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Ebselen attenuates cyclophosphamide-induced oxidative stress and DNA damage in mice

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Abstract
The role of selenium, a trace element in the human diet, has been extensively studied against cancer, immunity and infectious/inflammatory diseases. The purpose of the present study was to investigate the beneficial effect of ebselen, an organo-selenium compound, against cyclophosphamide-induced oxidative stress and DNA damage in mice. Malondialdehyde and total glutathione were estimated in the liver to detect the oxidative stress induced by cyclophosphamide. Standard and modified comet assays (the latter incorporated lesion-specific enzymes, formamidopyrimidine-DNA glycosylase and endonuclease-III) were used to detect the normal and oxidative stress-induced DNA damage by cyclophosphamide in the mouse bone marrow and the peripheral blood lymphocytes. In addition, a micronucleus assay capable of detecting DNA damage was also carried out in the mouse bone marrow and the peripheral blood reticulocytes induced by cyclophosphamide. The results confirm that pre-treatment with ebselen (2.5, 5 and 10 mg/kg) for 5 consecutive days decreased the oxidative stress induced by cyclophosphamide (100 mg/kg) based on the restoration in concentration of malondialdehyde and glutathione in the liver and decreased DNA damage and micronuclei count in the bone marrow and the peripheral blood. It is concluded that pre-treatment with ebselen attenuates cyclophosphamide-induced oxidative stress and the resultant DNA damage in mice.

Keywords: *Cyclophosphamide, ebselen, endonuclease-III, FPG, comet assay, micronucleus assay*

Abbreviations: *CP, cyclophosphamide; MDA, malondialdehyde; GSH, glutathione; ROS, reactive oxygen species; End-III, endonuclease-III, FPG, formamidopyrimidine-DNA glycosylase; AP, apurinic/apyrimidinic; PCEs, polychromatic erythrocytes; NCEs, normochromatic erythrocytes; MNPCEs, micronucleated polychromatic erythrocytes; MNRETs, micronucleated reticulocytes*

Introduction

Selenium is an essential trace element involved in several key metabolic activities via selenoproteins, enzymes that are essential for protection against oxidative damage and the regulation of immune function. Ebselen, [2-phenyl-1,2-benzisoselenazol-3(2*H*)-one] is a heterocyclic seleno-organic compound possessing anti-inflammatory, antioxidant, anti-atherosclerotic, cytoprotective and glutathione peroxidase-mimetic properties [1]. The antioxidant activity of ebselen is attributed to its direct free radical scavenging and glutathione peroxidase-mimetic properties [2–4]. The beneficial effects of ebselen in stroke have been demonstrated in experimental animal

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models [5,6] and clinical trials [7,8]. A survey of the literature reveals the protective effects of ebselen against gentamicin- and cisplatin-induced renal damage [9,10], aflatoxin B1-induced cytotoxicity in hepatotcytes [11], oxidative stress-induced damage in platelets and erythrocytes [12,13], oxyesterol-induced damage on rat arterial wall [14], daunorubicin-induced cardiomyopathy in rats [15], cadmium-induced testicular damage in mice [1], 4-hydroxynonenal-induced neuronal death [16], haloperidol-induced orofacial dyskinesia in rats [17] and chronic alcohol-induced damage in hippocampus and functional impairment in rats [18].

Cyclophosphamide (CP) is an alkylating agent used extensively in the treatment of various forms of cancers and in rheumatoid arthritis. CP is metabolized in the liver mainly by CYP-3A4 and CYP-2B6 and generates active metabolites phosphoramide mustard and acrolein [19,20]. Phosphoramide mustard is primarily responsible for the anti-cancer activity, while acrolein produces unwanted toxic effects. Urotoxicity is one of the major adverse effects of CP due to its glutathione (GSH) depletion activity and various thiols were used as a means of chemoprotective agent for its amelioration [21]. Acrolein produces oxidative stress resulting in the production of intracellular reactive oxygen species and nitric oxide [22,23]. These reactive oxygen and nitrogen species damage cellular lipid, proteins and DNA, leading to cell death [24,25]. Given that ebselen is an antioxidant, cytoprotective and glutathione peroxidase-mimetic, we examined its possible beneficial effects on the CP-induced oxidative stress and the resultant DNA damage.

Materials and methods

Animals

All animal experiment protocols were approved by the Institutional Animal Ethics Committee (IAEC). Experiments were performed on male Swiss mice (weight range 20–25 g) procured from the central animal facility of the institute. The animals were kept at room temperature ($22 \pm 2^{\circ}$C), with $50 \pm 10\%$ humidity and an automatically controlled cycle of 12 h light and 12 h dark. Standard laboratory animal feed (purchased from Tetragone Chemi Pvt. Ltd. Bengaluru, India) and water (aquapure) were provided *ad libitum*. Animals were acclimatized to the experimental conditions for a period of 1 week before the start of the experiment.

Chemicals

Cyclophosphamide (CAS no. 6055-19-2), 1,1,3,3-tetramethoxypropane (CAS no. 102-52-3), 2-thiobarbituric acid (CAS no. 504-17-6), Trizma (CAS no. 77-86-1), 5,5′-dithiobis(2-nitro-benzoic acid) (CAS no. 69-78-3), acridine orange (CAS no 10127-02-3), ebselen (CAS no. 60940-34-3), endonuclease-III (End-III), formamidopyrimidine DNA glycosylase (FPG) and SYBR Green I (CAS no. 163795-75-3) were purchased from Sigma-Aldrich Chemicals (Saint Louis, MO). GSH reduced, DMSO, normal melting agarose (NMA), low melting agarose (LMA), triton-X 100, EDTA, foetal bovine serum (FBS) and Hanks balanced salt solution (HBSS) buffer were obtained from Hi-media Laboratories Ltd. (Mumbai, India).

