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Modulatory effects of gentisic acid against genotoxicity and hepatotoxicity induced by cyclophosphamide in Swiss albino mice

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Abstract

Objectives This study evaluated the protective effects of gentisic acid (GA) against genotoxicity and hepatotoxicity induced by cyclophosphamide (CP) in Swiss albino mice.

Methods Mice were pretreated with GA orally at doses of 50 and 100 mg/kg for 14 consecutive days before the administration of a single intraperitoneal dose of 50 mg/kg CP. The ameliorative effect of GA on genotoxicity was studied using the in-vivo bone marrow micronuclei induction test, DNA integrity and alkaline unwinding assay. The activity of various oxidative stress enzymes were estimated in hepatic tissue.

Key findings A single intraperitoneal administration of CP in mice increased the malondialdehyde level, depleted the glutathione content and antioxidant enzyme activity (glutathione peroxidase, glutathione reductase, catalase and quinone reductase), and induced DNA strand breaks and micronuclei induction. Oral pretreatment with GA at both doses caused a significant reduction in malondialdehyde and glutathione levels, restoration of antioxidant enzyme activity, reduction in micronuclei formation and DNA fragmentation. Serum toxicity marker enzymes such as aspartate aminotransferase, alanine aminotransferase and lactate dehydrogenase were increased after CP treatment but restored in GA pretreated groups.

Conclusion The results support the protective effect of GA against CP induced genotoxicity and hepatotoxicity.

Introduction

Cancer therapy and its management involves the consistent use of antineoplastic agents. These agents kill the rapidly proliferating cells and are responsible for the destruction of neoplastic tissue in cancer patients. However, because of their low therapeutic index, they also damage normal tissue. Therefore, chronic use of these agents causes excessive damage and is a matter of great concern.[1] Monitoring the mutagenic potential of anticancer agents helps minimize their destructive effects on genetic material.

Cyclophosphamide (CP) is a chemotherapeutic drug and immunosuppressive agent, widely used for the treatment of various cancers and autoimmune diseases. There is concern about the chronic carcinogenic side-effects of alkylating and neoplastic agents due to the use of combination drug therapies. There are a number of studies demonstrating the mutagenic effects of CP in humans and animals.[2,3] Chemically reactive metabolic products of CP cause cytotoxicity that alkylates DNA and protein, leading to the production of crosslinks in DNA.[4] Antineoplastic and toxic effects such as apoptosis, necrosis and oncosis of CP are linked with two active metabolites, namely acrolein and phosphoramide.[5] CP produces carbonium ions that react with the electron-rich centres of nucleic acids and proteins. It is known to cause mutations, DNA damage, micronuclei induction as well as the production of reactive oxygen species (ROS). The high reactivity of free radicals causes cellular damage through various mechanisms.[6] The most deleterious effects of free radicals lead to DNA damage that causes cancer.[7] It has been

reported that exposure to CP causes biochemical and physiological disturbances due to oxidative stress.[8–10] Various studies show that antioxidant intake can control the reaction to chemotherapy and also minimize the adverse side-effects of antineoplastic drugs.[11]

Epidemiological studies have shown that there is a relationship between the intake of foods rich in phenolic acids and protection from various diseases.[12] These phenolic compounds possess excellent antioxidant and chemoprotective properties *in vivo*.[13] Several studies have focused on the role of herbal plant extracts and their constituents. The present study focuses on gentisic acid (GA), a constituent of the plant *Hibiscus rosa sinensis*, which has been reported to have cancer preventive activity.[14] GA is a 2,5-dihydroxybenzoic acid. It is mainly an adduct product of salicylic acid and hydroxyl radicals, and its formation occurs *in vivo*.[15] There is evidence indicating that GA has biological activity such as antioxidant, anti-inflammatory and antimutagenic properties.[16,17] Although there have been a number of studies on GA, so far its antigenotoxic and hepatoprotective effects have not been investigated.

