Gallic acid protects rat liver mitochondria ex vivo from bisphenol A induced oxidative stress mediated damages

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ABSTRACT

Humans are often exposed to bisphenol A (BPA), the monomer of polycarbonate plastics and epoxy resins, through BPA contaminated drinking water, beverages and foods, packaged in polycarbonate plastic bottles and cans coated with epoxy resins due to leaching. Several research groups have reported that BPA may cause damage of mitochondria in liver, kidney, heart and brain cells by inducing oxidative stress. The antioxidant efficacy of gallic acid (GA), a polyphenol compound obtained from plants, against different toxicants induced oxidative stress has been well established. The aim of the present study was to examine the protective efficacy of GA against BPA induced oxidative damages of the rat liver mitochondria ex vivo. In our study, we have found a significant decrease in the intactness of mitochondria; a significant increase (P≤0.001) in the levels of lipid peroxidation end product (i.e. malondialdehyde) and protein carbonylation product; and also a significant decrease (P≤0.001) in the reduced glutathione content; when mitochondria were incubated with BPA (160 μM/ml) only. These results indicate that BPA probably causes damage to the cellular macromolecules through oxidative stress. We have observed significant counteractions (P≤0.001) against BPA induced alterations in mitochondrial intactness, lipid peroxidation and protein carbonylation products formation and reduced glutathione content when mitochondria were incubated with BPA and GA (20 μg/ml/ 40 μg/ml/ 80 μg/ml) in combination in a dose-dependent manner. Gallic acid also showed significant restorations (P≤0.001) of the activities of antioxidant enzymes, Krebs cycle enzymes, respiratory chain enzymes and thioredoxin when mitochondria were incubated with BPA and dosage of GA (20 μg/ml/ 40 μg/ml/ 80 μg/ml) in combination compared to BPA incubated mitochondria. Furthermore, GA significantly (P≤0.001) counteracted the BPA induced decrease in tryptophan and NADH auto-fluorescence levels in mitochondria. This result suggests that GA protects the mitochondria probably by reducing the oxidative stress. Besides, GA protects the mitochondrial surface from BPA induced oxidative damages as viewed under the scanning electron microscope. Considering all the results, it can be concluded that GA shows potent efficacy in protecting the rat liver mitochondria ex vivo from BPA induced oxidative stress mediated damages.

1. Introduction

Gallic acid (3,4,5-trihydroxybenzoic acid, GA) (Fig. 1) is a low molecular weight natural triphenolic compound possessing antioxidant activity present in various plants, especially in green tea, grapes, mangoes, walnuts and wine [1]. GA is the main component of various therapeutic herbal medicines and cosmetics. It received much attention because of its potential property of scavenging reactive oxygen species (ROS), like superoxide anions, hydrogenperoxide (H2O2), hydroxyl radicals (OH·) and hypochlorous acid (HOCl), ameliorating oxidative stress, especially heavy metal-induced oxidative stress [2–5]. Already the anti-diabetic, anti-bacterial, anti-inflammatory, anti-angiogenic, anti-oxidant, and anti-cancer activity of GA has been reported [6–10]. Furthermore, the neuro-protective effect of GA in rats has been established [11]. It has been established that GA has the ability in lowering the systolic blood pressure in rats with essential hypertension, and in providing the protection against NOX2-induced oxidative stress response [12]. Earlier study reveals that GA can provide protection against renal oxidative stress by reducing peroxidation intensity [13]. Besides, high fat diet-induced dyslipidaemia, hepatosteatosis and oxidative stress also can be protected by GA as studied in rats [14]. GA exerted a protective effect against toxicity induced by various environmental contaminants such as sodium fluoride and lead [15,15].

Nowadays BPA has become a most important focus of intense public...
studies have been defined as 50 μg/kg per day, while the lowest observable adverse effect level (LOAEL) for ubiquitously are being exposed to BPA via various consumer products. Human beings scrutiny and also its association with various human disorders such as obesity, diabetes, reproductive disorders, and cancer etc.

2. Materials and methods

2.1. Chemicals

All the chemicals used including bisphenol A (BPA), gallic acid (GA), the solvents, were of analytical grade obtained from Sigma, St. Louis, MO, USA, Sisco Research Laboratories (SRL), Mumbai, India, Qualigens (India/Germany), SD fine chemicals (India), Merck Limited, Delhi, India.

2.2. Animals

Male Charles Foster rats (body weight 120–140 gms) were procured and were fed with standard diet and handled with care in Animal House as per the recommended guidelines of the Kalyani University Animal Ethics Committee.

2.3. Experimental design

2.3.1. Animal sacrifice and collection of tissue samples

The animals were sacrificed through cervical dislocation. After careful opening of the abdominal cavity surgically the liver was collected. The collected tissues were rinsed well in cold saline and soaked properly with a piece of blotting paper and stored in sterile vials at −20°C for preparing mitochondria.

2.3.2. Preparation of liver mitochondria

Liver mitochondria were isolated according to the procedure of Dutta et al., (2015) with some modifications [28]. Two grams of tissue was placed in 10 ml of sucrose buffer [0.25 (M) sucrose, 0.001 (M) EDTA, 0.05 (M) Tris–HCl (pH 7.8)] at 25°C. Then, by using a Potter Elvenjem glass homogenizer (Belco Glass Inc., Vineland, NJ, USA) the tissue was homogenized for 1 min at low speed. Then the homogenate was centrifuged at 1500 rpm for 10 min (at 4°C). The supernatant was then collected and kept in ice. Then, the supernatant was centrifuged at 4000 rpm for 5 min (at 4°C). The supernatant, thus, obtained was further centrifuged at 14,000 rpm for 20 min at 4°C. The final pellet was collected and resuspended in sucrose buffer and was stored at −20°C for further assay and the final supernatant obtained was discarded.

