more adequate in pre-treated groups as compare to post treated groups in alleviating the arsenic induced increased MN formation. Pre-treatment of EGCG at higher dose (50 mg/kg b.wt.) was found to be more efficient (9.821 ± 0.823c*) as compared to lower dose of 25 mg/kg b.wt. (13.825 ± 0.727) in averting arsenic educed micronucleation (Fig. 5a, b). 38.5% (25 mg/kg b.wt.) and 57.5% (50 mg/kg b.wt.) inhibitory indices were observed among pre-EGCG treatments whereas, post treatments showed less inhibitory indices of 13% and 17% with both the treatments. Among all EGCG treatments, pre-treatment with 50 mg/kg b.wt. was found to be most effective having the highest inhibitory index of 57.5% against arsenic induced micronucleation.

Comet assay

Control groups revealed basal or minimal comet tailing parameters viz. % DNA tail (2.324 ± 0.432), tail moment (0.268 ± 0.133) and olive tail moment (0.945 ± 0.113). An extremely significant ($p < 0.0001$, a*) increase in various comet assay parameters such as % DNA in tail (25.038 ± 1.289), tail moment (2.778 ± 0.158) and olive tail moment (6.878 ± 0.504) were observed in blood lymphocytes of mice treated with arsenic when compared with normal control mice (Fig. 6a–d). Pre-administration of EGCG at both doses (25 mg and 50 mg/kg b.wt.) significantly ($p < 0.0001$, b*) reduced comet tailing as compared to arsenic treated mice. Pre-EGCG treatments (25 mg and 50 mg/kg b.wt.) reduced all the comet parameters to 15.894 ± 1.059 and 10.65 ± 0.47 in case of % DNA tail, 1.75 ± 0.109 and 0.942 ± 0.107 in tail moment and 3.932 ± 0.421 and 2.803 ± 0.375 in olive tail moment respectively (Fig. 6a–d). However, post treatments were not much effective in attenuating arsenic educed comet



(A)



(B)

Fig. 5 **a** Representative images of the formation of micronuclei in bone marrow cells of control, arsenic, EGCG-25 mg, Pre-EGCG (25 mg), Post-EGCG (25 mg), EGCG-50 mg, Pre-EGCG (50 mg) and Post-EGCG (50 mg) treated groups. **b** Graph showing percentage frequency of micronuclei/1000 cells in bone marrow cells of control, arsenic, EGCG (25 mg), Pre-EGCG (25 mg), Post-EGCG (25 mg), EGCG (50 mg), Pre-EGCG (50 mg) and Post-EGCG (50 mg) treated groups. Values are shown as Mean ± S.D (n = 5). Levels of significance: # ≤ 0.005 (statistically significant); * ≤ 0.0001 (very statistically significant). a = comparison with control; b = comparison with arsenic; c = comparison between Pre-EGCG (25 mg) and Pre-EGCG (50 mg)

formation in mice lymphocytes. Whereas, among the pre-treatments of EGCG (25 mg and 50 mg/kg b.wt.), higher dose (50 mg/kb b.wt.) is statistically more effective in reducing comet tailing (Fig. 6a–d).

Pre-EGCG treatment at lower dose (25 mg/kg b.wt.) exhibited inhibitory index of 40.4% on % DNA tail, 41% on tail moment and 49.7% on olive tail moment whereas, higher dose of 50 mg/kg b.wt. revealed inhibition of 63.4% on % DNA tail, 73.2% on tail moment and 68.7% on olive tail moment. Post treatments with both the doses of EGCG showed moderate inhibitory indices. Pre-treatment with higher dose (50 mg/kg b.wt.) was found to be most effective, having the maximum inhibitory index in attenuating arsenic induced comet tailing (Table 5).