Dose selection, time schedule, chemical preparation and animal treatment

The doses of ebselen (2.5, 5 and 10 mg/kg ip) were selected on the basis of two studies in which ebselen protected against: (a) nitrogen mustard-induced apoptosis in normal and transformed lymphocytes in BALB/c mice [26] and (b) gentamicin-induced renal damage in rats [9]. Ebselen was dissolved in a mixture of DMSO and PBS (1:4). The mice were given either vehicle (DMSO-PBS mixture) or ebselen in doses of 2.5, 5 and 10 mg/kg body weight (bw) by intraperitoneal (ip) route for 5 consecutive days prior to the administration of CP. CP (100 mg/kg bw) was dissolved in distilled water and administered through ip on the 5^{th} day, 1 hour after ebselen treatment. It has already been reported that pre-conditioning with ebselen increases the GSH metabolism and stress protein expression, which are crucial in reducing oxidative injury [27]. Pre-treatment with ebselen prepares the cell better to fight against oxidative stress because ebselen inside the body reacts with thioredoxin reductase system and forms ebselen diselenide, which acts as a reservoir of ebselen [28]. The animals were killed 24 h after the treatment with CP.

Estimation of lipid peroxidation

Lipid peroxide in the liver was measured according to the method described by Okhawa et al. [29] with some modifications. The liver was rinsed in ice-cold physiological saline, minced and a 10% homogenate was prepared in phosphate buffer (pH 7.4) containing EDTA (1 mM). The sample was centrifuged at 700 g for 10 min and the supernatant was used for the determination of lipid peroxidation. The supernatant (100 µl) was added to SDS (100 µl of 8.1%), followed by acetic acid (20%, 750 µl, pH 3.4) and thiobarbituric acid (0.8%, 750 µl). The volume was made up to 2 ml with distilled water and heated on a water bath at 95°C for 60 min. The mixture was then cooled under the tap water and was centrifuged at 10 000 rpm for 10 min. The supernatant was removed and the absorbance was measured at 532 nm. The protein content in the tissue homogenate supernatant was determined as described by Lowry et al. [30].

Lipid peroxidation was calculated from a standard curve using the 1,1,3,3-tetraethoxypropane (97%) and was expressed as nM MDA/mg of protein.

Estimation of glutathione in the liver homogenate

Reduced GSH in the liver homogenate was estimated as described by Moron et al. [31] with some modifications. The liver was rinsed with ice-cold physiological saline, minced and a 10% homogenate was prepared in phosphate buffer (pH 7.4), containing EDTA (1 mM). It was then centrifuged at 700 g for 10 min and the supernatant was used for the determination of GSH. The supernatant (0.5 ml) was mixed with sulphosalicylic acid (0.5 ml, 10%) and kept in ice for 20 min for the precipitation of the protein. The resulting mixture was then centrifuged (10 000 g for 5 min) at 4°C and the supernatant (50 µl) was mixed with phosphate buffer (450 µl) and 5,5′-dithiobis(2-nitro benzoic acid) (1.5 ml, 0.1 mM). It was then incubated at 37°C (10 min) followed by measurement of the optical density at 412 nm using reduced GSH as the external standard. The protein content in the tissue homogenate supernatant was determined as described by Lowry et al. [30].

Single cell gel electrophoresis (SCGE:comet) assay

Standard alkaline comet assay. The comet assay was performed as described by Singh et al. [32] with some modifications. All the procedures were conducted in the dark to avoid possible photo-induced DNA damage. The blood was collected from the retro-orbital plexus under light ether anaesthesia and the lymphocytes were isolated by using Ficoll histopaque solution. A final cell-agarose suspension (100 µl) was prepared containing $\approx 1 \times 10^{4,}$ lymphocytes/ml in 0.5% LMA. The bone marrow cells were isolated as described by Tice et al. [33] with some modifications. The femur was perfused with 1 ml cold HBSS containing EDTA (20 mM) and DMSO (10%). From the bone marrow suspension ($2–5 \times 10^4$ cells/ml), 5 µl was removed and mixed with 95 µl of 0.5% LMA to prepare the final cell-agarose suspension. From the final cell-agarose suspension 80 µl were spread over a microscope slide (75×25 mm glass slides with 19 mm frosted ends, Axiva Sichem Pvt. Ltd, Delhi, India), pre-coated with 1% NMA. The cells were then lysed in a buffer containing 2.5 M NaCl, 100 mM EDTA, 10 mM Tris (pH 10.0) with freshly prepared 1% Triton X-100 and 10% DMSO for 24 h at 4°C. After lysis the slides were rinsed three times in de-ionized water to remove the salt and detergent. The slides were then coded and were placed in a specifically designed horizontal electrophoresis tank (Model, CSLCOM20, Cleaver Scientific Ltd., UK) and the DNA was allowed to unwind for 20 min in an alkaline solution containing 300 mM NaOH and 1 mM EDTA (pH > 13) and was then electrophoresed for 30 min at 300 mA and 20 V (0.70 V/cm). The slides were then neutralized with 0.4 M Tris (pH 7.5), stained with SYBR Green I (1:10 000) for 1 h and covered with cover slips.

Modified alkaline comet assay. The alkaline comet assay using lesion-specific enzymes was used to detect oxidized purines and pyrimidines (that are generated as a result of oxidative stress-induced DNA damage by CP) as described by Collins et al. [34] with some modifications. The cell-agarose suspension slides were prepared as described above for the standard comet assay. After lysing, the slides were washed three times with the enzyme buffer (40 mM HEPES, 100 mM KCl, 0.5 mM EDTA and 0.2 mg/ml BSA) at room temperature and were incubated at 37°C for 30 min with: (i) End-III (1:1000, 30 min), (ii) FPG (1:1000, 45 min) and (iii) enzyme buffer (control). End-III recognizes oxidized pyrimidines, while FPG recognizes oxidized purines, specifically 8-oxo-guanine. Slides were coded and placed in a specifically designed horizontal electrophoresis tank and the DNA was allowed to unwind for 20 min in alkaline solution containing 300 mM NaOH and 1 mM EDTA, pH > 13. The DNA was electrophoresed for 30 min at 300 mA and 20 V (0.70 V/cm). The slides were then neutralized with 0.4 M Tris (pH 7.5), stained with SYBR Green I (1:10 000) for 1 h and covered with cover slips.