2.3.3. Incubation of mitochondria with BPA

The incubation mixture of control group containing mitochondria (protein; 1.6 mg/ml), 50 mM potassium phosphate buffer (pH 7.4) and DMSO (12%) in a final volume of 1.0 ml was incubated at 37°C in incubator for the time duration of 1 h. On the other hand, the incubation mixture of BPA-treated groups containing mitochondria (protein; 1.6 mg/ml), 50 mM potassium phosphate buffer (pH 7.4), and BPA (160 μM/ml) (dissolved in 12% DMSO) in a final volume of 1.0 ml was incubated at 37°C in incubator for the time duration of 1 h, also [29].

2.3.4. Protection of BPA induced toxic injury to mitochondria by GA

The rat liver mitochondria were co-incubated with BPA and three different concentrations of GA (20, 40, 80 μg/ml). After incubation, the intactness of mitochondria, activities of reactive nitrogen species, the biomarkers of oxidative stress, activities of antioxidant enzymes, Krebs cycle enzymes, mitochondrial swelling, ATPase activity, conjugated diene level, tryptophan level, thiolase activity, DNA damage, di-tyr- osine level, scanning electron microscopy were determined.

2.4. Determination of mitochondrial intactness by using Janus green B stain

After incubation, by using 50 mM phosphate buffer (pH 7.4) the mitochondrial sample was diluted (1:200). Then, the diluted mitochondria were spread and dried on slide. The mitochondrial was left to dry for 5–10 minutes for staining after putting few drops of Janus green stain on the slide. After 5 min of staining, the mitochondrial samples were rinsed once with distilled water to drain out the extra amount of stain. Then a cover slip was used to mount the mitochondria by using a drop of distilled water. After that the mounted mitochondrial samples were imaged with a laser scanning confocal system (Zeiss LSM 510 META, Germany) and the stacked images were captured by phase contrast microscope also [30].

2.5. Measurement of mitochondrial lipid peroxidation (LPO) level, reduced glutathione (GSH) content and level of protein carbonyl (PCO)

The lipid peroxidation level in the incubated mitochondria were determined according to the method of Buege et al., (1978) [31] with some modification as adopted by Dutta et al., (2014) [32]. The thiobarbituric acid–trichloro acetic acid (TBA–TCA) reagent was mixed with incubated mitochondria with thorough shaking and heated at 80°C for 20 min. The samples were then cooled down to room temperature. Then the samples were centrifuged at 8000 rpm for 10 min at room temperature. The absorbance of the pink chromogen present in the clear supernatant was measured at 532 nm using a UV–VIS spectrophotometer.

The reduced glutathione content (GSH) in the incubated mitochondrial samples was estimated by its reaction with DTNB (Ellman’s reagent) following the method of Sedlak et al., (1968) [33] with some
2.2 × 10⁻⁴ M⁻¹ cm⁻¹. The protein carbonyl content was estimated by DNPH assay (Levine et al., 1994) [35] with some modifications by Dutta et al., (2014) [36]. In each tube 0.5 ml DNPH in 2.0 (M) HCl and 0.25 ml of incubated mitochondrial suspension were taken mixed well. The tubes were vortexed every 10 min in the dark for 1 h. Then 30% TCA was added to each tube to precipitate out the proteins by centrifugation at 2000 rpm for 10 min. The pellet collected was washed three times carefully by using 1.0 ml of ethanol: ethyl acetate (1:1, v/v). One ml of guanidine HCl (6.0 M) in 20 mM potassium dihydrogen phosphate (pH 2.3) was used to dissolve the finally collected pellet. The absorbance was determined spectrophotometrically at 370 nm. The protein carbonyl content was calculated using a molar absorption coefficient of 2.2 × 10⁻⁴ M⁻¹ cm⁻¹.

2.6. Measurement of the activities of Mn-superoxide dismutase (Mn-SOD), glutathione reductase (GR) and glutathione peroxidase (GPx) of rat liver mitochondria

Pyrogallol autoxidation method [37] with some modifications by Dutta et al., (2014) [38] was adapted to measure the activity of manganese superoxide dismutase (Mn-SOD). To four thirty microliters of 50 mM Tris–HCl buffer (pH 8.2) and 20 μl of 2 mM pyrogallol, 50 μl of the mitochondrial sample was added. An increase in absorbance was measured at 420 nm for 3 min by using UV/VIS spectrophotometer. Fifty percent inhibition of the rate of autoxidation of pyrogallol as determined by change in absorbance/minute at 420 nm is equal to one unit of enzyme activity.

The method of Krohne-Ehrich et al., (1977) [39] with some modifications by Mukherjee et al., (2015) [40] was adapted to measure the activity of glutathione reductase (GR). The final volume of 3 ml assay mixture contained 50 mM phosphate buffer, 1 mM EDTA and water. The blank was set by using this mixture. Then, the suitable amount of incubated mitochondria (as the source of enzyme) and 0.1 mM NADPH was added together into the cuvette. One milimole oxidized glutathione (GSSG) was used to initiate the reaction. After that spectrophotometrically at 340 nm a decrease in NADPH absorption was measured.

The method of Paglia et al., (1967) [41] with some modifications as adopted by Dutta et al., (2014) [42] was used to measure the activity of glutathione peroxidase (GPx). The final volume of 1 ml assay system contained 0.05 (M) phosphate buffer, 2 mM EDTA (pH 7.0), 0.025 mM sodium azide, 0.25 mM NADPH, and 0.15 mM glutathione. Hydrogen peroxide (0.36 mM) was added to start the reaction. The linear decrease in absorbance was recorded using a UV/VIS spectrophotometer at 340 nm.