The aim of this study was to determine if GA protects against genotoxicity and hepatotoxicity induced by CP. The extent of the protective effect of GA against CP induced genotoxicity and its hepatoprotective effects were determined by studying markers of genotoxicity (alkaline unwinding assay, DNA fragmentation assay and micronuclei induction test) and biochemical estimation of antioxidant enzymes in Swiss albino mice.

Materials and Methods

Chemicals

GA, CP, EDTA, Tris, reduced glutathione (GSH), oxidized glutathione, nicotinamide adenine dinucleotide phosphate (NADPH), bovine serum albumin, bisbenzamide; ProteinaseK, EDTA, SDS, phenol, chloroform, isoamyl alcohol and RNase were all obtained from Sigma (St Louis, MO, USA). All other reagents and solvents used were of a high analytical grade.

Animals

Adult male Swiss albino mice (8 weeks old, 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. Animals received humane care in accordance with the Guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals, Government of India, and prior permission was sought from the Institutional Animal Ethics Committee (IAEC no:173/ CPCSEA, 2000).

Experimental design

For the study of biochemical parameters and the micronuclei assay, 30 male Swiss albino mice were divided into five groups. Group 1 served as the saline treated control. Group 2 was given a single intraperitoneal injection of CP (50 mg/kg) freshly dissolved in distilled water. GA was orally administered at two doses (50 and 100 mg/kg) to Groups 3 and 4, respectively, for 14 consecutive days. Group 5 received only a high dose of GA for 14 consecutive days. On Day 14 of pretreatment, a single intraperitoneal dose of CP (50 mg/kg) was given to the animals in Groups 2, 3 and 4. All the animals were killed 24 h after treatment with CP. Liver and femur bones were removed and processed for enzyme estimation and the micronuclei assay.

Micronucleus assay

The air-dried slides containing bone marrow aspiration were stained with May-Grunwald and Giemsa.[18] A total of 1500–2000 polychromatic erythrocytes (PCE) and normochromatic erythrocytes (NCE) were scored per animal by the same observer to determine the frequency of micronucleated polychromatic erythrocytes (MnPCE). To detect possible cytotoxic effects, the PCE : NCE ratio in 200 erythrocytes per animal was calculated according to Gollapudi and McFadden.[19]

DNA isolation

DNA extraction from liver was done by a standard chloroform isoamyl method.[20] The quality and quantity of DNA extracted was measured using a Thermoscientific Nanodrop spectrophotometer 2000 C (Wilmington, DE, USA) and 1% agarose gel electrophoresis. The amount of DNA was quantitated spectrophotometrically at 260 and 280 nm.

Alkaline unwinding assay

The fluorescence of double stranded DNA was determined by placing a 100-mmol DNA sample, 100 ml NaCl (25 mM) and 2 ml SDS (0.5%) in a prechilled test tube, followed by the addition of 3 ml 0.2 M potassium phosphate pH 9 and 3 ml 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 single stranded DNA was determined as above but using a DNA sample that had been boiled for 30 min to completely unwind the DNA. NaOH (50 ml, 0.05 N) was rapidly mixed with 100 ml of a DNA sample in a pre-chilled test tube. The mixture was incubated on ice in the dark for 30 min followed by rapid addition and mixing of 50 ml HCl (0.05N). This was followed

immediately by the addition of 2 ml SDS (0.5%) and the mixture was forcefully passed through a 21-G needle six times. Fluorescence of the alkaline unwound DNA sample was measured as described above. The ratio between double stranded DNA to total DNA (*F* value) was determined as follows:

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

where auDNA, ssDNA and dsDNA are the degree of fluorescence from the partially unwound, single stranded and double stranded DNA determinations, respectively.[21,22]

Gel electrophoresis and DNA fragmentation

The sample was mixed with 10 ml 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 Tris Borate EDTA (TBE) buffer. The DNA was visualized using ethidium bromide and photographed using a digital camera.

