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MANGIFERIN PROTECTS RENAL IMPAIRMENT AGAINST BENZO (A) PYRENE INDUCED TOXICITY BY REGULATING MITOCHONDRIAL AND DNA INTEGRITY

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ABSTRACT

Contaminated food and water ingestion and inhalation of polycyclic aromatic hydrocarbon are the main sources of human exposure to benzo(a)pyrene leading to cause renal injury and provoking a nephropathic response. Mangiferin is a highly potent antioxidant present in mango and known for several medicinal properties. The present study was undertaken to evaluate the renoprotective effect of mangiferin against benzo(a)pyrene induced toxicity. Benzo(a)pyrene was administered at a dose of 120.0 mg/kg once alone or in combination with mangiferin at 10.0 and 20.0 mg/kg for seven days in Swiss albino mice. Exposure to benzo(a)pyrene decreased the activities of glutathione peroxidase and increased glutathione-S-transferase level in the kidneys of mice. Moreover, DNA alkaline unwinding assay exhibit a significant decrease in F-value for the toxic control group in comparison to the normal mice, which is a marker for alteration in DNA integrity. Benzo(a)pyrene treatment also revealed an increased number of micronuclei in polychromatic erythrocytes in comparison with the control group indicating chromosomal damage in erythrocytes of bone marrow. Mangiferin pretreatment significantly improved the renal mitochondrial antioxidant status and restored the renal DNA integrity, thus demonstrating the protective effect in benzo(a)pyrene-treated mice. Dietary inclusion of mangiferin could exert protective effects against renal toxicity resulting from benzo(a)pyrene exposure.

Keywords: Benzo(a)pyrene, GPx, GST, Mangiferin, Micronucleus

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INTRODUCTION

Benzo(a)pyrene [B(a)P] is a polycyclic aromatic hydrocarbon known worldwide for its environmental contaminant property. Incomplete combustion of carbonaceous materials by humans for energy and barbeque, industrial ignition products and diesel exhaust are the chief ecological sources of B(a)P.[1-3] The ingestion of contaminated food and water and inhalation of polycyclic aromatic hydrocarbon are the main source of human exposure to B(a)P leading to cause renal injury[4] and provoking a nephropathic response.[5] Nanez *et al.* (2005) reported renal changes in female Sprague Dawley rats following once weekly injections of 10 mg/kg B(a)P for 16 weeks.[6] Previous toxicological studies demonstrated the possible metabolic activation of B(a)P to reactive intermediates similar to the liver cells. Moreover, B(a)P-induced nephropathy was demonstrated to involve disruption of glomerular cell–cell and cell–matrix interactions in rats. A single oral administration of B(a)P at the dose of 125 mg/kg caused renal toxicity and loss of DNA integrity in mice.[7] Renal toxicity in rodents induced by B(a)P treatment has become popular paradigm to study protective efficacy of various novel possible therapeutic candidates.

There is considerable scientific evidence to suggest that compounds isolated from plants can inhibit the process

of many diseases effectively.[8] Xanthones are naturally synthesized in various biological systems such as plants, lichens and fungi and are stored as byproducts. In addition to taxonomic significance, they are also important in the treatment/management of a number of human disorders. Mangiferin belongs to xanthones, known as a privileged class of secondary metabolites. Initially, it was isolated primarily from the *Mangifera indica* but it is also present in the plants of 16 other families including Anacardiacae, Gentianaceae and Iridaceae. It is considered one of the main active constituents in more than 40 polyherbal formulations in traditional Chinese medicine.[9] Mangiferin has proven its effectiveness as an antioxidant, analgesic, antidiabetic, antiproliferative, chemopreventive, radioprotective, cardiotonic, immunomodulatory and diuretic agent. It is a potent antioxidant agent and also stimulates mitochondrial respiration and suppresses oxidative and inflammation damage.[10] Keeping this in mind, mangiferin was chosen to evaluate its protective effect on B(a)P-induced renal toxicity. A series of indices related to oxidative stress, renal and DNA integrity were chosen to elucidate the protective effect of mangiferin on B(a)P-induced toxicity in rodents to provide a means of protection against environmental B(a)P-induced renal damage and to generate more comprehensive and reliable data for toxicological risk evaluation and protection by mangiferin.

