ORIGINAL ARTICLE

UDC: 615.099:615.03]::616.36-092.9
DOI: 10.2298/VSP1505405C

Relationship of hepatotoxicity and the target tissue dose of decabrominated diphenyl ether in subacutely exposed Wistar rats

Odnos hepatotoksičnosti i doze dekabromovanog difeniletra u ciljnom tkivu kod subakutno izloženih Wistar pacova

Marijana Ćurčić*, Sladjana Tanasković†, Sanja Stanković‡, Saša Janković§, Marko Antunović‖, Snežana Djordjević‖¶, Vesna Kilibarda‖¶, Slavica Vučinić‖¶, Biljana Antonijević*

***Department of Toxicology "Akademik Danilo Soldatović", Faculty of Pharmacy, University of Belgrade, Belgrade, Serbia; †Faculty of Pharmacy, University of Belgrade, Belgrade, Serbia; ‡Center for Medical Biochemistry, Clinical Center of Serbia, Belgrade, Serbia; §Institute of Meat Hygiene and Technology, Belgrade, Serbia; ‖National Poison Control Center, Military Medical Academy, Belgrade, Serbia; ¶Faculty of Medicine of the Military Medical Academy, University of Defence, Belgrade, Serbia**

Abstract

Background/Aim. Based on numerous studies in animals, the most prominent toxic effects of decabrominated diphenyl ether (BDE-209) are observed in the liver, thyroid hormone homeostasis, reproductive and nervous systems. BDE-209 exhibits its toxic effects partly through the aryl hydrocarbon (Ah) receptor and consequent induction of hepatic microsomal enzymes. The aim of this study was to assess the hepatotoxic effect *vs* target tissue dose of BDE-209 in the subacutely orally exposed Wistar rats. **Methods.** Effects were examined on male Wistar rats, weighing 200–240 g, exposed to doses of 1,000, 2,000 or 4,000 mg BDE-209/kg body weight (bw)/day by gavage during 28 days. Animals were treated according to the decision of the Ethics Committee of the Military Medical Academy, No 9667-1/2011. Evaluation of the hepatotoxic effect was based on: relative liver weight water and food intake, biochemical parameters of liver function [aspartate amino transferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), gama glutamyl transferase (γ-GT)], and oxidative stress parameters in liver homogenates [malondialdehyde (MDA), superoxide dismutase (SOD), -SH] and morphological and pathohistological changes in the liver. For the assessment of internal dose – response relationship, lower confidence limit of Benchmark dose (BMDL) of 5% or 10% i.e. $BMDL_5$ or $BMDL_{10}$, were calculated using PROAST software. **Results.** After the application of 1,000, 2,000 or 4,000 mg BDE-209/kg bw/day, the concentrations of BDE-209 measured in liver were 0.269, 0.569 and 0.859 mg/kg of liver wet weight, (ww) respectively. Internal doses correlated with external ($r = 0.972$; $p < 0.05$) according to equation: internal dose (mg BDE-209/kg of liver ww) = 0.0002 × external dose (mg/kg bw/day) + 0.0622. Hepatotoxicity was demonstrated based on significant increase in AST and γ-GT activities and the degree of histopathological changes. The lowest $BMDL_5$ of 0.07228 mg BDE-209/kg of liver ww, correlating to external dose of 39 mg/kg/day, indicated the increase of AST activity as the most sensitive biomarker of BDE-209 hepatotoxicity in subacutely exposed rats. **Conclusion.** The results of the present work add up to the issue of BDE-209 toxicity profile with a focus on relationship between internal dose and hepatotoxicity. Critical internal dose for the effect on AST of 0.07 mg/kg of liver ww, corresponding to external dose of 39 mg/kg/day, is the lowest dose ever observed among the studies on BDE-209 hepatotoxicity. For the persistent substances with low absorption rate such as BDE-209, critical effect based on internal dose in majority of cases is considered as more precisely defined than the effect established based on external dose, particularly.

Key words:
halogenated diphenyl ethers; liver; toxicity test; rats.

Apstrakt

Uvod/Cilj. Prema podacima iz brojnih studija na životinjama, dekabromovani difeniletar (BDE-209) najznačajnije toksične efekte ispoljava na jetri, homeostazi hormona štitaste žlezde, reproduktivnom i nervnom sistemu. BDE-209 ispoljava toksične efekte delom preko receptora za aromatične ugljovodonike (Ah) i posledične indukcije mikrozomalnih enzima jetre. Cilj rada bio je procena hepatotoksičnog efekta u odnosu na dozu BDE-209 u ciljnom tkivu kod su-

Correspondence to: Marijana Ćurčić, Department of Toxicology, "Akademik Danilo Soldatović" Faculty of Pharmacy, University of Belgrade, Vojvode Stepe 450, 11 221, Belgrade, Serbia. Phone.: +381 11 3151 248. E-mail: makitox@pharmacy.bg.ac.rs