Postmitochondrial supernatant preparation

The liver was homogenized in chilled phosphate buffer (0.1 M, pH 7.4) containing KCI (1.17%) using a Potter Elvehjen homogenizer. The homogenate was filtered through muslin cloth and was then centrifuged at 800*g* for 5 min at 4°C using a REMI cooling centrifuge (Cat. No. RQ127 A, Remi Motors, Mumbai, India) to separate the nuclear debris. The sample obtained was centrifuged at 12 000*g* rev/min for 20 min at 4°C to obtain the postmitochondrial supernant (PMS).[23]

Reduced glutathione estimation

A 1-ml sample of PMS was precipitated with 1.0 ml of sulfosalicylic acid (4%). The samples were kept at 4°C for 1 h and then centrifuged at 1200*g* for 20 min at 4°C. The assay mixture contained 0.1 ml filtered sample, 2.7 ml phosphate buffer (0.1 M, pH 7.4) and 0.2 ml 1,2-dithiobisnitrobenzoic acid (100 mM) in a total volume of 3.0 ml. The yellow colour developed was read at 412 nm.[24]

Glutathione reductase activity

The reaction mixture consisted of 1.65 ml phosphate buffer (0.1 M, pH 7.6), 0.1 ml EDTA (0.5 mM), 0.05 ml oxidized GSH (1 mM), 0.1 ml NADPH (0.1 mM) and 0.1 ml 10% PMS in a total volume of 2 ml. Enzyme activity was quantitated at 25°C by measuring the disappearance of NADPH at 340 nm.[25]

Glutathione peroxidase activity

The reaction mixture consisted of 1.49 ml phosphate buffer (0.1 M, pH 7.4), 0.1 ml EDTA (1 mM), 0.1 ml sodium azide (1 mM), 0.05 ml glutathione reductase (1 IU ml/1), 0.05 ml GSH (1 mM), 0.1 ml NADPH (0.2 mM), 0.01 ml H_2O_2 (0.25 mM) and 0.1 ml 10% PMS in a total volume of 2 ml. The disappearance of NADPH at 340 nm was recorded at 25°C.[26]

Malondialdehyde formation

The reaction mixture in a total volume of 1.0 ml contained 0.60 ml phosphate buffer (0.1 M, pH 7.4), 0.2 ml microsomes and 0.2 ml ascorbic acid (100 mM). The reaction mixture was incubated at 37°C in a shaking water bath for 1 h. The reaction was stopped by adding 1.0 ml 10% trichloroacetic acid. Following the addition of 1.0 ml 0.67% thiobarbituric acid, all tubes were placed in a boiling water bath for 20 min and then transferred to a crushed ice-bath before centrifuging at 2500*g* for 10 min. The amount of malondialdehyde (MDA) formed in each of the samples was assessed by measuring the optical density of the supernatant at 535 nm against a reagent blank.[27]

Catalase activity

The reaction mixture consisted of 1.95 ml phosphate buffer (0.1 M, pH 7.4), 1.0 ml hydrogen peroxide (0.019 M) and 0.05 ml 10% PMS in a final volume of 3 ml. Changes in absorbance were recorded at 240 nm. Catalase activity was calculated as nmol H_2O_2 consumed/min per mg protein.[28]

Quinone reductase activity

The 3-ml reaction mixture consisted of 2.13 ml Tris-HCl buffer (25 mM, pH 7.4), 0.7 ml bovine serum albumin, 0.1 ml flavin adenine dinucleotide, 0.02 ml NADPH (0.1 mM) and 50 µl (10%) PMS. The reduction of dichloroindophenol was recorded calorimetrically at 600 nm and enzyme activity was calculated as nmol of dichloroindophenol reduced/min per mg protein using a molar extinction coefficient of 2.1×10^4 M/cm.[29]

