

STUDY OF OXIDATIVE STRESS INDUCTION AFTER EXPOSURE TO BISPHENOL A AND METHYLPARABEN IN RATS

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Abstract

Long exposure to small concentrations of xenoestrogens (XEs) seems to be responsible for the increase of certain hormone-dependent diseases. The results of the research concerning the study of potentially synergic interactions between XEs are few and contradictory and need further studies. The oxidative stress is involved in the generation of several pathologies, such as cancer. We studied the oxidative stress induction in rats after exposure to bisphenol A (BPA) and/or methylparaben, two xenobiotics identified as XEs. Malondialdehyde (MDA), a lipid peroxidation end-product, and 2,3-dihydroxybenzoic acid (2,3-DHBA), a marker of *in vivo* hydroxyl radical production, were used to evaluate the oxidative stress generation. The levels of MDA in rat plasma were determined by HPLC-UV (detection at 307 nm) after derivatization with dinitrophenylhydrazine. 2,3-DHBA was analyzed by LC-MS/MS in multiple reaction monitoring (MRM) mode using an ion trap mass spectrometer with electrospray negative ionization. The ion transition monitored was m/z 153 $\rightarrow$ m/z (109). The results indicated that both studied xenobiotics have the potential of oxidative stress induction in rats but simultaneous exposure does not increase the intensity of effects.

Rezumat

Expunerea prelungită la concentrații mici de xenoestrogeni (XE) pare a fi responsabilă de creșterea incidenței anumitor boli hormono-dependente. Rezultatele cercetărilor privind studiul unor potențiale interacțiuni synergice între XE sunt puține și contradictorii și necesită efectuarea unor noi investigații. Stresul oxidativ este implicat în generarea mai multor patologii, inclusiv a cancerului. S-a studiat inducerea stresului oxidativ la șobolani după expunere la bisfenol A (BPA) și/sau metilparaben, două xenobiotice identificate ca fiind XE. Pentru evaluarea producerii stresului oxidativ s-a determinat concentrația plasmatică de malondialdehidă (MDA), un produs final al peroxidării lipidelor, și a acidului 2,3-dihidroxibenzoic (2,3-DHBA), un marker al

producerii de radicali hidroxil *in vivo*. Nivelele de MDA în plasma de șobolan s-au determinat prin HPLC-UV (dectecție la 307 nm) după derivatizare cu dinitrofenilhidrazină, iar cele de 2,3-DHBA prin LC-MS/MS în modul MRM (*multiple reaction monitoring*) folosind un spectrometru de masă cu trapă ionică și ionizare negativă prin *electrospray*. S-a monitorizat tranziția m/z 153 $\rightarrow$ m/z (109). Rezultatele au indicat faptul că ambele xenobiotice studiate au potențial de a induce stres oxidativ la șobolani, dar în cazul expunerii simultane nu s-a observat o creștere a intensității efectelor.

Keywords: Oxidative stress, bisphenol A, methylparaben, xenoestrogens.

Introduction

Long exposure to small concentrations of xenoestrogens (XEs) seems to be responsible for the increase of certain hormone-dependent diseases (breast or prostate cancer) [9, 10, 25]. The results of the research concerning the study of potentially synergic interactions between XEs are few and contradictory and need further investigations [6, 14, 18, 30, 33].

Bisphenol A (BPA) and parabens are xenobiotics identified as xenoestrogen molecules. Bisphenol A is a monomer of polycarbonate and epoxy resins largely used in packaging industry for food and drinks. It can be released from packages, becoming a food contaminant [10, 21, 25]. Parabens are esthers of 4-hydroxybenzoic acid used as antibacterial agents in numerous foods, cosmetics and pharmaceutical products [8]. Human exposure to these substances is not negligible and must be reevaluated in the case of subjects diagnosed with breast cancer or with increased risk of developing breast cancer.

At present, it is well known that oxidative stress is involved in the pathophysiology of several diseases including cancer [24, 29]. Moreover, the oxidation of estrogens determines the formation of some metabolites with different estrogenic and genotoxic potential [3, 13].

We studied the oxidative stress induction in rats after short exposure to Bisphenol A and/or methylparaben (MP) (Figure 1).