bakutno oralno eksponovanih Wistar pacova. **Metode.** Efekti su ispitivani na mužjacima Wistar pacova, mase 200–240 g, koji su putem oralne sonde primali doze od 1 000, 2 000 ili 4 000 mg BDE-209/kg telesne mase (tm) dan, tokom 28 dana. Životinje su tretirane u skladu sa odlukom Etičkog komiteta Vojnomedicinske akademije u Beogradu br. 9667-1/2011. Procena hepatotoksičnih efekata bazirana je na merenju relativne mase jetre, unosa vode i hrane, biohemijskih parametara funkcije jetre [aspartat aminotransferaza (AST), alanin aminotransferaza (ALT), alkalna fosfataza (ALP), gama glutamil transferaza (γ-GT)], parametara oksidativnog stresa u homogenatima jetre [malondialdehid (MDA), superoksid dizmutaza (SOD), -SH)] i morfoloških i histoloških promena na jetri. Za procenu odnosa interna doza – odgovor izračunavana je donja granica pouzdanosti granične Benchmark doze (BMDL) od 5% ($BMDL_5$) ili 10% ($BMDL_{10}$) primenom PROAST softvera. **Rezultati.** Koncentracije BDE-209 iznosile su 0,269, 0,569 i 0,859 mg/kg jetre nakon aplikacije 1 000, 2 000, odnosno 4 000 mg BDE-209/kg tm/dan. Interna doza u našoj studiji korelisala je sa eksternom dozom prema jednačini: interna doza (mg BDE-209/kg jetre) = 0,0002 × eksterna doza (mg/kg tm/dan) + 0,0622 (r = 0,972; p < 0,05). Hepatotoksičnost je potvrđena na osnovu nalaza o povećanju aktivnosti enzima AST i γ-GT, kao i stepena patohistološkog oštećenja jetre. Najniža $BMDL_5$ u eksperimentu od 0,07228 mg BDE-209/kg jetre, koja koreliše sa eksternom dozom od 39 mg/kg tm/dan izračunata je za aktivnost AST i ukazuje na to da je aktivnost AST ujedno i najosetljiviji biomarker hepatotoksičnosti BDE-209 kod subakutno eksponovanih pacova. **Zaključak.** Rezultati prezentovane studije daju doprinos pitanju toksikološkog profila BDE-209 sa fokusom na odnos između interne doze i hepatotoksičnih efekata. Kritična interna doza za efekat na AST od 0,07 mg/kg jetre, koja koreliše sa eksternom dozom od 39 mg/kg tm/dan, jeste ujedno i najniža kritična doza do sada definisana za hepatotoksične efekte BDE-209. Kritičan efekat koji se bazira na dozi u ciljnom tkivu u većini slučajeva može se smatrati preciznije definisanim od kritičnog efekta definisanog na bazi oralno primenjene doze, naročito za nedegradabilne supstance sa niskim stepenom apsorpcije, kao što je BDE-209.

Ključne reči:
difeniletri, halogenovani; jetra; toksičnost, testovi; pacovi.

Introduction

Polybrominated diphenyl ethers (PBDEs) are considered to be extremely efficient flame retardants [1, 2]. Decabrominated diphenyl ether (BDE-209) is a major component of deca-BDE commercial mixture [3–6] that can migrate from the product, get into the environment and pose a risk for the human health. The main source of exposure to BDE-209 is from the diet, however inhaled air could also contribute to its entire body burden [7, 8]. After entering the organism, BDE-209 exhibits its toxic effects partly through the aryl hydrocarbon (Ah) receptor and consequent induction of hepatic microsomal enzymes. It may also induce production of reactive oxygen species [7] found to be related to DNA damage. Based on numerous studies [8–17] on animals, the most prominent toxic effects of PBDEs are observed in the liver, thyroid hormone homeostasis, reproductive and nervous systems.

While the liver is one of the main target organs of its toxicity, the aim of this study was to assess the hepatotoxic effect of BDE-209 in the subacutely orally exposed Wistar rats. Considering that BDE-209 has poor solubility the level of its absorption and the amount reaching target tissues are difficult to predict. We assumed that the dose measured at target site is more appropriate than orally given dose, allowing us to more accurately perceive the liver changes from the relationship of internal dose and intensity of effects. Evaluation of hepatotoxic effects was based on serum liver enzymes activity: aspartat aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyl transferase (γ-GT), alkaline phosphatase (ALP), degree of histopathological changes, as well as oxidative stress parameters in liver homogenates: malondialdehyde (MDA), activity of total superoxide dismutase (SOD) and content of sulfhydryl (-SH) groups.

Methods

Chemicals

BDE-209, 98% (Sigma-Aldrich, St. Louis, MA, SAD) and dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MA, SAD) were purchased from commercial sources.

Experimental animals

Male albino Wistar rats, weighing 200 g to 240 g were obtained from a disease-free stock bred at the Military Medical Academy in Belgrade, Serbia. The animals were housed in plastic cages with wire mesh top, in a climate-controlled facility with a constant 12-hour day and night cycle at 20°C to 24°C and relative humidity between 40% and 60%. The animals had free access to food and water throughout the study and were treated according to the guidelines for animal studies (No. 9667-1/2011), issued by the Academy's Ethical Committee.

Experimental protocol

After a quarantine period of 14 days, each group of animals (n = 8 *per* group) was receiving treatment solution by gavage in a volume of 0.5 mL/kg body weight (bw) day for 28 days. Control animals were receiving water (the control group), while rats in the vehicle control group were receiving DMSO alone the (DMSO group). Three groups were receiving BDE-209 as a suspension in DMSO in the doses of 1,000, 2,000, or 4,000 mg/kg bw/day (groups assigned as: $BDE\text{-}209_{1000}$, $BDE\text{-}209_{2000}$, and $BDE\text{-}209_{4000}$, respectively). The doses of BDE-209 were chosen based on literature data [18, 19]. Bws, water and food intake were recorded weekly. Clinical signs of poisoning were observed each day of the experiment.

After the period of exposure, animals were sacrificed, and samples of blood and liver were taken. Liver weight and observed morphological changes were recorded immediately. Liver enzyme activity was measured in serum samples. Samples of the liver were taken for histopathological analysis as well as for homogenisation, and these samples were stored at -80°C prior to the analysis of the oxidative stress parameters. The rest of the liver had been stored at -20°C before the analysis of BDE-209 concentration could be performed.