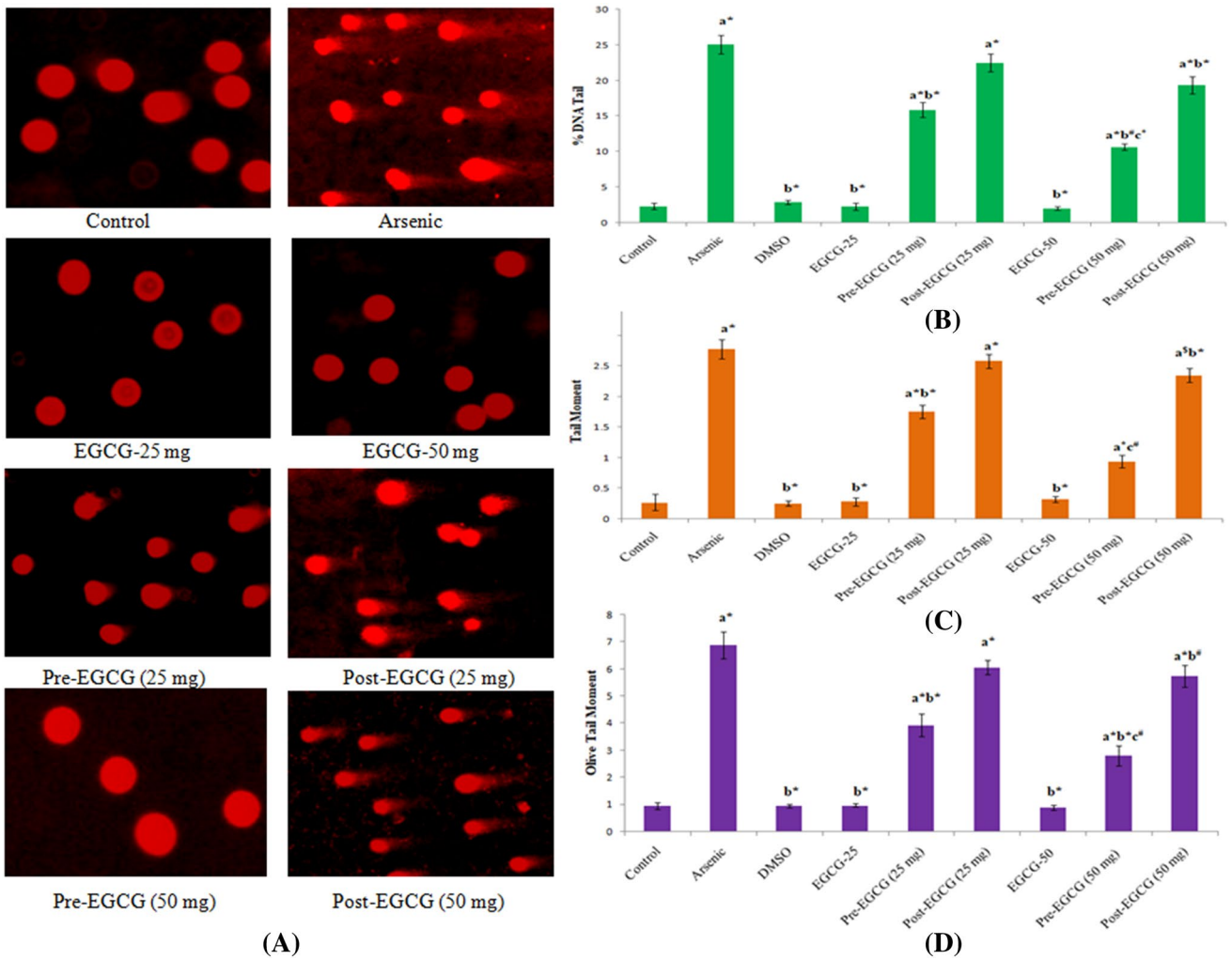


Fig. 6 **a** Photomicrographs of DNA migration patterns in blood lymphocytes of control, arsenic, EGCG-25 mg, Pre-EGCG (25 mg), Post-EGCG (25 mg), EGCG-50 mg, Pre-EGCG (50 mg) and Post-EGCG (50 mg) treated groups. **b** Graph showing % DNA in tail in control, arsenic, EGCG-25 mg, Pre-EGCG (25 mg), Post-EGCG (25 mg), EGCG-50 mg, Pre-EGCG (50 mg) and Post-EGCG (50 mg) treated groups. **c** Graph showing the tail moment in control, arsenic, EGCG (25 mg), Pre-EGCG (25 mg), Post-EGCG(25 mg), EGCG (50 mg), Pre-EGCG (50 mg) and Post-EGCG (50 mg) treated groups. **d** Graph showing the olive tail moment in control, arsenic, EGCG-25 mg, Pre-EGCG (25 mg), Post-EGCG(25 mg), EGCG-50 mg, Pre-EGCG (50 mg) and Post-EGCG (50 mg) treated groups. Values are shown as Mean ± S.D. (n = 5). Levels of significance: @ ≤ 0.05; $ ≤ 0.01; # ≤ 0.005; * ≤ 0.0001. a = comparison with control; b = comparison with arsenic; c = comparison between Pre-EGCG (25 mg) and Pre-EGCG (50 mg)

Table 5 Inhibitory indices of different treatments of EGCG (25 and 50 mg/kg b.wt.) in different comet parameters

Groups	% DNA tail	Tail moment (%)	Olive tail moment (%)
Pre-EGCG (25 mg)	40.4	41	49.7
Post-EGCG (25 mg)	11.35	7.95	14
Pre-EGCG (50 mg)	63.4	73.2	68.7
Post-EGCG (50 mg)	25.2	17.1	19.3

Discussion

