Original Article

Influence of Genista Tinctoria L or Methylparaben on Subchronic Toxicity of Bisphenol A in Rats*

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Abstract

Objective To evaluate the influence of an extract of *Genista tinctoria* L. herba (GT) or methylparaben (MP) on histopathological changes and 2 biomarkers of oxidative stress in rats subchronicly exposed to bisphenol A (BPA).

Methods Adult female Wistar rats were orally exposed for 90 d to BPA (50 mg/kg), BPA+GT (35 mg isoflavones/kg) or BPA+MP (250 mg/kg). Plasma and tissue samples were taken from liver, kidney, thyroid, uterus, ovary, and mammary gland after 30, 60, and 90 d of exposure respectively. Lipid peroxidation and *in vivo* hydroxyl radical production were evaluated by histological analysis along with malondialdehyde and 2,3-dihydroxybenzoic acid detection.

Results The severity of histopathological changes in liver and kidneys was lower after GT treatment than after BPA or BPA+MP treatment. A minimal thyroid receptor antagonist effect was only observed after BPA+MP treatment. The abnormal folliculogenesis increased in a time-dependent manner, and the number of corpus luteum decreased. No significant histological alterations were found in the uterus. The mammary gland displayed specific estrogen stimulation changes at all periods. Both MP and GT revealed antioxidant properties reducing lipid peroxidation and BPA-induced hydroxyl radical generation.

Conclusion GT L. extract ameliorates the toxic effects of BPA and is proved to have antioxidant potential and antitoxic effect. MP has antioxidant properties, but has either no effect or exacerbates the BPA-induced histopathological changes.

Key words: Food contaminant; Phytoestrogen; Morphological effect; Lipid peroxidation; Hydroxyl radical

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INTRODUCTION

Xenoestrogens (XEs) are xenobiotics with endocrine activity and can bind to estrogen receptors showing estrogenic or/and antiandrogenic effects and disruption of cellular signalling pathways.

Humans and wildlife are frequently exposed to mixtures of XEs in various doses, often below the lowest-observable effect level (LOEL)[1]. The exposure to small concentrations of XEs for long periods affects the development[2] and evolution of certain hormone-dependant diseases, including breast cancer or prostate cancer[3-4]. Only a small number of studies are available on potential synergistic interactions between XEs and their results are contradictory, requiring therefore further investigations[1,5-12].

Bisphenol A (BPA) and methylparaben (MP) are xenobiotics known as xenoestrogen molecules that are common in bodily fluids at low concentrations with adverse health consequences[13]. BPA, a 4,4'-(propane-2,2-diyl) iphenol, is used in manufacturing polycarbonate plastics, epoxy resins, food packaging and coatings, dental sealants, rubber chemicals and flame retardants. The general population may be exposed to BPA via inhalation of ambient air, ingestion of food and drinking water and direct exposure via interaction with consumer products containing this compound[4,14]. At present, it is considered that exposure to BPA is almost ubiquitous and over 90% of Americans have detectable levels of BPA in their bodies[15-16]. The toxicity of BPA has been intensively studied since the 1970s, and showed only modest estrogenic activity[17]. It is not mutagenic but can exhibit genotoxic activity and induce oxidative stress[18-22]. Although it mainly has reproductive and developmental toxicity[23-25], it can induce liver[26], kidney[27], and brain damage[21,28] or disturb glucose homeostasis[29-31] in animals. Human exposure to BPA was associated with male sexual dysfunction[32], heart disease[33] and hypertension[34], diabetes[35] or low-grade albuminuria[36]. The European Food Safety Authority (EFSA) and US Environmental Protection Agency estimate a no-observed-adverse-effect-level (NOAEL) for BPA in rats and mice of 5 mg/kg bw/day and a lowest- observed-adverse-effect-level (LOAEL) of 50 mg/kg bw/day[37-38].

Isoflavones are the most common form of phytoestrogens (PEs), non-steroidal polyphenolic plant metabolites, which have structural similarities to 17-beta-estradiol and the ability to bind to estrogen receptors having estrogenic and/or anti-estrogenic effects. In natural state, the isoflavones exist primarily as glycosides and biologically inactive forms. After oral ingestion, they are hydrolyzed by intestinal bacteria to their active aglycone and then absorbed[44]. *Genista* species (*Fabaceae* family) show interesting biological properties due to their high content in isoflavones. Previous study has demonstrated some of their pharmacological properties such as hypoglycemic, anti-inflammatory, antiulcer, spasmolytic, antioxidant, estrogenic and cytotoxic activity against various cancer cell lines[45]. GT L. (dyer's greenweed), widely spread in Romanian flora, is especially rich in the glycosides genistin and daidzin, and in their aglycone, genistein and daidzein, respectively[46-48]. This plant has not been studied much until present. Previous study showed that PEs can modify the toxicological behaviour of other XEs, and the interactions may be complex and difficult to predict based only on their *in vitro* affinity for steroid receptors[7].