Measurement of liver toxicity markers

Each substrate, 0.5 ml (2 mM α-ketoglutarate and either 200 mM L-alanine or L-aspartate) was incubated for 5 min at 37°C in a water bath. Then, 0.1 ml serum was added and the volume was adjusted to 1.0 ml with 0.1 M, pH 7.4 phosphate buffer. The reaction mixture was incubated for exactly 30 and 60 min at 37°C for alanine aminotransferase (ALT) and aspartate aminotransferase (AST), respectively. Then, 0.5 ml of 1 mM DNPH was added to the reaction mixture. After

Table 1 Effects of pretreatment with gentisic acid on serum toxicity marker enzymes after cyclophosphamide treatment in mouse liver

Group	Aspartate aminotransferase (IU/l)	Alanine aminotransferase (IU/l)	Lactate dehydrogenase (nmol NADH oxidised/min per mg protein)
Group 1 (control)	35.65 ± 0.44	33.98 ± 0.39	201.02 ± 20.35
Group 2 (CP only)	77.40 ± 0.73***	60.61 ± 1.17***	417.91 ± 12.06***
Group 3 (GA 50 mg/kg + CP)	62.46 ± 0.10###	48.78 ± 0.48###	285.66 ± 17.38###
Group 4 (GA 100 mg/kg + CP)	50.54 ± 0.59###	35.24 ± 0.52###	216.89 ± 14.58###
Group 5 (GA only 100 mg/kg)	35.79 ± 0.24	34.39 ± 0.39	206.31 ± 10.03

CP, cyclophosphamide GA, gentisic acid. Results represent mean ± SE of six animals per group. ***$P < 0.001$, CP treatment led to a significant elevation in the serum marker enzymes in Group 2 compared with Group 1. ###$P < 0.001$, pretreatment with GA restored the activity of these enzymes in Groups 3 and 4 significantly as compared with Group 2.

30 min at room temperature, the colour was developed by the addition of 5.0 ml of 0.4N NaOH and the product was read at 505 nm.[30]

Lactate dehydrogenase activity

The assay mixture consisted of serum, 0.02 M NADH, 0.01 M sodium pyruvate, 0.1 M, pH 7.4 phosphate buffer and distilled water in a total volume of 3 ml. Lactate dehydrogenase (LDH) activity was recorded at 340 nm and was calculated as nmol NADH oxidized/min per mg protein.[31]

Estimation of protein concentration

The protein concentration in all samples was determined by the method of Lowry *et al*.[32]

Histopathological examination

After the mice were killed, the livers were quickly removed and preserved in 10% neutral buffered formalin for histopathological processing. Sections were stained with hematoxyline and eosin before being observed under an Olympus microscope at 400× magnification.

Statistical analysis

Differences between groups were analysed using analysis of variance followed by Dunnet's multiple comparisons test. All data points are presented as the treatment groups mean ± SE.

Results

Effect of GA pretreatment on serum AST, ALT and LDH activity

A protective effect of GA on serum AST, ALT and LDH levels was observed. A significant protective effect ($P < 0.01$, $P < 0.001$) on these marker enzymes was observed in the GA pretreatment groups; GA was found to be effective in the normalization of these markers when compared with the CP treated group (Table 1). Group 5 (GA only) showed no significant difference compared with the control group.

Effect of GA pretreatment on hepatic GSH level

A protective effect of GA on the hepatic GSH level was observed. The GSH level was significantly depleted ($P < 0.001$) in the CP treated group compared with the control group. The GSH level in the GA pretreated groups was significantly increased ($P < 0.01$, $P < 0.001$) compared with the CP treated group. Group 5 (GA only) showed no significant changes in GSH level compared with the control group (Table 2).

