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Original Research

Characterization of Genotoxic Activity of Phthalates and Their Metabolites by the UmuC *in vitro* Assay

Radosław Czernych[1]*, Milena Chraniuk[2], Lidia Wolska[2]

[1]Department of Hygiene and Epidemiology, Faculty of Medicine, Medical University of Gdańsk, Dębinki Str 7, 80-211 Gdansk, Poland
[2]Department of Environmental Toxicology, Faculty of Health Sciences, Medical University of Gdańsk, Dębowa Str 23A, 80-204 Gdansk, Poland

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Abstract

The presented study investigates and compares the genotoxic potential of four commonly used diesters of phthalic acid (phthalates) in the presence and absence of S9 metabolic activation by using the SOS/umuC assay on *Salmonella typhimurium* TA1535 [pSK1002]. Phthalates are plasticizers with a wide range of applications for manufacturing plastic and non-plastic products. Therefore, phthalates are identified as contaminants in a variety of consumer products. The results showed that phthalates did not induce mutations without S9 metabolic activation (with the exception of BBzP). The genotoxic activity was further studied with the S9 metabolic activation. Assessment of the genotoxic potential indicates a significant ($p<0,05$ for DEP, DBP and BBzP) and highly significant ($p<0,01$ for DEHP) increase of this potential for each phthalate sample.

Keywords: diphthalates, metabolites, metabolic activation, genotoxicity, *in vitro* assay

Introduction

Phthalates are broadly used plasticizers that have a wide range of applications. They are used for manufacturing plastic (PVC) as well as non-plastic products. Since phthalates are not chemically bonded with polymer they can easily migrate to almost any environment they get in contact with. Therefore, their presence has been monitored in food, indoor air, cosmetics as well as medical devices [1-4].

After absorption, phthalates undergo biotransformation into monoesters (Fig. 1). This process usually occurs in a gastrointestinal tract, kidneys and liver. Low molecular weight phthalates such as DMP, DEP, DBP or BBzP are converted to monoesters (ester hydrolysis) in the first phase biotransformation. Studies show that low molecular weight phthalates are excreted as metabolites in approximately 80-90% [5, 6]. The first phase biotransformation process is catalyzed by lipases and esterases and takes place in the intestines and liver parenchymal cells [6, 7]. High molecular weight phthalates are initially metabolized to monoesters, followed by the second phase, i.e. enzymatic oxidation of alkyl chain (hydrophilic metabolites) and are

*e-mail: r.czernych@gumed.edu.pl

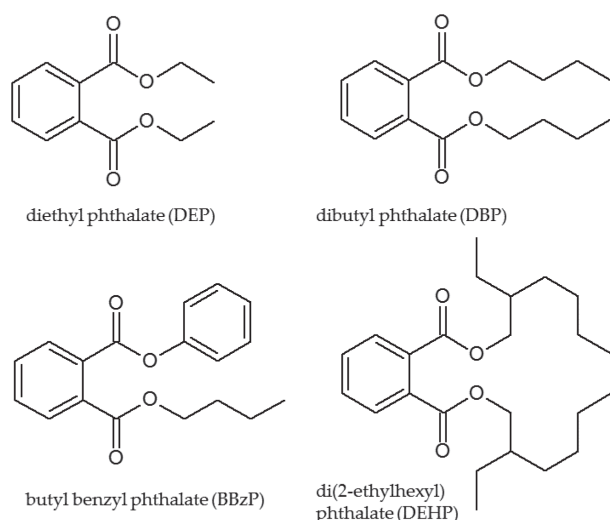

Fig. 1. Chemical structures of: DEP, DBP, BBP, DEHP.

conjugated with glucuronic acid in the presence of uridine-5'-diphoso-glucuronyltransferase. In this way, hydrophilic glucuronide conjugates (oxidative metabolites) are formed [8]. Some phthalates have more than one metabolite (Table 1).

