

Cyclophosphamide-induced nephrotoxicity, genotoxicity, and damage in kidney genomic DNA of Swiss albino mice: the protective effect of Ellagic acid

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Abstract Cyclophosphamide (CPM), an alkylating agent is used as an immunosuppressant in rheumatoid arthritis and in the treatment of several cancers as well. In this study, Ellagic acid (EA), a naturally occurring plant polyphenol, was evaluated for its antigenotoxicity and antioxidant efficacy against the CPM-induced renal oxidative stress and genotoxicity in Swiss albino mice. The mice were given a prophylactic treatment of EA orally at a dose of 50 and 100 mg/kg body weight (b wt) for seven consecutive days before the administration of a single intraperitoneal (i.p.) injection of CPM at 50 mg/kg b wt. The modulatory effects of EA on CPM-induced nephrotoxicity and genotoxicity were investigated by assaying oxidative stress biomarkers, serum kidney toxicity markers, DNA fragmentation, alkaline unwinding assay, micronuclei (MN) assay, and by histopathological examination of kidney tissue. A single intraperitoneal administration of CPM in mice increased malondialdehyde level with depletion in glutathione content, antioxidant enzymes activities, viz. glutathione peroxidase, glutathione reductase, catalase, quinone reductase, induced DNA strand breaks, and MN induction. EA oral administration at both doses caused significant reduction in their levels, restoration in the activities of antioxidant enzymes, reduction in MN formation, and DNA fragmentation. Serum toxicity marker enzymes like BUN, creatinine, and LDH were also increased after CPM treatment which was significantly

decreased in EA pretreated groups. Present findings suggest a prominent role of EA against CPM-induced renal injury, DNA damage, and genotoxicity.

Keywords Cyclophosphamide · DNA damage · Ellagic acid · Genotoxicity · Nephrotoxicity

Abbreviations

EA	Ellagic acid
CPM	Cyclophosphamide
GSH	Reduced glutathione
GPx	Glutathione peroxidase
GR	Glutathione reductase
XO	Xanthine oxidase
MDA	Malondialdehyde
BUN	Blood urea nitrogen
LDH	Lactate dehydrogenase

Introduction

Natural products have been shown to be an excellent and reliable source for the development of new drugs [1]. Epidemiologic studies have showed that there is an association between the intake of phenolic foods and the protection from various diseases [2]. These phenolic compounds have tremendous antioxidant and chemoprotective properties in vivo [3]. Phenolic compounds are the most promising anticarcinogenic agents in plants [4]. Ellagic acid (EA) has been reported to inhibit peroxy-nitrite-induced oxidation and nitration reactions [5]. EA is a potent dietary antioxidant found in variety of fruits, nuts, and many other food sources. EA is reported to exhibit antioxidant, antiproliferative, radical scavenging, antiapoptotic, antiatherogenic,

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antiasthmatic, estrogen receptor modulator, and chemopreventive, in a variety of tissues and cells. It has been also shown to inhibit cancer caused by several types of chemical carcinogens including polycyclic aromatic hydrocarbons, *N*-nitrosamines, aflatoxin B1, and aromatic amines [6–15]. EA also finds application in cosmetology as a skin whitening agent as it inhibits radiation induced melanogenesis. EA contains four hydroxyl groups and two lactone groups in which hydroxyl group is known to increase antioxidant activity in lipid peroxidation and protect cells from oxidative damage [16] and few more reported that EA decrease hepatic cytochrome P₄₅₀ activity and also increase the activities of several phase II enzymes [17–19].

Cyclophosphamide (CPM) is a well-known bi-functional alkylating agent widely used in cancer chemotherapy and expresses its genotoxicity when metabolically activated [20, 21]. It is extensively used for the treatment of various cancers as well as an immunosuppressant in organ transplantation, rheumatoid arthritis, systemic lupus erythematosus, multiple sclerosis, and other benign diseases [22, 23]. According to the International Agency for Research on Cancer (IARC), CPM is widely used as reference mutagen and has been classified as carcinogenic for animals and humans [24]. Reactive metabolites of CPM chemically alkylate DNA as well as protein, producing cross-links which are responsible for its cytotoxic effect [25]. Normal tissues injury or damage is the major limitation of using CPM, which gives rise to numerous side effects, CPM treatment also results in the production of reactive oxygen species (ROS), which cause peroxidative damage to kidney and other vital organs [26]. Antineoplastic and toxic effects like necrosis, apoptosis, and oncosis of CPM are linked with two active metabolites, i.e., phosphoramidate and acrolein. Further Tripathi and Jena [27] reported CPM to be toxic in germ cells of mice, it has also been reported that CPM treatment for non-Hodgkin's lymphoma leads to the induction of secondary cancers in bladder and kidney [28]. The important factor for therapeutic and toxic effects of CPM is the requirement of metabolic activation by hepatic microsomal cytochrome P₄₅₀ mixed functional oxidase system [29, 30]. On activation, CPM generates active alkylating metabolites such as 4-hydroxycyclophosphamide, acrolein, and aldophosphamide mustard, which hamper with cellular DNA synthesis in fast dividing cells and ultimately lead to cell death [31]. Bagley and Bostick [32] have reviewed the toxicity and pharmacology of CPM.