Effect of GA pretreatment on antioxidant enzyme activity

In the CP treated group, hepatic antioxidant enzymes (glutathione peroxidase, glutathione reductase, quinone reductase and catalase) were significantly depleted compared with the control group ($P < 0.001$). Pretreatment with GA before CP administration was found to be significantly effective in restoring the activity of these enzymes at both GA doses used ($P < 0.001$). There was no significant difference in the activity of these antioxidant enzymes between the control group and Group 5 (GA only) (Tables 2 and 3)

Effect of GA pretreatment on MDA formation

MDA formation was measured to demonstrate lipid peroxidation (LPO) in the liver of Swiss albino mice with CP induced toxicity. A significant ($P < 0.001$) increase in MDA formation was found in the CP treated group compared with the control group. It was observed that pretreatment with GA at both doses (50 and 100 mg/kg) led to significant ($P < 0.001$ and $P < 0.001$, respectively) prevention of membrane damage by reducing the elevated levels of LPO in the liver when compared with CP treated group (Table 4). No significant differ-

Table 2 Effects of pretreatment with gentisic acid on antioxidant enzymes after cyclophosphamide administration in mouse liver

Group	Reduced glutathione (nmol CDNB conjugate formed/g tissue)	Glutathione reductase (nmol NADPH oxidized/min per mg protein)	Glutathione peroxidase (nmol NADPH oxidized/min per mg protein)
Group 1 (control)	0.619 ± 0.011	223.42 ± 23.8	349.6.1 ± 16.93
Group 2 (CP only)	0.378 ± 0.002***	85.65 ± 6.57***	123.04 ± 11.7**
Group 3 (GA 50 mg/kg + CP)	0.494 ± 0.004$^{\#\#\#}$	174.45 ± 2.66$^{\#}$	261.56 ± 12.5$^{\#}$
Group 4 (GA 100 mg/kg + CP)	0.57 ± 0.002$^{\#\#\#}$	220.84 ± 5.0$^{\#\#}$	331.64 ± 20.83$^{\#\#}$
Group 5 (GA only 100 mg/kg)	0.618 ± 0.01	220.79 ± 9.14	342.61 ± 22.91

CP, cyclophosphamide, GA, gentisic acid. Results represent mean ± SE of six animals per group. $^{**}P < 0.01$ and $^{***}P < 0.001$, CP treatment led to a significant depletion in the activity of antioxidant enzymes in Group 2 as compared with Group 1. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$, pretreatment with GA restored the activity of these enzymes in Groups 3 and 4 significantly as compared with Group 2.

Table 3 Effects of pretreatment with gentisic acid on the activity of quinone reductase, catalase and DNA strand breaks after cyclophosphamide administration in mouse liver

Group	Quinone reductase (nmol NADPH oxidized/min per mg protein)	Catalase (nmol H_2O_2 consumed/min per mg protein)	DNA strand breaks (*F* value)
Group 1 (control)	341.26 ± 5.57	32.36 ± 1.72	0.907 ± 0.01
Group 2 (CP only)	146.02 ± 6.34***	12.09 ± 1.08***	0.434 ± 0.05***
Group 3 (CP + GA 50 mg/kg)	241.14 ± 11.43$^{\#}$	20.61 ± 0.92$^{\#\#}$	0.762 ± 0.07$^{\#}$
Group 4 (CP + GA 100 mg/kg)	339.76 ± 29.69$^{\#\#\#}$	29.13 ± 0.104$^{\#\#\#}$	0.939 ± 0.02$^{\#\#\#}$
Group 5 (GA only 100 mg/kg)	340.96 ± 2.50	29.74 ± 3.42	0.901 ± 0.02

CP, cyclophosphamide GA, gentisic acid. Results represent mean ± SE of six animals per group. $^{***}P < 0.001$, CP treatment led to a significant depletion in the activity of antioxidant enzymes and DNA strand breaks in Group 2 as compared with Group 1. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$, pretreatment with GA restored the activity of these enzymes and DNA strand breaks in Groups 3 and 4 significantly as compared with Group 2.