The most studied mode of action for arsenic toxicity is the formation of reactive oxygen species (superoxide anion, hydroxyl radical, hydrogen peroxide) and nitrogen species which further leads to oxidative stress in the system [34]. Arsenic induced ROS effects basic cellular processes by altering cell proliferation, signal transduction and genotoxicity [35]. There are no evidence based treatments available to treat arsenic induced cellular insults, but antioxidants

have been encouraged. Epigallocatechin gallate is a well-documented anti-oxidant of green tea. In the present study, pre administration of EGCG markedly reduced the ROS generation and LPO, restored the antioxidant levels and significantly attenuated the alterations in different genotoxic indices.

According to the various studies, arsenic induced ROS generation is pre-requisite for metal induced redox imbalance and other cellular toxic havoc. In the present study, 10 days arsenic exposure caused marked elevation in the ROS generation in blood lymphocytes creating huge load of oxidative stress in the biological system. These observations are in conformity with the studies of Maheshwari et al. who have documented the excessive generation of ROS during arsenic exposure in human red blood cells [36]. Moreover, Dutta et al. has also observed arsenic induced enhanced ROS generation in renal tissue of mice [37]. The plausible mechanism underlying is the methylation of arsenic compounds during its metabolism which leads to production of large number of free radical species. Methylated forms of arsenic also release redox-active iron from ferritin (Fe^{3+}) which plays a central role in generating harmful oxygen species by promoting Haber–Weiss reaction [38].