Data scoring and photomicrographs of comet assay. The fluorescence-labelled DNA was visualized (magnification 200 ×) using an automated AXIO Imager M1 fluorescence microscope (Carl Zeiss, Germany) and the images were captured with image analysis software (Comet Imager V.2.0.0). Duplicate slides were prepared for each treatment and were independently coded and scored without knowledge of the code. The parameters for the DNA damage analysis include:% tail DNA (TDNA), tail length (TL, in µm) and tail moment (TM) [33]. The TDNA is the best parameter since it covers the widest range of damage and is linearly related to the break frequency. On the other hand TL is an informative parameter at low level of DNA damage and loses its linearity at high level. TM has been promoted as a way of expressing both the TL and the tail intensity in a single value, but causes deviation from linearity in the dose-response curve at low doses and is also devoid of any standard units [35]. The edges of the slides, any other damaged part of the gel, any debris, superimposed comets and comets without distinct head ('hedgehogs' or 'ghost' or 'clouds') were not considered for the analysis.

Bone marrow micronucleus assay

The bone marrow slides were prepared as described by Jena et al. [36] with some modifications. The bone marrow was isolated from the femur bone with the help of a 22 gauge needle fitted to a syringe and was homogenized with FBS. The suspension was centrifuged and the pellet was suspended in the residual FBS and a drop of it was smeared on to a clean grease-free slide and fixed in absolute methanol for 5 mins [37]. The slides were stained with acridine orange (AO) and were washed twice with phosphate buffer (pH 6.8) as described before [38]. Cytotoxicity of CP was determined from PCEs-to-NCEs ratio. In total, 200 cells per animal were counted [39]. The slides were examined by fluorescence microscopy (Olympus Fluorescent microscope, Model BX 51) connected to digital photomicrograph software (OLYSIA Imaging System, 2001).

Peripheral blood micronucleus assay

The peripheral blood smears were prepared as described by Holden et al. [40] with some modifications. The blood was collected from tail tip and the smears were prepared on pre-cleaned slides. The smear was allowed to dry at room temperature and fixed in absolute methanol for 5 min. After fixation, the slides were stained with acridine orange (AO) and were washed twice with phosphate buffer (pH 6.8) as described in Hayashi et al. [38]. The slides were read as described above.

Statistical analysis

Results were shown as mean ± SEM for each group. Statistical analysis was performed using Jandel Sigma Stat (Version 2.03) statistical software. Significance of difference between two groups was evaluated using Student's *t*-test. For multiple comparisons, one-way analysis of variance (ANOVA) was used. In cases where ANOVA showed significant differences, post-hoc analysis was performed with Tukey's test. A value of $p < 0.05$ was considered statistically significant.

Results

Effects on oxidative stress

CP (100 mg/kg) produced a significant reduction in GSH (5.84 ± 0.25) in the liver relative to the control (7.12 ± 0.32). Groups receiving ebselen (5 or 10 mg/kg/day) showed a significant and dose-dependent restoration in GSH concentration in the liver (see Figure 1). CP (100 mg/kg) also significantly increased malondialdehyde (MDA) relative to the control, which was significantly restored in animals pre-treated with ebselen (5 or 10 mg/kg/day) as opposed to those receiving only CP (see Figure 2).

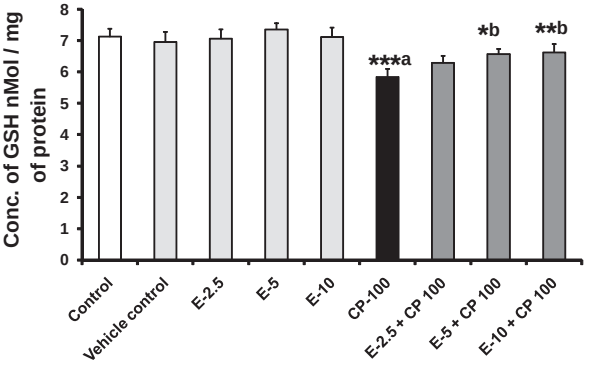

Figure 1. Effect of ebselen (5 day pre-treatment) on GSH level in liver. All the values are expressed as Mean ± SEM ($n = 5$), *** $p <$ 0.001 and * $p < 0.05$, (A) vs Control, (B) vs CP 100.

Effects on DNA damage observed from standard and modified comet assays

DNA damage, including strand breaks, apurinic/apyrimidinic (AP) sites and oxidized purines and pyrimidines were detected by the alkaline comet assay modified with lesion-specific enzymes FPG and End-III. The standard alkaline comet assay only measures strand breaks and AP sites, represented as SB, whereas the comet assay modified with the lesion-specific enzymes End-III/FPG measures strand breaks (SB) and oxidized pyrimidines/purines, represented as SB + End-III and SB + FPG, respectively. The CP-treatment led to a significant increase in the SB, SB + End-III and SB + FPG values as compared to the negative control ($p < 0.001$). Ebselen pre-treatment followed by CP significantly and dose-dependently reduces the SB, SB + End-III and SB + FPG values as opposed to the group receiving CP only (see Figures 3 and 4). Within the modified comet assay, lesion-specific enzyme-FPG cleaves the various oxidized purines, specifically 8-oxo guanine, while treatment with End-III, another lesion-specific enzyme, cleaves the various oxidized pyrimidines and results in an increase in DNA damage (see Figure 5).