Parabens are esters of 4-hydroxybenzoic acid used as preservative and antibacterial agents in numerous foods, cosmetics and pharmaceutical products[39]. MP, the methyl ester of p-hydroxybenzoic acid, and a stable and non-volatile compound, is considered to be safe[40-41]. A temporary acceptable daily intake (ADI) of 10 mg/kg bw/day has been established by EFSA for the sum of methyl and ethyl p-hydroxybenzoic acid esters and their sodium salts on the basis of NOAEL of 1000 mg/kg bw/day determined in rats[39,42]. MP has estrogenic activity through its main metabolite, p-hydroxybenzoic acid. It was reported that parabens contribute to aberrant estrogen signalling in the human breast and adversely influence the incidence of breast cancer[13,43].

As already mentioned above, xenoestrogen may intervene in carcinogenesis, modulating the evolution of hormone-dependant cancers, and simultaneous exposure to several xenoestrogens has an additive or potential effect. However, the mechanism of xenoestrogens underlying carcinogenesis is not limited to their estrogenic effect. One of the mechanisms is oxidative stress, which is involved in the pathophysiology of several diseases, including cancer.

The first aim of this study was to determine the histopathological changes that characterize the subchronic toxicity of BPA in adult female Wistar rats

and its potential to induce oxidative stress via lipid peroxidation, or by forming hydroxyl radicals *in vivo*, as possible mechanisms involved in toxicity. The second aim was to study the influence that an extract of GT L. herba or MP has on identified toxic effects caused by BPA in simultaneous subchronic exposure in order to identify possible additive or potential interactions between them.

MATERIALS AND METHODS

Chemicals

Bisphenol and methylparaben of analytical-reagent grade (≥99%) were purchased respectively from Sigma-Aldrich (SUA) and Merck (Darmstadt, Germany). HPLC-grade acetonitrile, methanol, formic acid, ammonium acetate, and hexane were purchased from Merck (Darmstadt, Germany). All other chemicals with analytical reagent grade were obtained from Merck (sodium hydroxide, hydrochloric acid, 2,4-dinitrophenylhydrazine (DNPH), acetaldehyde, sodium salicylate, ethanol). Deionised water was prepared using a Milli-Q water purification system (Millipore, Milford, MA, USA).

Preparation of Extract of GT L. Herba (GT)

Aerial part of GT L. (dyer's greenweed) was collected in the Cluj area, Romania, in July 2010, and authenticated by Botanical Department of the Faculty of Pharmacy Cluj-Napoca. Voucher specimens were deposited in the Herbarium of the Faculty of Pharmacy, "Iuliu Haţieganu" University of Medicine and Pharmacy Cluj-Napoca at nr. 1276.

The GT extract was prepared by percolation from dry herba, using 1000 mL of 70 % (v/v) ethanol for 57.32 g of powdered plant material. The powdered plant material was uniformly moistured with 50 mL 70% (v/v) ethanol and packed into the percolator. The material was macerated for 24 h with approximately 200 mL 70% (v/v) ethanol at room temperature. After that, the outlet was opened and solvent was percolated at a controlled rate with continuous addition of fresh volume. The percolate extract was filtrated and completed then at 1000 mL.

The GT extract was analyzed by UPLC-MS/MS to determine the isoflavone content. The sum of genistin, genistein, daidzin, daidzein and formononetin was quantified. The determined isoflavone content (IF) of GT extract was of 827.42 mg/L (14.44 mg/g dry herba), with a very high content in genistin and genistein (92.1%)[47].

Experimental Protocol

The experimental protocol was in compliance with the general guidelines for the use and care of animals used for scientific purposes[53] and approved by the Ethics Committee of the "Iuliu Haţieganu" University of Medicine and Pharmacy Cluj-Napoca (Romania). Adult nulliparous female Wistar albino rats aged 3 months (130±14 g) were supplied by the Practical Skills and Experimental Medicine Centre of the "Iuliu Haţieganu" University of Medicine and Pharmacy Cluj-Napoca (Romania). The rats were housed in large polypropylene cages (6 per cage) and maintained under standard conditions (25±3 ˚C, 35%-60% humidity, 12 h light/12 h dark cycle) with free access to standard dry pellet diet (Cantacuzino Institute, Bucharest, Romania) and water throughout the experiment.

The rats were divided into 3 experimental groups and a negative control group (18 in each group). The substances were administered daily for 90 days, in food, except GT extract that was administrated by gastric intubation. After an acclimation period of 7 d, the animals were randomly divided into BPA (50 mg/kg bw) +GT (35 mg IF/kg bw) group, BPA (50 mg/kg bw) group, BPA (50 mg/kg bw) +MP (250 mg/kg bw) group , and negative control group receiving standard food without substances. Since the presence of ethanol in the plant extract can influence the animal response to the action of tested substances, GT extract was concentrated by evaporation in a water bath at 40˚C and the concentrate thus obtained was diluted with distilled water so that rats were given a dose of 35 mg IF/kg bw (3.5 mg IF/100 g bw/mL).

Plasma Samples

Sodium salicylate (equivalent dose of 500 mg salicylic acid/kg bw) was administered orally (by gastric intubation) to six animals of each groups as *in vivo* hydroxyl radical trap on the 30th, 60th, and 90th d of the experiment. The tested substances were administered after 30 min and the blood was collected from the retro-orbital sinus in the presence of sodium fluoride as anticoagulant after other 60 min, under diethyl ether anesthesia. Plasma sampling was performed by a veterinary specialist at the Practical Skills and Experimental Medicine Center of University. Plasma separated by centrifugation (3000 g/15 min) was stored for further analysis at -20 °C.

