Food and Chemical Toxicology 133 (2019) 110750

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Di-n-butyl phthalate, butylbenzyl phthalate, and their metabolites exhibit different apoptotic potential in human peripheral blood mononuclear cells

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ARTICLE INFO

Keywords:
Phthalates
Human peripheral blood mononuclear cells
Apoptosis
Caspase activation
Cytosolic calcium ion level
Transmembrane mitochondrial potential

ABSTRACT

Human peripheral blood mononuclear cells (PBMCs) are one of the main cell models used in studies concerning the exposure of humans (*in vitro*) to various chemical substances. Changes in PBMCs may reflect the general reaction of the organism regarding the effect of xenobiotics.

The aim of this work was to evaluate the effect of di-n-butyl phthalate (DBP), butylbenzyl phthalate (BBP) and their metabolites: mono-n-butylphthalate (MBP), mono-benzylphthalate (MBzP) upon the induction of apoptosis in human peripheral blood mononuclear cells *in vitro*. PBMCs were incubated with the studied compounds at concentrations from 1 to 100 μg/mL for 12 h and/or 24 h.

In order to clarify the mechanism of phthalates-induced programmed cell death, the changes in the calcium ions (Ca^{2+}) level, alterations in the transmembrane mitochondrial potential ($\Delta\Psi m$) and caspase-8, -9, -3 activity as well as externalization of phosphatidylserine have been determined.

An increased Ca^{2+} level and a reduction of the $\Delta\Psi m$ were observed in PBMCs incubated with all of the studied compounds, and particularly with DBP and BBP. Phthalates caused an increase of caspases activity. The most pronounced increase was observed for caspase −9. The most pronounced pro-apoptotic changes were caused by DBP followed by BBP and then by their metabolites.

1. Introduction

A relationship between environmental exposure to endocrine-disrupting chemicals (EDCs) and the development of various diseases has been a subject of particular interest to scientists in recent years. Phthalates are one example of EDCs. Epidemiological studies have indicated a significant exposure to these substances, as they are used in large quantities as plasticizers in many branches of industry [Heudorf et al., 2007]. Phthalates migrate from various products to water, food (oils, spices, rice, fruits, vegetables, fish) [Serrano et al., 2014; Lee et al., 2014] and the air [Pie et al., 2013; Kolena et al., 2014]. Food is the major source of people's exposure to phthalates. The amount of consumed DBP and BBP was estimated at 7–10 μg/kg/day [Kavlock et al., 2002]. Patients using coated medicinal products containing DBP (estimated daily consumption of 1–233 μg/kg/day) and humans occupationally exposed to those compounds (0.1–76 μg/kg/day) are also at risk [Hernández-Díaz et al., 2009; Bahadar et al., 2014; Hines et al., 2011]. Humans are also exposed to phthalates from the air and suspended dust particles [Dodson et al., 2012]. Phthalates are not covalently bonded to polymer chains, but rather their molecules are embedded between the polymer chain molecules, so they can leach out or evaporate into the air or become a component of dust and airborne particles [Jaakkola and Knight, 2008; Schettler, 2006]. The phthalates that are the most often associated with air and dust include DEHP, BBP, and DBP. The BBP air concentration may range from 0.058 ng/m^3 to 3.97 mg/m^3, while the DBP level may reach 1.5–270 ng/m^3 [Kolarik et al., 2008; Pie et al., 2013; Kolena et al., 2014].

Phthalates do not bioaccumulate but are metabolized within a few hours into their corresponding monoesters [Frederiksen et al., 2007]. After entering the organism, BBP is decomposed to monoesters, such as mono-n-butylphthalate (MBP or MnBP) and mono-benzylphthalate (MBzP) in the reactions catalyzed by lipases and esterases. The same enzymes metabolize DBP to MBP [Takahara et al., 2014] (Table 1).

Significant levels of phthalates and their metabolites have been detected in body fluids. In venous blood, the DBP level was determined from 0.051 to 7.67 μg/mL, and in cord blood from 0.0197 to 5.71 μg/mL. BBP levels from 0.82 to 1.97 ng/mL were found in blood serum [Wan et al., 2013]. MBP was detected in samples of amniotic fluid at a concentration of 3.53 μg/L and MBzP at 0.16 μg/L [Huang et al., 2016]. Phthalates have been detected in urine from children and adults all over the world, and their concentrations are from 85.1 to 119.6 nmol/g of creatinine (sum of di-n-butyl phthalate metabolites), while MBzP is in the range from 7.4 to 9.5 ng/mL [Axelsson et al., 2015; Hartmann et al., 2015; Perng et al., 2017]. In Poland, the urinary MBP level of 4.6 μg/g

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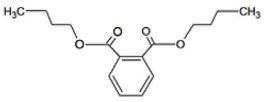
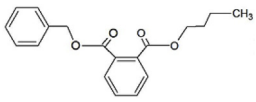
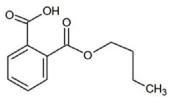
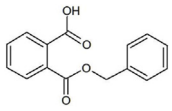
https://doi.org/10.1016/j.fct.2019.110750
Received 27 November 2018; Received in revised form 23 July 2019; Accepted 2 August 2019
Available online 04 August 2019
0278-6915/

Table 1
Physico-chemical characteristic of the studied phthalates (DBP, BBP) and their metabolites (MBP, MBzP).

Phthalate	DBP	BBP	MBP	MBzP
Structure				
Chemical formula	$C_{16}H_{22}O_4$	$C_{19}H_{20}O_4$	$C_{12}H_{14}O_4$	$C_{15}H_{12}O_4$
Molecular weight	278.34	312.36	222.24	256.25
Log P	4.83	5.00	2.72	2.90

creatinine was detected in the study of the female population [Polanska et al., 2016]. Moreover, DBP has been found in breast milk in the range of concentrations from 0.0223 to 0.0932 μg/mL [Chen et al., 2008; Lin et al., 2008; Fromme et al., 2011].