2.7. Measurement of the activities of pyruvate dehydrogenase and some of the Krebs cycle enzymes

The method of Chretien et al., (1995) [43] with some modifications was adapted to measure the activity of pyruvate dehydrogenase (PDH) spectrophotometrically. In this reaction we followed the reduction of NAD⁺ to NADH at 340 nm using 50 mM phosphate buffer (pH 7.4), 0.5 mM NAD⁺ and 0.5 mM sodium pyruvate as the substrate in addition to the enzyme.

Isocitrate dehydrogenase (ICDH) activity was measured by following the method of Duncan et al., (1979) [44] with some modifications by Dutta et al., (2014) [45]. In this reaction we measured the reduction of NAD⁺ to NADH at 340 nm by using UV-VIS spectrophotometer. The suitable amount of incubated mitochondria as the source of enzyme, 50 mM phosphate buffer, pH 7.4, 0.1 mM MnSO₄, 0.1 mM NAD⁺ and 0.5 mM isocitrate were present in one millilitre assay volume.

The activity of alpha-ketoglutarate dehydrogenase (α-KGDH) was measured according to the method of Duncan et al., (1979) [44] with some modifications [45] spectrophotometrically. In this reaction, the reduction of 0.35 mM NAD⁺ to NADH was measured at 340 nm using 0.1 mM α-ketoglutarate as the substrate, 50 mM phosphate buffer, pH 7.4 as the assay buffer and incubated mitochondria as the source of enzyme.

A process of reduction of potassium ferricyanide [K₃Fe (CN)₆] was used to measure the activity of succinate dehydrogenase (SDH) spectrophotometrically at 420 nm according to the method of Veeger et al., (1969) [46]. One ml assay mixture contained 2% (w/v) BSA, 4 mM succinate, 2.5 mM K₃Fe(CN)₆, 50 mM phosphate buffer, pH 7.4, and a suitable amount of the incubated mitochondria as the source of enzyme.

2.8. Measurement of the activities of respiratory complex enzymes

The method of Goyal et al., (1995) [47] was adapted to measure the activity of NADH-Cytochrome C oxidoreductase of mitochondria spectrophotometrically by following the reduction of oxidized cytochrome C at 565 nm. One ml of assay mixture contained the suitable amount of mitochondrial suspension as the source of enzyme, 50 mM phosphate buffer, 0.1 mg BSA, 20 mM oxidized cytochrome C and 0.5 (M) NADH.

The activity mitochondrial cytochrome C oxidase was determined spectrophotometrically by following the oxidation of reduced cytochrome C at 550 nm according to the method of Goyal et al., (1995) [47]. One ml of assay mixture contained 50 mM phosphate buffer, pH 7.4, 40 mM reduced cytochrome C and a suitable aliquot of the mitochondrial suspension as the source of enzyme.

2.9. Measurement of reactive nitrogen species (RNS) in mitochondria

The method of Fiddler (1977) [48] by using Griess reagent [49] the concentrations of nitric oxide in the incubated mitochondria were measured spectrophotometrically at 548 nm. The reaction mixture in a spectrophotometer cuvette (1 cm path length) contained 100 μL of Griess Reagent, 700 μL of the mitochondrial sample and 700 μL of distilled water.

2.10. Measurement of mitochondrial swelling

Mitochondrial swelling was measured by detecting spectrophotometrically the changes in the absorbance of the suspension at 520 nm (A) [50]. The incubation medium for this assay contained 250 mmol/L sucrose, 0.3 mmol/L CaCl₂ and 10 mmol/L Tris (pH 7.4). Mitochondria (0.5 mg protein) were suspended in 3.6 ml of phosphate buffer. To both sample and reference cuvette, 1.8 ml of this suspension was added. But 6 mmol/L succinate was added to the sample cuvette only. The changes in absorption were recorded continuously at 25 °C for 10 min at 520 nm wavelength. Swelling of mitochondria was evaluated according to decrease in values of in absorption at 520 nm.

2.11. Measurement of conjugated dienes

Conjugated diene formation was determined from the absorbance ratio of A₂₃₃/A₂₁₅. For this assay the mitochondria was dispersed (20 μg/mL protein) with 10 mmol/L phosphate buffer containing 1% Lubrol [51]. Fifty microgram proteins per ml, 10 mmol/L HEPES, 100 mmol/L KCl (pH 7.0) were added to the assay mixture and changes in the fluorescence were measurements at 25°C by using a spectrofluorimeter.
2.12. Measurement of acetoacetyl CoA thiolase activity (potassium-sensitive)

The enzymatic activity of mitochondrial acetoacetyl CoA thiolase was assayed in the presence of 50 mM KCl (total activity) and in its absence (K⁺ replaced by an equivalent concentration of Na⁺). The cytosolic thiolase activity was represented by the latter one. The activity of mitochondrial acetoacetyl CoA thiolase was calculated by subtracting the rate of enzyme activity in the absence of K⁺ from that in the presence of K⁺. When measuring K⁺ activation, care was taken to ensure that neither substrate nor enzyme solution contained K⁺, this ion being replaced by Na⁺. The rate of the reaction due to formation of acetyl CoA was assessed spectrophotometrically at 303 nm. The reaction mixture contained 100 mM Tris–HCl (pH 8.1), 50 mM CoA, 25 mM MgCl₂, 50 mM KCl and 10 mM acetoacetyl CoA [52].