Determination of BDE-209 in liver

The method was based on multistep extraction from homogenised liver, extract cleaning and determination of BDE-209 by gas chromatography (GC) – electron capture detection (GC – ECD) and GC – mass spectrometry (MS). Homogenised sample of 0.5–2 g was dried and fragmented into fine powder and transferred in a glass centrifuge tube. A total of 8 mL of mixture n-hexane:dichlormethane [(8:2; (V/V)] was added to the powder, and the total amount was vortexed, sonificated and centrifuged. Supernatant was separated, dried in air flow and than resolved in 5 mL of acetonytrile. Further cleaning of sample was done using QuEChERSs (Quick Easy Cheap Effective Rugged Safe) (ENVIRO-CLEAN r EUMIV50CT, amchro GmbH, Hattersheim, Germany). After shaking, the content was centrifuged and aliquot was transfered into the tube for solvent evaporation. The dried residue was reconstituted in 1 mL of n-hexane and injected in GC. The retention time (Rt) and mass spectra (m/z values) were used for determination, and the pik area was used for quantification. Quality control of the analytical method was performed using certified reference material CIL-EDF-2524 Clean Fish (slurry) - Organic contaminants (LGC standards).

Determination of liver enzymes activities

Activity of AST, ALT, γ GT, and ALP in serum samples was determined on automatic analyser using commercial tests (Roche Elecsys 2010-cobas c 111 analyser, Roche Diagnostics, Mannheim, Germany).

Histopathological analysis of liver tissue

The liver sample was excised and fixed with 10% neutral formaldehyde. Dehydratation of tissue samples was done with graded aethanol and embedded in paraffin blocks. For semiquantitative tissue analysis, sections in 2 μm thick paraffin were stained by hematoxylin and eosin (HE) method and analyzed (Olympus-2 microscope).

Following criteria were used for semiquantitative evaluation of histopathological changes: 0 – unchanged liver`s parenchyma; 1 – single cells with intracellular oedema, vasodilatation of blood vessels and the appearance of inflammatory response cell; 2 – groups of cells with cytoplasmic vacuolation, distinct vasodilatation of numerous blood vessels (over 50%) and the accumulation of cellular infiltrate in the surrounding tissue; 3 – most cells with prominent vascular degeneration and karyopyknosis, focal accumulation of cellular infiltrate, 4 – vacuolar degeneration and karyolysis in all cells, single cell necrosis and diffuse accumulation of cell infiltrate, and 5 – massive and diffuse necrosis, foam cells and tissue degeneration. For determine these parameters, whole longitudinal and cross-sectional tissue was analysed and observed under high magnification (40×).

Determination of oxidative stress parameters

Oxidative stress parameters were measured after homogenisation of liver samples in saccharose medium. Following parametres were determined: concentration of MDA, activity of SOD and concentration of -SH groups. Proteins were measured in homogentes by Bradford [20] method.

The level of MDA in liver homogenates was based on reaction of MDA with thiobarbituric acid under acidic condition for 15 min at 95°C in termostatic water bath. In this reaction light yellow to pink colour complexes were formed depending on the concentration of MDA in tissue homogenates. Intensity of colour was measured at 523 nm and 600 nm.

Activity of SOD, EC 1.15.1.1. in homogenates was measured using the method of Misra and Fridovich [21], based on ability of SOD to inhibit spontaneous autooxidation of adrenaline under alkaline condition (pH 10.2).

The total content of -SH groups in liver homogenates was determined by the Ellman's [22] method which is based on the reaction of 2,2-dinitro-5,5-dithio-benzoic acid (DTNB) with alifatic thiol compounds in alkaline condition (pH 9.0).

Statistical analysis

A significance of the difference among the data in the groups for certain effect was determined by analysis of variance (ANOVA) and post-hoc Tukey test ($p < 0.05$) (Statistica 7.0).

PROAST software was used (RIVM, Bilthoven, Netherland) for the determination of dose-response relationship and calculation of benchmark dose lower confidence limit [(BMDL i.e. $BMDL_5$ if 5% of change in effect was considered as critical effect size – (CES)]. Moreover, associated benchmark dose to critical dose was assigned as critical effect dose (CED) [23–25].

Correlation analysis was used to estimate the relationship between external and internal dose ($p < 0.05$). The following equation was derived: internal dose [mg BDE-209/kg of liver wet weight (ww) = 0.0002 × external dose (mg/kg bw/day) + 0.0622

Results

In life toxicology

During the study period no clinical signs of poisoning were observed. Water consumption during the period of exposure decreased after the first week (Figure 1) and at

the end of study was significantly lower in rats treated with medium and the highest dose of BDE-209 (Table 1).

During the experiment there were no significant changes in food intake related to DMSO group (Figure 1).

Animals body weights were increasing during the period of exposure, but statistically significant differences in weight gain were not observed (Figure 2).

After the application of 1,000, 2,000 or 4,000 mg BDE-209/kg/day, the concentrations of BDE-209, measured in the liver, were 0.269, 0.569 and 0.859 mg/kg of the liver ww, respectively. In the control and the DMSO group concentrations of BDE-209 were below the quantification limit.

The lowest concentration of BDE-209 in the liver of Wistar rats, resulted in a significant increase in relative liver weight comparing to the control, while two highest concentrations, resulted in a significant decrease in relative liver weight compared to the results recorded in the DMSO group (Table 1). Relative liver weight was uniformly changing in the dose dependant manner, and calculated BMD_5 for internal dose of BDE-209 was 0.224 mg/kg of liver ww, corresponding to external dose of 971.4 mg BDE-209/kg/day (Table 1).