MATERIALS AND METHODS

Chemicals

Genistein (purity: ≥98%) and B(a)P (purity: ≥96%), were purchased from Sigma-Aldrich Chemicals Pvt. Ltd., Bangalore, India. All other chemicals and reagents used were of analytical grade. Mangiferin was solubilized in 1% DMSO and B(a)P in corn oil. All other chemicals were dissolved in double distilled water. The doses for all freshly prepared drug solutions were expressed in terms of their free bases.

Subjects

Swiss albino male mice (25-30 g and 8 weeks or older) were used in this study. Animals were grouped six mice per cage and maintained at standard conditions of humidity (55 ± 5%), temperature 24 ± 2 °C under 12:12 h light/dark cycle. Animals have free access to rodent chow and tap water *ad libitum*. Animal studies were conducted after getting approval from Institutional Animal Ethics Committee.

Treatment schedule

Animals were divided into five groups consisting six animals each.

Group I [Normal Control]: animals of this group were treated with corn oil (p.o.) for seven days.

Group II [Toxic Control]: animals of this group were administered single oral dose of B(a)P (125.0 mg/g) on the first day of experimental period to induce renotoxicity [7], followed by a single oral dose of B(a)P (125.0 mg/g) on the eighth day.

Group III [B(a)P + M (10)]: animals of this group were pretreated with mangiferin (10.0 mg/kg, i.p.) for seven days, followed by a single oral dose of B(a)P (125.0 mg/g) on the eighth day.

Group IV [B(a)P + M (20)]: animals of this group were pretreated with mangiferin (20.0 mg/kg, i.p.) for seven days, followed by a single oral dose of B(a)P (125.0 mg/g) on the eighth day.

Group V [M (20)]: animals of this group were pretreated with mangiferin (20.0 mg/kg, i.p.) for seven days.

The above-mentioned doses of mangiferin were selected based on preliminary studies carried out in our laboratory. Acute and chronic toxicity studies and pharmacological studies[11] conducted on mangiferin were used as reference in deriving the current doses.

Assessment of mitochondrial integrity

Post-mitochondrial supernatant and microsome preparation

Tissue processing and preparation of post-mitochondrial supernatant (PMS) were done as described by Athar and Iqbal (1998). After the treatment period, 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 KCl (1.17%) using homogenizer. The homogenate was filtered through muslin cloth and was centrifuged at 800 g for 5 min at 4 °C to separate the nuclear debris. The aliquot so obtained was centrifuged at 12000 rpm for 20 min at 4 °C to obtain post-mitochondrial supernatant (PMS), which was used as a source of enzymes. A portion of the PMS was centrifuged for 60 min by ultracentrifuge at 34000 rpm at 4 °C. The pellet was washed with phosphate buffer (0.1 M, pH 7.4) containing KCl (1.17%). All the biochemical determination were completed within 24 h of animal sacrifice.[12]

Assay for glutathione peroxidase activity (Phase I enzyme)

Glutathione peroxidase (GPx) activity was measured by the method of Mohandas *et al.* (1984). The reaction mixture consisted of 1.44 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), 0.05 ml reduced glutathione (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. Enzyme activity was calculated as nmol NADPH oxidized/min/mg protein using a molar extinction coefficient of 6.22 × 10^3/M/cm.[13]

Assay for glutathione-S-transferase activity (Phase II enzyme)

Glutathione-S-transferase (GST) activity was assayed by the method of Habig (1974). The reaction mixture consisted of 1.475 ml phosphate buffer (0.1 M, pH 6.5), 0.2 ml reduced glutathione (1 mM), 0.025 ml, 1,chloro-2,dinitrobenzene (CDNB) (1 mM) and 0.3 ml PMS