2.13. Measurement of di-tyrosine and tryptophan level of rat liver mitochondria

Emission spectra of di-tyrosine which is a product of tyrosine oxidation were recorded in range 380 to 440 nm (5 nm slit width) at excitation wavelength of 325 nm (5 nm slit width). Emission spectra of di-tyrosine were determined at the range of emission spectra from 425 to 480 nm (5 nm slit width) with excitation of 365 nm (5 nm slit width) [53]. The fluorescence emission spectra (from 300 to 450 nm, 5 nm slit width) of tryptophan were measured by excitation at 295 nm (2 nm slit width) [54].

2.14. Determination of mitochondrial nicotinamide adenine dinucleotide-reduced (NADH) level

The mitochondrial pyridine nucleotide, nicotinamide adenine dinucleotide-reduced (NADH), was monitored by measuring its auto-fluorescence with excitation and emission wavelengths of 360 nm and 450 nm, respectively according to the method described earlier. Mitochondria (2 mg protein) were added to 1.8 ml of phosphate buffer containing 6 mmol/L succinate and the auto-fluorescence of NADH was determined [55].

2.15. Determination of mitochondrial (mt) DNA damage with agarose gel electrophoresis

The incubated mitochondria with or without BPA, BPA plus GA and GA only were lysed with 3 ml of TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) containing 0.5% SDS and 0.3 mg/ml of proteinase K overnight at 37 °C. Mitochondrial DNA was isolated using extraction with 1 (M) NaCl for 10 min at room temperature and purified twice with chloroform/isoamyl alcohol, 24:1. After that, the samples were precipitated and dissolved in TE buffer, and the DNA, thus obtained, gave an average 260/280 absorbance ratio of 2.25. The obtained DNA samples were then mixed with 6X loading dye and resolved in 0.8% agarose gel. The gel was stained with ethidium bromide and DNA bands detected in a Gel-Doc apparatus (Biorad, Hercules CA) [28].

2.16. Altmann’s technique for staining of mitochondria

The Altmann’s staining technique was used to stain the mitochondria to identify the changes in mitochondria induced by BPA quantitatively as well as qualitatively. After incubation, by using 50 mM phosphate buffer (pH 7.4) the mitochondrial sample was diluted (1:200). Then, the diluted mitochondria were spread and dried on slide. The slide was then flooded with aniline-acid fuchsin solution. After that the slide was rinsed with tap water followed by differentiator solution 1 and differentiator solution 2 to remove the excess red colour. The slide was then dehydrated rapidly in two changes of absolute ethanol and then it was cleaned with xylene and was mounted with the help of non-aqueous mounting medium (DPX) [56].

2.17. Scanning electron microscopy of mitochondria

Two fifty microlitre of mitochondrial suspension was centrifuged, and the pellet was collected. The pellet was fixed overnight with 2.5% glutaraldehyde. Then, the pellet was dehydrated for 10 min at each concentration of a graded ethanol series (50, 70, 80, 90, 95 and 100%), after washing three times with PBS. The pellet was mixed in pure tert-butyl alcohol and was then placed into a 4 °C refrigerator until the tert-butyl alcohol solidified. The frozen samples were dried by placing them into a vacuum bottle. Mitochondrial morphology was evaluated by scanning electron microscopy (SEM; Zeiss Evo 18 model EDS 8100) [40].

2.18. Estimation of protein

The protein content of the isolated mitochondria was determined by the method of Lowry et al., (1951) [57].

2.19. Statistical evaluation

Each experiment was repeated at least three times. Data are presented as means ± S.E. Significance of mean values of different parameters between the treatment groups were analyzed using one way post hoc tests (Tukey’s HSD test) of analysis of variances (ANOVA) after ascertaining the homogeneity of variances between the treatments. Pairwise comparisons were done by calculating the least significance. Statistical tests were performed using Microcal Origin version 7.0 for Windows.

3. Results

3.1. Effect of GA on the intactness of mitochondria

Fig. 2(A–F) depicts a significant decrease in the mitochondrial intactness following the incubation of mitochondria with BPA. This decrease in mitochondrial intactness were found to be significantly protected from being altered when the mitochondria were co-incubated with BPA and GA (20, 40, 80 μg/ml), dose-dependently. However, GA alone could not produce any alterations in mitochondrial intactness.

3.2. Effects of GA on the oxidative stress biomarkers

A significant increase in mitochondrial LPO level following the incubation of mitochondria with BPA (96.55% compared to control, P ≤ 0.001) was observed (Table 1). This elevated level of LPO were found to be significantly protected from being increased (50.88% protection compared to BPA incubated group, P ≤ 0.001) when the mitochondria were co-incubated with BPA and GA (80 μg/ml). But mitochondrial LPO level was not found to be altered when mitochondria was incubated only with GA.

On the other hand, significant decrease in mitochondrial GSH content following the incubation of mitochondria with BPA (62.89% compared to control, P ≤ 0.001) was observed. The GSH content was found to be significantly protected from being decreased (1.7 folds protection compared to BPA treated group, P ≤ 0.001) when the mitochondria were co-incubated with BPA and GA (80 μg/ml) (Table 1). We did not observe any alterations in mitochondrial GSH content when mitochondria were incubated with GA only.

The protein carbonyl assay showed a significant increase in mitochondrial PCO level following the incubation of mitochondria with BPA (4.09 folds compared to control, P ≤ 0.001). A significant protection against the elevated level of PCO level was observed (81.50% protection compared to BPA incubated group, P ≤ 0.001) when the
mitochondria were co-incubated with BPA and GA (80 μg/ml) (Table 1). GA alone could not produce any alterations in mitochondrial PCO level.