The findings of current study showed increased levels of hepato-renal LPO (an indicator of tissue injury) in arsenic intoxicated mice. Lipid peroxidation is one of the main consequences of augmented oxidative stress associated with arsenic toxicity which occurs when the dynamic balance between pro-oxidant and antioxidant mechanism is impaired [39]. The enhanced LPO stimulated mitochondrial respiration which increased free radical release into the cytoplasm, resulted in elevation of oxidative stress [40]. Thus, in the present study the most probable reason for enhanced LPO could be due to increased oxidative stress and this elevated LPO further enhanced ROS production which disturbed membrane fluidity, damaged protein and DNA systems. In addition to this, hepato-renal GSH stores are also consumed for detoxification of arsenic induced ROS and peroxides. GSH plays an important role in detoxifying arsenic species and has been considered to be an important intracellular reductant for arsenic methylation and transport, which in turn helps in the removal of arsenic from the body. Depletion of hepatic and renal GSH facilitates accumulation of arsenic and causes oxidative stress [41]. The observed decline in the hepato-renal levels of GSH caused the accumulation of arsenic in tissues (data not shown) and further augmented the ROS production and supports the present observations of enhanced ROS production. Oxidative damage of hepatic and renal tissues is further validated by decreased activities of antioxidant enzymes (SOD, CAT, GST and GR). Most plausibly, arsenic mediated massive ROS generation depletes the intracellular stores of antioxidants. Reduced activity of catalase caused accumulation of H_2O_2 and enhanced production of hydroxyl radical via Fenton reaction whereas, decreased SOD levels indicate reduced dismutation of O_2^- to H_2O_2. Moreover, decreased levels of GST resulted in lowering the process of detoxifying xenobiotics, carcinogens and other toxic moieties from the body [42]. These observations lend support from studies of Muthumani and Miltonprabu, Al-Brakati et al. and Meharzadi et al. who have also investigated the arsenic mediated increased lipid peroxidation and loss of hepatic and renal antioxidant defense system [43–45]. These findings further support the view that the oxidative stress forms the basis of cellular redox imbalance leading to the structural and functional malformations in major biomolecules viz. DNA, proteins and lipids.

Arsenic induces DNA damage at multiple levels causing adverse impact on DNA repair mechanism, gene expression alterations via epigenetic modifications and aneuploidogenesis [46]. The present investigations revealed that arsenic augmented the genomic DNA fragmentation in hepatic and renal tissue of mice. These observations are in conformity with the findings of Dua et al. who have also noticed arsenic induced genomic DNA fragmentation in hepato-renal tissues of mice [41]. Cooke et al. also documented that reactive free radical species like hydroxyl radical reacted with thiamine and removed a hydrogen atom from methyl group and led to DNA breakage which could be the most plausible reason for DNA damage [47].

Many studies demonstrated that arsenic induced reactive oxygen species are involved in the DNA strand breaks and evoke chromosomal aberrations [7]. In our previous study Chopra et al., we have already correlated heavy metal induced oxidative stress and chromosomal aberrations [48]. The present study has also revealed elevated structural, physiological, numerical chromosomal aberrations and enhanced micronuclei formation in bone marrow cells of arsenic intoxicated mice. Sankar et al. and Odunula et al. have also observed arsenic induced chromosomal alterations such as breaks, reunion aneuploidy and enhanced micronucleation in bone marrow cells [49, 50]. Many human studies have also revealed that arsenic exposure is responsible for enhanced micronucleation in sputum cells, bladder cells, buccal cells and lymphocytes [51–53]. Many findings suggest that arsenite acts as aneugen at low doses [54] by interfering with spindle formation and functions as clastogen at higher doses [55]. Thus the observed chromosomal aberrations and micronucleation could be a result of inhibition of DNA repair enzymes and disarrangement of spindle apparatus.

Furthermore, the present data of arsenic induced chromosomal aberration and micronucleation is supported by quantification of DNA damage in the individual cells by studying the pattern of DNA migration. Ten days arsenic intoxication elicited an increase in DNA damage using single cell gel electrophoresis. The present observations are in agreement with Balakumar et al. who have also described

increased genomic damage in the arsenic administered rats [56]. The observed DNA damage could develop directly due to large number of accumulated free radicals and/or indirectly by interaction of arsenic with DNA by forming DNA adducts [9].

Many studies have documented the extensive pharmacological properties of EGCG in in vivo and in vitro investigations. EGCG reduces the risks related to heavy metal poisoning epidemiologically and experimentally [57]. Pre-administration of EGCG at both doses (25 and 50 mg/kg b.wt,) effectively decreased the ROS generation, reduced levels of LPO and enhanced the antioxidants levels in liver and kidney tissue. Whereas, post treatments of EGCG were found to be moderately effective in attenuating arsenic induced genotoxicity probably due to large ROS load and already initiated cellular damage. EGCG due to its free radical scavenging ability prevented the initiation and propagation of lipid peroxidation process and controls the tissue oxidative damage [58]. Furthermore, EGCG also has anti-lipid peroxidative property [59] that shields the membrane from free radicals attack [60]. However, EGCG also reduced the oxidative stress in both the tissues due to its antioxidant enhancing property [57] which regulates the pro-antioxidants balance in liver and kidney tissues of mice [61] and exerts a beneficial action against arsenic induced oxidative hepatic stress.