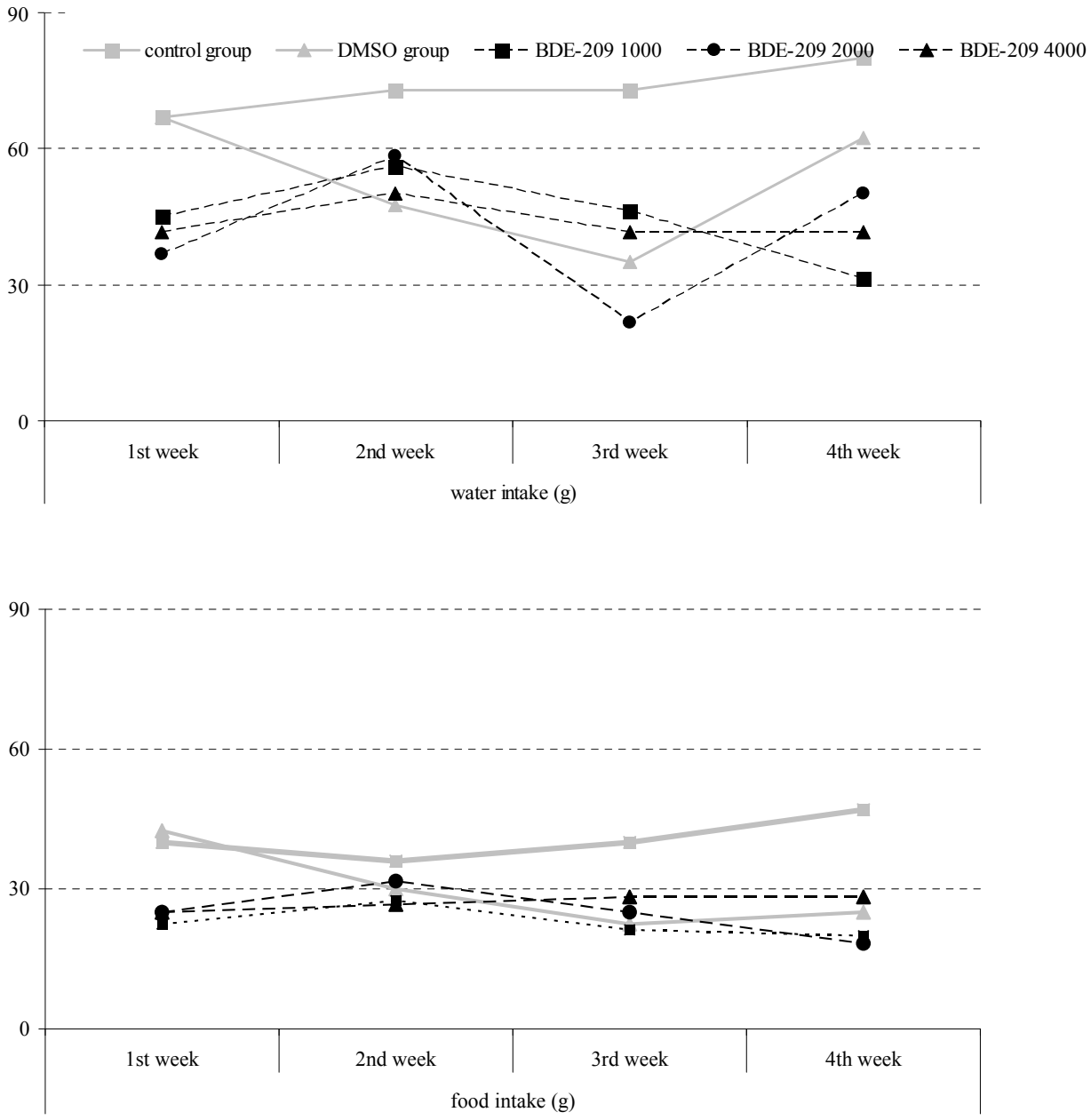


Fig. 1 – Weekly records on water and food intake in *Wistar* rats subacutely exposed to decabrominated diphenyl ether (BDE-209); DMSO – dimethyl sulfoxide.

Table 1

Influence of increasing doses of decabrominated diphenylether on liver weight, relative liver weight, and water and food intake in subacutely exposed Wistar rats

Internal dose (mg/kg)/ Dose (mg/kg bw/day)	Liver weight (g)	Relative liver weight (%)	Water intake[§] (g)	Food intake[§] (g)
0/0 $_{control}$	8.50 ± 0.38	2.72 ± 0.26	73.25 ± 5.32	40.75 ± 4.50
0/0 $_{DMSO}$	9.11 ± 0.77	3.63* ± 0.37	53.00 ± 14.61	30.00 ± 8.89
0.269/1000	10.93 ± 1.21	3.44* ± 0.29	44.69 ± 10.28	22.81* ± 3.29
0.569/2000	8.39 ± 0.83	2.75[#] ± 0.16	41.68* ± 16.02	25.00* ± 5.47
0.859/4000	6.90 ± 1.02	2.38[#] ± 0.24	43.78* ± 4.15	27.08* ± 1.58
		Results on benchmark dose and model internal dose / external dose		
BMD/BMDL	nt	<10 / <10	-	-
dose response	nt	(+) / (+)	(-)	(-)
Model	nt	E3 / E5	-	-
BMD_5	nt	0.2244 / 971.4	-	-

*** – A statistically significant difference from the control group; [#] – A statistically significant difference from the DMSO group (ANOVA, post-hoc Tukey test); [§] – Average obtained from 4 measurements, recorded weekly, during the 4-week of experiment; nt – dose response relationship was not tested; (+) or (-) – dose response relationship confirmed or not confirmed; E1-E5 – type of dose-response model given by PROAST software; BMD – benchmark dose; BMDL – 95% lower confidence limit of BMD; DMSO – dimethyl sulfoxide.**