3.3. Effects of GA on the activities of antioxidant enzymes

Results presented in the Table 2 indicates a highly significant increase (2.57 folds compared to control, \( P \leq 0.001 \)) in the activity of Mn-SOD following incubation of mitochondria with BPA. But the activity of this enzyme was found to be significantly protected from being increased when the mitochondria were co-incubated with BPA and GA (70.13% protection; \( P \leq 0.001 \)) compared to BPA incubated group, at the dose of 80 μg/ml). The activity of Mn-SOD was not altered when mitochondria was incubated with GA only.

Results of Table 2 also indicate a highly significant decrease in the activities of GR (67.25% compared to control, \( P \leq 0.001 \)) and GPx (86.39% compared to control, \( P \leq 0.001 \)) following incubation of mitochondria with BPA. The activities of GR (2.16 folds protection; \( P \leq 0.001 \)) compared to BPA incubated group, at the dose of 80 μg/ml) and GPx (6.46 folds protection; \( P \leq 0.001 \)) compared to BPA incubated group, at the dose of 80 μg/ml) were found to be significantly protected from being decreased when the mitochondria were co-incubated with BPA and GA. But we did not find any alterations in the activities of GR and GPx when mitochondria were incubated with GA alone.

3.4. Effects of GA on the activities of pyruvate dehydrogenase and some of the Krebs cycle enzymes

Incubation of mitochondria with BPA decreased the activities of PDH (75.59% compared to control, \( P \leq 0.001 \)), ICDH (82.79% compared to control, \( P \leq 0.001 \)), α-KGDH (83.65% compared to control, \( P \leq 0.001 \)) and SDH (70.96% compared to control, \( P \leq 0.001 \)). When the mitochondria were co-incubated with BPA and GA, the activities of these enzymes, however, were found to be significantly protected compared to the activity observed in the BPA-incubated group \( (2.65 \text{ folds protection (PDH)}, 5.78 \text{ folds protection (ICDH)}, 5.38 \text{ folds protection (α-KGDH)} \text{ and 2.59 folds protection (SDH); } P \leq 0.001\text{ compared to BPA incubated group, at the dose of 80μg/ml}). But the activities of these enzymes were not altered when mitochondria was incubated with GA alone (Table 3).
The values are expressed as Mean ± SE for six replicates in the experiment; *P ≤ 0.001 compared to control values using ANOVA; ^P ≤ 0.001 compared to bisphenol A-incubated values using ANOVA.

Table 1

| Groups       | Lipid peroxidation level (nmol TBARS/mg of protein) | Reduced glutathione content (μM GSH/mg of protein) | Protein carbonylation level (nmol/mg of protein) |
|--------------|----------------------------------------------------|---------------------------------------------------|--------------------------------------------------|
| Control      | 0.578 ± 0.004                                      | 19.00 ± 0.12                                      | 2.90 ± 0.03                                       |
| BPAa         | 1.135 ± 0.029                                      | 7.05 ± 0.09*                                      | 14.76 ± 0.03*                                    |
| GA20b         | 0.580 ± 0.0035                                     | 18.60 ± 0.17                                      | 2.79 ± 0.03                                       |
| GA40c         | 0.581 ± 0.017                                      | 19.07 ± 0.03                                      | 2.79 ± 0.03                                       |
| GA80d         | 0.584 ± 0.0015                                     | 19.07 ± 0.09                                      | 2.92 ± 0.05                                       |
| BPA-GA20e     | 0.960 ± 0.058                                      | 9.30 ± 0.06                                       | 10.82 ± 0.05                                     |
| BPA-GA40f     | 0.735 ± 0.0031                                     | 14.10 ± 0.17                                      | 6.49 ± 0.04                                       |
| BPA-GA80g     | 0.556 ± 0.0043                                    | 19.00 ± 0.23*                                     | 2.73 ± 0.09*                                    |

Table 2

| Groups       | Mn-superoxide dismutase (units/min/mg of protein) | Glutathione reductase (units/min/mg of protein) | Glutathione peroxidase (nmol NADPH produced/mg of protein) |
|--------------|---------------------------------------------------|-------------------------------------------------|----------------------------------------------------------|
| Control      | 3.05 ± 0.03                                       | 2.29 ± 0.07                                      | 11.90 ± 0.17                                              |
| BPAa         | 10.88 ± 0.22*                                     | 0.75 ± 0.02*                                    | 1.62 ± 0.03*                                              |
| GA20b         | 3.32 ± 0.04                                      | 2.13 ± 0.07                                      | 12.56 ± 0.19                                              |
| GA40c         | 3.44 ± 0.03                                      | 2.25 ± 0.05                                      | 12.89 ± 0.37                                              |
| GA80d         | 3.20 ± 0.08                                      | 2.29 ± 0.02                                      | 12.88 ± 0.51                                              |
| BPA-GA20e     | 8.78 ± 0.06                                      | 1.05 ± 0.03                                      | 4.90 ± 0.17                                               |
| BPA-GA40f     | 6.40 ± 0.12                                      | 1.81 ± 0.06                                      | 8.75 ± 0.00                                               |
| BPA-GA80g     | 3.25 ± 0.00*                                     | 2.37 ± 0.02*                                    | 12.09 ± 0.36*                                             |