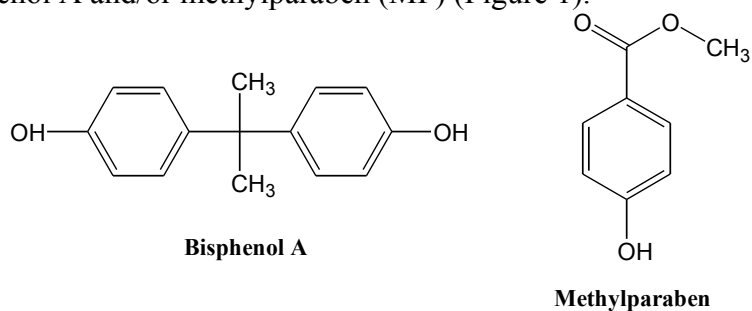


Figure 1

Chemical structures of bisphenol A and methylparaben

Malondialdehyde (MDA), a lipid peroxidation end-product, and 2,3-dihydroxybenzoic acid (2,3-DHBA), a marker of *in vivo* hydroxyl radical production, were used to evaluate the oxidative stress generation [31]. A LC-UV assay for MDA in rat hepatic microsomes was adapted to allow the quantification of the same biomarker in rat plasma samples [16, 17]. In order to determine the *in vivo* generation of hydroxyl free radicals, salicylic acid was administered as their trapping agent. The plasmatic level of 2,3-dihydroxybenzoic acid, a specific metabolite of salicylic acid formed only in the presence of hydroxyl radicals, was determined [28].

The aim of this study was to determine to what extent the exposure to BPA and MP generates oxidative stress through these two mechanisms and if there is any interaction in the case of simultaneous exposure to BPA and MP.

Materials and Methods

Reagents and materials

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

Animals

Female Wistar rats (body weight 146 ± 18 g) were supplied from the Practical Skills and Experimental Medicine Centre of the University of Medicine and Pharmacy "Iuliu Hațieganu" Cluj-Napoca (Romania). The rats were kept in temperature, humidity, day/night cycle standard conditions and they had access to food and water *ad libitum* throughout the experiment.

Experimental protocol

The experimental protocol was in compliance with the institutional and national guidelines for laboratory animal experiments, being approved by the Ethics Committee of the University. The protocol included 3 experimental groups and a negative control group, each consisting of 10 rats. The substances were administered for 10 days in food as following: group I – a daily dose of 50 mg/kg bw BPA and 250 mg/kg bw MP; group II

- a daily dose of 50 mg/kg bw BPA; group III - a daily dose of 250 mg/kg bw MP. Group IV was the negative control group and received standard food without substances.

Plasma samples

Blood was collected from the retro-orbital sinus in the presence of sodium fluoride as anticoagulant on the 10th day of the experiment. 90 min before the blood sampling sodium salicylate (58 mg/100g/mL, an equivalent dose of 500 mg salicylic acid / kg bw) was administered orally (by gastric intubation) to all animals, as *in vivo* hydroxyl radical trap. 30 min after the administration of sodium salicylate, 60 min before the blood sampling, the animals from group I received by gastric intubation BPA (50 mg/kg bw) and MP (250 mg/kg bw), the animals from group II only BPA (50 mg/kg bw) and the animals of group III only MP (250 mg/kg bw). Plasma separated by centrifugation (3000 g / 15 min) was stored at -20°C until the analysis.

Sample preparation

In order to quantify total MDA, plasma samples (75 µL) were treated with 1% H₂SO₄ (75 µL) and 6M NaOH (25 µL) in a water bath for 30 min at 60°C. After the hydrolysis of MDA bound to proteins, a solution of acetaldehyde (100 µL) as internal standard (IS) was added, followed by a deproteinisation step with 36% HClO₄ (63 µL). After vortex-mixing (10 s) and centrifugation (10 min at 10000 rpm), the supernatants (150 µL) were derivatized with 5mM DNPH in 2M HCl (20 µL) at room temperature, protected from light. The obtained hydrazone was extracted in hexane (1.2 mL) and the organic layer was evaporated in a centrifugal concentrator at 30°C. The residues were dissolved in mobile phase (150 µL) and 50 µL were injected into the HPLC system.

In order to quantify 2,3-DHBA, plasma samples (0.2 mL) were deproteinized with acetonitrile (0.6 mL). After vortex-mixing (10 s) and centrifugation (2 min at 12000 rpm), the supernatants (0.15 mL) were transferred in autosampler vials and 4 µL were injected into the HPLC system.