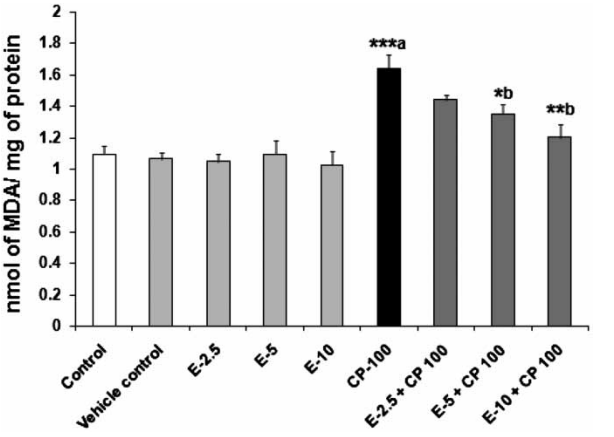

Figure 2. Effect of ebselen (5 day pre-treatment) on MDA level in liver. All the values are expressed as Mean ± SEM ($n = 5$), ***$p < 0.001$ and * $p < 0.05$, (A) vs Control, (B) vs CP 100.

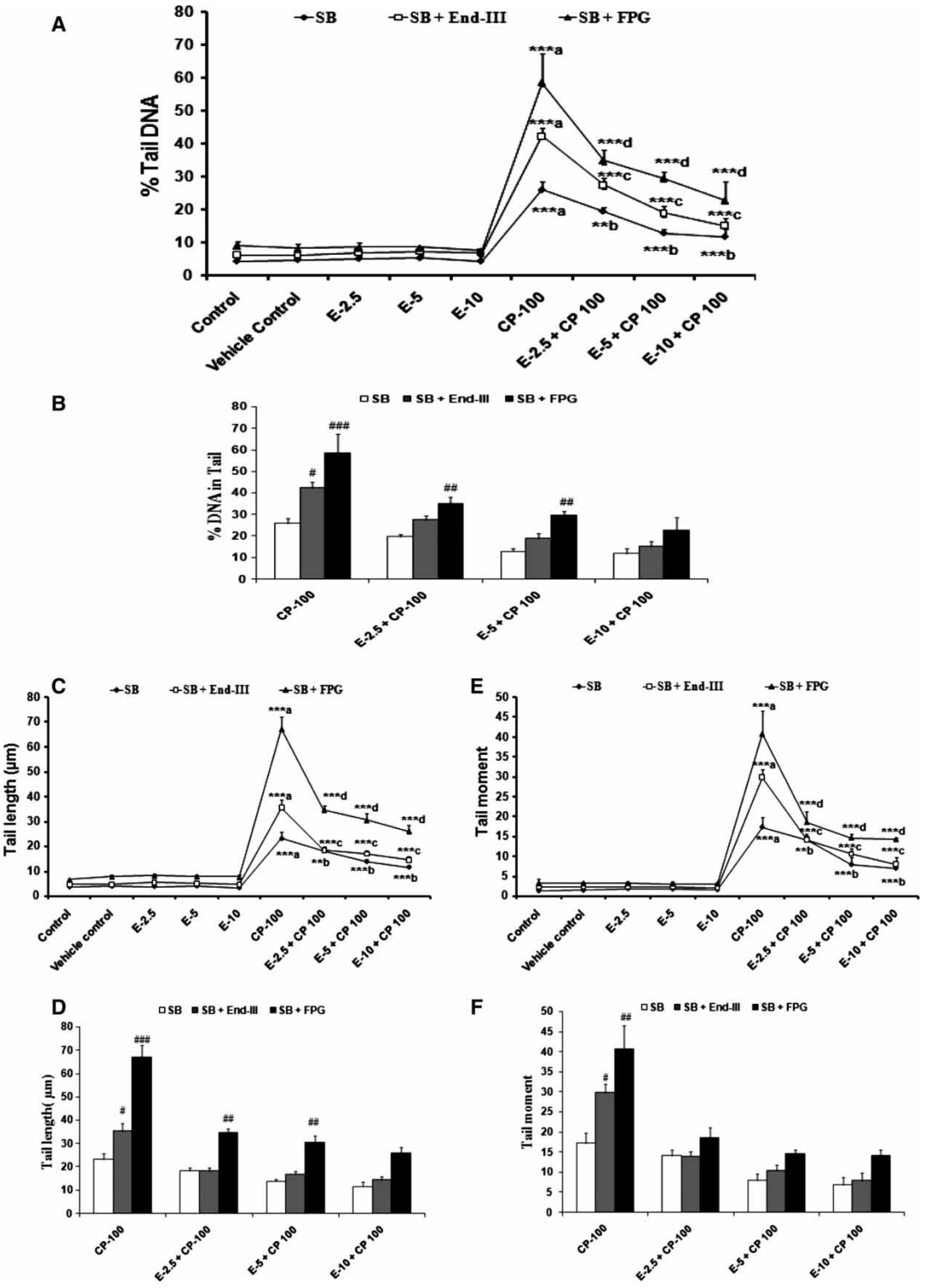

Figure 3. Effect of ebselen (5 day pre-treatment) against CP-induced oxidative DNA damage in bone marrow cells of mice by standard and modified comet assay using lesion specific enzyme End-III and FPG.% Tail DNA was considered as main index of DNA damage while other comet parameters such as tail length and tail moment also show a similar pattern. Standard comet is represented as strand breaks (SB) while modified comet assay is represented as SB+End-III and SB+FPG for the enzyme End-III and FPG, respectively. In (A), (C) and (E) the comparisons were made between the control vs CP-100 and CP-100 vs Ebselen+CP-100. In (B), (D) and (F) the comparisons were made between the SB vs SB+End-III and SB vs SB+End-III. All the values are expressed as Mean±SEM ($n=5$), *** $p<0.001$, * $p<0.05$, ### $p<0.001$, ## $p<0.01$ and # $p<0.05$, (A) vs Control, (B) vs CP 100, (C) vs CP 100+End-III and (D) vs CP 100+FPG.