Table 3

| Groups       | Pyruvate dehydrogenase (units/min/mg of protein) | Isocitrate dehydrogenase (units/min/mg of protein) | α-ketoglutarate dehydrogenase (units/min/mg of protein) | Succinate dehydrogenase (units/min/mg of protein) |
|--------------|-------------------------------------------------|--------------------------------------------------|----------------------------------------------------------|--------------------------------------------------|
| Control      | 54.08 ± 3.19                                     | 5.23 ± 0.23                                      | 26.00 ± 3.61                                              | 91.84 ± 4.67                                     |
| BPAa         | 13.20 ± 0.23*                                    | 0.90 ± 0.00*                                    | 4.25 ± 0.00*                                              | 26.67 ± 0.00*                                    |
| GA20b         | 58.64 ± 0.04                                     | 5.03 ± 0.12                                      | 26.12 ± 1.02                                              | 95.29 ± 0.00                                     |
| GA40c         | 56.86 ± 0.99                                     | 5.23 ± 0.23                                      | 26.67 ± 0.00                                              | 93.47 ± 1.06                                     |
| GA80d         | 54.67 ± 0.83                                     | 4.98 ± 0.18                                      | 26.50 ± 0.10                                              | 91.69 ± 4.80                                     |
| BPA-GA20e     | 19.30 ± 0.40                                     | 2.52 ± 0.03                                      | 11.83 ± 0.29                                              | 47.50 ± 1.44                                     |
| BPA-GA40f     | 33.00 ± 0.43                                     | 3.41 ± 0.12                                      | 19.72 ± 0.17                                              | 58.57 ± 0.83                                     |
| BPA-GA80g     | 46.20 ± 1.84                                     | 6.10 ± 0.07                                      | 27.13 ± 4.11                                              | 95.82 ± 2.42                                     |

The values are expressed as Mean ± SE for six replicates in the experiment; *P ≤ 0.001 compared to control values using ANOVA; †P ≤ 0.001 compared to bisphenol A-incubated values using ANOVA.

3.5. Effects of GA on the activities of enzymes of oxidative chain

A significant decrease was observed in the activities of NADH cytochrome C oxidoreductase (84.83% compared to control, *P ≤ 0.001) and cytochrome C oxidase activity (94.89% compared to control, †P ≤ 0.001) when mitochondria were incubated with BPA (Table 4). But co-incubation of mitochondria with BPA and GA significantly protects the activities of these enzymes [6.12 folds protection (NADH cytochrome C oxidoreductase) and 19.13 folds protection (cytochrome C oxidase); ‡P ≤ 0.001 compared to BPA incubated group, at the dose of 80 μg/ml]. But, GA alone could not produce any alterations in the activities of NADH cytochrome C oxidoreductase and cytochrome C oxidase.

3.6. Effect of GA on the status of reactive nitrogen species (RNS)

The level of NO in mitochondria of BPA-incubated group was found to be increased significantly (Fig. 3A) compared to control group by 2.68 folds (P ≤ 0.001). However, a dose-dependent protection of the level of NO was observed when the mitochondria were co-incubated with BPA and increasing concentrations of GA. At 80 μg/ml, GA was found to maximally protect the level of mitochondrial NO from being altered (72.49% protection; P ≤ 0.001 compared to BPA incubated group). We did not observe any alterations in the level of NO when the mitochondria were incubated with GA only.
3.7. Effect of GA on the status of mitochondrial swelling

The decrease in absorbance of mitochondria incubated with BPA was found to be lower compared to the control group (Fig. 3B); indicating that the incubation of mitochondria with BPA caused mitochondrial dysfunction which is responsible for mitochondrial swelling. The absorbance was found to be significantly increased when the rat liver mitochondria were co-incubated with BPA and GA (80 μg/ml) compared to mitochondria incubated with BPA only.

3.8. Effect of GA on the status of conjugated diene

A highly significant increase in the level of conjugated diene was observed when mitochondria were incubated with BPA (31.91% compared to control, \( P \leq 0.001 \)). Co-incubation of mitochondria with BPA and GA (80 μg/ml) significantly protects the conjugated diene level (28.67% protection; \( P \leq 0.001 \) compared to BPA incubated mitochondria). But we did not observe any alterations in the conjugated diene levels of mitochondrial membranes when mitochondria were incubated with GA only (Fig. 3C).

3.9. Effect of GA on the status of acetoacetyl CoA thiolase activity

We observed a significant decrease in the activity of acetoacetyl CoA thiolase when mitochondria were exposed to BPA (68.39% compared to control, \( P \leq 0.0001 \)). This alteration was found to be protected from being decreased when the mitochondria were co-incubated with BPA and GA (80 μg/ml) (2.46 folds protection; \( P \leq 0.0001 \) compared to BPA incubated mitochondria), significantly. GA, by itself, has no effect on the activity of acetoacetyl CoA thiolase (Fig. 4A).

3.10. Effects of GA on the status of di-tyrosine and tryptophan fluorescence intensity

The effect of the free radical-generating system on protein structure was examined by measuring di-tyrosine and tryptophan fluorescence study. That BPA induced oxidative stress has a direct effect on the oxidation level of amino acid is evident from an increased di-tyrosine formation (Fig. 4B) and a reduced tryptophan level (Fig. 4C) studied through the measurement of basal auto-fluorescence of the amino acids. We observed a protection against the alterations in the levels of di-tyrosine and tryptophan when mitochondria were co-incubated with BPA and GA.

3.11. Effect of GA on the mitochondrial DNA (mtDNA) damage

Incubation of rat liver mitochondria with BPA caused damage to mtDNA. However, when the mitochondria were co-incubated with BPA and GA (at 80 μg/ml dose), the mtDNA damage was found to be almost completely protected (Fig. 4E).
3.13. Effect of GA on the number and structure of mitochondria

We observed a significant decrease in the number of mitochondria and also mitochondrial structural deformities when mitochondria were treated with BPA. These alterations in the number of mitochondria and also in the structure of mitochondria were found to be significantly protected when the mitochondria were co-treated with BPA and GA (20, 40, 80 μg/ml), dose-dependently. However, GA alone had no effect on the number and structure of mitochondria [Fig. 5(A–H)(100X) and Fig. 5(I–P)(400X)].