Table 4 Effects of pretreatment with gentisic acid on lipid peroxidation and micronuclei induction after cyclophosphamide administration in mouse liver

Group	LPO (nmol MDA formed/h per g tissue)	MnPCE : 1000 PCE	PCE : NCE ratio
Group 1 (control)	2.92 ± 0.03	7.4 ± 0.40	1.11 ± 0.01
Group 2 (CP only)	5.72 ± 0.08***	18.0 ± 0.60***	0.67 ± 0.04***
Group 3 (GA 50 mg/kg + CP)	3.52 ± 0.23$^{\#\#\#}$	12.4 ± 1.18$^{\#\#}$	0.93 ± 0.02$^{\#\#\#}$
Group 4 (GA 100 mg/kg + CP)	2.66 ± 0.02$^{\#\#\#}$	8.0 ± 0.74$^{\#\#\#}$	1.07 ± 0.01$^{\#\#\#}$
Group 5 (GA only 100 mg/kg)	2.51 ± 0.35	7.8 ± 1.03	1.08 ± 0.03

CP, cyclophosphamide, GA, gentisic acid. Results represent mean ± SE of six animals per group. $^{***}P < 0.001$, CP treatment led to a significant elevation in the lipid peroxidation (LPO) level, an increase in the MnPCE/1000 PCE ratio and an increase in the PCE : NCE ratio in Group 2 as compared with Group 1. $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$, pretreatment with GA decreased the LPO level, MnPCE : 1000 PCE ratio and PCE : NCE ratio in Groups 3 and 4 significantly as compared with Group 2.

ence was found in the MDA level between control and only the groups receiving the higher dose of GA (100 mg/kg).

Effect of GA pretreatment on genotoxicity

In the DNA alkaline unwinding assay (Table 4), a concurrent decrease in the *F* value was marked compared with the control group, whereas there was a significant increase in the *F* value at both doses of GA. Similarly, it is clear from Table 4 that there was higher induction of micronuclei in the CP only treated group, and the level was decreased by pretreatment with GA at both doses. DNA damage was evaluated in terms of smearing and lack of intact band control; only the GA pretreated groups showed less smearing and an intact band was also observed. The estimated PCE : NCE ratios in bone marrow preparations in Table 4 show a statistical decrease in hematopoiesis in the CP only treated group compared with the control group. Pretreatment with GA at both doses increased the PCE : NCE ratio by reversal of the cytotoxic effects caused by CP. In Figure 1, the results indicate that there was significant DNA fragmentation in the CP treated group compared with the control group; there was less fragmentation in GA pretreated groups. GA pretreatment restored DNA integrity.

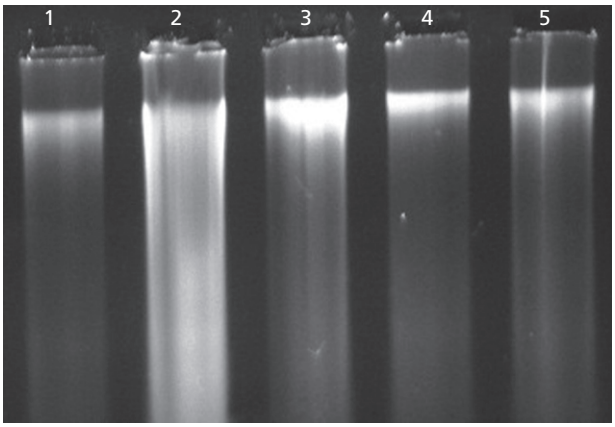

Figure 1 Agarose gel electrophoresis of DNA obtained from mouse liver. Lane 1, control; lane 2, cyclophosphamide only; lane 3, gentisic acid (50 mg/kg) and cyclophosphamide; lane 4, gentisic acid (100 mg/kg) and cyclophosphamide; lane 5, gentisic acid only (100 mg/kg). Cyclophosphamide treatment caused DNA fragmentation as indicated by smearing of DNA compared with the control. There was a decrease in DNA smearing as a result of gentisic acid pretreatment at both doses. Results represent mean ± SE of six animals per group. $^{***}P < 0.001$, results were significantly different compared with the control group. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$, results were significantly different compared with the cyclophosphamide treated group.