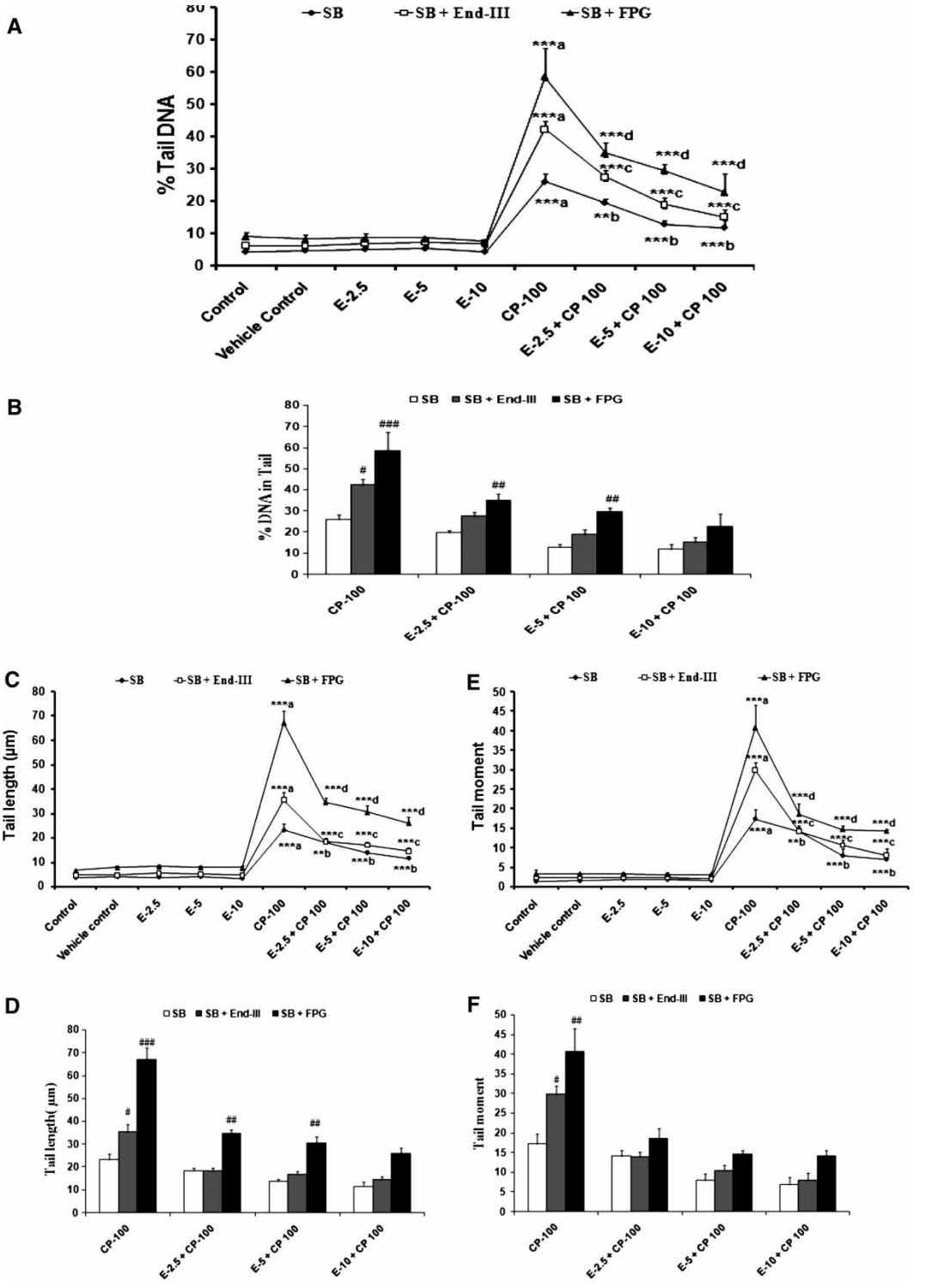

Figure 4. Effect of ebselen (5 day pre-treatment) against CP-induced oxidative DNA damage in lymphocytes by standard and modified comet assay using lesion-specific enzyme End-III and FPG.% Tail DNA was considered as main index of DNA damage while other comet parameters such as tail length and tail moment also show a similar pattern. Standard comet is represented as strand breaks (SB) while modified comet assay is represented as SB+End-III and SB+FPG for the enzyme End-III and FPG, respectively. In (A), (C) and (E) the comparisons were made between the control vs CP-100 and CP-100 vs Ebselen+CP-100. In (B), (D) and (F) the comparisons were made between the SB vs SB+End-III and SB vs SB+End-III. All the values are expressed as Mean ± SEM ($n = 5$), *** $p < 0.001$, * $p < 0.05$, ### $p < 0.001$, ## $p < 0.01$ and # $p < 0.05$, (A) vs Control, (B) vs CP 100, (C) vs CP 100+End-III and (D) vs CP 100+FPG.