3.14. Scanning electron microscopy (SEM)

Significant changes in mitochondrial surface were observed in scanning electron microscopy study after the treatment of mitochondria with BPA [Fig. 5(N–U) (25.00KX)]. The mitochondrial membrane showed convolutions with the perforated surface. However, we did not observe any membrane convolutions, perforations and blebs formation in the mitochondria of BPA and GA (80 μg/ml) co-treated group. The figure shows a perforated surface with convoluted membranes.

4. Discussion

Atoms containing odd (unpaired) number of electrons are known as free radicals. In the physiological system free radicals are being produced in mitochondria by various metabolic pathways including oxidative phosphorylation [58]. Complexes I and III of respiratory chain in inner mitochondrial membrane [59] and monoamine oxidase in the outer mitochondrial membrane are the major sites of free radical generation. In the physiological system, the imbalance between ROS generation and scavenging of ROS through antioxidant defense mechanisms is responsible for the generation of oxidative stress. The ROS also causes the peroxidation of lipids and oxidative damages to DNA and proteins [60]. The free radicals can be scavenged by various extrinsic organic substances such as polyphenol, flavonoids etc. which are naturally found in different tropical plants [61]. Through the removal of ROS and other harmful oxidative products in cell the oxidative stress can be minimized or subsided. And so, we did not observe any kind of membrane degenerations as stated above.

Gallic acid, a natural polyhydroxy benzoic acid, is predominantly found in the form of free acids, esters, catechin derivatives and hydrolysable tannins in different plant substances (Fig. 6A-D). A few reports say about the antioxidant properties of gallic acid and its derivatives [61–64]. Antimicrobial and anti-fibrotic activities of gallic acid have been established against human pathogens and plant pathogen and also against human pathogenic yeast [65,66,1]. A few earlier studies reported that GA acts as a scavenger of electrophilic mutagens [67]. In our study we have tested the efficacy of GA in the protection of BPA-induced oxidative stress in rat liver mitochondria.

The foods become contaminated by BPA after leaching out from the epoxy resin inner coating of cans and from various polycarbonate plastic products (polycarbonate tableware, food storage containers, water bottles, and baby bottles). Recent studies have also suggested that people may be exposed to BPA by handling cash register receipts which is made up of components containing BPA as one of the constituents [16].

Glutathione provides a first line of defense against ROS, as it can scavenge free radicals and reduce H₂O₂ formation in cell. BPA produces

Fig. 4. Protective effect of gallic acid against bisphenol A-induced decrease in the activity of (A) acetoacetyl CoA thiolase, levels of (C) tryptophan and (D) NADH auto fluorescence and decrease in (B) di-tyrosine level and also (E) damages in mitochondrial DNA of rat liver mitochondria. BPA = bisphenol A- incubated mitochondrial group; GA20-80 = mitochondrial groups incubated with gallic acid at the dose of 20–80 μg/ml respectively; BPA-GA20-80 = mitochondrial groups co-incubated with bisphenol A and gallic acid at the dose of 20–80 μg/ml respectively; The values are expressed as Mean ± SE for six replicates in the experiment; *$P \leq 0.001$ compared to control values using ANOVA; †$P \leq 0.001$ compared to bisphenol A- incubated values using ANOVA; ‡$P \leq 0.0001$ compared to control values using ANOVA; ††$P \leq 0.0001$ compared to bisphenol A- incubated values using ANOVA.
various quinol and semiquinone intermediates which can react with glutathione and to produce glutathione-conjugates. Glutathione-conjugates in turn may produce oxidative stress [29]. MDA level is assayed to determine the extent of oxidative damages involving mainly membrane lipids. Free radicals are being produced by oxidative stress which can easily react with lipids of the cell membrane and thereby initiating oxidative stress.

Fig. 5. Protective effect of gallic acid against bisphenol A-induced alterations in (A–H) mitochondrial quantity (100X) and (I–M) mitochondrial quality (400X); Scanning electron microscopic study also depicts the changes in cyto-architecture of mitochondria (N–U); Arrow heads indicate the furrow formation on the surface of mitochondria by the treatment of bisphenol A; BPA = bisphenol A- incubated mitochondrial group; GA20-80 = mitochondrial groups incubated with gallic acid at the dose of 20–80 μg/ml respectively; BPA-GA20-80 = mitochondrial groups co-incubated with bisphenol A and gallic acid at the dose of 20–80 μg/ml respectively.

Fig. 6. Different forms of gallic acids which are present in the plant materials.
a chain reactions leading to the production of lipid peroxides [68]. In the present study, a significant protection of the levels of both GSH was discernible in mitochondria co-incubated with BPA and GA in comparison with the level of GSH in BPA exposed mitochondria. The carbonylation of proteins is an indicator of oxidative stress. Most of the oxidized proteins are functionally inactive and are rapidly removed from the body but some are gradually accumulated and may contribute to damage [69]. Carbonyl formations are considered as an early and stable marker for protein oxidation [70]. This present study suggests that when mitochondria were co-incubated with BPA and GA then the levels of LPO and PCO were found to be protected in comparison to the levels of LPO and PCO in the mitochondria of only BPA treated group.