(10% w/v) in a total volume of 2.0 ml. The changes in the absorbance were recorded at 340 nm and enzyme activity was calculated as nmol CDNB conjugate formed per minute per mg protein using a molar extinction coefficient of 9.6×10^6/M/cm.[14]

Assessment of DNA integrity

DNA Isolation

DNA was extracted from approximately 500 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 10 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. It was then centrifuged for 5 min at 13000 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 RNAase (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 2 volumes of absolute ethanol and 1/10 volume of 3 M sodium acetate, pH 5.2. The sample was then centrifuged (13000 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. Two 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.[15]

Alkaline unwinding assay

The procedure used alkaline unwinding was essentially the same as that outlined by Shugart (1988) with slight modifications. In the alkaline unwinding assay, the rate of transition of double stranded DNA (dsDNA) to single stranded DNA (ssDNA) under pre-defined 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 and from its fluorescence, 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 were 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 auDNA. The fluorescence of initial or dsDNA was determined by placing 100 mmol DNA sample, 100 ml NaCl (25 mM) and 2 ml SDS (0.5%) in a pre-chilled test tube, followed by 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 darkness for 15 min to allow fluorescence to stabilize. The fluorescence of the sample was measured using a spectrofluorimeter (E_x: 360 nm, E_m: 450 nm). The fluorescence of single stranded DNA was determined as above but using the DNA sample that had already been boiled for 30 min to completely unwinds the DNA. The amount of 0.50 ml NaOH (0.05 N) was rapidly mixed with 100 ml of the DNA sample in a pre-chilled test tube. The mixture was incubated on ice in darkness for 30 min [15] followed by rapid addition and mixing of 50 ml HCl (0.05 N). This was followed immediately by addition of 2 ml SDS (0.5%) and the mixture was forcefully passed through a 21 G needle six times. Fluorescence of alkaline unwounded DNA sample was measured as described above. Measurement of the alkaline unwounded sample was performed in triplicate and the average was reported. The ratio between dsDNA to total DNA (F-value) was determined as follows:

F value = (auDNA – ssDNA)/(dsDNA – ssDNA)

Where, auDNA, ssDNA and dsDNA were the degrees of fluorescence from the partially unwound, single stranded and double stranded determinations, respectively. The F value was inversely proportional to the number of strand breaks present and thus could be used as an indicator of DNA integrity.[16]

Micronucleus test

The mouse bone marrow micronucleus test was carried out according to the method of Schimid (1975). For this test, mice were sacrificed 24 h after treatment with single oral dose of B(a)P. Femur bones for bone marrow were collected for micronucleus assay and kidney tissue for enzymatic assay. The cells were smeared on glass slide, air-dried and then stained successively with May-Gruenwald and Giemsa stain. Per animal, 2000 polychromatic erythrocytes (PCEs) were scored to determine the frequency of micronucleated polychromatic erythrocytes (MNPCEs) and the ratio of PCE/NCE was counted in 200 normochromatic erythrocytes (NCEs). A total of 2500-3000 polychromatic erythrocytes (PCEs) were scored per animal by the same observer for determining the frequencies of micronucleated polychromatic erythrocytes (MnPCEs).[17]

Statistical analysis

Differences between groups were analyzed using analysis of variance (ANOVA) followed by Tucky's multiple comparisons test. All data points are presented as the treatment groups mean ± standard error of the mean (SEM). Probability values less than 0.05 were considered statistically significant in all the cases.

RESULTS

Assessment of mitochondrial integrity

There was a significant drop ($p< 0.0001$) in levels of GPx and significant increase ($p< 0.0001$) in GST levels in renal tissue of mice induced with B(a)P toxicity as compared to normal control mice. Chronic treatment with mangiferin (10.0 and 20.0 mg/kg) followed by single dose of B(a)P (125.0 mg/kg) significantly increased ($p< 0.0001$) GPx level and significantly decreased GST level ($p< 0.0001$) in renal tissue compared to toxic control mice. In addition, chronic

treatment with mangiferin alone (20.0 mg/kg) in mice significantly increased (p< 0.0001) GPx levels and significantly decreased (p< 0.0001) GST level in renal tissue (Table 1).