In this study, we have made an attempt to investigate the beneficial effects of EA against CPM-induced nephrotoxicity and genotoxicity. The experimental end points for nephrotoxicity included biochemical estimation of antioxidant enzymes and histopathological measurement for the determination of oxidative stress. Genotoxicity end points included evaluation of micronuclei (MN), DNA fragmentation, and alkaline unwinding assay in terms of DNA

strand breaks. Our results reveal that EA decreased the CPM-induced oxidative stress and subsequent oxidative DNA damage and genotoxicity in Swiss albino mice.

Materials and methods

Chemicals

EA, EDTA, Tris, reduced glutathione (GSH), oxidized glutathione, nicotinamide adenine dinucleotide phosphate reduced (NADPH), CPM, bovine serum albumin, bisbenzamide; Proteinase K, EDTA, SDS, phenol, chloroform, isoamyl alcohol and RNase were from Sigma St. Louis. All other reagents and solvents were of a high analytical grade.

Animals

Eight-week-old adult male Swiss albino mice (20–25 g) were obtained from the Central Animal House Facility of Hamdard University, New Delhi and were housed in a ventilated room at 25 ± 5°C under a 12-h light/dark cycle. The animals were acclimatized for 1 week before the study and had free access to standard laboratory feed (Hindustan Lever Ltd., Bombay, India) and water ad libitum. The study was approved by the Committee for the Purpose of Control and Supervision of Experimental Animals.

Experimental design

For study of biochemical parameters and MN assay, 25 male Swiss albino mice were divided into five groups. Group I served as a saline-treated control. Group II served as toxicant and was given single intraperitoneal injection of CPM (50 mg/kg b wt i.p.) freshly dissolved in distilled water. EA was orally given at two doses, 50 and 100 mg/kg b wt, [33, 34] to groups III and IV, respectively, for seven consecutive days. Group V received only a high dose of EA for seven consecutive days. On the seventh day (last day) of pretreatment, single intraperitoneal dose of CPM (50 mg/kg b wt i.p.) was administered to the animals in groups II, III, and IV. All the animals were killed after 24 h of intoxication with CPM. Kidneys and femur bones were removed and processed for enzyme estimation and MN assay. The dose of CPM was selected on the basis of previous reports from our lab and others [35–37].

Micronucleus assay

The micronucleus test (mouse bone marrow) was performed according to the method of Schmid [38]. In brief,

the air-dried slides were stained with May–Grunwald and Giemsa as described by Schmid, made permanent, and coded. A total no. of 1,500–2,000 Normochromatic erythrocytes (NCE) and polychromatic erythrocytes (PCEs) were scored per animal by the same viewer for determining the frequencies of micronucleated PCE (MnPCEs). To detect possible cytotoxic effects, the PCE:NCE ratio in 200 erythrocytes per animal was calculated according to Gollapudi and McFadden [39]. Coded slides were scored with a light microscope at a $\times 1000$ magnification.

DNA isolation

DNA extraction was done by standard chloroform isoamyl method. DNA was extracted from approximately 400 mg of kidney tissue by homogenizing the tissue in 5 mL TNE buffer (50 mM Trisma, 100 mM EDTA, 0.5% SDS, pH 8.0) in a 2-mL ground glass homogenizer. Each sample was homogenized with ten standardized strokes of the pestle to minimize any potential effect on DNA integrity introduced by the homogenization procedure. An equal volume of buffered phenol/chloroform/isoamyl alcohol (PCI) (25:24:1, v/v/v, pH 8.0) was then added to the sample. The sample was gently mixed and allowed to settle for 5 min and then centrifuged for 5 min at 13,000 rpm at 4°C. The aqueous layer was transferred to a new micro centrifuge tube and PCI extraction was repeated. The aqueous layer was then digested by 5 mL of RNase (10 mg/mL) for 30 min at 37°C and the digest was extracted once by PCI and once by 500 mL of chloroform. DNA was precipitated from the resulting aqueous layer by adding two volumes of absolute ethanol and 1/10 volume of 3 M sodium acetate, pH 5.2. The sample was then centrifuged (13,000 rpm, 15 min), and the resulting pellet rinsed with 500 mL of 70% ethanol and air-dried. The amount of DNA was quantitated spectrophotometrically at 260 and 280 nm. 2 mg/mL of DNA sample was dissolved in 1 mL of TE buffer (10 mM Trisma, 1 mM EDTA) and subsequently used in the DNA alkaline unwinding assay.