Histopathological Studies

On days 30, 60, and 90 of the experiment, the 6 animals of each group were sacrificed by cervical spine dislocation. Plasma and tissue samples were taken immediately from liver, kidney, thyroid, ovary, uterus, and mammary gland and fixed in 10% neutral buffered formalin (Chempur, Poland) for paraffin embedding. Briefly, following fixation and washing in distilled water, the samples were dehydrated in ascending concentrations of ethanol (70% - 1 h, 90% - 2 times and 1 h for each and 100% - 2 times and 1 h for each), immersed in a mixture of 50% alcohol and 50% xylene for ½ h, the xylene was changed 2 times, 1 h for each, and immersed in liquid Histowax (Histo-Lab. Ltd, Gothenburg, Sweden). The paraffin-embedded tissues were cut into 5 µm-thick sections which were stained with H&E and photographed[54]. Slides were interpreted by 2 pathologists blinded for any clinical or laboratory data as required. Image acquisition and processing were performed using an Olympus BX51 microscope equipped with Olympus Cell B computer software.

Liver Histology

Stained liver sections were examined for possible toxic effects by grading a previously described system adapted from Knodell histology and activity index[55-56] using the necro-inflammatory score (periportal±bridging necrosis, intralobular degeneration and focal necrosis, portal inflammation) and the fibrosis score.

Kidney Histology

Renal lesions were histopathologically graded by light microscopy and graded into 5 categories using a scale from 0 to 5 as previously described[57]. Briefly, the scale consists of 0 (normal histomorphology), 1 (tubular epithelial cell degeneration, without significant necrosis/apoptosis), and 2-5 (25%, 50%, 75%, and 100% of the tubules showing tubular epithelial cell necrosis/apoptosis, respectively, accompanied by other concomitant alterations).

Thyroid Histology

Thyroid sections were evaluated for any morphological alterations, hypertrophy of follicular epithelial cells, follicular distention due to colloid accumulation and follicular epithelial hyperplasia[61].

Reproductive Ttract Histology

After removal of the ovaries and uterine horns, the sectioning and trimming were performed in accordance with the goRENI standards[58]. Longitudinal sections through the uterus and transversal section through the midhorn were prepared. Ovaries were also sectioned longitudinally. For each rat, both ovaries were examined for histological abnormalities. From each animal and ovary, the number of corpus luteum (CL) was calculated and averaged to obtain a value for statistical analysis. It was reported that CL count is a reliable indicator for successful ovulation[59]. Although the morphological appearance of CL varies during the estrus cycle, its overall number does not[60]. Mammary gland tissue was examined for any morphological alteration by looking at the number, size and type of ducts, terminal ducts, and alveolar buds as previously described[24]. Warfarin-treated skin was taken 24 and 72 h respectively following the last application, trimmed free of subcutaneous tissue and cut into 1 cm^2 pieces, which were finaly minced with scissors and homogenized in 0.25 mol/L saccharose-phosphate buffer (pH 7.2) with IKA T18 basic homogenizer (IKA Works Inc, Wilmington NC, USA). Skin homogenates were sonicated (3x15 seconds) on ice at 30% of maximum intensity amplitude with Bandelin Electronic UW 2070 sonicator (Bandelin Electronic, Berlin, Germany, EU). Lipid peroxidation was evaluated by the thiobarbituric acid reaction as previously described[27]. In brief, tissue homogenates were mixed with thiobarbituric acid-trichloroacetic acid (TCA) reagent and Tris-Cl (pH 7.4) and heated for 60 min at 100 °C. The absorbance of the supernatant obtained by centrifugation was measured at 535 nm using a spectrophotometer (Shimadtzu Corporation, Lakewood, USA). MDA content in skin homogenates was measured following the reference to a standard curve plotted according to the known amount of MDA. Data were expressed as nanomols of MDA/g of freshly explanted skin tissue and nanomols of MDA/g of protein.

Measurement of Total MDA Levels

Total MDA levels were measured by HPLC[62]. In brief, plasmatic MDA bound to proteins was hydrolyzed with 6 mol/L NaOH and derivatized with 5 mmol/L DNPH in 2 mol/L HCl at room temperature and protected from light. The resulted hydrazone was extracted in hexane and the residues obtained after evaporation were dissolved in mobile phase, injected into a HPLC system (2695 Waters Alliance HPLC - Waters, USA), secerated by chromatography

on a Spherisorb ODS column (250 mm×4 mm, 5 μm) with a Spherisorb ODS (20 mm×4 mm, 3 μm) guard column, maintained at 25 °C, and a mixture of 1% formic acid/acetonitrile (62/38, v/v) was used as the mobile phase. The flow rate was 1mL/min and the absorbance of the eluent was monitored at 307 nm. Data obtained were processed using Empower 2 software (Waters, USA).

