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Endocrine adverse effects of mono(2-ethylhexyl) phthalate and monobutyl phthalate in male pubertal rats

Gözde Karabulut[1] and Nurhayat Barlas[2]

[1] Dumlupınar University Faculty of Science, Department of Biology, Kütahya, Turkey
[2] Hacettepe University Faculty of Science, Department of Biology, Ankara, Turkey

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Considering that research of adverse effects of mono(2-ethylhexyl) phthalate (MEHP) and monobutyl phthalate (MBP), two key metabolites of the most common phthalates used as plasticisers in various daily-life products, has been scattered and limited, the aim of our study was to provide a more comprehensive analysis by focusing on major organ systems, including blood, liver, kidney, and pancreas in 66 male pubertal rats randomised into eleven groups of six. The animals were receiving either metabolite at doses of 25, 50, 100, 200, or 400 mg/kg bw a day by gavage for 28 days. The control group was receiving corn oil. At the end of the experiment, blood samples were collected for biochemical, haematological, and immunological analyses. Samples of kidney, liver, and pancreas were dissected for histopathological analyses. Exposure to either compound resulted in increased liver and decreased pancreas weight, especially at the highest doses. Exposed rats had increased ALT, AST, glucose, and triglyceride levels and decreased total protein and albumin levels. Both compounds increased MCV and decreased haemoglobin levels compared to control. Although they also lowered the insulin level, exposed rats had negative islet cell and insulin antibodies, same as control. Treatment-related histopathological changes included sinusoidal degeneration in the liver, glomerular degeneration in the kidney, and degeneration of pancreatic islets. Our findings document toxic outcomes of MEHP and MBP on endocrine organs in male pubertal rats but also suggest the need for additional studies to better understand the mechanisms behind adverse effects in chronic exposure.

KEY WORDS: diabetes; endocrine disrupting chemicals; histopathological changes; metabolites; toxicity

Like many other endocrine disrupting chemicals (such as industrial solvents, oils, polychlorinated biphenyls, polybrominated biphenyls, dioxins, bisphenol A, pesticides, fungicides, and various pharmaceuticals), phthalates tend to accumulate in the adipose tissue and can be more toxic when metabolised (1, 2). For years, di(2-ethylhexyl) phthalate (DEHP) had been the most important plasticiser used in the production of polyvinyl chlorides (3, 4). Second to DEHP in terms of exposure is di-*n*-butyl phthalate (DBP) used in adhesives, varnishes, paints, printing inks, softeners, solvents, and different cosmetic products. Because of their persistence in the environment and adverse effects on human health, they have been classified as priority pollutants by several international health and environment organisations (5, 6). Both phthalates owe much of their toxicity to their metabolites, most notably mono(2-ethylhexyl) phthalate (MEHP) and monobutyl phthalate (MBP), respectively (7, 8), yet many of their harmful effects are still poorly documented. In addition, among many recent studies, none investigated the effects of these metabolites in a way that would involve hormonal, biochemical, haematological, and histopathological analysis to give more comprehensive profiles of MEHP and MBP in developing organisms.

Corresponding author: Nurhayat Barlas, Hacettepe University Faculty of Science, Department of Biology, Beytepe Campus, Ankara, Turkey
E-mail: *barlas@hacettepe.edu.tr*

The aim of our study was therefore to complete the scattered and limited information gathered so far by looking into a wide range of effects caused by a 28-day exposure to these two metabolites on endocrine organs of pubertal male rats. To estimate the extent of damage, we ran an array of biochemical, haematological, and immunological tests and detailed histopathological analyses.

MATERIALS AND METHODS

Chemicals and reagents

Mono(2-ethylhexyl) phthalate (CAS No. 4376-20-9, 97 % purity) and monobutyl phthalate (CAS No. 131-70-4, 97 % purity) as well as the following kits – alanine aminotransferase (ALT) activity assay (catalogue No. MAK052), aspartate aminotransferase (AST) activity assay (catalogue No. MAK055), urea assay (catalogue No. MAK266), triglyceride quantification (catalogue No. MAK006), bromocresol purple albumin assay (catalogue No. MAK125), creatinine assay (catalogue No. MAK080), total protein kit (catalogue No. TP0100), and glucose assay kit (catalog No. CBA086) – were obtained from Sigma-Aldrich (Steinheim, Germany). To measure insulin levels, we used the insulin rat ELISA Kit (Thermo-Fisher Scientific, Waltham,

USA). Rat islet cell antibody (catalogue No. BG-RAT11369) was obtained from Novatein Biosciences (Woburn, MA, USA), and rat insulin antibody (catalogue No. RAB0904) from Sigma-Aldrich.