Instrumentation and chromatographic conditions

The analysis of total MDA was performed using the method developed and validated by the authors [17] on a 2695 Waters Alliance HPLC system (Waters, Milford, MA, USA) composed of a quaternary pump, autosampler, column heater and solvent degasser. The HPLC unit was connected to a 996 PDA (photodiode array) detector and a 2475

fluorescence detector (Waters, Milford, MA, USA). Chromatographic separation was achieved on a Spherisorb ODS column 250mmx4mm, 5 μ m) with a Spherisorb ODS (20mmx4mm, 3 μ m) guard column, maintained at 25°C. The mobile phase consisted in a mixture of 1% formic acid/acetonitrile (62/38, v/v). The flow rate was 1mL/min and the absorbance of the eluent was monitored at 307nm, corresponding to the maximum in the UV spectra of the MDA hydrazone obtained after derivatization. Data acquisition and processing were performed using Empower 2 software (Waters, USA) [17].

The analysis of 2,3-DHBA was performed using the method developed and validated by the authors [28]. The HPLC system used was an 1100 series Agilent Technologies model (Darmstadt, Germany) consisting of a G1312A binary pump, an in-line G1379A degasser, a G1329A autosampler, a G1316A column thermostat and an Agilent Ion Trap Detector 1100 SL (Darmstadt, Germany). Chromatographic separation was performed on a Luna HILIC (100 x 2.0 mm, 4 μ m) column (Phenomenex Inc., USA), maintained at 15°C, under isocratic conditions, using a mixture of 50 mM ammonium acetate in water (pH 4.5)/acetonitrile as the mobile phase (94:6, v/v) with a flow rate of 0.5 mL/min. Chromatograms were processed using Quant Analysis software (Agilent Technologies). The detection was performed in multiple reaction monitoring (MRM) mode using an ion trap mass spectrometer equipped with an electrospray ionization ion source (ESI), operated in negative mode: dry gas nitrogen at 12 L/min, dry gas temperature 350°C, nebulizer 60 psi (nitrogen), capillary 3500 V. Helium was used as collision gas and the collision potential was 1.1 V. The ion transition monitored was m/z 153 $\rightarrow$ m/z (109) for both analytes [28].

Statistical analysis

The results are presented as mean values $\pm$ standard deviation and the experimental groups were compared with the control group and between them by ANOVA analysis of variance using Origin 4.1 computer software.

Results and Discussion

a) Oxidative stress induction by lipid peroxidation

A representative chromatogram obtained from a rat treated orally with 50 mg/kg bw/day bisphenol A for 10 days (group II) and 500 mg salicylic acid /kg bw 90 min before the blood sampling is shown in Figure 2.

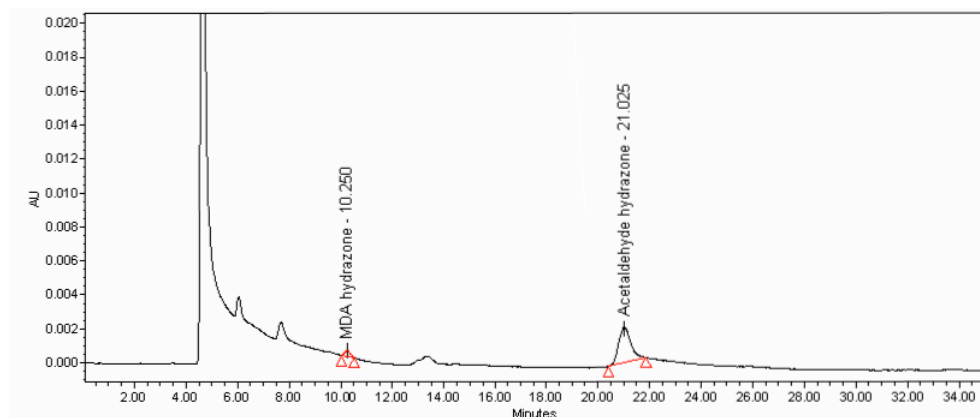


Figure 2

Quantification of total MDA. Chromatogram of sample plasma from a rat after oral administration of BPA (50 mg/kg bw /day for 10 days) and salicylic acid (500 mg/kg bw, 90 min before the blood sampling)

The MDA levels were significantly high for all treated animal groups comparatively with the control group ($p < 0.05$ in the case of simultaneous exposure to BPA and MP; $p < 0.01$ in the case of exposure to BPA or MP) (Figure 3). Consequently, the exposure of rats to BPA and MP, respectively, generates oxidative stress due to lipid peroxidation. The obtained results are in accordance with the data published in the literature and can be partially explained based on the metabolism pathways of the two tested substances.