Measurement of 2,3-DHB A Levels

2,3-DHBA levels were measured by validated LC-MS/MS[63] using a 1100 HPLC system, Agilent Technologies model (SUA) and an Agilent Ion Trap Detector 1100 SL (Agilent Technologies, SUA). In brief, plasma samples were deproteinized with acetonitrile. After vortex-mixing and centrifugation, the supernatants transferred in autosampler vials were analyzed, separated by chromatographyon a Luna HILIC (100×2.0 mm, 4 μm) column and maintained at 15 °C , using a mixture of 50 mmol/L ammonium acetate in water (pH 4.5) / acetonitrile (6:94, v/v) with a flow rate of 0.5 mL/min. The multiple reaction monitoring (MRM) mode was detected using an ion trap mass spectrometer equipped with an electrospray ionization ion source (ESI), operated in negative mode. The ion transition monitored was m/z 153 → m/z (109). Chromatograms were processed using Quant analysis software (Agilent Technologies, SUA).

Statistical Analysis

The data are presented as mean±SE and analyzed by one-way ANOVA analysis of variance followed by Fisher's protected least significant difference (PLSD) test as a post hoc test for comparison between two groups using Origin 4.1 and GraphPad computer softwares. $P \leq 0.05$ was considered to be statistically significant.

RESULTS

Histopathology

All animals survived throughout the entire experimental period and no significant difference was observed in body weight between the control group and different treatment groups (Figure 1).

None of the animals from the control group showed any histological change in the liver 30, 60, and 90 d after exposure. The BPA and GT extract group showed minimal liver changes after 30 days, including slight congestion and isolated focally

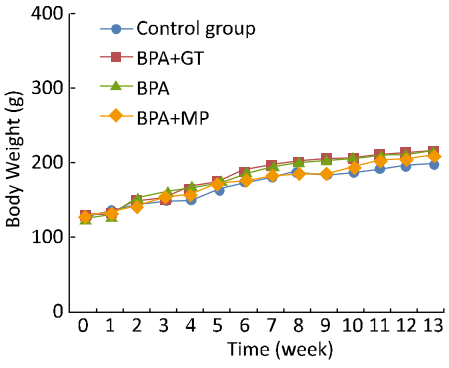

Figure 1. Body weight of female Wistar rats at different time points throughout the experiment.

necrotic hepatocytes after 60 and 90 d. The presence of a chronic inflammatory infiltrate composed of mononuclear cells, mainly lymphocytes, was also noticed and a vacuolated aspect of the cytoplasm (probably composed of both lipids and glycogen) was observed in one animal after 90 d of exposure, suggesting the administered products have a mild to moderate toxic effect and the changes are more visible after 60 or 90 d of exposure. In the case of animals treated with only, the microscopical lesions in BPA group were similar to those in BPA+GT group. However, acute congestion-hyperemia was observed BPA+GT group in addition to necrosis of isolated hepatocytes in BPA+GT group after 30 d of exposure. Moreover, the presence of isolated foci of necrosis and focal areas of minimal to mild fibrosis in the portal area, accompanied by moderate biliary hyperplasia (atypical regenerating liver damage, probably due to the toxic effect) was observed after 90 d of BPA exposure. The characteristics and time course of liver lesions in BPA+MP group were similar to those in BPA+GT group and BPA group. Moderate hyperemia, microvesicular lipidosis (liver fatty degeneration) and the presence of focally mononuclear cell inflammatory infiltrates (nonspecific reactive hepatitis), even with sequestration of hepatocytes in necrosis were observed after 30 d of exposure. The mild hepatocyte necrosis was maintained after 60 and 90 d of exposure. Regenerative atypical lesions (moderate biliary hyperplasia and mild portal fibrosis) were observed in BPA+GT group after 90 d of exposure, which were similar to those in BPA group (Figure 2). The histopathological lesions from liver sections are summed up in Table 1.

No morphopatological change was found in kidneys of control group. Mild to moderate degenerative lesions were observed in different exposure groups (Figure 3). Renal degenerative lesions (granulovacuolar degeneration mainly in the cortex) were observed in BPA+GT group after 60 and 90 d of exposure . The lesions with necrosis in the uriniferous tubular epithelium (cells with eosinophilic cytoplasm, pycnotic nuclei, or even areas in some tubules with absence of nuclei) in BPA group were similar to those in BPA+GT group after 30 d of exposure. Moderate dystrophic lesions (granulovacuolar degeneration) were observed at all time points. The morphological changes in the kidney sections are summarized in Table 2.