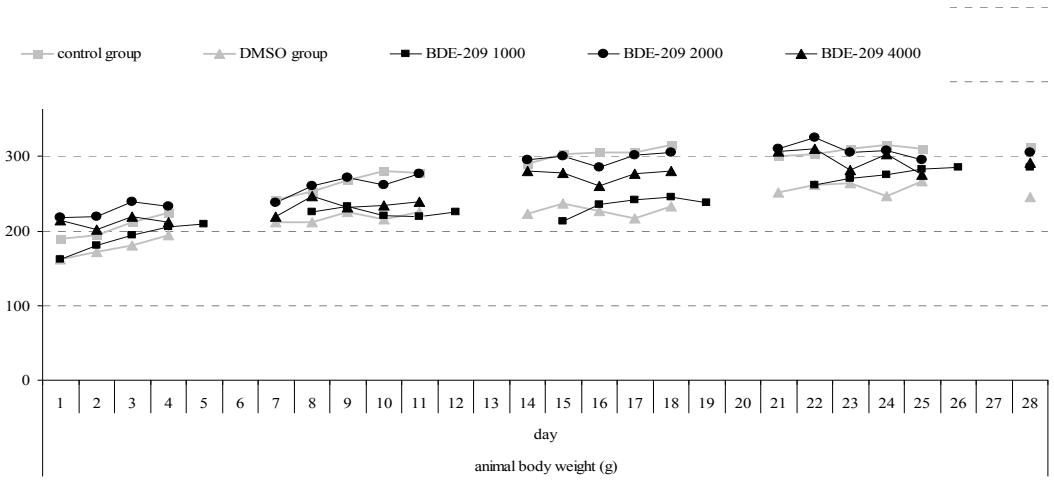


Fig. 2 – Daily records (Records were made five times a week) on body weight (g) of Wistar rats subacutely exposed to decabromineted diphenyl ether (BDE-209); DMSO – dimethyl sulfoxide.

Liver enzymes

As for the results of liver enzyme activity, a significant increase of the AST was induced by the medium and the highest concentrations, while a significant increase of γ-GT was induces by the lowest and the highest liver BDE-209 concentrations (Table 2).

The dose-response relationship was confirmed only for these two effects on liver enzymes. For the effect on AST, calculated $BMDL_5$ was 0.07228 mg BDE-209/kg of liver ww and

Table 2

Influence of increasing doses of decabrominated diphenylether on serum liver enzyme activity of subacutely exposed Wistar rats

Internal dose (mg/kg)/ dose (mg/kg bw/day)	AST (U/L)	ALT (U/L)	ALP (U/L)	γ-GT (U/L)
0/0 $_{control}$	211.0 ± 47.9	76.3 ± 21.2	295.5 ± 27.3	2.2 ± 1.0
0/0 $_{DMSO}$	221.0 ± 15.6	61.3 ± 4.11	173.5* ± 54.8	1.8 ± 0.5
0.269/1000	218.1 ± 17.4	63.4 ± 5.7	250.0 ± 37.7	4.9*,[#] ± 1.1
0.569/2000	330.0* ± 73.0	85.2 ± 22.7	166.0 ± 48.9	2.0 ± 1.3
0.859/4000	295.2* ± 33.9	72.2 ± 10.8	184.8 ± 31.4	4.4*,[#] ± 2.3
	Results on benchmark dose and model internal dose / external dose			
BMD/BMDL	<10 / -	- / -	- / -	- / -
dose response	(+) / (-)	(-) / (-)	(-) / (-)	(+) / (+)
Model	E2 / -	- / -	- / -	E1 / E1
BMD_5	0.07228/ -	- / -	- / -	- / -

*** – A statistically significant difference from the control group; [#] – A statistically significant difference from the dimethyl sulfoxide (DMSO) group (ANOVA, post-hoc Tukey test); (+) or (-) – dose-response relationship confirmed or not confirmed; E1-E5 – type of dose-response model given by PROAST software; BMD – benchmark dose; BMDL – lower confidence limit of BMD; AST – aspartat aminotransferase; ALT –alanine aminotransferase; ALT – alanine aminotransferase; ALP – alkaline phosphatase; γ-GT – gamma glutamyl tranferase.**

the curve representing the target tissue dose-response relationship is shown in Figure 3. The dose of 0.07228 mg BDE-209/kg of liver ww, corresponding to external dose of 39 mg/kg/day, was the lowest $BMDL_5$ among all the calculated benchmark doses indicating the effect on AST as the most sensitive biomarker of BDE-209 hepatotoxicity in subacutely exposed rats.

Histopathology

Tissue sections of the rat liver from the control and the DMSO group did not show any deviation from the normal histological structure (Figure 4, A and B).

With the lowest concentration of 0.269 mg/kg, normal histology of the liver was preserved only in the central parts. Edema of hepatocytes, mild hyperemia and small focal hemorrhages in the sinusoids were observed. Sinusoids were slightly narrowed because the moderate edema of the most hepatocytes. Cytoplasm was very eosinophilic and filled with small vacuoles. Nucleus in these cells were rounded, irregular and hyperchromatic, and nucleoli were difficult to be observed (Figure 4C). Numerous pathological mitoses were present, in most hepatocytes, particularly in the group where internal dose of BDE-209 was 0.569 mg/kg of liver ww (Figure 4D). All blood vessels were dilated, with moderate to severe polymorphonuclear cell infiltration (Figure 4E). A response higher than 10% was considered as extra risk for the degree of histopathological changes and therefore $BMDL_{10}$ was calculated. The values of the degree of histological damage as ordinal type of the variable were converted in quantal type of the variable [23, 24]. Dose response was confir-