Phthalates are a common chemical compounds used to enhance plasticity of industrial polymers [9]. They are present in many types of products e.g. cosmetics, building materials, furnishings, pharmaceuticals etc. [10]. The exemplary chemical structures of commonly used phthalates are presented in Fig. 2. Humans can be exposed to phthalates in various ways (food, inhalation and dermal way) [11] but health effects (especially genotoxicity) caused by phthalates and its monoester metabolites are still not clearly understood. Literature mostly focuses on genotoxic effects of DEHP and MEHP [7, 12, 13] which is considered as metabolite with the highest genotoxicity. But not only MEHP is present in urine samples collected from people exposed to DEHP. R. Hauser et al. mentions 2 additional metabolites: mono-(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP) and mono-(2-ethyl-5-oxohexyl) phthalate (MEOHP) which had higher concentrations in urine than MEHP [14]. Moreover, Third National Report on Human Exposure to Environmental Chemicals [15] informs about metabolite of diethyl phthalate (DEP) - monoethyl phthalate (MEP) which was found in samples collected from people exposed to mixtures of phthalates. Gray et al. reported MEP has no genotoxic properties [16] but next research presents that this metabolite can damage sperm DNA which was confirmed in COMET assay [14]. DNA damages after exposures to phthalates can occur in many ways. DEHP and MEHP are responsible for chromosomal break in human lymphocytes [17]. In addition DEHP can stimulate polyploidy and aneuploidy in human fetal lung cells [18]. Possible is phthalates interaction with estrogenic receptors [19] which can cause changes in genes expression and promote of hepatic carcinogenesis in rodents [20]. Some of phthalates can occur antiandrogenic activity – they block hAR activation [21]. More effects can be observed for modifications in HPRT gene in chromosome X which is a model of DNA damages in mammalian cell lines. A good example is dibutyl phthalate (DBP) which causes increase of mutated HPRT gene amount [22]. But not every phthalate and phthalate metabolite [23] which interfere in DNA is responsible for HPRT gene modification, e.g. MEHP causes chromosome damage,

Table 1. Diester phthalates and their metabolites [44].

Phthalate (abbreviation)	Metabolite (abbreviation)
dimethyl phthalate (DMP)	monomethyl phthalate (MMP)
diethyl phthalate (DEP)	monoethyl phthalate (MEP)
di-n-butyl phthalate (DBP)	monobutyl phthalate (MBP)
diisobutyl phthalate (DiBP)	monoisobutyl phthalate (MiBP)
butylbenzyl phthalate (BBzP)	monobenzyl phthalate (MBzP)
di(2-ethylhexyl) phthalate (DEHP)	mono(2-ethylhexyl) phthalate (MEHP)
	mono(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP)
	mono(2-ethyl-5-oxohexyl) phthalate (MEOHP)
	mono(2-ethyl-5-carboxypentyl) phthalate (MECPP)
	mono(2-carboxymethylhexyl) phthalate (MCMHP)
diizononyl phthalate (DiNP)	monoisononyl phthalate (MiNP)
	mono(hydroxyisononyl) phthalate (MHiNP)
	mono(oxoisononyl) phthalate (MOiNP)
	mono(carboxyisooctyl) phthalate (MCiOP)