Alkaline unwinding assay

In the alkaline unwinding assay, the rate of transition of double stranded DNA (dsDNA) to single stranded DNA (ssDNA) under predefined alkaline denaturing condition was proportional to the number of breaks in the phosphodiester backbone and thus was used as a measure of DNA integrity. Bisbenzamide was used as a DNA-binding dye from the fluorescence of which various types of DNA were quantitated. For the fluorescence determination of dsDNA, ssDNA, and partially unwound DNA (au-DNA), three equal portions of diluted DNA sample was prepared. The amount of dsDNA was obtained from the fluorescence of a

sample without any treatment; while ssDNA was determined from the sample that had been boiled for 30 min. Fluorescence of the DNA sample which had been subjected to alkaline treatment (pH 12.2) on ice for 30 min provided an estimate of the amount of au-DNA. The fluorescence of initial or dsDNA was determined by placing 100 μ mol DNA sample, 100 μ L NaCl (25 mM) and 2 μ L SDS (0.5%) in a prechilled test tube, followed by the addition of 3 mL 0.2 M potassium phosphate pH 9, and 3 μ L bisbenzamide (1 mg/mL). The contents were mixed and allowed to react in the dark for 15 min to allow the fluorescence to stabilize. The fluorescence of the sample was the measured using a spectrofluorimeter (Ex: 360 nm, Em: 450 nm). The fluorescence of ssDNA was determined as above but using the DNA sample that had already been boiled for 30 min to completely unwind the DNA. 50 μ L NaOH (0.05 N) was rapidly mixed with 100 μ L of DNA sample in a prechilled test tube. The mixture was incubated on ice in the dark for 30 min followed by rapid addition and mixing of 50 μ L HCl (0.05 N). This was followed immediately by an addition of 2 μ L SDS (0.5%) and the mixture was forcefully passed through a 21 G needle five times. Fluorescence of alkaline unwound DNA sample was measured as described above. Measurement of the alkaline unwound sample was performed in triplicate and the average was reported. The ratio between dsDNA to total DNA (*F* value) was determined as follows:

$$F \text{ value} = (\text{auDNA} - \text{ssDNA}) / (\text{dsDNA} - \text{ssDNA})$$

where au-DNA, ssDNA, and dsDNA were the degrees of fluorescence from the partially unwound, single stranded, and double stranded determinations, respectively. The *F* value was inversely proportion to the number of strand breaks present and thus could be used as an indicator of DNA integrity.

Gel electrophoresis and DNA fragmentation (smearing)

The sample was mixed with 10 μ L of loading solution (10 mM EDTA (pH 8.0), 1% (w/v) bromophenol blue, and 40% (w/v) sucrose) preheated to 70°C. The DNA samples were loaded onto a 1.8% (w/v) agarose gel and sealed with 0.8% (w/v) low melting point agarose. The DNA fragments were separated by electrophoresis at 25 V for 12 h at 4°C in TBE buffer. The DNA was visualized using ethidium bromide and photographed by digital camera.

Postmitochondrial supernatant preparation

Kidneys were removed quickly, cleaned free of extraneous material, and immediately perfused with ice-cold saline (0.85% sodium chloride). The kidneys were homogenized in chilled phosphate buffer (0.1 M, pH 7.4) containing

potassium chloride (KCl; 1.17%) using a Potter Elvehjem homogenizer. The homogenate was filtered through muslin cloth and was centrifuged at $800\times g$ for 5 min at 4°C by REMI Cooling Centrifuge to separate the nuclear debris. The aliquot so obtained was centrifuged at 12,000 rpm for 20 min at 4°C to obtain postmitochondrial supernatant which was used as a source of enzymes. All the biochemical estimations were completed within 24 h of killing the animal [40].

GSH estimation was done by the method of Jollow et al. [41].

Glutathione reductase (GR) activity was estimate by the method of Carlberg and Mannervik [42].

Glutathione peroxidase (GPx) activity was performed by Mohandas et al. [43].

Glutathione-S-transferase activity was estimated by Habig et al. [44].

Malondialdehyde (MDA) formation was estimated by Wright et al. [45].