The percentage tail DNA was plotted against the dose of ebselen and the area under curve (AUC) was calculated between the CP (100 mg/kg) and ebselen (2.5, 5 and 10 mg/kg) + CP (E + CP) treated groups in both the bone marrow and the peripheral blood lymphocytes from standard and modified comet assays. Ebselen pre-treatment significantly reduced the AUC as opposed to CP only in both standard and modified

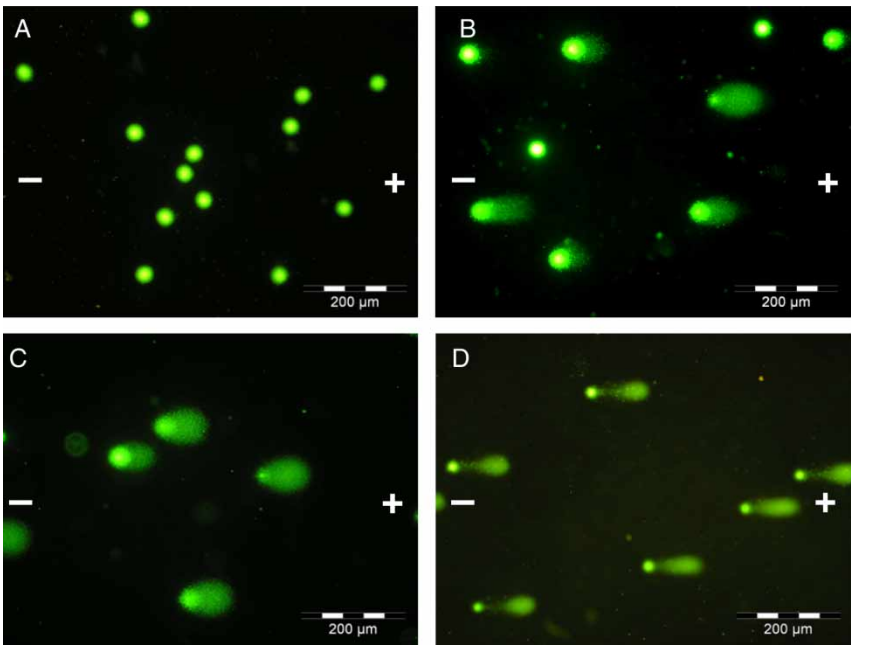

Figure 5. Photomicrograph showing the DNA migration pattern in mice, (A) lymphocytes isolated from mice received normal saline, (B) lymphocytes isolated from mice received CP 100 mg/kg, (C) lymphocytes isolated from mice received CP 100 mg/kg and incubated with End-III and (D) lymphocytes isolated from mice received CP 100 mg/kg and incubated with FPG. The symbols '+' and '−' represent the anode and cathode, respectively, during electrophoresis. Bar length 200 µm represents the magnification 200 ×.

comet assay carried out in the bone marrow and peripheral blood lymphocytes (see Figures 6 and 7).

Effects on micronuclei formation and mitotic index

The potential for inducing micronuclei in the bone marrow and peripheral blood is widely used in the assessment of clastogenic or aneugenic potential of chemicals. CP at a dose of 100 mg/kg induced statistically significant increase in the number of micronuclei in both bone marrow and peripheral blood (see Figure 8). Five days of ebselen pre-treatment significantly and dose-dependently reduced the CP-induced increase in the number of

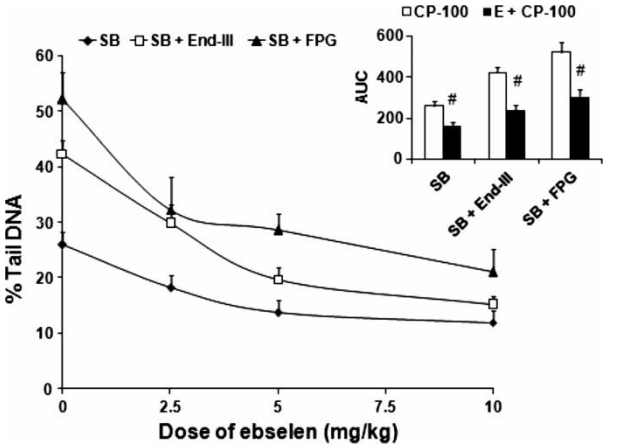

Figure 6. Effect of ebselen (5 day pre-treatment) against CP-induced oxidative DNA damage in bone marrow cells of mice by standard and modified comet assay using lesion-specific enzyme FPG and End-III. The decrease in the DNA damage was observed with increase in the dose of ebselen. The area under curve was calculated between the CP (100 mg/kg) treated group and ebselen (2.5, 5 and 10 mg/kg) and CP (E+CP) treated group in standard and modified comet assay. The Bar diagram shows the AUC between only CP treatment group and E+CP treated group in standard and modified alkaline comet assay using lesion specific enzyme END-III and FPG. All the values are expressed as Mean ± SEM ($n=5$), # $p<0.001$.

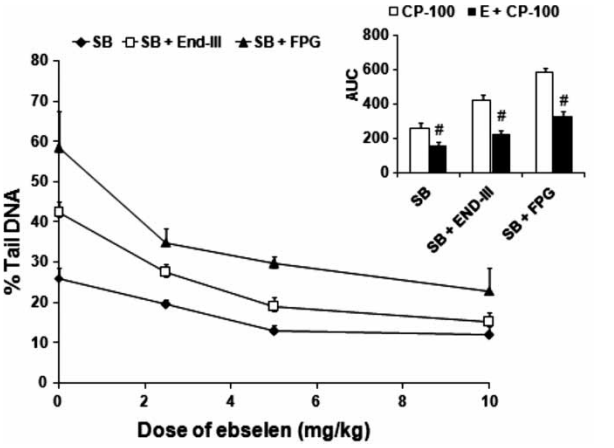

Figure 7. Effect of ebselen (5 day pre-treatment) against CP-induced oxidative DNA damage observed in peripheral blood lymphocytes of mice by standard and modified comet assay using lesion-specific enzyme FPG and End-III. The decrease in the DNA damage was observed with increase in the dose of ebselen. The area under curve was calculated between the CP (100 mg/kg) treated group and ebselen (2.5, 5 and 10 mg/kg) and CP (E+CP) treated group in standard and modified comet assay. The Bar diagram shows the AUC between only CP treatment group and E+CP-100 treated group in standard and modified alkaline comet assay using lesion-specific enzyme END-III and FPG. All the values are expressed as Mean ± SEM ($n=5$), # $p<0.001$.