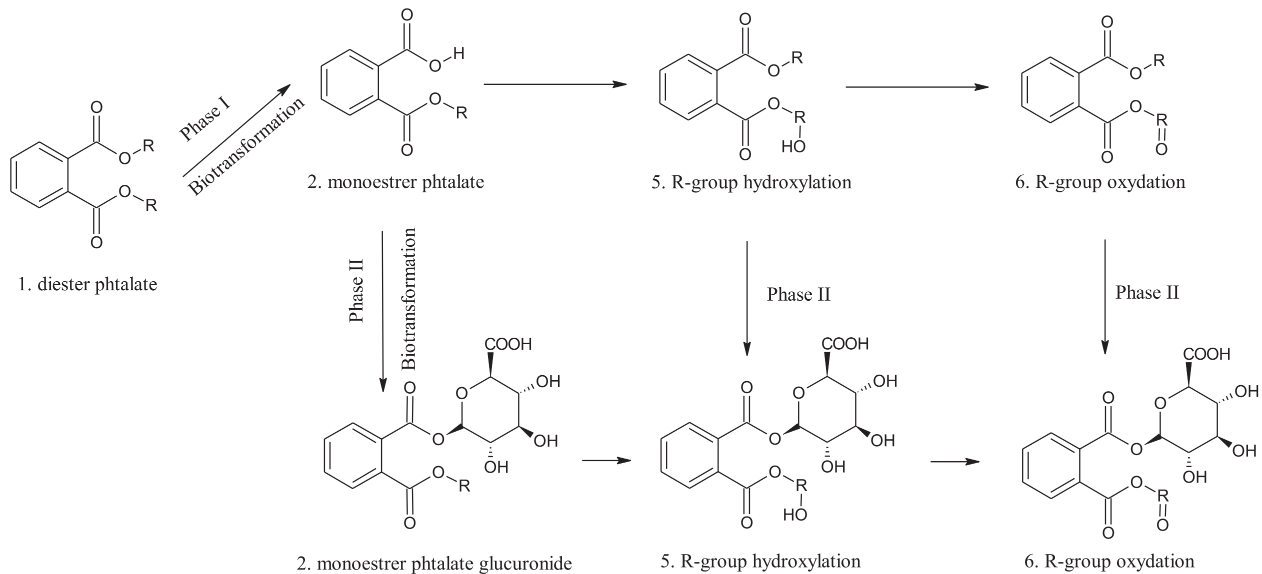

Fig. 2. Metabolic pathway of diester phthalates.

but does not affect HPRT genes in Chinese hamster (CH) cells. Genotoxicity assessment of phthalates is difficult, results can be different and depend on type of test also research model (cell line, tissue, organism). Phthalates can provide various results for investigated DNA damage which can be seen in Table 2.

Most of research of phthalates genotoxicity is carried *in vitro*. Application of *in vitro* assays offer several advantages. *In vitro* bioassays provide high-throughput, short-term, and low-cost measurements of genotoxic potential of new chemicals before marketing, thus preventing possible massive use of hazardous substances and materials. The assays can also be applied to identify genotoxic or mutagenic activity of chemicals that are already in use in order to identify the cause of an endocrine effect observed either in wildlife or in humans. Finally, the *in vitro* assays can also be used to assess genotoxic potential of environmental samples (environmental monitoring) regardless to its composition.

The purpose of this investigation was to provide new and comprehensive insight into the genotoxic

Table 2. The effects phthalates on DNA damage using *in vitro* assays.

Phthalate	Type of experiment	Research model	Overview	Conclusion	References
DEHP	*in vitro*	✓ human lymphocytes ✓ human fetal lung cells.	✓ chromosomal breaks, increases or decreases in chromosomal gaps decrease in mitotic rate in human lymphocytes ✓ poliploidy and aneuploidy in human ✓ fetal lung cells	DEHP caused chromosomal damages and changed number of chromosomes	[45]
DEHP	*in vitro*	✓ *Salmonella typhimurium*, ✓ mouse lymphoma ✓ rat hepatocytes	DEHP did not cause any changes in genetic material.	DEHP is non-genotoxic	[46]
MEHP	*in vitro*	✓ CHO cells ✓ RL4 liver cells	chromosomal aberrations in CHO cells and RL4 liver cells.	MEHP caused chromosomal aberrations	[46]
BBP	*in vitro*	✓ CHO cells ✓ Mouse bone marrow cells ✓ *Salmonella typhimurium*,	✓ chromosomal aberrations in Mouse bone marrow cells after 17h. ✓ BBP did not cause any changes in Mouse bone marrow cells after 36h, in *Salmonella typhimurium* and CHO cells genetic material.	BBP can cause chromosomal aberrations	[31]
DBP and DiBP	*in vitro*	oropharyngeal and nasal mucosa	DBP and DiBP induce DNA damages	DBP and DiBP have genotoxic effects	[47]

effects of often used phthalates and their metabolites by applying commercially available UmuC Easy CS (SOS/*umu*) assay. The SOS/*umu* test is based on the ability of DNA-damaging agents to induce *umu*C gene expression in *Salmonella typhimurium* strain TA1535/pSK1002 [24]. This assay has been frequently used to detect the genotoxic effects of chemicals and environmental samples [25-27].