Xanthine oxidase (XO) estimation was done by Stripe and Della Corte [46].

Catalase activity was measured by Claiborne [47].

Lactate dehydrogenase (LDH) activity was measured by the method of Kornberg [48].

Estimation of protein concentration was done by Lowry et al. [49].

Estimation of blood urea nitrogen (BUN), γ -glutamyl transferase and creatinine was done by commercially available kit from Rekon diagnostics Pvt Limited.

Histopathological examination

The kidneys were quickly removed after killing the mice and preserved in 10% neutral buffered formalin for histopathological processing. The kidneys were embedded in paraffin wax and longitudinally sectioned under microtome. Hematoxylin and eosin staining of the above sections was done and observed under Olympus microscope.

Statistical analysis

Differences between groups were analyzed using analysis of variance (ANOVA) followed by Dunnet's multiple comparisons test. All data points are presented as the treatment groups mean \pm standard error of the mean (S.E.M).

Results

Effects of pretreatment of EA on renal glutathione, its dependent enzymes, and antioxidant enzyme system

Table 1 shows that CPM administration leads to significant depletion of renal GSH ($p < 0.001$). In addition, there was

marked inhibition of GR enzyme by ($p < 0.001$), GST ($p < 0.001$), and GPx ($p < 0.001$) as compared to control as shown in Table 1. Pretreatment with EA augmented renal GSH content by both the doses ($p < 0.05$, $p < 0.001$), GR ($p < 0.01$), GST ($p < 0.01$, $p < 0.001$), GPx ($p < 0.05$, $p < 0.001$). Similarly CPM treatment as shown in Table 1 inhibited catalase which was increased by EA prophylactic treatment at both doses. No significant difference was found in the only D2 group compared with control in any of the parameters.

Table 1 also shows that CPM administration leads to the significant enhancement in the levels of XO, MDA, and GGT ($p < 0.001$) in comparison to control. Pretreatment with EA improved renal XO ($p < 0.001$), MDA ($p < 0.05$, $p < 0.001$) formation and GGT ($p < 0.01$, $p < 0.001$).

Effects of pretreatment of EA on renal toxicity marker enzymes

The effect of EA administration on CPM-mediated leakage of kidney toxicity markers (BUN, LDH, and creatinine) were shown in Figs. 1, 2, 3. Figures 1 and 2 showed that mice treated with CPM showed a significant increase in BUN ($p < 0.001$) and creatinine ($p < 0.001$) levels when compared with control. On treatment with EA marked inhibition was observed in BUN at both the doses ($p < 0.001$) (Fig. 1) and also in the creatinine level at both the doses ($p < 0.01$) (Fig. 2). Similar type of results was observed for LDH levels (Fig. 3). No significant difference was found in the only D2 group compared with control.

Effects of EA pretreatment on genotoxicity

Table 2 shows that there was higher induction of MN in CPM-treated group ($p < 0.001$) and this level was decreased by prophylactic treatment of EA at low dose ($p < 0.05$) and more effectively at higher dose ($p < 0.001$). DNA damage is estimated in terms of smearing and lack of intact band control, only the EA treated groups at both doses showed less smearing and an intact band was also observed (Table 2). Similarly, in the DNA alkaline unwinding assay, a simultaneous significant decrease in F value ($p < 0.001$) on treatment with CPM was noted as compared to control group, whereas results indicate that there was significant increase in the F value at both the doses of EA ($p < 0.05$, $p < 0.001$).

The estimated PCE:NCE ratio in bone marrow preparations in the Table 2 shows a statistical decrease ($p < 0.001$) in hematopoiesis as a result of CPM treatment as compared to control. The pretreatment of EA at both the dose significantly increased the PCE:NCE ratio, indicating reversal of the cytotoxic effects caused by CPM administration to mice. In the Fig. 4, results indicate that there was

Table 1 Results of pretreatment of EA on MDA formation, antioxidant, and related enzymes like GSH, GST, GR, GPX, catalase, XO, and γ -GGT on CPM-induced renal redox imbalance