The cellular antioxidant system against oxidative stress is composed of tripeptide glutathione and a few enzymes regulating its metabolism. Glutathione peroxidase (GPx) uses reduced glutathione (GSH) to remove peroxides produced due to oxidative stress [71]. On the other hand, glutathione reductase (GR) reduces the oxidized glutathione back to GSH using NADPH [72]. The elevated levels of activity of Mn-SOD following incubation of mitochondria with BPA was found to be protected when mitochondria were co-incubated with BPA and GA. GSH depletion along with NADPH oxidation and altered redox homeostasis plays an important role in the alteration of antioxidant defense leading to elevated levels of oxidative stress. Cellular macro-molecules like lipids and proteins are being affected by the enhanced ROS/RNS along with compromised antioxidants. We have observed that when the mitochondria were co-incubated with BPA and GA then activities of MnSOD, GR, GPx were found to be protected in comparison to the mitochondria incubated only with BPA.

Pyruvate dehydrogenase has been demonstrated as a sensitive molecule in the production of oxygen free radicals, which might be a cause of decrease in the enzyme activity [73]. Isocitrate dehydrogenase is a major NADPH producer in the mitochondrial Krebs cycle pathway and thus plays a key role in cellular defense against oxidative stress-induced damage [74]. During marked elevation in ROS generation, DNA fragmentation, and also during a reduction in the ATP level, the activity of isocitrate dehydrogenase was observed to be decreased [75]. In our present study when the mitochondria were co-incubated with BPA and GA, the activities of these enzymes, however, were found to be significantly protected compared to the activity observed in the BPA incubated mitochondria.

Study of the mitochondrial respiratory parameters provides an important indicator to understand mitochondrial physiology and the potential role of mitochondrial pathologies in cellular damages. Mitochondrial uncoupling can arise from oxidative damage of the mitochondrial membrane as induced by increased ROS concentrations [76]. Mitochondrial production of ROS is thought to play an adverse role in many pathologic disorders. When the mitochondria were co-incubated with BPA and GA in our study, the GA can protect the activities of NADH cytochrome C oxidoreductase and cytochrome C oxidase from being decreased due to BPA incubation.

Nitric oxide also can produce a free radical (ONOO⁻) which plays an important role in the pathogenesis of pain, inflammation, neural signal transmission, immune response, control of vasodilatation and blood pressure [77,78]. Nitric oxide (NO), one of the reactive nitrogen species (RNS), is a molecular mediator of many physiological processes including vasodilatation, immunity and neurotransmission. We observed when mitochondria were co-incubated with BPA and GA then the concentration of NO was found to be decreased significantly in a dose-dependent manner in comparison to the concentration of NO of the mitochondria incubated with BPA.

Paradies et al. (1998) found a close correlation between LPO and inhibition of the cytochrome C oxidase activity in mitochondria exposed to oxidizing agent suggesting that lipid-mediated damage plays an important role in protein modification [79]. To evaluate the role of lipid-mediated protein modification we measured the fluorescence of conjugated dienes formed by LPO end-products, such as malondialdehyde, and free amino-groups of proteins [80]. In the present study we have observed that BPA induced increase in conjugated dienes level were found to be significantly protected when mitochondria were co-incubated with BPA and GA, dose-dependently.

It has been observed that reduction in mitochondrial absorbance at certain wavelengths (520 nm) helps to detect mitochondrial swelling which was caused by pH change and high calcium. It is well established that energized mitochondria supplemented with high Ca²⁺ concentrations swell in the presence of inorganic phosphate. In our present study GA counter acting the BPA induced swelling of mitochondria. This effect may be due to the blockage of calcium entrance through the inhibition of the calcium channels. The high intra-mitochondrial
concentration of calcium prevents the electron transport of the respiratory chain and then oxidative phosphorylation, or activates the principal enzymes responsible for ROS generation [81]. We propose that GA competitively inhibits calcium for binding to its receptor channels at the mitochondrial level at the transition pore for causing the swelling of mitochondria which was also established by morphological study of mitochondria by scanning electron microscopy (SEM). This result has also been corroborated by the results of scanning electron microscopic study. In scanning electron microscopic study, we have found that membrane convolutions, perforations and blebs formation in the mitochondria of only BPA incubated group were found to be protected significantly when mitochondria were co-incubated with BPA and GA. Thus, the results strongly support the hypothesis that GA prevents BPA induced mitochondrial swelling by providing competitive inhibition to Ca-binding.

The intrinsic fluorescence of tryptophan and di-tyrosine was measured to test the modification of specific amino acids by OH radicals. Tryptophan fluorescence is a sensitive marker of oxidative damage during ischemia and reperfusion [82]. BPA induced loss of tryptophan fluorescence, observed in the present study, and suggests a modification of this amino acid residue. The direct oxidation of mitochondrial proteins can be established by increased fluorescence of di-tyrosine [83]. The results of the current studies indicate that BPA induced a modification to the tryptophan and tyrosine residues in mitochondria. Our results are probably consistent with the results of Welch et al., (2001) [84]. Literature also reported us that the alterations of tryptophan and di-tyrosine also are being associated with the modifications (2001) [84]. Literature also reported us that the alterations of tryptophan and di-tyrosine also are being associated with the modifications 179 (2009) 118–124. [83]. The current study presents the modification of specific amino acids by ˙OH radicals. Our study suggests that co-incubation of mitochondria with BPA and GA protected the alterations of the levels of tryptophan and di-tyrosine from being taken place in case of the mitochondria of only BPA incubated group. The summary of the current work is presented in Fig. 7.

5. Conclusions

So, the results of the present study suggest that GA may provide protection against BPA induced damage of the mitochondria isolated from rat liver, ex vivo, probably by inhibiting the BPA induced oxidative stress, mitochondrial amino acids, by increasing the content of reduced glutathione, mitochondrial DNA damages, by decreasing the levels of LPO, PCO, by preventing the alterations in the activities of the enzymes of Krebs cycle and respiratory chain and also by preventing the BPA induced mitochondrial swelling by providing competitive inhibition to Ca-binding.

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