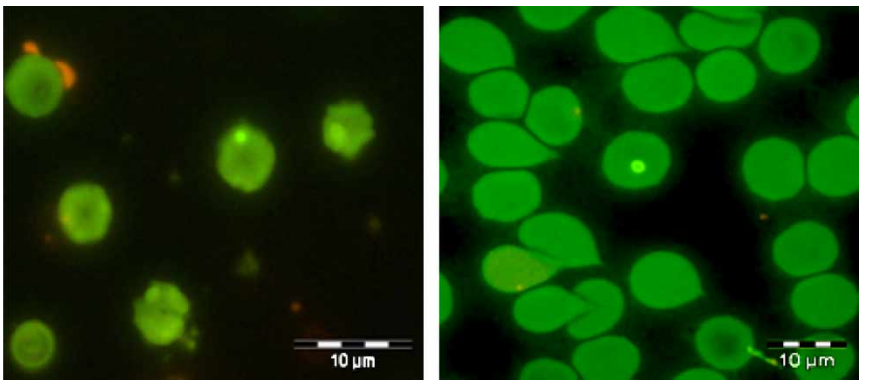

Figure 8. Representative photomicrograph of micronuclei (stained with acridine orange) of Bone marrow PCEs and peripheral blood RETs of mice. Arrow indicates the MNPCE in bone marrow (A) and MNRET in peripheral blood (B) of CP treated mice. Bar length 20 µm represent the magnification 1000 ×.

micronuclei in peripheral blood and bone marrow (see Table I). Furthermore, the cytotoxicity induced by CP as assessed by the ratio of PCEs-to-NCEs in the bone marrow was restored with ebselen pre-treatment and the ratio returned to the control level at the highest dose (see Table I). Linear regression analysis showed a strong and positive correlation (>0.91) between the comet assay (standard and modified) and the micronucleus assay (see Figure 9).

Discussion

Treatment with CP results in various adverse effects including genotoxicity and apoptosis in the normal cells [41]. The anti-neoplastic effect of CP is due to its ability to inhibit cell division by damaging the DNA of proliferating cancerous cells. However, at the same time it also damages the DNA of the healthy tissues with high cellular turnover such as the bone marrow, the gastro-intestinal tract (GIT) and the germ cells. During the course of metabolism, CP generates active metabolites, like 4-hydroxycyclophosphamide (4-OHCP), phosphoramide mustard and acrolein. These metabolites preferably alkylate N^7 position of the guanine residue of DNA and this leads to inter- and intra-strand cross-links, DNA strand breaks, cessation of DNA synthesis, DNA-protein cross-links and DNA adduct formation [42,43]. Recently it has been reported that 4-hydroperoxycyclophosphamide mediates caspase-independent T-cell apoptosis, which involves oxidative stress-induced nuclear relocation of mitochondrial apoptogenic factors, such as apoptotic inducing factor (AIF) and endonuclease-G (Endo-G) [44].

The present result indicates that in the liver CP manifested oxidative stress as observed from a significant increase in the MDA and decrease in the GSH concentration. The CP treatment led to a significant increase in the number of micronuclei in both the bone marrow as well as in the peripheral blood. The oxidative stress induced by CP significantly increased the DNA damage in both the bone marrow and the peripheral blood lymphocytes, as evident from the modified alkaline comet assay using lesion-specific restriction enzymes, FPG and End-III. FPG and End-III recognize and remove the oxidized purines (8-hydroxy guanine) and pyrimidines (thymine glycol, 5-hydroxycytosine), creating AP sites. The AP-sites are cleaved by AP lyase activity, creating a gap in the DNA strand (measured as additional strand breaks) that can be detected by the modified comet assay [45–47].

Table I. Effect of ebselen (5 days pre-treatment) on CP (100 mg/kg) induced MN frequency in bone marrow PCEs and peripheral blood RETs.

Treatments	Bone marrow (MN PCEs/1000PCEs)	Peripheral blood (MN RETs/1000RETs)	P/N ratio
Control	1.20 ± 0.37	1.40 ± 0.24	1.09 ± 0.04
Vehicle control	1.40 ± 0.40	1.00 ± 0.32	1.13 ± 0.14
E-2.5	1.60 ± 0.24	1.20 ± 0.20	1.10 ± 0.04
E-5	1.60 ± 0.40	1.00 ± 0.44	1.07 ± 0.05
E-10	1.40 ± 0.24	1.30 ± 0.32	1.11 ± 0.03
CP-100	55.20 ± 2.44***[a]	45.40 ± 2.24***[a]	0.87 ± 0.04**[a]
E-2.5 + CP-100	43.40 ± 2.24*[b]	34.80 ± 1.85*[b]	0.95 ± 0.02
E-5 + CP-100	36.00 ± 2.86***[b]	28.20 ± 2.15***[b]	0.98 ± 0.04
E-10 + CP-100	31.80 ± 2.52***[b]	23.00 ± 2.35***[b]	1.04 ± 0.05*[b]

All the values are expressed as Mean ± SEM (n = 5), *p < 0.05, **p < 0.01 and *** p < 0.001.
[a]vs Control, b vs CP 100.