Treatment regimen per group	GSH (nmol GSH/g tissue)	GST (nmol CDNB conjugate formed/min/mg protein)	GR (nmol NADPH oxidized/min/mg protein)	GPX (nmol NADPH oxidized/min/mg protein)	Catalase (nmol H ₂ O ₂ consumed/min/mg protein)	MDA (nmol MDA formed/g tissue)	XO (μ g of uric acid formed/min mg protein)	γ -GGT (nmoles <i>p</i> nitroaniline formed/min/mg protein)
Group I (control)	0.65 ± 0.04	130.4 ± 10.23	219.9 ± 8.42	280.0 ± 14.0	151.9 ± 6.98	2.83 ± 0.32	0.206 ± 0.01	488.0 ± 18.6
Group II (CPM only)	0.26 ± 0.03***	42.10 ± 2.62**	102.6 ± 2.82***	141.2 ± 6.73***	54.51 ± 4.40***	5.76 ± 0.11***	0.480 ± 0.03***	836.2 ± 10.9***
Group III (CPM + EA D1)	0.43 ± 0.03 [#]	93.65 ± 9.10 ^{##}	138.0 ± 11.4 ^{ns}	194.9 ± 6.43 [#]	118.2 ± 3.26 ^{###}	4.30 ± 0.36 [#]	0.340 ± 0.01 ^{###}	681.7 ± 42.3 ^{##}
Group IV (CPM + EA D2)	0.51 ± 0.03 ^{###}	104.2 ± 5.98 ^{###}	180.5 ± 23.3 ^{##}	230.8 ± 19.6 ^{###}	96.58 ± 6.09 ^{###}	3.13 ± 0.18 ^{###}	0.30 ± 0.00 ^{###}	569.8 ± 18.5 ^{###}
Group V (only EA D2)	0.63 ± 0.04	129.9 ± 9.46	202.5 ± 7.22	202.1 ± 4.67	148.7 ± 0.02	2.74 ± 0.36	0.25 ± 0.04	484.1 ± 20.7

EA Ellagic acid, D1 50 mg/kg b wt, D2 100 mg/kg b wt

Results represent mean ± SE of six animals per group. CPM administration resulted in significant decrease in the level of reduced glutathione, glutathione reductase, glutathione peroxidase, and catalase. Pretreatment with EA at both doses significantly modulated the alterations induced by CPM in mice kidney. CP treatment leads to significant depletion in the activities of antioxidant enzymes in II group as compared to I group (*** $p < 0.001$ and ** $p < 0.01$). Pretreatment of EA increased activity of these enzymes in the III and IV groups significantly as compared to CPM-treated II group (### $p < 0.001$), (## $p < 0.01$), ($p < 0.05$), and (^{ns} $p =$ not significant). In case of MDA formation, xanthine oxidase and GGT, CPM caused significant increase and Ellagic pretreatment was instrumental in bringing level of these parameters to normal

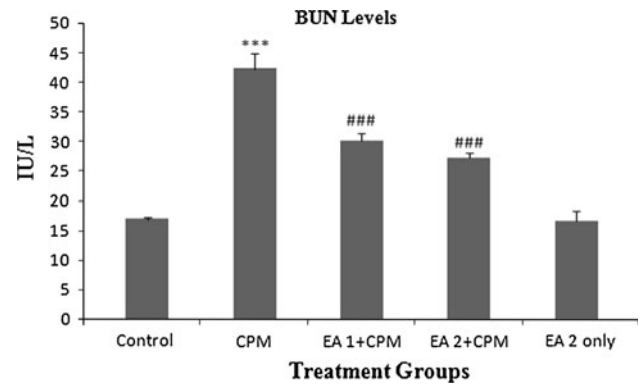


Fig. 1 Results represent mean ± SE of six animals per group. Results obtained are significantly different from control group (*** $p < 0.001$). Results obtained are significantly different from CPM-treated group ([#] $p < 0.05$), (^{##} $p < 0.01$), and (^{###} $p < 0.001$). EA Ellagic acid, D1 50 mg/kg b wt, D2 100 mg/kg b wt

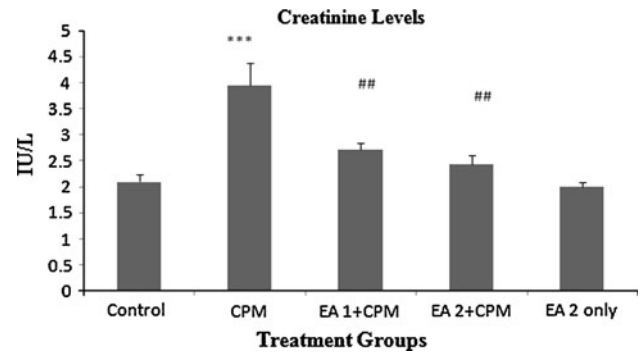


Fig. 2 Results represent mean ± SE of six animals per group. Results obtained are significantly different from control group (*** $p < 0.001$). Results obtained are significantly different from CPM-treated group ([#] $p < 0.05$), (^{##} $p < 0.01$), and (^{###} $p < 0.001$). EA Ellagic acid, D1 50 mg/kg b wt, D2 100 mg/kg b wt