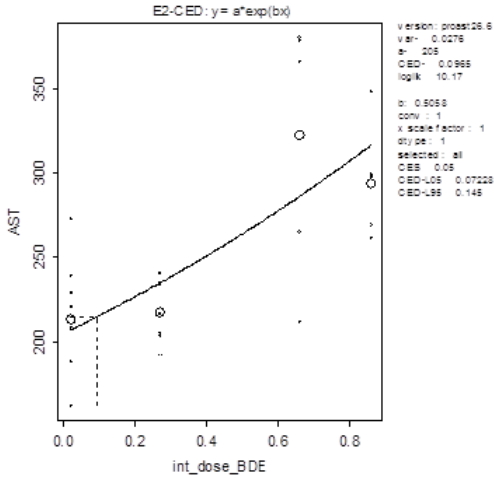
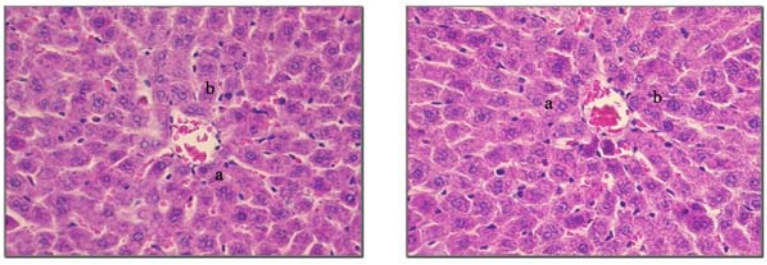
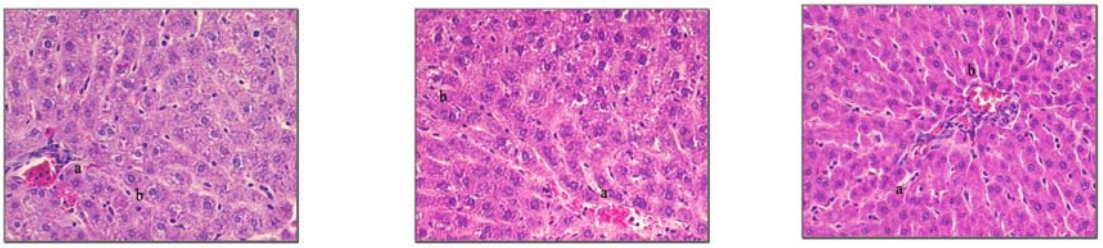


Fig. 3 – Dose-response curve for the effect on aspartat amino transferase (AST) activity against liver concentrations of decabromineted diphenyl ether (BDE-209); CED – critical effect dose.



A) Control group **B) Dimethyl sulfoxide group**



C) 0.269 mg BDE-209/kg liver **D) 0.569 mg BDE-209/kg liver** **E) 0.859 mg BDE-209/kg liver**

Fig. 4 – Histopathological changes in the liver induced by decabromineted diphenyl ether (BDE-209) after 28 days oral exposure; (HE, 40×): A) a – radially placed hepatocytes, b – unchanged hepatocyte; B) a – no histological lesions found, b – hepatocytes in different phases of mitosis; C) a – small number of polymorphonuclears around dilated blood vessel, b – local bleedings in sinusoids; D) a – moderate focal bleeding, b – pathological mitosis of hepatocytes; E) a – discontinuity of vascular wall, b – different phases of hepatocytes degenartion.

med for either internal or external dose of BDE-209 and corresponding $BMDL_{10}$ doses were 0.324 mg BDE-209/kg of liver ww and 812 mg/kg bw/day, respectively.

Oxidative stress parameters

A significant influence of BDE-209 on oxidative stress parameters, MDA, SOD, -SH, in liver homogenates was not confirmed, however internal dose-response relationship was confirmed as a linear model assigned as E1 according to PROAST software (Table 3).

Table 3

Influence of increasing doses of decabrominated diphenylether on oxidative stress parameters in liver homogenates of subacutely exposed Wistar rats

Internal dose (mg/kg)/ dose (mg/kg bw/day)	MDA (nmol/mg of proteins)	-SH groups (nmol/mg of proteins)	SOD (U/g of proteins)
$0/0_{control}$	108.71 ± 47.77	29.47 ± 8.92	103.03 ± 43.64
$0/0_{DMSO}$	127.44 ± 44.01	26.94 ± 14.23	102.75 ± 40.56
0.269/1000	90.91 ± 39.93	27.40 ± 12.94	100.88 ± 21.43
0.569/2000	114.84 ± 57.04	25.40 ± 9.99	100.50 ± 21.77
0.859/4000	161.99 ± 82.12	27.86 ± 3.70	97.69 ± 18.05
	Results on benchmark dose and model internal dose / external dose		
BMD/BMDL	- / -	- / -	- / -
dose response	(+) / (+)	(+) / (+)	(+) / (+)
Model	E1 / E1	E1 / E1	E1 / E1
BMD_5	- / -	- / -	- / -

ANOVA with post-hoc Tukey test was used for statistical analysis; (+) or (-) – dose-response relationship confirmed or not confirmed; E1-E5 – type of dose-response model given by PROAST software; MDA – malondialdehyde; SH – sulfhydryl; SOD – superoxide dismutase; BMD – benchmark dose; BMDL – lower confidence limit of BMD.

Discussion

Determining the relation between external and internal doses, and measurements of BDE-209 concentrations in the liver show that the ratio among the external doses (1 : 2 : 4) is different from the ratio among internal doses (1 : 2 : 3.5), implying the lower absorption when the highest dose is applied. Internal doses correlate with external ones ($r = 0.972$; $p < 0.05$) according to the equation: internal dose (mg BDE-209/kg of liver ww) = 0.0002 × external dose (mg/kg bw/day) + 0.0622. Hepatotoxicity is demonstrated based on significant increase in AST and γ-GT activities, while the lowest $BMDL_5$ is calculated for the serum AST activity. The value of 0.07228 mg BDE-209/kg of liver ww, corresponding to external dose of 39 mg/kg/day, indicates the increase of AST activity as the most sensitive biomarker of BDE-209 hepatotoxicity in subacutely exposed rats. The degree of histological damage increases in the dose-dependent manner, and the corresponding $BMDL_{10}$ dose is 0.324 mg BDE-209/kg of the liver.