Materials and Methods

Chemicals

DEP (>99% pure, CAS # 84-66-2), DBP (>99% pure, CAS # 84-74-2), BBP(>99% pure, CAS # 85-68-7), DEHP (>99% pure, CAS # 117-81-7), methanol (>99% pure, CAS # 67-68-5) were purchased from Sigma-Aldrich (Steinheim, Germany). The UmuC Easy CS assay kits for concentrated samples were obtained from Xenometrix AG (Allschwil, Switzerland). A special care was taken to avoid the contact of solvents and reagents with plastic materials, thus preventing possible interaction between phthalates while performing the assay. To minimize the risk of secondary contamination, glass materials were used in place of plastic materials anywhere it was possible. All solvents were checked for the presence of phthalates before use. In order to better dissolve samples, prepared solutions (dissolved in 2,5% methanol solution) were placed for 30 minutes in an ultrasonic bath. The standard solutions' genotoxicity was immediately evaluated after the preparation. Each time, after testing, the phthalates' solutions were refrigerated at 4ºC. The standard solutions were not kept for longer than a week.

UmuC Assay

The bacterium mutant *Salmonella typhimurium* TA1535 [pSK1002] is a test organism that is subjected to a 120 minute exposure to a potentially genotoxic compound at 4 concentration levels. In the presence of a genotoxic substance, the cell induces umuC genes. These genes allow the cell to continue DNA replication, despite the presence of damage to the matrix polynucleotide chain. This is the so-called SOS answer. In the pSK1002 plasmid, the umuC genes are associated with the lacZ reporter gene. Induction of the umuC gene simultaneously causes activation of the lacZ gene. The decoded lacZ gene decomposes the colorless ONPG solution to yellow o-nitrophenol in the presence of β-galaktosidase. The intensity of the o-nitrophenol coloration of the plate wells in which the bacteria were incubated in the presence of the test compound was proportional to the magnitude of lesions of the polynucleotides caused by the test compound, ie the genotoxic potential of the test compound. Measurement of the optical density (OD_{600}) before and after a 2-hour incubation period enables calculating the Induction Ratio (IR) and identifying toxic growth inhibitory effects. An IR≥2 was considered as a positive effect (for details, see ISO 13829). In addition, the growth inhibition factor (GF) of bacteria was calculated in order to indicate the cytotoxicity of the samples. For growth factors below 0.5 (50 % of growth inhibition), the results were not valid due to cytotoxicity. Evaluation of the genotoxic potential of the substance takes place directly in the presence or absence of the hepatic fraction S9, which enables the metabolic activation of the genotoxic properties of the test compound. Each phthalate was tested at four concentration levels in triplicate.

Data Analysis

For the analysis of UmuC Easy CS genotoxicity assay results, the MS Excel spreadsheets provided with the test kit by the test manufacturer (Xenometrix AG, Switzerland) were used. Resulting concentration-response curves for individual phthalates and their metabolites were calculated with the software GraphPad Prism 5 (GraphPad, San Diego, CA, USA) using the four-parameter logistic equation model following log transformation of the concentration values. Based on the curves, the EC_{50} toxicity parameter was calculated for each tested chemical. The significance of IR increase for samples incubated with S9 compared to those incubated without S9 has been calculated using Welch's t-test. The probability values of $p<0.05$ were considered significant, and $p<0.01$ was considered highly significant.

Results

The genotoxic properties of DEP, DBP, BBzP and DEHP and their metabolites mixture were assessed using umuC assay in the absence (-S9) and presence (+S9) of metabolic activation factor. In each of the test series, the positive control reached IR≥2. Bacterial growth control achieved the test criterion (mean OD600 value increased twofold after a 2-hour incubation period). Based on the results obtained from positive control and growth control, the test validity criteria are considered maintained.

Within the selected concentration range, none of the tested compounds showed cytotoxic effects, i.e. G values were >0.5 (Table 3). Of the four phthalates, DEP, BBzP and DEHP did not exhibit significant genotoxic activity in the absence of S9 fraction. The only exception was DBP, where the IR threshold was exceeded at the lowest dilution level. IR values for phthalates were 1,69, 1,87, 2,37, and 1,85 for DEP, DBP, BBP, and DEHP respectively. Assessment of the genotoxic potential of phthalate standard solutions incubated with S9 fraction (hepatic fraction) indicates a significant (p<0,05 for DEP, DBP and BBzP) and highly