Many epidemiological studies have shown the adverse effects of phthalates on human health. Studies conducted in Europe, Asia, and the US confirm that airborne phthalates DBP, BBP, and their metabolites (MBP, MBzP) are toxic and may cause allergies, asthma, eczema rhinitis, wheezing, and an increased inflammation of the respiratory tract [Bornehag et al., 2014; Kolarik et al., 2008; Just et al., 2015; Larsson et al., 2010; Shu et al., 2014; Whyatt et al., 2014; Just et al., 2012; Ferguson et al., 2014; Hoppin et al., 2013]. For that reason, in 2011, the European Chemical Agency (ECHA) imposed restrictions on the use of phthalates (DEHP, DBP, BBP, DiBP) in products intended for children. According to the EU Commission, HBCDD, DEHP, BBP, DBP, and other substances should be tested once again [Commission-delegated Directive (UE) 2015/863 of 31 March 2015; Commission Regulation (UE) 2015/326 of 2 March 2015].

To explain the associations between the exposure of humans to xenobiotics and changes in the function of organisms, alternative *in vitro* tests are recommended for the initial assessment of toxicity of the chemical substances as well as for the further identification of the underlying cellular mechanisms of their toxicity. A good research model is human peripheral blood because mononuclear blood cells are the first target for the exposure of humans to chemicals and it provides information about the body's overall response to xenobiotics [Dusinska et al., 2017].

Despite these data, there are only a few published studies focused on the explanation of the mechanism of the toxic action of DBP and BBP and their metabolites (MBP and MBzP) in human PBMCs. There are reports in which the expression, secretion of cytokines, ADAM33 gene expression, and methylation in PBMCs were evaluated in cells exposed to DBP, MBP, and MBzP [Glue et al., 2002; Hansen et al., 2015; Yang et al., 2018]. Some studies have shown that increased apoptosis of PBMCs may lead to changes in signaling pathways specific for the immune system [Hartung and Corsini, 2013]. This, in turn, causes changes in the expression of surface markers or cytokine production, which may interfere with the anti-tumor mechanisms of cellular immune response [Starska et al., 2009], activate inflammatory processes [RostamiRad et al., 2018], and consequently leads to a reduction in the immunity of the human body [Weinberg et al., 2004].

In order to achieve more information about the effect of phthalates, such as DBP and BBP and their metabolites (MBP, MBzP) on human PBMCs, the apoptotic and necrotic changes in PBMCs have been assessed. In addition, an attempt was made to explain the mechanism underlying the processes studied. The compounds that were studied were used in concentrations ranging from 1 to 100 μg/mL. Similar concentrations were detected in human blood (DBP approx. 0.02–8 μg/mL), which may reflect the level of the general population exposure [Wan et al., 2013; Chen et al., 2008; Lin et al., 2008]. The concentration of 1 μg/mL was used as the lowest because the exposure of zebrafish embryos to DBP at 0.5–1.7 μg/mL (*in vitro*) caused the reduced formation and function of the immune cells [Xu et al., 2013; Xu et al., 2015]. Higher concentrations of the compounds used in this study corresponded to DBP, MBP, and MBzP levels (2–200 μg/mL) that caused statistically significant changes in the immunological parameters in PBMCs (*in vitro*) [Glue et al., 2002; Hansen et al., 2015].

2. Materials and methods

2.1. Chemicals

Phthalates: di-n-butyl phthalate (DBP), butylbenzyl phthalate (BBP), phthalate metabolites: mono-n-butylphthalate (MBP), monobenzylphthalate (MBzP) (99–99.5% purity) were purchased from Sigma-Aldrich (USA).

Lymphocyte separation medium (LSM) (1.077 g/cm^3) and RPMI 1640 medium with L-glutamine were purchased from Cytogen (Germany). The HBSS solution, bovine serum albumin, bovine calf serum, penicillin streptomycin, pluronic F-127, valinomycin, camptothecin, propidium iodide, calcein-AM, caspase-3 and -8 fluorometric assay kits, caspase-9 chromogenic substrate, and caspase-9 inhibitor were purchased from Sigma-Aldrich (USA). MitoTracker Red CMXRos were purchased from Molecular Probes (USA). Fluo-3/AM was purchased from MoBiTec (Germany). FITC Annexin V Apoptosis Detection Kit (BD Pharmingen™) was purchased from BD Biosciences (USA).

2.2. Cell isolation and treatment

PBMCs were isolated from the leucocyte-buffy coat obtained from blood in the Blood Bank in Lodz, Poland. Blood was taken from 25 healthy volunteers (aged 20–45). Blood was collected from donors in accordance with the announcement of Minister of Health (June 9, 2017) in case of requirements of good practice of blood and its elements collection, analysis, preparation, storage, delivery, and transport for the organizational units of public blood service. The personal data of donors has not been accessible for us. Donors were disqualified in accordance with the criteria contained in the announcement of Minister of Health (June 9, 2017).

The research studies were approved by the Bioethics Committee of the University of Lodz No. 16/KBBN-UŁ/III/2014. PBMCs were isolated using LSM (1.077 g/cm^3) by centrifugation at 600*g* for 30 min at 20 °C. PBMCs were collected, suspended in erythrocyte lysis buffer (150 mM NH_4Cl, 10 mM $NaHCO_3$, 1 mM EDTA, pH 7.4), and incubated for 5 min at 20 °C. Then, PBS was added immediately, and the cells were centrifuged at 200 g for 15 min at 20 °C. The supernatant was decanted, and the cells were washed twice with RPMI with L-glutamine and 10% fetal bovine serum (FBS) at 200 g for 15 min at 20 °C. The cells were resuspended in RPMI medium with L-glutamine, 10% FBS. and penicillin-streptomycin (0.5%). The final PBMCs density used in the experiments (after the addition of the phthalates solution) was 1×10^6 cells/mL. The compounds were dissolved in ethanol. The final concentration of ethanol in negative control samples (without phthalates) and samples treated with individual phthalates was 0.2%. The concentration of ethanol used in the experiments (samples) were not toxic for PBMCs as analyzed by all of the parameters examined.