Many reports revealed that most of the catechins like EGCG possess antioxidant and free radical quenching properties due to the presence multiple structural components. Free radical scavenging ability of EGCG, is due to the presence of eight hydroxyl groups in a four ringed structure which enhances its antioxidant efficacy and makes it readily dissolvable in water and can be modified into many components like, stearic (SA), eicosapentaenoic (EPA), and docosahexaenoic acids (DHA) during EGCG metabolism, which enhances its ROS scavenging activity and can further reduce the oxidative stress [62]. The presence of the C ring gallate group promotes the free radical engulfing ability of this compound that helps in its direct binding with ROS and facilitate their elimination during the oxidation process [63].

EGCG efficiently reduced the DNA damage in hepatic and renal tissues, comet formation in blood lymphocytes, chromosomal anomalies and micronucleation in bone marrow cells of mice. EGCG has eight phenolic hydroxyl groups which are most potent scavengers of the free radicals and inhibit cellular DNA damage [64]. It also has metal chelating activity as it binds with metal and prevents oxidation of DNA [62]. EGCG, being a flavonoid may act as hydrogen donor (or single electron donor) to lessen the formation of reactive oxygen species, including superoxide and hydroxyl radicals generated by Fenton reaction [65]. Preventive efficacy of higher dose of EGCG is further

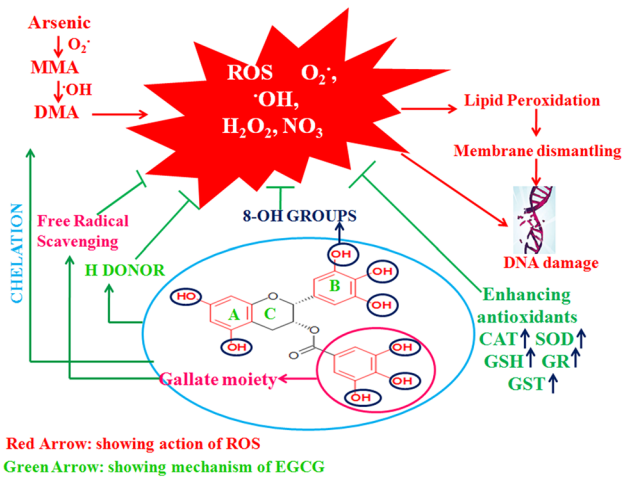


Fig. 7 Suggested mechanism of geno-protective action of EGCG. EGCG suppresses oxidative stress and exerts its geno-protective efficacy by employing its anti-radical and antioxidant properties

validated by inhibitory index analysis which reveals that pre-treatment of EGCG (50 mg/kg b.wt.) has the maximum inhibitory potential against the arsenic induced genotoxicity.

Hence it is proposed that EGCG due to its structural components like C ring gallate and eight hydroxyl groups in a four ringed structure make it a potent free radical scavenger, antiradical agent, a metal chelator, chain reaction halter and antioxidant. Due to these properties EGCG has efficiently reduced the arsenic bioaccumulation (data not shown) and arsenic generated free radical burden in the cells and tissues. By regulating the arsenic induced oxidative stress it eliminates ROS during the oxidation process and there by limits oxidation of major bio-molecules like DNA, proteins and lipids and exerts its geno-protective efficacy (Fig. 7). Thus this study suggests EGCG as a potential geno-protective agent against metal induced oxidative insults and further studies could be useful in deciphering a molecule of higher potency by using it as a whole or by modifying its components.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

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