Effect of GA on liver histology

Histological features of the control group showed normal hepatocytes. In the CP treated group, the liver showed loss of normal hepatocyte architecture, inflammatory cell infiltration and fatty changes. Pretreatment with GA at both doses reverted these changes and the liver looked normal, with few inflammatory cells and congestion of central veins as hepatic sinusoids appeared lined by endothelial cells in the GA pretreated groups (Figure 2).

Discussion

Although anticancer drugs have been used for many years for cancer treatment, the various mechanisms leading to the destruction of healthy cells are still unclear. Metabolic products and ROS produced during drug metabolism may be a contributing factor.[33,34] It is well known that the redox balance of a cell is disturbed during the production of ROS, causing oxidative stress, cancer and finally the destruction of cells.[35] During the metabolism of CP, ROS are produced and catalysed by cytochrome P-450, peroxidases and lipooxygenases.[36]

CP is the most common drug used for the treatment of a wide range of cancers. Acrolein and phosphoramide are the active compounds of CP that decelerate the growth of cancer cells when they interfere with cellular DNA. The mutagenic effect of CP is due to the formation of phosphoramide through the intermediate compounds hydroxycyclophosphamide and deschloroethylcyclophosphamide,[37,38] causing the induction of crosslinks and strand lesion in DNA.[39] The genotoxic effects of CP have been evaluated in in-vivo and in-vitro studies.[40] It has been reported that CP, along with various other chemotherapeutic drugs, cause gene mutations. In the search for new effective strategies for cancer chemotherapy much attention has been focused on the use of natural compounds and their products which modulate the toxicity of anticancer drugs.[41]

The results of the present study indicate that CP treatment is highly clastogenic and shows cytotoxicity. It is has been reported that various anticancer drugs induce micronuclei formation.[42,43] Pretreatment with GA inhibits micronuclei formation and the cytotoxicity induced by CP in a dose-dependent manner. It has been observed that a decrease in the PCE to mature erythrocyte ratio is responsible for the induction of bone marrow cytotoxicity.[44] In the present study, CP was found to decrease the ratio of PCE to mature erythrocytes, but this was normalized in the GA pretreated groups.

In the process of lipid peroxidation MDA is formed by the conversion of polyunsaturated fatty acid or lipid peroxides.[45] In the present study, we found that GA pretreatment significantly decreased MDA formation due to ROS in mice treated with CP. Pretreatment with GA restored the MDA level, suggesting that GA might be successful in quenching free radicals, thus inhibiting LPO and protecting against membrane damage from oxidative damage in mice. Modulation of antioxidant enzyme activity was also observed. Reduced glutathione, which is known to be a first line of defence, neutralizes the hydroxyl radical and plays a significant role against inflammatory responses and oxidative stress.[46] A significant, dose-dependent, restoration of glutathione and dependent enzymes, namely glutathione reductase and glutathione peroxidase, to normal levels in GA pretreated groups was found. Simultaneously, GA pretreatment was found to restore depleted levels of quinone reductase and catalase in GA pretreated groups. There was a good correlation between cellular damage and leakage of enzymes, as evidenced by the elevated levels of serum marker enzymes.[47] The levels of serum toxicity marker enzymes (AST, ALT and LDH) were increased in the CP treated group and were restored in the GA pretreated groups. It is evident from the present study that GA not only reduces cellular damage but also suppresses DNA fragmentation and MnPCE formation in-vivo compared with the CP treated group. The *F* value and PCE : NCE ratio were also restored to normal in the GA pretreated groups. Histopathological evaluations also support that GA pretreatment had a protective effect on liver morphology. More research is needed to explain the down-regulation pathways of the modulatory actions of GA against CP induced genotoxicity.