significant ($p<0,01$ for DEHP) increase of this potential for each phthalate sample (Table 3). The comparison of the induction ratio (IR) for plate with and without S9 indicates on average twofold increase of its value for the metabolised form of DBP and BBzP (plate with fraction S9). The IR value for the DEHP metabolites was about 4-fold greater than that of the parent compound. By using the sigmoid concentration-response function effective concentration values (EC_{50}) were calculated (Table 3). The lowest genotoxic potential was assessed for DEP (EC_{50} = 24,73 µM) whereas the highest was for DBP (EC_{50} = 0,36 µM). EC_{50} obtained for most of the tested diphthalates were from four up to twenty times higher compared to the EC_{50} values obtained for the same samples incubated with S9 fraction (Table 3).

Fig. 3 shows a graphical relationship of Induction Ratio (IR) of β-galactosidase activity and concentration of phthalates incubated with (+S9) and without (-S9) hepatic fraction. The concentration-response curves indicate that IR increases in a concentration-dependant manner. The IR for DEP, DBP and DEHP (+S9) samples is on average 2 up to 3 times higher compared with (-S9) samples. The only exception is BBP, where a more dynamic increase in IR with an increasing concentration (up to 4-fold increase in IR for highest concentration) can be observed. The degree of matching of sigmoidal concentration-response curve was additionally assessed in order to evaluate the eligibility of sigmoidal concentration-response model. The degree of matching was variable but satisfactory. The lowest degree of matching was observed for (-S9) DEHP and (+S9) DEHP (R^2 = 0.7143 and 0.9030, respectively), and the highest for and (+S9) DEP (R^2 = 0.9561).

The results obtained for phthalates incubated in the presence of fraction S9 indicate a significant increase in the genotoxic potential of these compounds. That drives the conclusion that phthalates' metabolic activation leads to the formation of genotoxic metabolites. The results suggest that phthalates can only react with genetic material after metabolic activation.

Discussion

The genotoxic potential of the four phthalates: DEP, DBP, BBzP and DEHP was evaluated using the UmuC Easy CS. The UmuC test uses the mutant strain *Salmonella typhimurium* TA1535 [pSK1002] as a test organism. A unique advantage of the test is the possibility of parallel studies of phthalate metabolites which, during incubation, undergo biotransformation in the presence of liver enzymes derived from the rat liver lyophilisate fraction S9. Incubation of the samples tested with S9 fraction is intended to map metabolic transformations that happen in the presence of liver enzyme (lipase) compounds.

Table 3. The genotoxic activity of phthalates in *Salmonella typhimurium* TA1535 [pSK1002] without (-)S9 and with (+)S9 metabolic activation determined with the UmuC assay.

	Sample concentration [µM]	(-) S9			(+)S9			P
		GF±SD	IR±SD	EC50	GF±SD	IR±SD	EC50	
DEP	0,40	1,50±0,32	0,60±0,09	24,73	0,73±0,14	1,69±0,14	5,37	<0,05
	0,80	1,93±0,21	0,45±0,06		0,71±0,20	1,95±0,08		
	1,66	1,14±0,11	1,16±0,09		0,82±0,17	**2,79± 0,14***		
	3,28	1,14±0,29	1,69±0,08		1,20±0,25	**3,66± 0,06***		
DBP	0,02	0,59±0,18	1,01±0,06	0,36	0,97±0,11	1,82±0,05	0,06	<0,05
	0,04	0,86±0,21	1,22±0,06		0,88±0,24	**2,20±0,10***		
	0,07	0,72±0,32	1,54±0,06		1,10±0,34	**2,93±0,06***		
	0,11	0,78±0,15	1,87±0,12		1,29±0,42	**3,11±0,07***		
BBP	0,03	0,65±0,12	0,90±0,09	1,68	0,57±0,21	1,83±0,10	0,08	<0,05
	0,06	0,78±0,24	1,13±0,07		0,72±0,37	**2,93±0,12***		
	0,10	0,53±0,13	1,33±0,06		0,59±0,15	**5,71±0,18***		
	0,22	0,73±0,22	**1,82±0,23***		0,71±0,26	**7,20±0,19***		
DEHP	0,01	0,93±0,28	0,25±0,07	0,91	0,82±0,29	1,69±0,08	0,24	<0,01
	0,03	1,07±0,08	0,58±0,10		1,26±0,41	1,88±0,11		
	0,05	1,15±0,13	1,47±0,11		1,40±0,36	**2,82±0,10***		
	0,10	1,02±0,20	1,85±0,1		1,09±0,21	**3,50±0,14***		