For decaBDE the United States Environmental Protection Agency provides no observed adverse effect level (NOAEL) dose of 2.22 mg/kg bw/day as a reference point, however this dose is related with neurobehavioral effects [26–28]. As for the liver, in a 2-year long NTP study (1986), male and female B6C3F1 mice, administered decaBDE in the diet at the doses of 0,3200 or 6,650 mg/kg bw/day for males and 0,3760 or 7,780 mg/kg bw/day for females, the following effects were recorded: liver granulomas and liver hypertrophy in males. Based on these results no NOAEL for liver effects is derived. However, the study identifies a NOAEL for a portal-of-entry effect for females of 3760 mg/kg bw/day [29]. The data sets from another NTP (1986) 2-year rat and mouse studies are selected for BMD modeling: thrombosis in the liver, liver degeneration, in male rats and centrilobular hypertrophy in livers of male mice. The lowest calculated $BMDL_{10}$ was 406 mg/kg bw/day for liver degeneration effect in male rats [29]. Only in one study internal dose, in site, was used for the assessment of BDE-209 subacute oral toxicity in rats [30]. In this experiment internal doses arround 0.25 mg/kg of liver ww induced slight centrilobular hypertrophy in the liver together with increased expression of hepatic CYP1A and CYP2B ($BMDLs_{10}$ 0.5–0.7 mg/kg bw/day) and dose-dependent decrease in serum ALP ($BMDL_{10}$ 0.6 mg/kg bw/day, corresponding to 0.222 mg/kg of liver ww). Histopathological changes seen in our experiment started from the concentration of 0.269 mg/kg of liver ww, which is very close to the value published by Van der Ven et al. [30]. Hepatotoxicity of BDE-209 either *in vivo* or *in vitro* is usually connected with: a) the increased formation of reactive oxygen species, oxidative stress and decreased efficacy of antioxidative defence system [7, 31–34]; induction of apoptosis related with oxidative stress in human hepatoma cells HepG2 [33]; aryl hydrocarbon receptor (AhR) binding particularly expression of luciferase reporter gene mediated by AhR in H4II hepatoma cell line [5]; induction of CYP1A1 mRNA and CYP1A1 protein mediated by AhR [5, 30]. The lack of effect on oxidative stress parameters in our experiment can be understood from the differences, since the values were measured at the end of exposure in which activation of adaptive mechanisms of enzyme or non-enzyme antioxidative defense could take place [35].

Conclusion

The results of the present work add up to the issue of decabrominated diphenyl ether toxicity profile with a focus on the relationship between internal dose and hepatotoxicity. Critical internal dose for the effect on AST of 0.07 mg/kg of liver ww,

corresponding to external dose of 39 mg/kg/day in our study is the lowest dose ever observed in the studies on decabrominated diphenyl ether hepatotoxicity indicating the increase of AST activity as the most sensitive biomarker of decabrominated diphenyl ether hepatotoxicity in subacutely exposed rats. Critical effect based on internal dose in the majority of cases is considered as more precisely defined than the effect established based on external dose, particularly for the persistent substances with low absorption rate, such as decabrominated diphenyl ether.

Aknowledgements

The results are the part of the Project III 46009, Ministry of Education, Science and Technological Development of the Republic of Serbia.