The final concentrations of the compounds used in the experiments were in a range from 1 to 100 μg/mL. The PBMCs were incubated with xenobiotics for 12 h and/or 24 h. The incubation was performed at 37 °C in a 5% CO_2 atmosphere in total darkness.

2.3. Cell viability calcein-AM/PI staining

The calcein-AM/propidium iodide (PI) viability test is commonly used in toxicity assays to quantify the number of viable cells. The calcein-AM is hydrolyzed to calcein, which has a negative charge and penetrates living cells, staining them green [Papadopoulos et al., 1994]. PI is one of the most widely used fluorescent markers for staining necrotic cells.

PBMCs were incubated with DBP, BBP, MBP, and MBzP in the final concentrations ranging from 2.5 to 100 μg/mL for 12 h and 24 h at 37 °C in total darkness. After incubation, the samples were centrifuged at 300 g for 5 min at 4 °C, the supernatant was decanted, and the cells were suspended in RPMI with L-glutamine and 10% FBS. The samples were treated with calcein-AM and PI in the final concentrations of 0.1 and 1 mM, respectively, and incubated for 15 min at 37 °C in total darkness. The analysis was performed by flow cytometry (Becton Dickinson, LSR II). FCM gate on PBMCs has been established for data acquisition, and the fluorescence was measured with excitation/emission maxima of 494/517 nm and 535/617 nm for calcein and PI, respectively. The data were recorded for a total of 10,000 events per sample.

2.4. Detection of apoptosis and necrosis Annexin V/propidium iodide (PI) staining

As a result of apoptosis, changes occur at the cell surface, which are related to the translocation of phosphatidylserine (PS) from the inner side of the plasma membrane to the outer layer. To detect apoptotic cells, fluorescein-conjugated Annexin V was used. Annexin has a high affinity for cells expressing PS on their surface. Propidium iodide (PI) was used for the detection of necrotic cells. The analysis was done according to the manufacturer's protocol (FITC Annexin V Apoptosis Detection Kit I, BD Pharmingen™).

The samples were incubated with DBP, BBP, MBP, and MBzP in the final concentrations ranging from 2.5 to 100 μg/mL for 12 h and 24 h at 37 °C in total darkness. The cells were stained with PI and fluorescein-conjugated Annexin V in Annexin-binding buffer for 15 min at room temperature in total darkness. The samples were analyzed by flow cytometry (LSR II, Becton Dickinson) with excitation at 488 nm to visualize fluorescein and PI fluorescence at a maxima of 525 nm and 617 nm, respectively. In the cells, apoptosis was induced with 10 μM of camptothecin (positive control). An FMC gate on PBMCs has been established for data acquisition, and the data was recorded for a total of 10,000 events per sample. The exemplary dot plots were made using the FlowJo 7.6.1 program.

2.5. Cytosolic calcium ions level

The analysis was carried out according to the manufacturer's protocol (Fluo-3/AM MoBiTec Germany). PBMCs were incubated with DBP, BBP, MBP, and MBzP in the final concentrations ranging from 1 to 50 μg/mL for 12 h at 37 °C in total darkness. After incubation, the cells were centrifuged at 300 g for 5 min at 4 °C, suspended in the Fluo-3AM solution (1 mM) and incubated for 20 min at 37 °C in total darkness. Then, HBSS (with 1% of BSA) was added to the PBMCs, and the cells were incubated for 40 min at 37 °C in total darkness. The cells were centrifuged at 300 g for 5 min at 4 °C and washed twice with a HEPES buffer. After centrifugation, the cells were suspended in HEPES buffer and incubated for 10 min at 37 °C in total darkness. Ionomycin (1 mM) was used in positive control samples. The samples were analyzed by flow cytometry (LSR II, Becton Dickinson) with excitation at 488 nm to visualize the Fluo-3 fluorescence at 525 nm. An FMC gate on PBMCs has been established for data acquisition, and the data was recorded for a total of 10,000 events per sample. An exemplary histogram was made using the FlowJo 7.6.1 program.

2.6. Mitochondrial transmembrane potential ($\Delta\Psi m$)

Mitochondrial transmembrane potential was indicated in red fluorescence intensity of MitoTracker Red CMXRos (excitation/emission maxima 579/599 nm). The analysis was done according to the manufacturer's protocol (MitoTracker™ Red CMXRos, Molecular Probes). This compound is a cell-permeable probe containing mildly thiol-reactive chloromethyl moiety for mitochondrial labeling. Nigericin and valinomycin (1 mM) were used as positive controls. PBMCs were incubated with DBP, BBP, MBP, and MBzP in the final concentrations ranging from 1 to 50 μg/mL for 12 h at 37 °C in total darkness. After that, the samples were centrifuged at 300 g for 5 min at 4 °C. The supernatant was decanted, and the cells were stained with MitoTracker CMXRos in PBS (1 mM) for 20 min at 37 °C in total darkness. The cells were centrifuged at 300 g for 5 min at 4 °C, suspended in PBS and analyzed. Changes in this parameter were assessed by flow cytometry (LSR II, Becton Dickinson). FMC gate on PBMCs has been established for data acquisition, and the data were recorded for a total of 10,000 events per sample. An exemplary histogram was made using the FlowJo 7.6.1 program.

2.7. Caspase-8, -9 -3 activation

Analysis of caspase-3 and-8 was carried out according to the manufacturer's protocols (Caspase-3 Assay Kit, Fluorometric, Sigma-Aldrich; Caspase-8 Assay Kit, Fluorometric Sigma-Aldrich). The caspases fluorometric assays were based on the hydrolysis of the peptide substrates acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (Ac-DEVD-AMC) by caspase-3 and acetyl-Ile-Glu-Thr-Asp-7-amino-4 methylcoumarin (Ac-IETD-AMC) by caspase-8, which resulted in the release of the fluorescent 7-amino-4-methylcoumarin (AMC) moiety. The excitation and emission wavelengths of AMC were 360 nm and 460 nm, respectively.