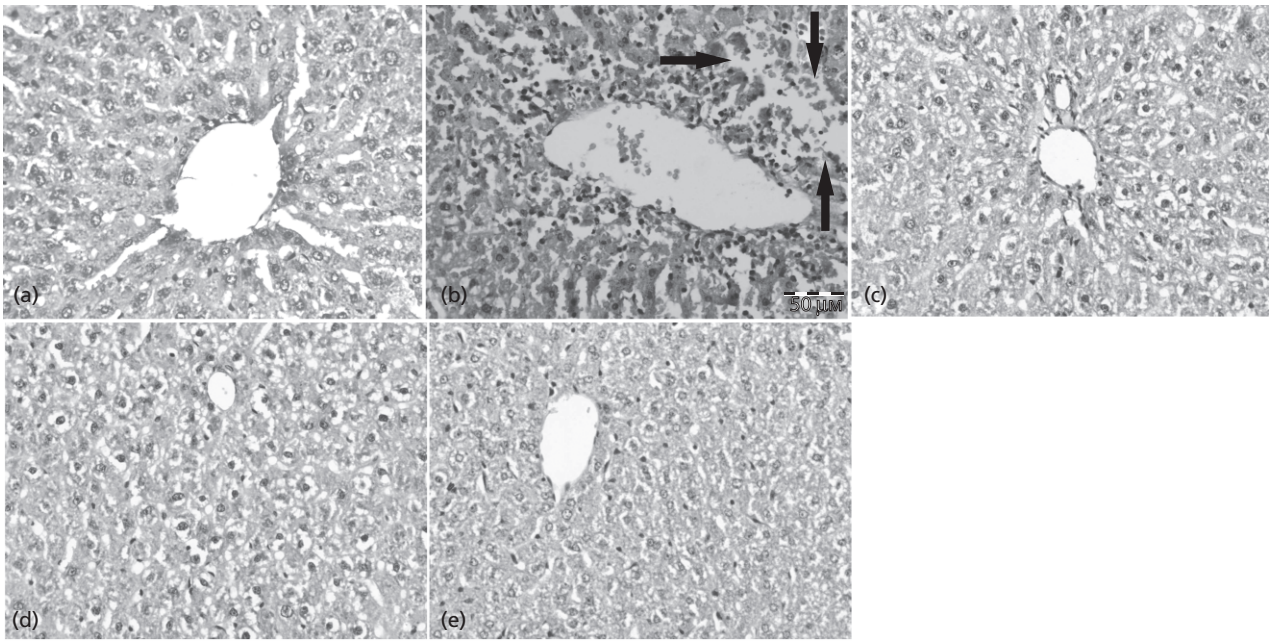

Figure 2 Liver histology. (a) Liver section from the control group shows hepatocytes with congested blood sinusoids. (b) Liver section from the cyclophosphamide only treated group showing loss of normal hepatic architecture, inflammatory cell infiltration and fatty changes with cell swelling indicated by arrows. (c) Liver section of the gentisic acid pretreated groups at the lower dose of GA (50 mg/kg) moderately prevented the cytotoxic damage induced by CP, as indicated by moderate swelling of the hepatic cells. (d) Liver section of the gentisic acid pretreated groups at the higher dose of gentisic acid (100 mg/kg) restored the morphology of the liver from the damage induced by cyclophosphamide as marked from the normal histology of control liver. (e) The higher dose of gentisic acid (100 mg/kg) did not cause any histological abnormalities in the liver. Magnification 400×.

Conclusions

GA showed a potent protective effect against CP induced hepatotoxicity and genotoxicity. The protective effect of GA can be attributed to its antioxidant properties, which enhance natural antioxidant enzymes and the cell membrane stabilizing property by inhibiting lipid peroxidation. GA can be used as a protective modulator against CP induced genotoxic and hepatic injury in patients undergoing CP chemotherapy. Our findings support the future research potential of the design and development of GA related modulatory compounds in combination with CP treatment. Such compounds might reduce the side-effects caused by widely used chemotherapeutic agents. Further studies are necessary before clinical application can be recommended.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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