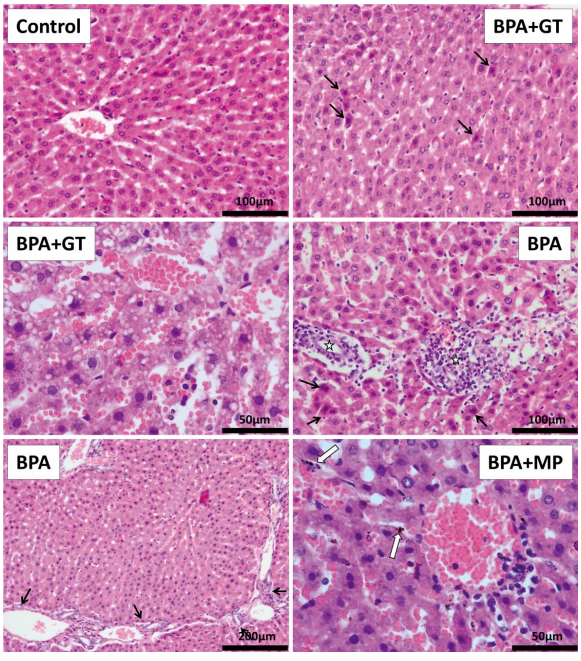

Figure 2. Histopathology showing normal architecture in control group; isolated hepatocytes undergoing necrosis and centrilobular microvesicular lipid steatosis (hepatocytes with foamy appearance) in BPA +GT group (black arrows); nonspecific portal hepatitis (stars), isolated hepatocytes undergoing necrosis (black arrows) and focal hepatocyte necrosis (middle right) in BPA group; and mild biliary hyperplasia (arrows) and mild portal fibrosis; BPA+MP-chronic liver congestion, presence of macrophages loaded with hemosiderin (siderophages, arrow), discrete proliferation around the centrilobular vein with mononuclear cells in BPA+MP after 90 d of exposure.

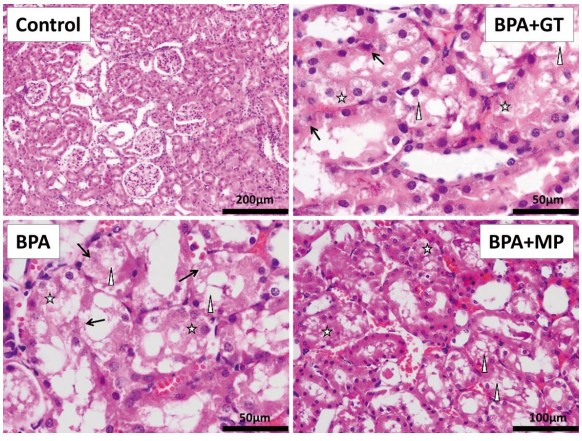

Figure 3. Histopathology showing normal morphology of cortical area in control group (HEx20); granulo-vacuolar degeneration, increased nephrocytes, granular nephrocyte cytoplasm (granular dystrophy, stars), foam cells (vacuolar dystrophy-arrowhead) and some nephrocytes undergoing necrosis (arrows) in BPA+GT group; granulo-vacuolar degeneration, increased nephrocytes and necrosis, loss of nuclei (arrows), haziness of nephrocyte cytoplasm (granular dystrophy, arrowhead), and foam cells (vacuolar dystrophy, stars) in BPA+MP group; granulo-vacuolar degeneration, nephrocytes and necrosis, loss of nuclei, haziness of nephrocyte cytoplasm (granular dystrophy); foam cells (vacuolar dystrophy), and intratubular protein casts in BPA group after 90 d of exposure.

Table 1. Histopathology Scores for Hepatic Necro-inflammation and Fibrosis Detected Using the Knodell Grading System (Mean±SE)

Groups	Necro-inflammation Score			Fibrosis Score		
	30 d	60 d	90 d	30 d	60 d	90 d
Control	0.05+0.02	0.07±0.03	0.05±0.02	0±0	0±0	0±0
BPA+GT	0.16±0.06*(P=0.023)	0.38±0.15	0.55±0.22$^{\#}$(P=0.027)	0±0	0±0	0±0*
BPA	0.66±0.27$^{\#\#}$(P=0.003)	0.55±0.2$^{\#}$(P=0.045)	0.72±0.29$^{\#\#}$(P=0.005)	0±0	0±0	0.50+0.20$^{\#}$(P=0.049)
BPA+MP	0.38±0.15$^{\#}$(P=0.044)	0.44±0.18$^{\#}$(P=0.011)	0.66±0.27$^{\#}$(P=0.012)	0±0	0±0	0.33±0.13

Note. $^{\#}$*vs* control group, P<0.05; $^{\#\#}$*vs* control group, P<0.01; **vs* BPA group (one-way ANOVA test and Fisher's PLSD test)

Only a change was observed in thyroid gland of BPA+MP group. The follicular volume increased in 1 out of 6 animals (16.6%) 30 d after BPA exposure and the nuclear volume decreased in 2 out of 6 animals (33.3%) 90 d after MP exposure, which are consistent with the reported data[64-65]. However, such effects were not observed in 3 exposure groups in which the epithelium featured small cuboidal cells and the follicular volume remained unchanged (Figure 4).

No morphological alterations were observed in the uterus. Histopathological analysis revealed no changes in mammary gland of control group with presence of moderate numbers of mammary gland lobules containing primary, secondary and tertiary ductules, as well as developing alveoli. On the other hand, specific lesions were found in the 3 observation periods of different exposure groups (Figure 5). The lesions observed initially in BPA+GT group were intraductal epithelial hyperplasia with columnar cells and hyperchromatic nuclei. Characteristic lesions were observed after 60 and 90 d of exposure to BPA and GT, represented by intraductal epithelial proliferations, sometimes occupying the entire ductal lumen. The lesions in BPA group were similar to those in BPA+GT group, including intraductal epithelial proliferations, epitheliosis aspect, ductal hyperplasia with the formation of new mammary ducts. Lesions were found starting on day 30 of exposure. The microscopical changes observed in BPA+MP group were more intense than those observed in the other groups. The common lesions were epitheliosis, and intraductal epithelium hyperplasia with the formation of new mammary ducts in the first period (30 d of exposure). Mammary adenosis with multiple foci of adenotic type, intraductal adenosis and ductal hyperplasia with the presence of numerous ducts of neoformation were observed in the second and third periods (after 60 and 90 d of exposure).