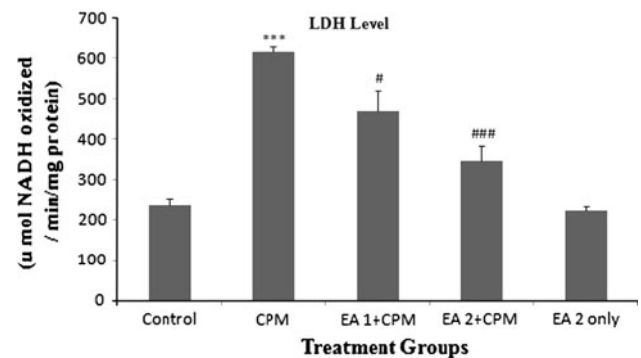


Fig. 3 Results represent mean ± SE of six animals per group. Results obtained are significantly different from control group (*** $p < 0.001$). Results obtained are significantly different from CPM-treated group ([#] $p < 0.05$), (^{##} $p < 0.01$), and (^{###} $p < 0.001$). EA Ellagic acid, D1 50 mg/kg b wt, D2 100 mg/kg b wt

Table 2 Results of modulatory effect of Ellagic acid on genotoxicity markers (MnPE/100 PCE, PCE/NCE, DNA strand breaks) on CPM-induced damage

Treatment regimen per group	MnPE/100 PCE	PCE/NCE	DNA strand breaks (<i>F</i> value)
Group I (control)	4.80 ± 0.86	1.26 ± 0.04	0.87 ± 0.03
Group II (CPM only)	20.4 ± 1.28***	0.70 ± 0.04***	0.37 ± 0.01***
Group III (CPM + EA D1)	15.4 ± 0.87 [#]	0.90 ± 0.03 [#]	0.51 ± 0.03 [#]
Group IV (CPM + EA D1)	9.2 ± 1.85 ^{###}	1.03 ± 0.05 ^{###}	0.76 ± 0.02 ^{###}
Group V (only EA D2)	3.2 ± 0.80	1.27 ± 0.05	0.87 ± 0.02

EA Ellagic acid, D1 50 mg/kg b wt, D2 100 mg/kg b wt, PCEs polychromatic erythrocytes, NCEs normochromatic erythrocytes, MnPEs micronucleated PCE

In case of genotoxicity parameters, viz., MN induction in bone marrow of mice; there was a marked increase (***) $p < 0.001$) in the micronuclei of the PCE in bone marrow of mice due to CPM administration as compared to control. However, pretreatment with EA reduced MN formation significantly at lower dose ([#] $p < 0.05$) and higher dose (^{###} $p < 0.001$). There was a significant (***) $p < 0.001$) decrease in the PCE:NCE ratio in CPM-treated group indicating the cytotoxicity in bone marrow cell. Pretreatment with EA at both the doses significantly (^{###} $p < 0.005$, [#] $p < 0.01$, ^{ns} $p =$ not significant) reduced the cytotoxic effects of CPM. Similar type of results were observed for DNA strand breaks in terms of *F* value. Only EA treated groups did not cause any toxic effect.

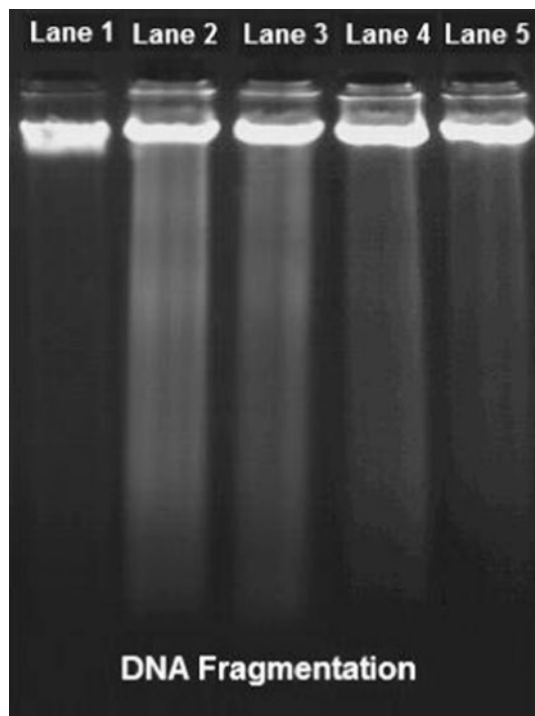


Fig. 4 Agarose (1.5%) gel electrophoresis of DNA obtained from mice kidney. Lane 1 control, Lane 2 CPM only, Lane 3 EA + CPM; D1, Lane 4 EA + CPM; D2, Lane 5 EA D2 only. From gel picture, it is marked that CPM treatment introduced DNA fragmentation by smearing of DNA when compared to control. There is decrease in DNA smearing as a result of EA pretreatment at both the doses in mice kidney. CPM cyclophosphamide, EA Ellagic acid, D1 50 mg/kg b wt, D2 100 mg/kg b wt

significant DNA fragmentation only in the toxicant group as compared to the control group while there was less smearing in EA pretreated groups, EA pretreatment inhibited DNA disintegration.