Analysis of caspase-9 was carried out according to the manufacturer's protocols (Caspase-9 Colorimetric Activity Assay Kit, LEHD Sigma-Aldrich). The caspase-9 colorimetric assay was based on the hydrolysis of the substrate acetyl-Leu-Glu-His-Asp-p-nitroaniline (Ac-LEHDpNA), which resulted in the release of p-nitroaniline (pNA) wherein absorbance was analyzed at 405 nm. Camptothecin (10 mM) was used as a positive control.

The analysis of caspase-3 and -8 activities was carried out using a fluorescent microplate reader (Fluoroskan Ascent FL, Labsystem), and the detection of caspase-9 activity was performed using an absorbance microplate reader (BioTek ELx808, Bio-Tek).

2.8. Statistical analysis

The results were shown as mean ± SD, achieved from 5 individual experiments (5 blood donors). For each individual experiment (donor), an experimental point was a mean value of 2–3 replications. Multiply comparisons among the group mean differences were analyzed by a one-way analysis of variance (ANOVA) followed by a Tukey's post-hoc test. When the p value was lower than 0.05, the differences were considered to be statistically significant. Finally, the 'sample size' and the 'power of test' for all the data was checked. Statistical analysis was conducted using STATISTICA software ver.13 (StatSoft Inc., Tulsa, OK USA).

Table 2
Changes in cell viability in human PBMCs incubated with DBP, BBP, MBP or MBzP in the concentrations range from 2.5 to 100 μg/mL for 12 and 24 h (*) Statistically significant different from control samples ($p < 0.05$). Each value represents the mean ± SD obtained from 5 independent experiments (5 donors).

Concentration	Viable cells (%)	12 h	12 h	12 h	Viable cells (%)	24 h	24 h	24 h
(μg/mL)	DBP	BBP	MBP	MBzP	DBP	BBP	MBP	MBzP
0	98.55 ± 1.87	98.55 ± 1.87	98.55 ± 1.87	98.55 ± 1.87	95.15 ± 2.36	95.15 ± 2.36	95.15 ± 2.36	95.15 ± 2.36
2.5	93.90 ± 4.33	93.58 ± 3.23	98.15 ± 1.40	97.61 ± 3.58	96.60 ± 2.47	93.60 ± 2.81	97.34 ± 3.36	97.77 ± 3.22
5	94.42 ± 3.97	93.92 ± 2.87	95.65 ± 2.73	95.15 ± 2.70	83.26 ± 4.84*	88.86 ± 3.58	90.43 ± 3.63	94.05 ± 3.44
10	95.94 ± 1.77	92.26 ± 3.31	91.74 ± 2.99	92.71 ± 4.94	82.05 ± 3.57*	86.90 ± 3.97*	86.51 ± 1.94	91.59 ± 2.50
20	91.24 ± 2.79	91.48 ± 1.90	90.09 ± 2.24	90.69 ± 3.56	72.73 ± 3.11*	78.08 ± 3.83*	80.86 ± 2.38*	88.97 ± 3.58*
50	89.07 ± 4.40	86.68 ± 4.09*	88.12 ± 6.38	86.29 ± 6.80	69.27 ± 4.29*	65.91 ± 5.50*	75.38 ± 1.99*	83.30 ± 2.49*
100	76.64 ± 4.42*	83.41 ± 3.08*	85.17 ± 5.89	84.64 ± 7.16	56.84 ± 2.95*	52.12 ± 5.61*	70.12 ± 1.75*	77.42 ± 2.41*

3. Results

3.1. Cell viability

Changes in the viability of PBMCs were assessed by flow cytometry with calcein-AM and propidium iodide (PI) staining. Two incubation times were used: 12 h and 24 h, with the phthalate concentrations range of 2.5–100 μg/mL. After 12 h of incubation, statistically significant changes were observed for DBP, starting from the concentration of 100 μg/mL, with viability reduced by approx. 23%. For BBP, statistically significant changes were observed from the concentration of 50 μg/mL, with the viability reduced by approx. 13%, and by 16% at the concentration of 100 μg/mL. No statistically significant changes were observed after that time of the PBMCs incubation with phthalate metabolites (Table 2).

After 24 h of incubation, the statistically significant reduction of cell viability was observed for all the compounds studied. DBP demonstrated the most pronounced activity, causing a 17% reduction of cell viability already at the concentration of 5 μg/mL. BBP caused similar changes at the concentration of 10 μg/mL. The statistically significant reduction of cell viability caused by metabolites was observed from 20 μg/mL upwards, and it was 19% and 11% for MBP and MBzP, respectively (Table 2).

3.2. Apoptotic/necrotic changes

Apoptotic and necrotic changes are associated with changes in the permeability of the PBMCs cellular membrane. They were assessed by flow cytometry with anexin-V and propidium iodide (PI). Two incubation times were used: 12 h and 24 h, with concentrations ranging from 2.5 to 100 μg/mL. After 12 h of incubation, a statistically significant increase of the number of apoptotic cells was observed starting from the concentration of 50 μg/mL for DBP and BBP. A minor increase of apoptotic cell count was observed starting from the 100 μg/mL for MPB and MBzP (Fig. 1). No statistically significant increase in the number of necrotic cells was observed for any phthalate after that incubation time (Fig. 1).