R E F E R E N C E S

1. BSEF. Bromine Science and Environmental Forum. What are brominated flame retardants. 2004. Available from: http://www.bsefsite.com/bromine/what_are_bfrs/index.php
2. *World Health Organization*. Environmental Health Criteria 162. Brominated diphenyl ethers. International Program on Chemical Safety. Geneva, Switzerland: World Health Organization; 1994.
3. *World Health Organization*. Environmental Health Criteria 192. Flame retardants: A general introduction. International Program on Chemical Safety. Geneva, Switzerland: World Health Organization; 1997.
4. *Alaee M, Arias P, Sjödin A, Bergman A*. An overview of commercially used brominated flame retardants, their applications, their use patterns in different countries/regions and possible modes of release. Environ Int 2003; 29(6): 683−9.
5. *ATSDR*. Toxicological Profile for Polybrominated Biphenyls and Polybrominated Diphenyl Ethers (PBBs and PBDEs). Atlanta, US: Agency for Toxic Substances and Disease Registry; 2004.
6. Environmental Protection Agency (EPA). Emerging Contaminants-Polybrominated Diphenyl Ethers (PBDE) and Polybrominated Biphenyls (PBB). 2008. Available from: nepis.epa.gov/Adobe/PDF/P1000L3S.PDF [cited 2008 April].
7. *European Food Safety Authority*. Scientific Opinion on Polybrominated Diphenyl Ethers (PBDEs) in Food. EFSA J 2011; 9(5): 2156−430.
8. *Ćurčić M, Antonijević B, Durgo K, Janković S, Jacević V*. Toxicological relevance and potential risk due to polybrominated diphenylethers exposure. Arch Pharm 2010; 60(3): 311−22.
9. *Alonso V, Linares V, Bellés M, Albina ML, Pujol A, Domingo JL*, et al. Effects of BDE-99 on hormone homeostasis and biochemical parameters in adult male rats. Food Chem Toxicol 2010; 48(8−9): 2206−11.
10. Environmental Protection Agency (EPA). Toxicological Rewiew of Decabromodiphenyl Ether (BDE-209). Washington, DC: Environmental Protection Agency; 2008. Available from: www.epa.gov/iris [cited 2008 June].
11. *Erratico CA, Moffatt SC, Bandiera SM*. Comparative oxidative metabolism of BDE-47 and BDE-99 by rat hepatic microsomes. Toxicol Sci 2011; 123(1): 37−47.
12. *Blanco J, Mulero M, Domingo JL, Sánchez DJ*. Gestational exposure to BDE-99 produces toxicity through upregulation of CYP isoforms and ROS production in the fetal rat liver. Toxicol Sci 2012; 127(1): 296−302.
13. *Ernest SR, Wade MG, Lalancette C, Ma Y, Berger RG, Robaire B*, et al. Effects of chronic exposure to an environmentally relevant mixture of brominated flame retardants on the reproductive and thyroid system in adult male rats. Toxicol Sci 2012; 127(2): 496−507.
14. *Morck A, Hakk H, Orn U, Klasson WE*. Decabromodiphenyl ether in the rat: absorption, distribution, metabolism, and excretion. Drug Metab Dispos 2003; 31(7): 900−7.
15. *Bondy GS, Gaertner D, Cherry W, MacLellan E, Coady L, Arnold DL*, et al. Brominated diphenyl ether (BDE) levels in liver, adipose, and milk from adult and juvenile rats exposed by gavage to the DE-71 technical mixture. Environ Toxicol 2011; 26(6): 677−90.
16. *Ćurčić M, Janković S, Jaćević V, Stanković S, Vučinić S, Durgo K*, et al. Combined effects of cadmium and decabrominated diphenyl ether on thyroid hormones in rats. Arh Hig Rada Toksikol 2012; 63(3): 255−62.
17. *Huwe JK, Hakk H, Birnbaum LS*. Tissue distribution of polybrominated diphenyl ethers in male rats and implications for biomonitoring. Environ Sci Technol 2008; 42(18): 7018−24.
18. *Frederiksen M, Vorkamp K, Thomsen M, Knudsen EL*. Human internal and external exposure to PBDEs-a review of level and sources. Int J Hyg Envir Heal 2009; 212(2): 109−34.
19. *Thuresson K, Bergman A, Jakobsson K*. Occupational exposure to commercial decabromodiphenyl ether in workers manufacturing or handling flame-retarded rubber. Environ Sci Technol 2005; 39(7): 1980−6.
20. *Bradford MM*. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72: 248−54.
21. *Misra HP, Fridovich I*. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. J Biol Chem 1972; 247(10): 3170−5.
22. *Ellman GL*. Tissue sulfhydryl groups. Arch Biochem Biophys 1959; 82(1): 70−7.
23. *Slob W*. Dose-Response Modeling of Continuous Endpoints. Tox Sci 2002; 66(2): 298−312.
24. *European Food Safety Authority*. Use of BMDS and PROAST software packages by EFSA Scientific Panels and Units for applying the Benchmark Dose (BMD) approach in risk assessment. EFSA J 2012; 113: 1−190.
25. *Ćurčić M, Janković S, Jacević V, Stanković S, Vučinić S, Durgo K*, et al. Use of proast software to assess the influence of decabrominated diphenyl ether and/or cadmium on thyroid hormones homeostasis in rats. Arch Pharm 2012; 62(1): 1−13.
26. U.S. EPA (Environmental Protection Agency). Toxicological review of 2,2',3,3',4,4',5,5',6,6'-decabromodiphenyl ether (BDE-209). Integrated Risk Information System (IRIS). Washington DC: National Center for Environmental Assessment; 2008. Available from: http://www.epa.gov/iris
27. *Viberg H, Fredriksson A, Jakobsson E, Orn U, Eriksson P*. Neurobehavioral derangements in adult mice receiving decabrominated diphenyl ether (PBDE 209) during a defined period of neonatal brain development. Toxicol Sci 2003; 76(1): 112−20.
28. *Viberg H, Fredriksson A, Eriksson P*. Changes in spontaneous behavior and altered response to nicotine in the adult rat, after neonatal exposure to the brominated flame retardant, decabrominated diphenyl ether (PBDE). Neurotoxicol 2007; 28(136): 209.
29. *National Toxicology Program*. NTP Toxicology and Carcinogenesis Studies of Decabromodiphenyl Oxide (CAS No. 1163-19-5) In F344/N Rats and B6C3F1 Mice (Feed Studies). Natl Toxicol Program Tech Rep Ser 1986; 309: 1−242.

30. *Van der Ven LT, van de Kuil T, Leonards PE, Slob W, Cantón RF, Germer S,* et al. A 28-day oral dose toxicity study in Wistar rats enhanced to detect endocrine effects of decabromodiphenyl ether (decaBDE). Toxicol Lett 2008; 179(1): 6−14.
31. *Chen J, Liufu C, Sun W, Sun X, Chen D*. Assessment of the neurotoxic mechanisms of decabrominated diphenyl ether (PBDE-209) in primary cultured neonatal rat hippocampal neurons includes alterations in second messenger signaling and oxidative stress. Toxicol Lett 2010; 192(3): 431−9.
32. *Zhang C, Liu F, Liu X, Chen D*. Protective effect of N-acetylcysteine against BDE-209-induced neurotoxicity in primary cultured neonatal rat hippocampal neurons in vitro. Int J Dev Neurosci 2010; 28(6): 521−8.
33. *He Y, Murphy MB, Yu RM, Lam MH, Hecker M, Giesy JP*, et al. Effects of 20 PBDE metabolites on steroidogenesis in the H295R cell line. Toxicol Lett 2008; 176(3): 230−8.
34. *Zhao A, Liu H, Zhang A, Wang X, Zhang H, Wang H*. Effect of BDE-209 on glutathione system in Carassius auratus. Environ Toxicol Pharmacol 2011; 32(1): 35−9.
35. *Matovic V, Dukic D, Plamenac-Bulat Z*. Influence of increased cadmium intake on antioxidative defence system. Yugoslav Med Biochem 2004; 23: 117−26. (Serbian)

Received on February 10, 2014.
Revised on March 18, 2014.
Accepted on March 26, 2014.