Histopathological examination

The control mice showed normal glomerular and tubular histology while CPM-administered mice were found to have necrosis and desquamation of epithelial cells, peritubular, and glomerular congestion and showed invasion of inflammatory cells in the cortical and medullary regions of kidneys. The nephrotoxicity was characterized by widespread degeneration of tubular architecture, tubular congestion, swelling and necrosis. In comparison, renal sections obtained from mice that were pretreated with EA at a dose of 100 mg/kg body weight demonstrated marked reduction of the histological character of renal injury. Prophylactic treatment of EA (50 mg/kg b wt) was also associated with a significant reduction in injury, but this reduction was less marked than in the higher dose EA pretreatment groups (Fig. 5).

Discussion

The kidney is the target of many xenobiotics toxicants, including drugs. There are many factors that contribute to the sensitivity of the kidney viz., presence of variety of metabolizing enzymes and xenobiotic transporters, large blood flow and concentration of solutes during urine production. Further physiological, anatomical, and biochemical features of the kidney make it particularly sensitive to many toxins and drugs. Present investigation was carried out with the aim of evaluating the possible role of EA in modulating the in vivo genotoxicity and oxidative renal injury of CPM.

The active metabolites of CPM are phosphoramidate and acrolein, and they slow down the growth of cancer cells as they interfere with the cellular DNA. Hydroxycyclophosphamide

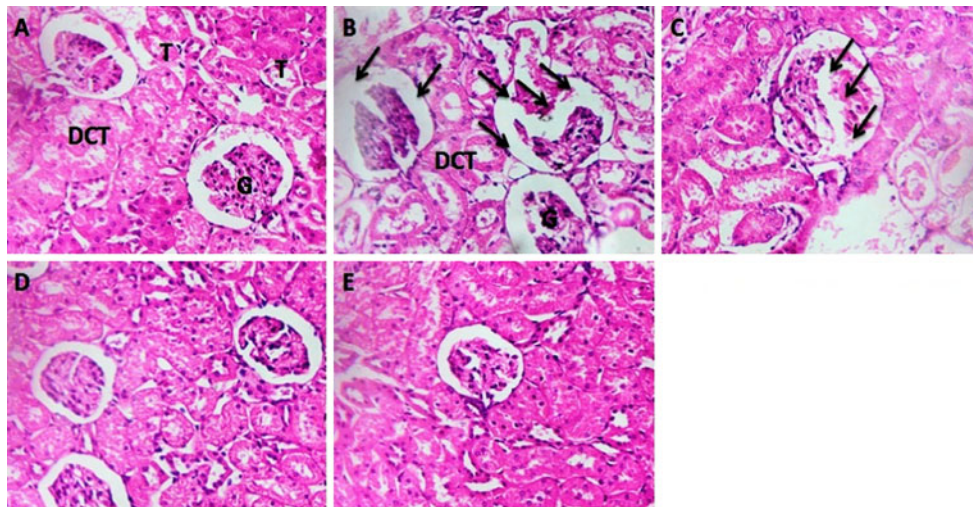


Fig. 5 Photomicrographs of kidney histology of Swiss albino mice ($\times 400$ magnifications). **A** Kidney section from control group shows normal renal histo-architecture. **B** Kidney section from only CPM-treated group shows loss of normal renal architecture, inflammatory cells infiltration, and fatty changes with cell swelling. **C** Kidney section of the EA pretreated groups at lower dose of EA (50 mg/kg b wt) moderately prevented the cytotoxic damage induced by CPM; as

indicated by moderate swelling of the renal epithelial cells. **D** Kidney section of the EA pretreated groups at higher dose of EA (100 mg/kg b wt) improved the morphology of the kidney from the damage induced by CPM. **E** Higher dose of EA administration did not showed any kind of histological abnormalities in the kidney tissue. CPM cyclophosphamide, EA Ellagic acid, D1 50 mg/kg b wt, D2 100 mg/kg b wt, G glomeruli, T tubule, DCT distal convoluted tubule