The 24 h incubation resulted in statistically significant apoptotic changes in the cell exposed to DBP, starting from a concentration of 5 μg/mL. The increase was approx. 6%. BBP caused a 7% increase in the number of apoptotic cells starting from 10 μg/mL. For metabolites, an increase in the number of apoptotic cells by 9% and 6% was observed from the level of 20 μg/mL, for MBP and MBzP, respectively. At the highest applied concentration of 100 μg/mL, the number of apoptotic cells increased by 34%, 26%, 15%, and 11% for DBP, BBP, MBP, MBzP, respectively (Fig. 1). The statistically significant increase of the number of necrotic cells after 24 h of incubation was observed first for BBP, by 3%, starting from the concentration of 20 μg/mL, and then for DBP, MBP, MBzP, by approx. 2.5%, from 50 μg/mL. At the highest applied concentration, the most pronounced necrotic changes were caused by BBP (an increase of 10%), followed by DBP (7%) and both metabolites (approx. 4%) (Fig. 1.)

3.3. Cytosolic calcium ion level

The Fluo-3/AM marker was used for the determination of the cytosolic calcium ion level. After binding to calcium ions, the marker exhibits a green fluorescence. Considering the fact that the calcium ion level increases earlier than the increase of phosphatidylserine, externalization was observed in the tests that were conducted after 12 h of incubation by using the concentrations of 1–50 μg/mL. Already at 1 μg/mL, a statistically significant 12% increase of the calcium ions level was observed for DBP. Other phthalates caused statistically significant changes in the parameter studied increase, starting from a concentration of 5 μg/mL, by 23%, 20%, 17% for BBP, MBP, and MBzP, respectively. At the highest concentration, the most pronounced increase of Ca^{2+} versus control was caused by DBP (by 73%), BBP (by 48%), MBP (by 31%), and MBzP (by 27%) (Fig. 2).

3.4. Changes in transmembrane mitochondrial potential ($\Delta\Psi m$)

Transmembrane mitochondrial potential was assessed using the MitoTracker Red CMXRos marker. Changes in the fluorescence intensity of that marker are associated with changes of $\Delta\Psi m$. The study demonstrated a reduction of $\Delta\Psi m$ for all researched phthalates. However, DBP was the first one to cause the statistically significant reduction of transmembrane mitochondrial potential by 10%, starting from the concentration of 1 μg/mL. Other compounds caused the reduction of the parameter examined starting from the 5 μg/mL, by 23%, 21%, and 6% for BBP, MBP, and MBP, respectively (Fig. 3).

3.5. Activation of caspase-9, -8, -3

The activity of selected caspases was determined after 24 h of incubation with phthalates and their metabolites at concentrations ranging from 2.5 to 50 μg/mL. All three experiments involved pre-incubation with an inhibitor of individual caspase. The addition of inhibitors caused a reduction in enzyme activity at or below control values (data not published).

The statistically significant increase of caspase-8 activity versus control was observed for all the phthalates studied, starting from a concentration of 20 μg/mL. The highest increase at that concentration was observed for BBP (by 33%). At a concentration of 50 μg/mL, DBP was observed to cause a higher increase of the enzyme activity – by 47% in relation to the control. For the phthalate metabolites MBP and MBzP, the highest increase of the enzyme activity was observed for the concentration of 20 μg/mL - by 25% and 27%, respectively (Fig. 4).

For caspase-9, statistically significant changes in relation to the control were observed starting from 2.5 μg/mL for phthalates and their metabolites. The highest increase was caused by DBP (by 28%), followed by BBP (by 22%), MBzP (by 18%), and MBP (by 12%). It should be emphasized that changes in caspase-9 activity after treatment with