*Significant genotoxic effect (IR >2)

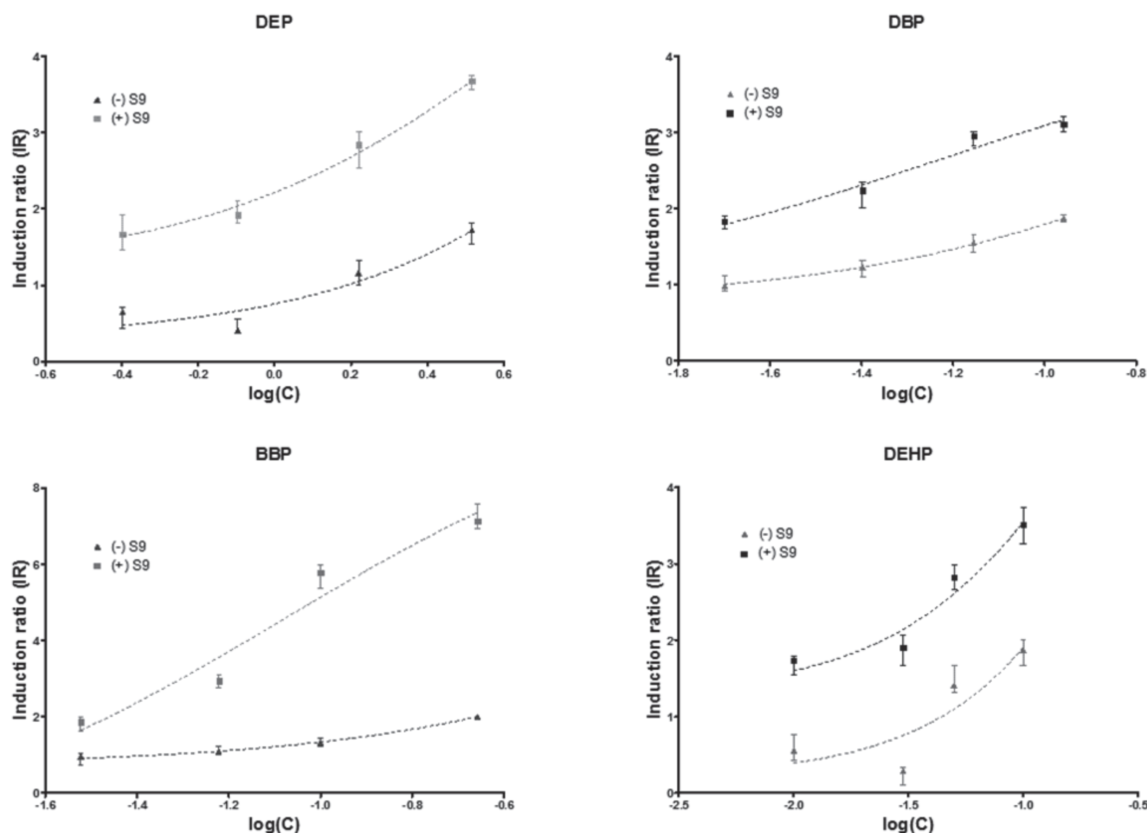

Fig. 3. Sigmoidal dose - response curves for genotoxic potential of phthalates incubated with (+S9) and without (-S9) hepatic fraction.

Genotoxicity studies of diesters of phthalic acid have shown a low potential of these compounds to cause damage to the genome. According to the test evaluation criteria the most concentrated samples (lowest dilution level) were characterized by rather low genotoxic potential. Ambiguous results were obtained for BBzP. Therefore, it is necessary to conduct further studies that exclude or confirm phthalates' genotoxic properties.