Qualitative analysis of ovarian morphology revealed normal healthy ovaries characterized by all stages of follicular development and the presence of healthy CL in control group. Although the animals

Table 2. Histopathology Scores of Kidney Lesions (Mean±SE)

Groups	30 d	60 d	90 d
Control	0.17±0.07	0.33±0.14	0.17±0.07
BPA+GT	$0.17\pm0.07^{**}$ (P=0.006)	$0.5\pm0.20^{**}$ (P=0.002)	$1.33\pm0.54^{\#\#,*}$ ($^{\#}P$=0.001) ($^{*}P$=0.016)
BPA	$1.6\pm0.72^{\#\#}$ (P=0.006)	$2.2\pm0.98^{\#\#}$ (P=0.001)	$2.6\pm1.16^{\#\#\#}$ (P=2.01E^{-4})
BPA+MP	$1.4\pm0.62^{\#\#}$ (P=0.002)	$1.8\pm0.8^{\#\#}$ (P=0.005)	$2.4\pm1.07^{\#\#\#}$ (P=2.82E^{-5})

Note. $^{\#}$*vs* control group, P<0.05; $^{\#\#}$*vs* control group, P<0.01; $^{\#\#\#}$*vs* control group, P<0.001, **vs* BPA group P<0.05; ***vs* BPA Group, P<0.01; (one-way ANOVA test and Fisher's PLSD test).

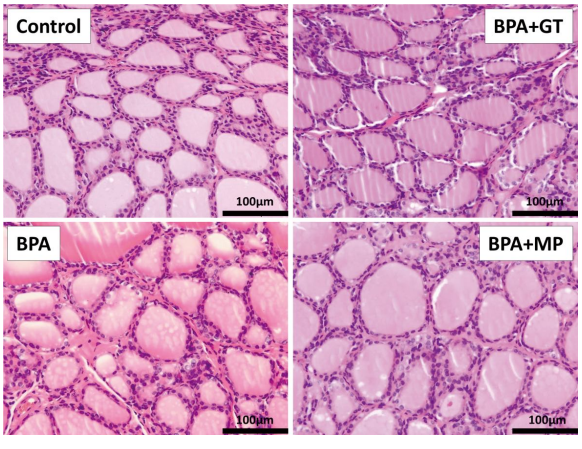

Figure 4. Histopathological analysis showing no visible morphological changes of thyroid gland in different exposure groups after 90 d of exposure.

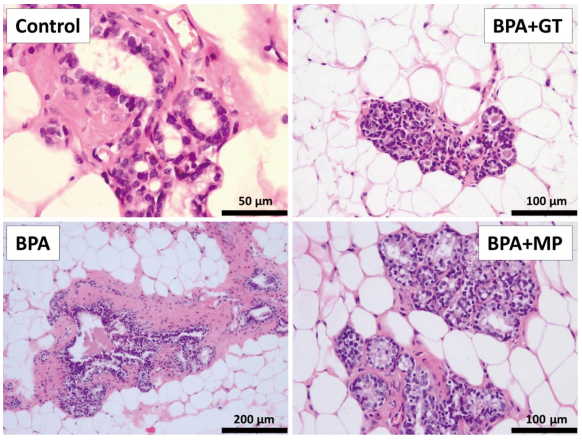

Figure 5. Histopathological analysis showing normal mammary tissue in control group, mammary duct hyperplasia and epitheliosis in BPA+GT group, intraductal epithelial proliferation and epitheliosis in BPA group, intraductal hyperplasia and mammary duct neoformation in BPA+MP after 90 d of exposure.

There in 3 treatment groups displayed all stages of follicular development, abnormal folliculogenesis and follicle degeneration were noticed at 3 intervals following the substance administration. One or more large antral-like follicles, structurally consistent with ovarian cysts, were observed in BPA group and sporadically in BPA+GT and BPA+MP groups. CL was counted in order to conduct a quantitative analysis of ovulatory capacity. No significant difference was found in the number of CL between control group and BPA group after 30, 60, and 90 d of exposure. The effect of substance administration was milder in both BPA+GT and BPA+MP groups than in BPA group.

Effect on Lipid Peroxidation

Total MDA level was used as biomarker of free radical-mediated lipid peroxidation injury[62,66-67] as is shown in Figure 6. The MDA level was higher in BPA group than in control group (P<0.01) and lower in BPA+GT group than in BPA group after 90 d of exposure. The lipid peroxidation induced by BPA was remarkably prevented by methylparaben in case of their co-administration and the MDA level was lower in BPA+GT group than in BPA group and control group after 30, 60, and 90 d of exposure (P<0.05, P<0.01).

Effect on Hydroxyl Radical Generation

The 2,3-DHBA level was measured as a biomarker for *in vivo* hydroxyl radical generation after administration of salicylic acid, a free radical scavenger[63,68] as is shown in Figure 7. The 2,3-DHBA level was higher in BPA group than in control group (P<0.05) and lower in MP+ BPA group than in BPA group after 30 days of exposure (P<0.05).