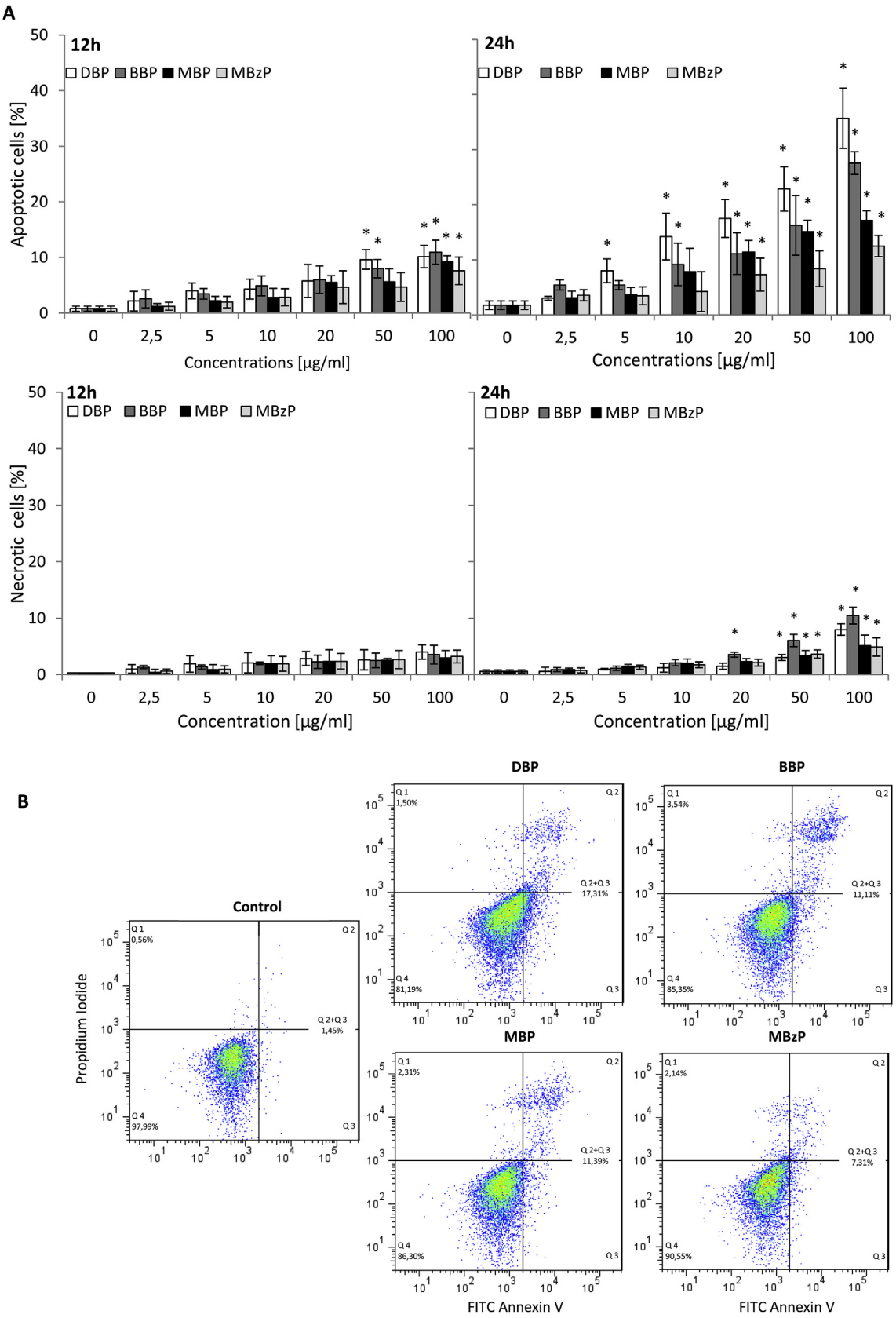


Fig. 1. Apoptotic and necrotic changes in human PBMCs incubated with DBP, BBP, MBP or MBzP in the concentrations range from 2.5 to 100 µg/mL for 12 and 24 h (A). Exemplary dot plot depicting apoptotic and necrotic changes in human PBMCs unexposed (control) and exposed to phthalates and their metabolites at 20 µg/mL for 24 h, Q_1 – necrotic cells, $Q_2 + Q_3$ – apoptotic cells, Q_4 – live cells (B). (*) Statistically significant different from control samples ($p < 0.05$). Each value represents the mean ± SD obtained from 5 independent experiments (5 donors).

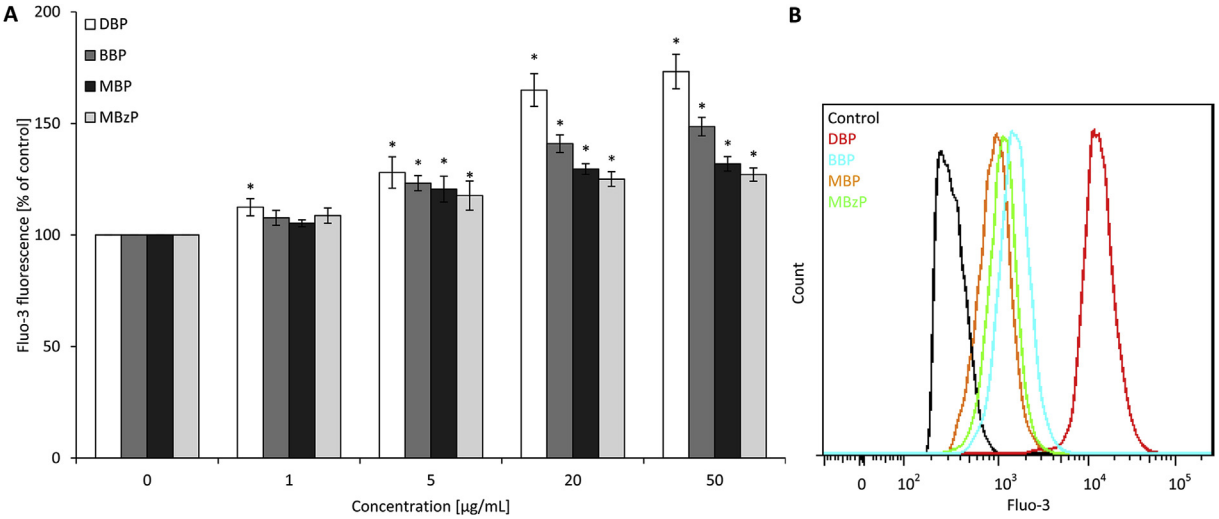


Fig. 2. Changes in cytosolic calcium ions level in human PBMCs incubated with DBP, BBP, MBP or MBzP in the concentrations range from 1 to 50 μg/mL for 12 h (A). Exemplary histogram depicting changes in Fluo-3 fluorescence (calcium ions level) in human PBMCs unexposed (control) and exposed to phthalates and their metabolites at 1 μg/ml for 12h (B). (*) Statistically significant different from control samples ($p < 0.05$). Each value represents the mean ± SD obtained from 5 independent experiments (5 donors).

phthalates at specified concentrations were statistically significant compared to changes in the caspase-8 activity at the same concentrations (Fig. 4).

The increase of caspase-3 activity in all cases depended on the concentration of applied phthalates. The higher increase of the activity of that enzyme was caused by DBP – already at a level of 2.5 μg/ml, by 17%, and at 50 μg/mL, the increase was already 89%. Slightly lesser changes, compared to DBP, were caused by BBP. In the case of that compound, the increase of the activity of caspase-3 was 15%, 54%, and 75%, respectively, for the concentrations of 2.5, 20, and 50 μg/mL. In case of the phthalate metabolites MBP and MBzP, the increase of caspase-3 activity was approx. 10% for the concentration of 2.5 μg/mL, and 54% and 47% for the concentration of 50 μg/mL, respectively (Fig. 4).