Table 1: Effect of mangiferin pretreatment on B(a)P mediated alteration in glutathione peroxidase and glutathione-S-transferase enzymes

Groups	Treatment	Glutathione peroxidase (nmol NADPH oxidized/min/mg protein)	Glutathione-S transferase (nmol CDNB conjugate formed/min/mg protein)
Normal Control	Corn oil (p.o., 7 d)	80.32 ± 0.59	208.63 ± 1.20
Toxic Control	B(a)P (125.0 mg/g, once)	39.81 ± 0.21*	432.74 ± 2.69*
B(a)P + M (10)	Mangiferin (10.0 mg/kg, i.p., 7 d) + B(a)P (125.0 mg/g, once)	54.53 ± 0.28#	335.12 ± 1.75#
B(a)P + M (20)	Mangiferin (20.0 mg/kg, i.p., 7 d) + B(a)P (125.0 mg/g, once)	71.86 ± 0.40#	263.39 ± 1.44#
M (20)	Mangiferin (20.0 mg/kg, i.p., 7 d)	98.45 ± 0.59#	212.37 ± 1.84#

Results are expressed as Mean ± S.E.M.; n = 6 in each group. Data was analyzed by one way ANOVA followed by Tukey's test. Significance: *p< 0.0001 when compared with normal control group; #p< 0.0001 when compared with toxic control group. B(a)P: benzo(a)pyrene; M: mangiferin.

Assessment of DNA integrity

DNA alkaline unwinding assay exhibit a significant decrease (p< 0.0001) in F-value for the toxic control group in comparison to the normal mice, which is a marker for alteration in DNA integrity. The F-value was inversely proportional to the number of strand breaks present and thus has been used as an indicator of DNA integrity. Chronic treatment with mangiferin (10.0 and 20.0 mg/kg) followed by single dose of B(a)P (125.0 mg/kg) significantly increased (p< 0.01 and p< 0.0001 respectively) F-values compared to toxic control mice. In addition, chronic treatment with mangiferin alone (20.0 mg/kg) in mice significantly increased (p< 0.0001) F-values compared to toxic control mice (Figure 1).

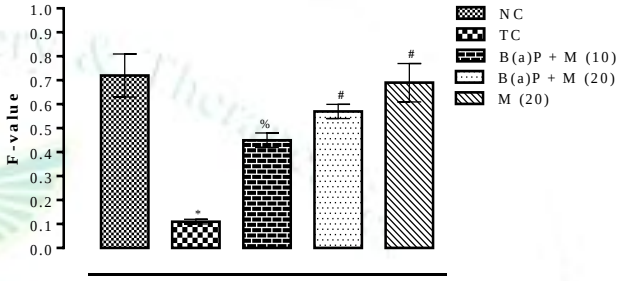

Figure 1: Effect of mangiferin pretreatment on B(a)P induced DNA damage in mouse renal tissue using alkaline unwinding assay

Results are expressed as Mean ± S.E.M.; n = 6 in each group. Data was analyzed by one way ANOVA followed by Tukey's test. Significance: *p< 0.0001 when compared with normal control group; %p< 0.01 when compared with toxic control group; #p< 0.0001 when compared with toxic control group. NC: normal control group, TC: toxic control group; B(a)P: benzo(a)pyrene; M: mangiferin.

The effect of pretreatment of mangiferin on B(a)P induced micronuclei formation in mouse bone marrow cells has been shown in Table 2. Mangiferin treatment at both doses (10.0 and 20.0 mg/kg) showed marked inhibition (p< 0.0001) in micronuclei formation at both doses (Table 2).