and deschloroethylcyclophosphamide are the two intermediate compounds that lead to the formation of phosphoramidate, the main agent responsible the mutagenic effect of CPM [4]. Phosphoramidate causes the induction of crosslink and strand lesion in DNA [50]. Studies have indicated that CPM has a pro-oxidant nature, and production of oxidative stress after CPM administration leads to decrease in the activities of antioxidant enzymes and increase in lipid peroxidation in different tissues of mice and rats [51, 52]. In our study also CPM treatment resulted in significant increases ($p < 0.001$) in the renal MDA with decrease in GSH (free thiols), GSH and its oxidized counterpart glutathione disulfide represent a major redox buffer system of the cell. GSH can act either as a non-enzymatic antioxidant by direct interaction of $-SH$ group with ROS or it can be implicated in the enzymatic detoxification reaction for ROS, as a cofactor or coenzyme. The depletion of GSH content may be attributed to the direct conjugation of CPM and its metabolites with free or protein bound $-SH$ groups and significant decreases ($p < 0.001$) in the GSH-Px and CAT activity in comparison to the control levels (Table 1). Moreover, Cooper et al. [53], Kehrer and Biswal [54] suggested that CPM treatment results in inflammation thus disturbing the overall renal redox cycling. CPM was also shown to cause decrease levels of other antioxidant enzymes like GR and glutathione-S-transferase. EA dose dependently increased the level of all the renal antioxidant enzymes to normal and this phenomenon was in agreement with the previously published

reports [55, 56]. Simultaneously the EA pretreatment also augmented the depleted levels of GGT and XO in these mice.

The marked increase in the levels of serum BUN and creatinine is a marker for the nephrotoxicity and kidney damage [57]. CPM also induced renal damage characterized by increases in the level of LDH, BUN, and creatinine. CPM-induced elevations in the levels of LDH, BUN, and creatinine observed in our study are in agreement with the previously published reports by Cagler et al. [57]. The elevation in the activities of these enzymes in the serum might be due to the leakage of these cytosolic enzymes into the circulatory system resulting from kidney damage after CPM administration. This is indicative of the onset of renal damage due to kidney dysfunction and disturbance in the biosynthesis of these enzymes, with alteration in the membrane permeability. Administration with EA prevented CPM-induced renal toxicity, as indicated by a steep decrease in serum BUN, creatinine levels and LDH activity, possibly by maintaining the renal cellular membrane integrity. This is an indicator of possible nephro-protective efficacy offered by EA compared with the untreated and CPM-intoxicated groups.

Histopathological studies also provided supportive confirmation for the biochemical parameters depicted by the photomicrographs. Kidney tissue (Fig. 5) of CPM-administered mice shows tubular necrosis and desquamation of epithelial cells, invasion of inflammatory cells in the cortical and medullary regions of kidneys was also

prominent. Lumen of tubules was observed to have collection of eosinophilic granular material. Another characteristic feature of CPM-induced toxicity is tubular renal epithelial cell hypertrophy which was observed in CPM-treated group and was in agreement with previously published report by Abraham et al. [58]. The key histological finding of this study was that EA pretreatment influenced the recovery of kidney architecture induced by CPM.

Experimental evidences have strengthened the concept that, DNA damage plays a crucial role in the initiation and subsequent promotion of carcinogenesis induced by different genotoxicants. Errors in the DNA molecules cause chromosomal aberrations. MN are well characterized biomarkers of structural and numerical chromosomal damage. The MN in young erythrocytes arise mainly from chromosomal fragments that are not incorporated into the daughter nuclei at the time of cell division in the erythropoietic blast cells [59]. Decrease in the PCE:NCE ratio is responsible for the induction of bone marrow cytotoxicity [60]. Results of our study reveal that CPM treatment shows cytotoxicity and has highly clastogenic nature. CPM was found to induce MN formation as it decreased PCE:NCE ratio and EA effectively brought this PCE:NCE ratio to the normal level. CPM also results in the formation of DNA strand breaks as a result of interaction of acrolein with DNA and cause DNA disintegration or smearing due to necrosis and MnPCEs formation. Prophylactic treatment of EA significantly and dose dependently restores normal level all these hallmarks of genotoxicity.

This study shows the protective effect of EA against CPM-induced genotoxicity and nephrotoxicity. In conclusion, our study provides evidence that EA pretreatment attenuates CPM-induced oxidative stress and the subsequent DNA damage in mice. The antigenotoxic potential of EA might be due to its antioxidant property. Future investigation targeting molecular pathways involved in modulatory action of EA on CPM-induced genotoxicity and nephrotoxicity are needed.

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