Animals

Six-week-old Wistar albino (*Rattus norvegicus*) male rats (N=66), each weighing 150–200 g were obtained from the Kobay Laboratory of Experimental Animals (Ankara, Turkey). They were housed in bisphenol A (BPA)-free polycarbonate cages (dimensions 20×40×22 cm) under standard laboratory conditions (22.5±1.5 °C temperature, 47.1±1 % relative humidity, 12:12 h light-dark cycle) and had free access to tap water and standard pellet food throughout the experiment.

The study was approved by the Hacettepe University Experimental Animals Ethics Committee (2020/06-04).

Experimental design

The study was designed as a 28-day subchronic toxicity study. The age of animals and duration of exposure were adjusted to the recommendations of the US EPA Endocrine Disruptor Screening and Testing Advisory Committee (9).

For the experiment, a total of 66 rats were randomly divided into eleven groups of six. Five groups were receiving five different MEHP doses or five different MBP doses, and the control group was receiving 1 mL of corn oil (used as phthalate solvent) by oral gavage every morning for 28 days. Exposed groups were receiving MEHP or MBP in daily doses of 25, 50, 100, 200, and 400 mg/kg bw by gavage. These doses are based on the no-observed-adverse-effect level (NOAEL) and the lowest-observed-adverse-effect level (LOAEL) of the parent phthalates. The NOAEL for DEHP is 5.8 mg/kg bw a day, and the LOAEL 29 mg/kg bw a day (10, 11). The NOAEL of DBP is 19.9 mg/kg bw a day, and the LOAEL 52 mg/kg bw a day (12). Since literature does not report NOAELs and LOAELs for the MEHP and MBP metabolites used in the experiment, the dose of 25 mg/kg bw a day was chosen as the lowest dose in the experimental protocol, taking into account toxicologically relevant values for DEHP and DBP, which should be between the NOAEL and LOAEL. Finally, our experimental protocol consisted of five daily doses, where 25 and 50 mg/kg bw of MEHP and MBP represented low doses, 100 mg/kg bw of MEHP and MBP represented a moderate dose, and 200 and 400 mg/kg bw of MEHP and MBP represented high doses.

The amount of consumed food and water was measured every morning before dosing. Animals were inspected daily to assess survival, clinical signs of toxicity, behavioural impairments, and body weight changes. Body weight of each animal (expressed in grams) was measured at the beginning and end of the experiment. The experiment was terminated after the final gavage at the end of day 28. Animals were humanely euthanized by cervical dislocation under ether anaesthesia.

Biochemical analysis

The blood for biochemical analysis was taken from the hearts with a sterile syringe and put into heparinised vacutainers to prevent clotting. To obtain serum, tubes were centrifuged at 3000 *g* and +4 °C for 25 min. At the end of centrifugation, serum aliquots were taken to measure ALT and AST, urea, total protein, glucose, albumin, and creatinine levels using commercial measurement kits specified above. The analyses were performed on an automatic SMT-120V chemistry analyser (Chengdu Seamaty Technology, Chengdu, China).

Haematological analysis

Blood samples for haematological analyses were also taken from the heart as described above. Samples were analysed on the same day using an automatic haematology analyser (DH36, Dymind Biotechnology Co., Shenzhen, China) that simultaneously measured leukocytes (count), neutrophils (%), lymphocytes (%), monocytes (%), red blood cell (count), haemoglobin, haematocrit, mean red blood cell haemoglobin (MCH), mean red blood cell haemoglobin concentration (MCHC), mean corpuscular volume (MCV), thrombocytes (count), and platelets (volume).

Immunological assays

Serum samples were also used in immunological assays to determine insulin levels and to detect islet cell and insulin antibodies. These measurements were performed on an ELISA reader (Shimadzu UV-1800 UV-Vis spectrophotometer, Kyoto, Japan) using commercial kits.