DISCUSSION

Subchronic exposure of the experimental animals to BPA in this study led to moderate toxic effects.

Hyperemia and necrosis of isolated hepatocytes were observed after 30 days of exposure and moderate liver fatty degeneration and regenerative atypical lesions were observed after 90 days of exposure. The histopathology scores for necro-inflamation were significantly higher in BPA group than in control group after 30 and 90 days of exposure (P<0.01). Minimal fibrosis was observed at the end of exposure in 3 out of 6 animals in BPA group (P<0.05, Table 1). Administration of GT and BPA delayed the toxic effects on necro-inflamation and fibrosis score, and attenuated their intensity.

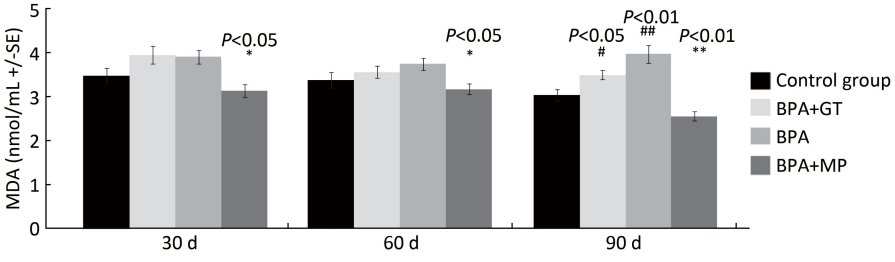

Figure 6. One-way ANOVA test and Fisher's PLSD test showing effect of bisphenol A (50 mg/kg) and combined GT L. herba extract (35 mg total isoflavones/kg) and methylparaben (250 mg/kg) on total MDA level in rat plasma (nmol/mL) (n=6). $^{\#}$*vs* control group, P<0.05; $^{\#\#}$*vs* control group, P<0.01; **vs* BPA group, P<0.05; ***vs* BPA grop, P<0.01; one-way ANOVA test and Fisher's LPD test.

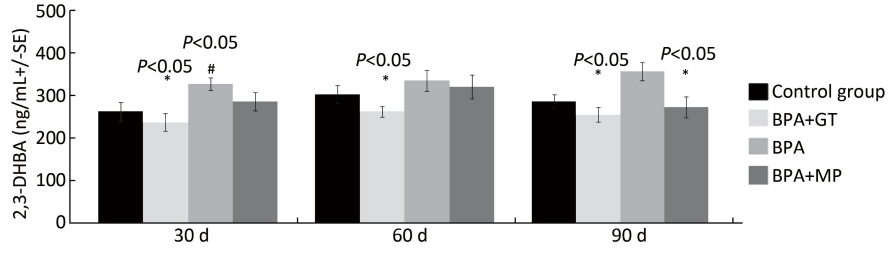

Figure 7. One-way ANOVA test and Fisher's PLSD test showing effect of BPA (50 mg/kg) and combined GT L. herba extract (35 mg total isoflavones/kg) and MP (250 mg/kg) on 2,3-DHBA levels in rat plasma (ng/mL) (n=6). $^{\#}$*vs* control group; **vs* BPA group.

Instead, simultaneous exposure to BPA and MP did not alter the nature of effects, but MP maintained the effect of BPA on necro-inflamation and fibrosis score (Table 1, Figure 2). The more renal degenerative lesions were severer in BPA group than in control group after 30, 60 and 90 d of exposure (P<0.01, Table 2). The granulovacuolar degeneration was severer in BPA+GT group and BPA+ MP group than in BPA group after 60 and 90 d of exposure (P<0.05, Figure 3).

The findings in the present study are consistent with the recent literature review data. Several studies showed that reactive oxygen species (ROS) can induce *in vivo* toxicity of BPA on liver[22,26,69-70] and kidney[22,27]. It was reported that high BPA dose (50 mg/kg) significantly increases the serum levels of ALT, AST and bilirubin and reduces the antioxidant gene expression in a rat model after 4 weeks of exposure[69]. Five and 10 ppm BPA cause oxidative damage in liver, kidney and reproductive organs after chronic exposure and female animals are more vulnerable than male animals[22]. Still, the effect of higher BPA dose is more pronounced and associated with increased lipid peroxidation, decreased glutathione level and activity of some antioxidant enzymes such as catalase, glutathione peroxidase, glutathione reductase and glutathione S-transferase. It was reported that oral BPA (25 mg/kg day) for 50 days causes oxidative damage in liver and kidney of male rats by disturbing the balance between ROS and antioxidant defence systems[26-27]. The activity of some hepatic enzymes (aspartate transaminase, alanine transaminase, lactate dehydrogenase) and lipid peroxidation level increased and the glutathione level decreased after exposure to BPA in this study. It was reported that hepatic necrosis and congestion occur in livers of rats exposed to BPA[26] and necrotic lesions, congestion, and mononuclear cell infiltration occur in their kidneys[27]. Administration of some antioxidants such as N-acetylcysteine[21] and vitamin C[70] reverses the low BPA dose-induced oxidative stress in rat liver. Vitamin C aggravates the oxidative stress injuries in vital organs of male rats when exposed to a higher BPA dose for a long period of time[23,26-28].