Genotoxicity studies using a strain of *Salmonella Typhimurium* have been the subject of research conducted by other research teams [28-30]. Studies have shown no direct genotoxic activity of BBzP and DEHP. According to other literature, most phthalate-induced genotoxic/mutagenic effects produce a negative test result [31]. With regard to carcinogenicity, DEP activity is questionable. DBP can take part in the promotion of benign neoplasms. In rodents exposed to DEHP, hepatocellular carcinoma and other effects such as peroxysomal and mitochondrial proliferation, increased activity of Cyp4A1 and PCoA, liver tissue proliferation and apoptosis suppression are observed [32]. Most of these effects are caused by the PPAR-alpha receptor induction. In mice deprived of the PPAR-alpha gene, administration of DEHP does not cause hepatocellular carcinoma. This is due to several reasons i.e. differences in PPAR-alpha density, signaling pathway regulation, harmful effects associated with PPAR receptor activation in rodents [33].

Examination of the genotoxic potential of selected phthalates incubated with fraction S9 showed a significant increase in the potential of each test compound. For the sample incubated with fraction S9, the test showed substantial genotoxic potential for all dilutions, including the highest (lowest concentrations). The study confirmed a significant increase in the genotoxic potential of phthalate metabolites produced by biological metabolism of parent compounds.

Given the information shown in Table 1 describing the phenomenon of phthalate degradation, it should be concluded that the genotoxic effect of samples incubated with fraction S9 should be attributed to a mixture of the parent compound (diester), present due to incomplete degradation, and the presence of its metabolite (monoester). Furthermore, for DEHP incubated with fraction S9, genotoxic potential should be attributed to a mixture of metabolites resulting from DEHP enzymatic degradation. DEHP metabolism has been best described in the literature. Due to the branched chain, DEHP has many metabolites. Nonetheless, it should be stressed that fraction S9 used in the UmuC test contains hepatic lipase, whereas phthalates are decomposed mainly by the action of lipase pancreas [9, 34].

The genotoxicity of DEHP and MEHP has been described by Chang et al. [34]. The clastogenic activity was evaluated based on Chinese hamster ovary cell culture. Chromosome damage was caused solely by

MEHP. Clostogenicity of MEHP suggests the role of this compound in the observed carcinogenicity of DEHP.

The study demonstrated the genotoxic potential of the mixture of phthalates and their metabolites. In the light of thebiomonitoring studies confirming the presence of metabolites of phthalates in the blood serum and breast milk, the outcome of the genotoxicity studies carry an extremely valuable information from the point of view of imposed health risk [35-38]. The risk of genotoxic potential of metabolites in this study may be attributed to the increased risk of gene mutations in women and children during prenatal and postnatal development. Recent epidemiological studies in Mexico have shown a positive relationship between the concentration of diethyl phthalate and the risk of breast cancer [39-42]. Studies in the United States and Taiwan have shown an increased risk of endometriosis and leiomyomata in women with elevated monohydrochloric acid and mono (2-ethylhexyl) phthalate in urine [43].

Conclusions

1. The use of the UmuC test provides a broad spectrum of information on the properties of a compound and its metabolites to interact with genetic material. The advantage of this test is the ability to assess the genotoxic potential of not only parent compounds but also their derivatives resulting from metabolic activation (incubation with hepatic fraction S9).
2. The analyzed diphthalates did not show significant genotoxic activity in conducted studies.
3. The results of the genotoxic potential study of phthalates suggest that phthalates can intercalate with genetic material to form DNA adducts only after metabolic activation. Eventually causing mutations and neoplasms in an exposed organism. Therefore, phthalate metabolites (monophthalates) can be regarded as compounds characterized by an increased genotoxic activity with respect to their parent compounds.
4. Therefore, for proper human risk assessment, further research on eukaryotic test systems is needed to elucidate whether phthalates and, more importantly, their metabolites can induce DNA damage especially in non-target cells and to understand possible initial effects and mechanisms involved in the adverse phthalates' and phthalates' metabolites genotoxic effects.

Abbreviations

BBzP – butyl benzyl phthalate
DMP – dimethyl phthalate
DEP – diethyl phthalate
DBP – dibutyl phthalate
DEHP – di(2-ethylhexyl) phthalate
DiNP – di-izo-nonyl phthalate
EC_{50} – effective concentration
EDC – endocrine disrupting chemical
GF – growth factor
IR – induction ratio
PVC – polyvinyl chloride

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Conflicts of Interest

The authors declare no conflict of interest.

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