4. Discussion

This study demonstrated that a decrease in PBMCs' viability depends on the time of incubation and the concentration of a tested xenobiotic. A time of 12 h of the incubation led to a reduction of cell viability first by BBP followed by DBP. After 24 h of incubation of PBMCs with the xenobiotics studied, DBP was the compound causing the earliest changes in cell viability, followed by BBP, and then by both metabolites (MBP and MBzP) (Table 2). Other studies regarded the effect of monobutyl phthalate (MBP) - a metabolite of DBP - on Sertoli cells. At a concentration of 10 mM, this compound caused a statistically significant reduction of cell viability following 24 h of incubation, and the EC_{50} value was 16.21 mM [Hu et al., 2012; Hu et al., 2014]. Other studies have demonstrated that DBP reduced the *de novo* formation of macrophages [Xu et al., 2013; Xu et al., 2015]. A decrease in PBMCs' viability was caused by monophthalates such as MBP and MBzP in the concentrations above 20 μg/mL [Glue et al., 2002]. Taking into

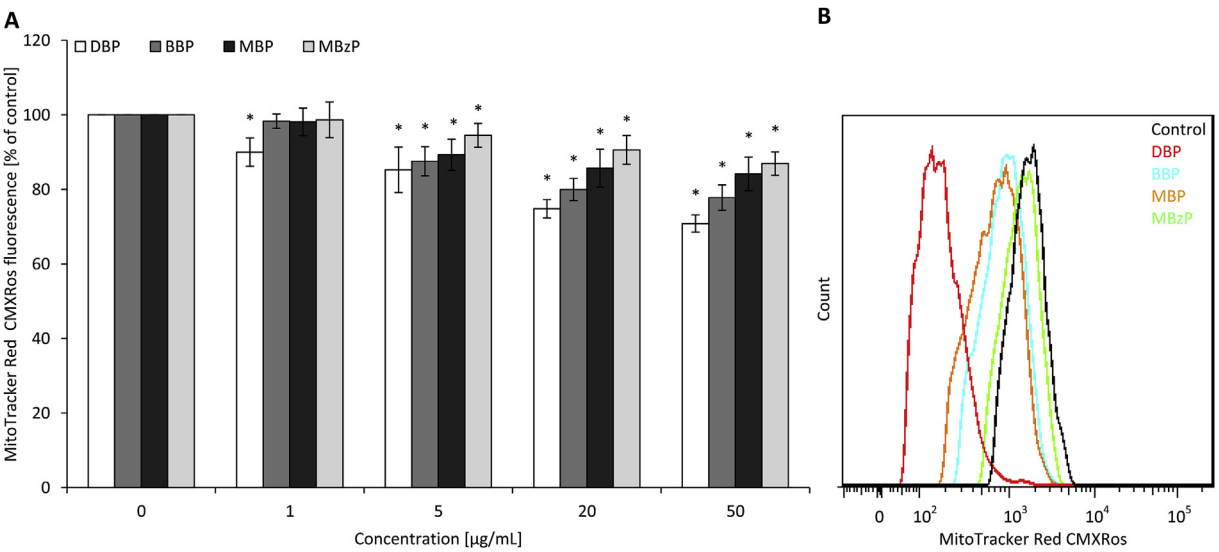


Fig. 3. Changes in transmembrane mitochondrial potential (ΔΨm) in PBMCs incubated with DBP, BBP, MBP or MBzP in the concentrations from 1 to 50 μg/mL for 12 h (A). Exemplary histogram depicting changes in ΔΨm in human PBMCs unexposed (control) and exposed to phthalates and their metabolites at 1 μg/ml for 12 h (B). (*) Statistically significant different from control samples ($p < 0.05$). Each value represents the mean ± SD obtained from 5 independent experiments (5 donors).

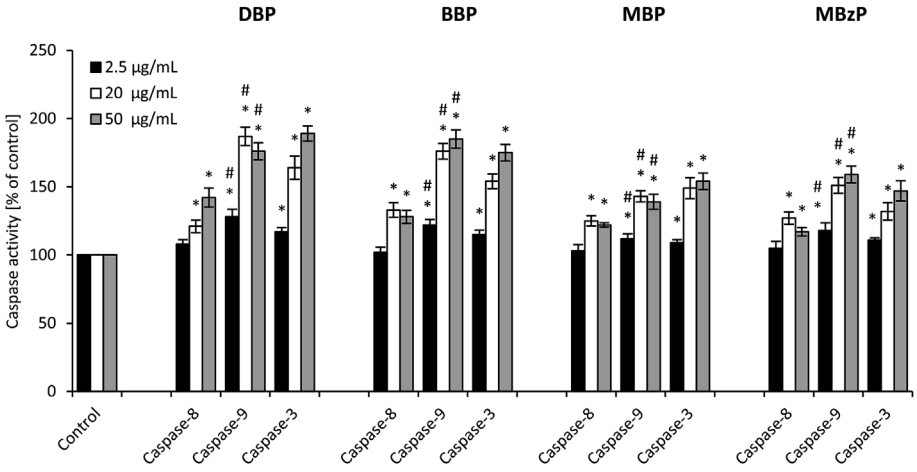


Fig. 4. Changes in the activity of caspase-8, −9 and −3 in human PBMCs incubated with DBP, BBP, MBP or MBzP in the concentrations range from 2.5 to 50 µg/mL for 24 h (*) Statistically significant different from control samples ($p < 0.05$). (#) Changes in caspase-9 activity statistically significant in relation to changes in caspase-8 activity at the same concentrations ($p < 0.05$). Each value represents the mean ± SD obtained from 5 independent experiments (5 donors).

consideration the literature data and the results of this study, it may be concluded that phthalates, which are often used in this industry, reduce cell viability.