Table 2: Effect of mangiferin pretreatment on B(a)P induced micronuclei formation in mouse bone marrow cells

Groups	Treatment	Number of counted nucleated cells	Number of PCE's	Number of PCE's with micronuclei	Percent of PCE's	Percent of PCE's with micronuclei
Normal Control	Corn oil (p.o., 7 d)	1947	412	26	21.16 ± 0.12	6.31 ± 0.03
Toxic Control	B(a)P (125.0 mg/g, once)	1980	551	67	27.82 ± 0.34*	12.15 ± 0.06*
B(a)P + M (10)	Mangiferin (10.0 mg/kg, i.p., 7 d) + B(a)P (125.0 mg/g, once)	1893	463	42	24.45 ± 0.11#	9.07 ± 0.05#
B(a)P + M (20)	Mangiferin (20.0 mg/kg, i.p., 7 d) + B(a)P (125.0 mg/g, once)	1954	431	30	22.05 ± 0.09#	6.96 ± 0.02#
M (20)	Mangiferin (20.0 mg/kg, i.p., 7 d)	1966	406	24	20.65 ± 0.13#	5.91 ± 0.03#

Results are expressed as Mean ± S.E.M.; n = 6 in each group. Data was analyzed by one way ANOVA followed by Tukey's test. Significance: *p< 0.0001 when compared with normal control group; #p< 0.0001 when compared with toxic control group. PCEs: polychromatic erythrocytes; B(a)P: benzo(a)pyrene; M: mangiferin.

DISCUSSION

The present study revealed that the treatment with B(a)P at the dose of 125.0 mg/kg significantly caused renal damage as assessed by monitoring mitochondrial and DNA integrity. B(a)P treatment significantly decreased GPx level and increased GST level in renal tissue. In addition, DNA alkaline unwinding assay exhibit a significant decrease in F-value and increased micronuclei formation in the B(a)P treated group. Pretreatment with mangiferin restored all the impaired conditions.

B(a)P is the most commonly studied mutagen with sources of exposure including occupation, diet and tobacco smoke. Our observations clearly indicate a compromised antioxidant defense system, inflammation and a state of oxidative stress in the renal tissues of B(a)P-exposed mice. The present observations are in agreement with the previous reports that B(a)P exposure induced oxidative stress and inflammation in rodents.[18] The covalent binding of carcinogens to DNA is an important step in the cancer initiation process, with B(a)P requiring metabolic activation for DNA adduct formation to occur.[19] The activation of carcinogens is primarily catalyzed by phase I enzymes, protection may be accomplished by inhibition of activating enzymes and/or by induction of phase II enzymes which leads to detoxification and accelerated excretion of carcinogens.[20] Hence, both phase 1 enzyme (GPx) and phase II enzyme (GST) have been considered in present study.

Ample biomarkers existing for assessing genotoxicity, which is often measured as the representative of cancer. Previous studies indicate that that B(a)P treatment leads to genotoxicity, chromosomal abbreviations, micronuclei induction, DNA adduct formation, strand breaks, etc. in a rodent model of experiment.[15] It is evident from this study that mangiferin was suppressed the formation of micronuclei polychlorinated erythrocytes *in vivo*, which are the hallmarks of B(a)P induced genotoxicity.[7] Mouse bone marrow micronucleus assay is a widely used genotoxic assay to detect both clastogenic and aneugenic potencies of genotoxic agents or radiation.[21] Chromosomal modifications with development of micronuclei may serve as an effective biomarker to assess cancer risk. This study revealed an increased number of micronuclei in PCE in comparison with the control group indicating that B(a)P produces chromosomal damage in erythrocytes of bone marrow and this damage is associated with the appearance and/or progression of tumors with adverse reproductive and developmental outcomes. These changes are in agreement with previous studies.[22] In present study, it is evident from the results that mangiferin reduced the number of micronuclei in comparison with the B(a)P toxic group.

In conclusion, mangiferin amelioration of B(a)P-induced renal toxicity is attributed to the enhancement antioxidant defense mechanism and improvement in the genotoxic procedure. Thus, supplementation and/or treatment with mangiferin could exert protective effects against renal toxicity resulting from B(a)P exposure. However, further studies are required in order to gain more insight at molecular level.

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