BPA is rapidly and almost completely absorbed (>95%) in human digestive tract and extensively metabolized in intestinal wall and liver mainly by glucuronidation with the conjugated metabolites rapidly excreted in urine[71]. A minor route of BPA metabolism is the oxidation by hydroxylation to a cathecol, 3-hydroxy-bisphenol A, followed by further transformation to an o-quinone, bisphenol A-3,4-quinone. The catechol/o-quinone couple is capable of redox cycling with generation of ROS and oxidative stress[19,72].

BPA exposure caused oxidative stress both by lipid peroxidation and by *in vivo* hydroxyl radical generation in the present study. Hydroxyl radical with a half-life of nanoseconds can induce lipid peroxidation in membrane phospholipids affecting ts cell membrane structure and functions, participate in propagation of other free radical generation , attack and damage all cellular constituents and DNA. The lipid peroxidation products at the membrane level can modify covalently other bimolecular constituents and increase cellular damage. The lipid peroxidation is related to liver and kidney injury[73-77]. Hence the toxic effects of BPA observed in the liver and kidney may be explained by cellular oxidative stress injury.

GT L, known as dyer's greenweed, is a species of the *Fabaceae* family rich in isoflavones and especially in genistin and genistein[47]. The antioxidant properties of genistein are well known. Genistein acts as a scavenger of ROS and reduces the expression of stress-related genes, thus protecting cells from damage[84-85]. In the present study, the the MDA level was lower in BPA+ GT group than in BPA group and no significant change was observed in the 2,3-DHBA level between BPA+GT group and control group. Moreover, histopathological analysis showed that GT attenuated the BPA-induced toxic lesions. The lesions were less severe and occurred later in BPA+GT group than in BPA group, suggesting a protective effect of GT extract. It was reported that chronic exposure to a high genistein concentration (500 mg/kg bw/day) can also cause hepatocellular hypertrophy and bile duct proliferation in rats[44]. In the present study, subchronic exposure to BPA and GT did not lead to any additive or synergic interaction, but decreased their toxicity.

The levels of lipid peroxidation and *in vivo* hydroxyl radical generation were significantly lower in BPA+MP group than in BPA group after 90 d of exposure (P<0.01, P<0.05). MP is quickly and completely absorbed in the gastrointestinal tract, hydrolyzed to p-hydroxybenxoic acid by esterases, and conjugated to p-hydroxyhippuric acid[78]. Its main metabolite, p-hydroxybenzoate, is a trapping agent of hydroxyl radicals *in vivo*, similar to salicylate (2-hydroxybenzoate), forming 3,4-dihydroxybenzoate[79-80] and can prevent lipid peroxidation. Recently, Kopalli *et al.* also reported that MP can

inhibit lipid peroxidation though neuronal cells[81].

However, the histopathological changes in liver and kidney in BPA+MP group were similar to those in BPA group. Therefore, even if MP has antioxidant properties, it cannot protec kidney or liver against injury. MP induces membrane permeability transition. Mitochondrial depolarization and depletion of cellular ATP through uncoupling of oxidative phosphorylation[78,82], and disruption of cytosolic calcium homeostasis[83] in a concentration- and time-dependent manner[82].

BPA caused intraductal epithelial proliferation and ductal hyperplasia in the mammary gland with the formation of new mammary ducts. It was recently reported that BPA may stimulate some cellular responses as estradiol and moreover and influence multiple endocrine-related pathways[2]. Co-administration of BPA with GT extract did not modify the effects, but delayed their appearance. Data from literature review showed that dietary PEs reduce or even cancel the effects of synthetic XEs[1]. Simultaneous exposure to BPA and MP caused the same types of lesions as BPA, suggesting that the toxicity of BPA increases when administered with MP. Acute, subchronic and chronic studies in rodents indicate that parabens are practically non-toxic[78]. MP and its main metabolite, p-hydroxybenzoic acid, induce estrogenic responses in human breast cancer cells[86]. It was recently reported that esters of p-hydroxybenzoic acid (parabens) are intact in human breast cancer tissues[87]. Exposure to parabens can increase the risk of breast cancer, interfere with male reproductive functions and modify malignant melanoma influenced by estrogenic stimulation[88]. In the present study, BPA and MP accentuated the estrogen stimulating effects, intraductal hyperplasia and formation of new mammary ducts after 30 and 90 d of exposure. These findings are very important because intraductal hyperplasia is considered as the precursor of carcinoma both in rodents and in humans[25].

In conclusion, we believe that several mechanisms are involved in subchronic toxicity induced by BPA. Some histopathological changes can be explained by estrogenic activity of BPA, others can be explained by different pathways which induce oxidative stress. GT L. herba extract rich in isoflavones, especially in genistein and genistin, reduced the BPA-induced histopathological changes in the liver, kidney and mammary gland of adult female rats following subchronic exposure rather than its antioxidant action, and MP maintained or even exacerbated the histopathological effects of BPA on the liver, kidney, and mammary gland in this study. Its influence on BPA toxicity was not mediated by oxidative stress but by its cytotoxic mechanism and/or xenoestrogenic properties. Specific estrogen stimulating effects observed in mammary gland, accentuated by exposure to BPA and MP, confirmed that patients with breast cancer should avoid the exposure to synthetic XEs.

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