Histopathological analyses

During necropsy, liver, kidney and pancreas were dissected and weighed. Some parts of tissue samples were fixed in Bouin solution for 8 h and put into 70 % ethanol. Other parts were fixed in 10 % formalin for 24 h, passed through the ethanol and xylene series, and then embedded in paraffin. Paraffin blocks were cut into 5 µm thick sections using a microtome (Accu-Cut® SRM™ 200 Sakura Finetek, Tokyo, Japan). The slides were stained with haematoxylin and eosin and examined under a light microscope (Zeiss Axio Scope A1, Jena, Germany) using the ZEN imaging software (Zeiss, Jena, Germany). Histopathological findings were photographed at magnifications of 200× and 400×.

Statistical analysis

The obtained results are reported as means ± standard deviations. Statistical analyses were performed using MedCalc® (MedCalc Software Ltd., Ostend, Belgium). Normal distribution of variables was tested with the Kolmogorov-Smirnov test, while further comparisons were made with one-way analysis of variance (ANOVA) and *post-hoc* Tukey's test. Comparisons between groups of variables that did not have normal distribution were made with the Kruskal-Wallis H test and the Mann-Whitney *U* test. Fisher's

exact test was used to compare the frequencies of histopathological lesions found in the tissues. The level of significance was set to $p<0.05$.

RESULTS

Food and water intake

There were no differences in water consumption between the MEHP-treated groups and control. However, food consumption significantly increased in rats receiving MEHP doses of 200 and 400 mg/kg bw a day compared to control and the groups receiving MEHP doses of 25 and 50 mg/kg bw a day (Table 1).

The MBP-exposed groups showed similar findings (Table 2).

Body and organ weights

Even though food intake was higher in the treated groups, we found no significant treatment-related increase in body weights in either MEHP or MBP groups (Tables 3 and 4, respectively).

As for individual organs, mean liver weight was significantly higher in groups receiving MEHP doses of 100, 200, and 400 mg/kg bw a day than control. Kidney weights did not differ significantly. Pancreas weight significantly decreased in rats treated with MEHP doses of 200 and 400 mg/kg bw a day compared to control and groups exposed to MEHP doses of 25, 50, 100 mg/kg bw a day (Table 3).

Similar to MEHP, rats receiving MBP doses starting with 100 mg/kg bw a day had significantly higher liver weight than control, whereas kidney weights did not differ. Absolute pancreas weight significantly decreased in the group treated with the MBP dose of 400 mg/kg bw a day compared to control. We also noticed a significant decrease in relative pancreas weight in the group receiving the MBP dose of 200 mg/kg bw a day (Table 4).

Blood biochemistry

Compared to control, ALT, AST, triglyceride, and glucose levels significantly increased in rats treated with MEHP doses of 100, 200, and 400 mg/kg bw a day, whereas total protein, albumin, and urea levels significantly dropped in those receiving 200 and 400 mg/kg bw a day of MEHP (Table 5).

MBP findings were similar to those of MEHP; ALT and AST levels rose significantly with 100, 200, and 400 mg/kg bw a day, both compared to control and the groups treated with lower doses (Table 6). Glucose and triglycerides also increased in the rats receiving MBP doses of 200 and 400 mg/kg bw a day compared to control and the 25 mg/kg bw a day group. Significant drops in total protein, albumin, creatinine, and urea were also observed at the highest MBP doses.

Haematological findings

The group receiving the highest MEHP dose had significantly higher MCV than control and the other dose groups, while haemoglobin, leukocyte, and monocyte counts were significantly lower. Other blood parameters did not differ significantly between the MEHP-exposed groups and control (Table 7).

Treatment with MBP showed similar findings (Table 8), as the highest dose significantly increased MCV and decreased haemoglobin compared to the other groups. Other blood parameters did not differ significantly.

Insulin findings

The highest two doses of both MEHP and MBP significantly lowered insulin levels compared to the other groups (Tables 9 and 10, respectively). However, all blood islet and insulin antibody tests were negative in all groups (Table 11).