Cell viability reduction is inseparably associated with cellular death. Two main mechanisms responsible for cellular death are apoptosis and necrosis. The factors triggering both processes may be similar, but the course of necrosis and apoptosis, their biochemical pathways, and morphological changes are largely different. Those changes lead to cell phagocytosis by macrophages [Elmore, 2007]. Therefore, the next stage of this study focused on the assessment of the level of apoptotic and necrotic changes in PBMCs exposed to the examined phthalates. A higher rate of apoptosis was observed after 12 h of incubation of the cells with DBP and BBP, than with their metabolites. No necrotic changes were observed after that time of incubation for any of the compounds studied. After 24 h, DBP also caused the most significant apoptotic changes, but the strongest necrotic properties were demonstrated by BBP (Fig. 1). Studies on other cell types confirm the higher pro-apoptotic properties of DBP compared to other phthalates. In murine macrophages, DBP caused a concentration-dependent apoptosis. A similar effect was not observed for BBP [Naarala and Korpi, 2009]. Studies concerning the effect of DBP and its metabolite MBP on murine antral follicles demonstrated that DBP, at a concentration of ≥10 µg/mL, caused a decreased growth of antral follicles and increased cytotoxicity associated with the activation of cyclin-dependent kinase inhibitor-induced inhibition of cell-cycle progression and the initiation of apoptosis, but a similar effect was not observed for MBP [Craig et al., 2013; Rasmussen et al., 2017]. Sen et al. (2015), studying the effect of DBP on the cell death of antral follicles in mice, observed the increased level of mRNA coding pro-apoptotic genes (Bax, Bad, and Bid). Other scientists studying the effect of DBP *in vitro* on Sertoli cells suggested two mechanisms of apoptosis: the first one – associated with increased intracellular calcium, ROS, and caspase-3 levels, and the second one – associated with the PI3K/AKT/mTOR signaling pathway [Wang et al., 2017; Alam et al., 2010]. According to the literature data, the second phthalate – BBP – is also able to induce apoptosis [Alam et al., 2010; Alam and Kurohmaru 2016]. It was also demonstrated that BBP caused the necrosis of human granulosa cells through CYP1B1 gene expression (regulates the cell cycle, apoptosis, and cell adhesion) and the AhR transcription factor [Chen et al., 2012].

It has been proven that the key role in the process of apoptosis is played by calcium ions that induce calcium-susceptible pro-apoptotic enzymes. Calcium is accumulated in mitochondria, endoplasmic reticulum (ER), and the nucleus. Disturbance of calcium homeostasis leads to the outflow of Ca^{2+} ions from cellular compartments into the cytosol. The consequence of changes in the calcium ion level is programmed cell death [Hajnoczky et al., 2003; Kroemer and Reed, 2000]. In this study, DBP caused a statistically significant increase of the calcium ion level, even at a concentration of 1 µg/mL, while BBP and metabolites changed this parameter at a concentration of 5 µg/mL (Fig. 2). Zhang et al. (2016) also observed an increase of the intracellular calcium ions level following the action of DBP on male germ cells. Despite the authors having no direct evidence on the association between the exposure to DBP and the ER stress in male germ cells, they supposed that the increased intracellular Ca^{2+} and ROS levels contributed to the induction of the ER stress and apoptosis. Dysfunction of ER may lead to the dysfunction of mitochondria. Calcium ions released from ER may produce some specific Ca^{2+} microdomains located between mitochondria and ER. Consequently, the mitochondrial uptake and accumulation of Ca^{2+} leads to the reduction of the transmembrane mitochondrial potential [Kaufman et al., 2002]. This study demonstrated that all the compounds studied caused a reduction of $\Delta\Psi m$ in PBMCs. However, parent phthalates, and particularly DBP, demonstrated a stronger effect (Fig. 3). The calcium ion level outflow from mitochondria and the reduction of the transmembrane mitochondrial potential are responsible for the release of pro-apoptotic factors, including the apoptosis-inducing factor (AIF), cytochrome *c*, smac/DIABLO, or caspases into cytosol [Giorgi et al., 2012]. For that reason, changes in the activity of caspases including caspase-3, -8, and -9 were assessed.

The study demonstrated the activation of caspase-3 under the influence of phthalates used in the study. Parent compounds demonstrated a stronger activity (with DBP causing changes earlier than BBP) compared to their metabolites (Fig. 4.). Similar changes were observed by other researchers. Pan et al. (2014) demonstrated that DBP induced a greater apoptosis *via* caspase-3 activation in keratinocytes and fibroblasts than DEP and DEHP. In addition, DBP demonstrated lower stimulation of the inflammatory process compared to other phthalates, which suggested, for that particular phthalate, that apoptosis, not necrosis, was the key process in the removal of the cells. Other studies have shown that DBP, at micromolar concentrations, stimulated the activity of caspase-3 in mouse neocortical neurons in primary cultures [Wójtowicz et al., 2017]. To find a preliminary explanation of whether the process of phthalate-induced apoptosis involved the internal pathway (mitochondrial) or the external one (receptor), the activity of caspase-9 and -8 was assessed. This study demonstrated the increased activity of both enzymes studied in PBMCs exposed to phthalates. More pronounced changes were observed for DBP and BBP. Moreover, a higher activity of caspase-9 was demonstrated, compared to caspase-8, which suggested that mainly mitochondrial pathways were involved in the phthalates induced apoptosis (Fig. 4.).

This study demonstrated that phthalates (DBP, BBP) caused apoptotic changes earlier and at lower concentrations than their metabolites (MBP, MBzP). The observed higher toxicity of parent compounds, compared to their metabolites, may be associated with the lipophilicity

of these compounds. Cellular membranes are easily penetrated by lipophilic substances characterized by a high logP coefficient (the logarithm of the octanol/water partition coefficient). LogP for DBP and BBP is higher than for MBP and MBzP (Table 1), which may explain a faster effect of those compounds at lower concentrations [Zhang et al., 2015]. Other studies on phthalates have found that their toxic effect may be associated with their alkyl side chain length [Glue et al., 2005; Jepsen et al., 2004; Larsen et al., 2002]. These data could explain the stronger toxic potential of DBP and BBP (as they possess a higher number of carbon atoms in side chains and an additional aromatic ring) compared to their metabolites.

5. Conclusion

This study demonstrated for the first time that DBP, BBP, and their metabolites MBP and MBzP caused a reduction of PBMCs viability and induced apoptosis and necrosis in this cell type. These compounds exhibited different apoptotic and necrotic activity, as DBP had the higher pro-apoptotic potential, while BBP mainly had the pro-necrotic one. The mitochondrial pathway was mainly involved in the process of apoptosis. Phthalate metabolites induced lower changes in PBMCs compared to their parent compounds.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was granted from the Research 505/387/R admitted for the Department of Biophysics of Environmental Pollution University of Lodz.

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