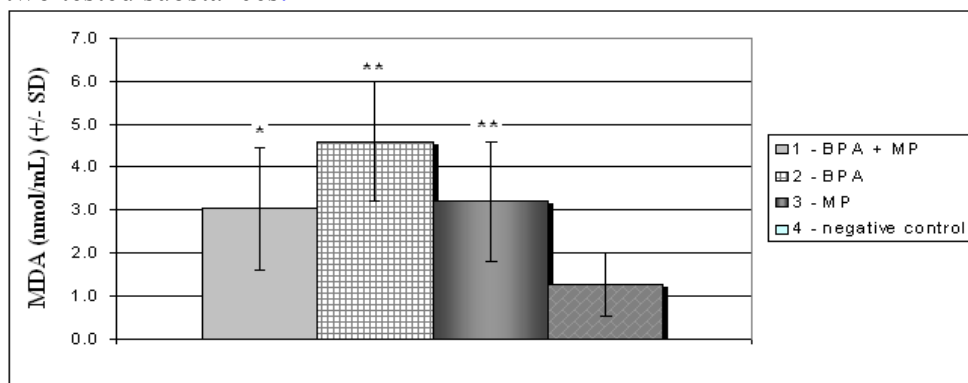


Figure 3

Total MDA levels in rat plasma after exposure to bisphenol A (BPA) and/or methylparaben (MP) (*, $p < 0.05$; **, $p < 0.01$)

There are numerous reports on the toxicity of BPA on liver [7, 19], central nervous system (CNS) [4, 22], reproductive system [5, 26] and kidney, mediated by reactive oxygen species (ROS) induction [15]. Thus,

oral exposure of male rats to BPA (25 mg/kg bw/day) caused oxidative damage in liver by disturbing the balance between ROS and antioxidant defense systems. The activity of some hepatic enzymes (aspartate transaminase, alanine transaminase, lactate dehydrogenase) and thiobarbituric acid-reactive substances (TBARS) were increased, and glutathione level was decreased in the group treated with BPA. Hepatic necrosis and congestion were observed in livers of rats exposed to BPA [19]. BPA generates significantly high concentrations of MDA in brain and sperm of male rats treated orally (25 mg/kg bw/day) for 45 days and causes oxidative damage in the brain and testes of rats [4, 5]. The production of ROS increases in a dose-dependent manner in sperm of rats after short term exposure (1 week) [26]. The main metabolic pathway of BPA is detoxification by glucuronidation. A minor route is the oxidation by hydroxylation to a catechol followed by further transformation to an o-quinone. The catechol-o-quinone couple is capable of redox cycling with generation of reactive oxygen species (ROS) and oxidative stress [20].

Parabens can induce cytotoxicity by mitochondrial failure dependent on induction of membrane permeability transition accompanied by the mitochondrial depolarization and depletion of cellular ATP through uncoupling of oxidative phosphorylation [32]. After oral administration, MP is quickly and completely absorbed from the gastrointestinal tract. It is hydrolyzed to *p*-hydroxybenzoic acid by esterases and then conjugated to *p*-hydroxyhippuric acid, ester and ether glucuronides and an ethereal sulfate [32]. Paraben (*p*-hydroxybenzoic acid) induced lipid peroxidation in the liver of mice *in vivo* [1] and in liver and kidney homogenates *in vitro* [2]. In skin cells, parabens may induce oxidative stress after conversion to glutathione conjugates of hydroquinone by reacting with singlet oxygen and glutathione [27]. Lipid peroxidation was significantly increased in MP-treated human skin keratinocytes only after UVB exposure [12].

In our study the degree of increase in MDA levels was not significantly different between the animals treated with BPA or MP alone and those treated with the combination of BPA and MP. Simultaneous exposure to BPA and MP did not result in additive effect, synergism or potentiation in terms of lipid peroxidation.

b) Oxidative stress induction by hydroxyl radical generation

A representative chromatogram obtained from a rat orally treated with 50 mg/kg bw/day bisphenol A for 10 days (group II) and 500 mg salicylic acid /kg bw 90 min before the blood sampling is shown in Figure 4.