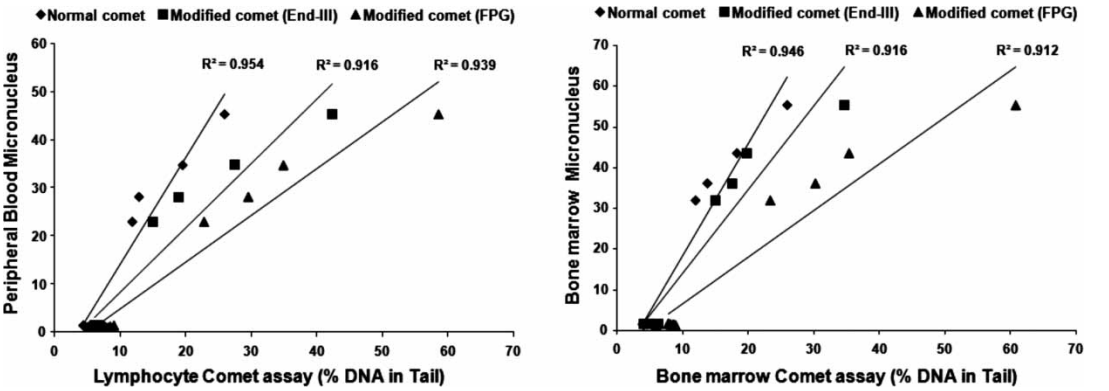

Figure 9. Linear regression analysis showing the correlation between the micronucleus count vs% DNA in tail obtained by standard and modified comet assay (End-III and FPG). (A) peripheral blood lymphocyte comet vs peripheral blood micronuclei and (B) bone marrow comet vs bone marrow micronuclei.

Acrolein, another metabolite of CP, is responsible for the unwanted toxic effects in the normal cells. It causes bladder damage and haemorrhagic cystitis, which are serious problems associated with CP therapy [48]. Korkmaz et al. [25] reported that nitric oxide and peroxynitrite are involved in the pathogenesis of CP-induced cystitis. Acrolein rapidly enters into the uroepithelial cells and activates intracellular ROS and produces nitric oxide (either directly or through NF-κB and AP-1) and peroxynitrite. The increased levels of peroxynitrite damages lipids (lipid peroxidation), proteins (protein oxidation) and DNA (strand breaks) leading to activation of PARP, a DNA repair enzyme. Over-activation of PARP leads to depletion of oxidized nicotinamide-adenine dinucleotide and adenosine triphosphate and consequently necrotic cell death [24]. CP also inactivates the enzyme thioredoxin reductase (TrxR) activity *in vitro* [49] and *in vivo* [50]. The Thioredoxin (Trx) system is responsible for the redox balance of the organism and regulates several vital functions (proliferation, growth, DNA synthesis, transcription, detoxification and apoptosis) that are required in the maintenance of cellular integrity [51]. Thus, a reduction in TrxR activity by CP in a non-specific manner results in the induction of cytotoxicity to normal cells. Acrolein and peroxynitrite have been reported to bind directly to the selenocysteine [52] residue located at the C-terminus of TrxR and as a result it deeply suppresses TrxR activity [53]. Acrolein also reacts directly with GSH, causing its depletion from the intracellular storage sites [54]. Furthermore, it produces inflammation by promoting accumulation of neutrophils, which ultimately leads to the generation of ROS [55]. Acrolein interacts with amino acids of proteins, causing structural and functional changes in the enzyme system (e.g. SOD), rendering them incapable to scavenge free radicals [56]. In summary, acrolein depletes GSH, inhibits Trx-R activity and promotes the formation of reactive oxygen species inside the cells, impairing the redox balance of the system.

Ebselen pre-treatment decreased the oxidative stress, as evident from a significant decrease in MDA and increase in GSH concentration in the liver. Pre-treatment with ebselen significantly attenuated the CP-induced DNA damage in a dose-dependent manner, as evident from the results of standard and modified comet assays. Ebselen at the highest dosage (10 mg/kg) significantly reduced the DNA damage ($\approx$ 54% DNA strand breaks; $\approx$ 58% DNA having oxidized pyrimidines and $\approx$ 62% DNA having oxidized purines). These findings were confirmed from the micronucleus assay. MN are well characterized biomarkers of structural and numerical chromosomal damage; they arise from acentric chromosome fragments or lagging whole chromosome(s) that fail to incorporate into daughter nuclei after the nuclear division [57]. Pre-treatment with ebselen at the highest dose (10 mg/kg) significantly reduced the CP-induced MNPCEs ($\approx$ 42.39%) and MNRET ($\approx$ 49.34%) in the mouse bone marrow and in the peripheral blood, respectively. Studies have been performed to elucidate the mechanism of DNA damage and to correlate the extent of DNA damage by employing both the comet and the micronucleus assay as the end points of evaluation [58–61]. In our study, a strong positive correlation of DNA damage was noted between the micronucleus assay and comet assay (standard and modified).

Selenium supplementation inhibits carcinogen-induced covalent DNA adduct formation and reduces the oxidative damage to DNA, lipids and proteins [62]. Mechanistic studies have shown that ebselen inhibits pro-oxidant enzyme NADPH oxidase [63] and decreases the free radical production by activated macrophages [64]. The glutathione peroxidase mimetic activity of ebselen reduces oxidative stress and augments peroxidase activity by inducing nrf-2-dependent transcription pathway [65]. Ebselen acts as a substrate for the mammalian thioredoxin system (e.g. thioredoxin, thioredoxin reductase and $NADPH + H^+$), resulting in the formation of ebselen

selenol. The latter-reacts with ebselen to form ebselen diselenide, which acts as a storage site for ebselen and reactivated by the thioredoxin system [28]. The ebselen selenol is more potent than thioredoxin itself for the reduction of various substrates, e.g. H_2O_2, smaller organic hydroperoxides as well as membrane-bound phospholipid and cholesterylester hydroperoxide. Since ebselen is effective against membrane hydroperoxides, it inhibits both enzymatic and non-enzymatic lipid peroxidation in the cells. Thus, in part, the above discussed mechanisms may be responsible for the attenuation of CP-induced oxidative stress and the subsequent DNA damage.

The present study provides for the first time evidence that ebselen attenuates CP-induced oxidative stress and the subsequent DNA damage in both the peripheral blood and the bone marrow cells of mice. The anti-genotoxic effect of ebselen might be due to its antioxidant and cytoprotective activity. However, additional studies using other end points with possible mechanistic evidence are required to elucidate the precise mechanism of protection offered by ebselen.

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