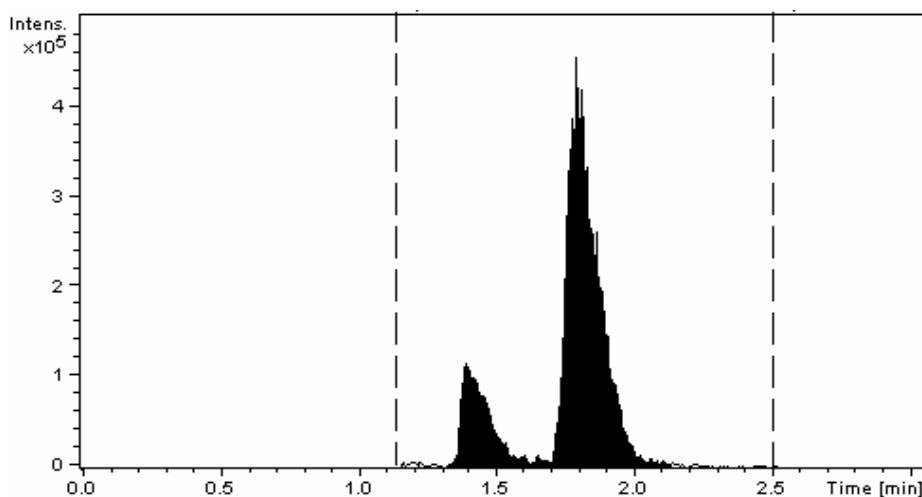


Figure 4

Quantification of 2,3-DHBA. Chromatogram of sample plasma from a rat after oral administration of BPA (50 mg/kg bw/day for 10 days) and salicylic acid (500 mg/kg bw 90 min before the blood sampling); retention times: 2,3-DHBA - 1.4 min, 2,5-DHBA - 1.85 min.

The 2,3-DHBA levels were significantly decreased for MP-treated animal groups (groups I and III) compared with the control group ($p < 0.01$) (Figure 5).

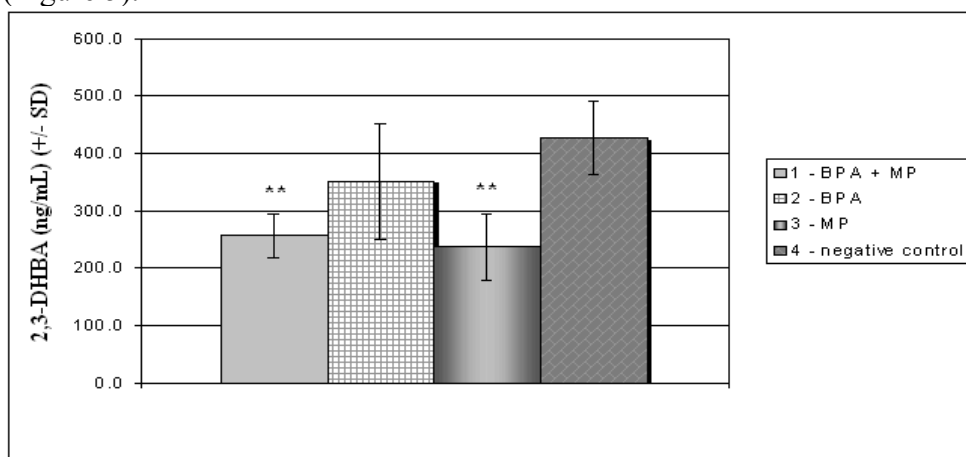


Figure 5

2,3-DHBA levels in rat plasma after exposure to bisphenol A (BPA) and/or methylparaben (MP) (**, $p < 0.01$)

BPA did not induce oxidative stress by hydroxyl radical generation. Regarding MP, its main metabolite, p-hydroxybenzoate, is a trapping agent of hydroxyl radicals *in vivo*, similarly to salicylate (2-hydroxybenzoate),

forming 3,4-dihydroxybenzoate [11, 23]. This explains why the 2,3-DHBA levels decreased in animals treated with MP.

Conclusions

The exposure of rats to BPA and MP, respectively, generates oxidative stress due to lipid peroxidation. Simultaneous exposure to BPA and MP did not lead to the appearance of any type of interaction (additive, synergism or potentiation) in the generation of lipid peroxidation. The generation of hydroxyl radicals was not identified as a mechanism of oxidative stress induction by BPA. MP acts as a trapping agent for hydroxyl radicals by its metabolite, p-hydroxybenzoate, showing a certain protection against the deleterious effects of these radicals. Therefore, simultaneous exposure to BPA and MP did not have a negative impact on the cellular oxidative status.

Acknowledgements

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