Table 1 Food and water consumption by rats in the oil control and experimental groups treated with mono(2-ethylhexyl) phthalate (MEHP)

Groups	Control	MEHP (mg/kg bw a day)				
		25	50	100	200	400
Food (g)	10.70±3.60	11.12±1.01	12.14±2.29	15.43±4.37	**20.06±6.51**[a,b,c,f]	**30.05±5.72**[a,b,c,d,e]
Water (mL)	9.82±2.76	10.21±0.12	10.32±0.78	11.63±2.95	11.53±1.39	12.13±3.81

Data are expressed as means ± standard deviations (n=6). The level of significance was set to $p<0.05$. [a] significantly different from control group; [b] significantly different from the group receiving MEHP dose of 25 mg/kg bw a day; [c] significantly different from the group receiving MEHP dose of 50 mg/kg bw a day; [d] significantly different from the group receiving MEHP dose of 100 mg/kg bw a day; [e] significantly different from the group receiving MEHP dose of 200 mg/kg bw a day; [f] significantly different from the group receiving MEHP dose of 400 mg/kg bw a day

Table 2 Food and water consumption by rats in the oil control and experimental groups treated with monobutyl phthalate (MBP)

Groups	Control	MBP (mg/kg bw a day)				
		25	50	100	200	400
Food (g)	10.70±3.60	12.18±1.03	13.67±2.32	14.23±4.37	**18.07±6.51**[a]	**23.10±5.72**[a,b,c,d]
Water (mL)	9.82±2.76	10.01±0.54	10.34±0.33	10.23±2.95	12.53±1.39	13.53±3.81

Data are expressed as means ± standard deviations (n=6). The level of significance was set to $p<0.05$; [a] significantly different from control group, [b] significantly different from the group receiving MBP dose of 25 mg/kg bw a day, [c] significantly different from the group receiving MBP dose of 50 mg/kg bw a day, [d] significantly different from the group receiving MBP dose of 100 mg/kg bw a day

Table 3 Body and absolute and relative organ weights of rats in the oil control and experimental groups treated with mono(2-ethylhexyl) phthalate (MEHP)

Groups	Control	MEHP (mg/kg bw a day)				
		25	50	100	200	400
Baseline body weight (g)	220.8±1.0	219.5±0.2	221.1±0.1	222.3±0.9	217.8±0.1	218.9±0.1
Final body weight (g)	330.9±1.2	340.4±0.3	340.3±0.5	333.2±0.7	340.5±0.4	348.7±1.1
Weight gain %	50.02±0.2	52.03±0.5	51.02±0.1	51.09±0.8	55.01±0.3	54.06±0.4
Liver						
Absolute weight (g)	10.5±1.0	12.4±1.2	14.2±0.1	**16.5±0.9**[a]	**16.9±0.1**[a]	**17.48±0.1**[a]
Relative weight (mg/g)	34.8±1.2	32.1±0.2	33.1±0.4	32.7±0.7	32.9±0.4	33.1±1.1
Kidney						
Absolute weight (g)	1.07±0.1	1.07±0.1	1.09±0.2	1.09±0.9	1.09±0.2	1.10±0.1
Relative weight (mg/g)	3.2±0.1	3.1±0.2	3.2±0.2	3.1±0.2	3.1±0.1	3.0±0.2
Pancreas						
Absolute weight (g)	0.253±0.1	0.240±0.2	0.242±0.4	0.212±0.2	**0.198±0.3**[a,b,c]	**0.160±0.4**[a,b,c,d]
Relative weight (mg/g)	0.74±0.1	0.73±0.2	0.72±0.1	0.71±0.2	**0.68±0.3**[a,b,c,d]	**0.59±0.5**[a,b,c,d]

Data are expressed as means ± standard deviations (n=6). The level of significance was set to $p<0.05$; [a] significantly different from control group, [b] significantly different from the group receiving MEHP dose of 25 mg/kg bw a day, [c] significantly different from the group receiving MEHP dose of 50 mg/kg bw a day, [d] significantly different from the group receiving MEHP dose of 100 mg/kg bw a day

Table 4 Body and absolute and relative organ weights of rats in the oil control and experimental groups treated with monobutyl phthalate (MBP)

Groups	Control	MBP (mg/kg bw a day)				
		25	50	100	200	400
Baseline body weight (g)	220.8±1.0	220.4±0.1	221.3±0.2	224.4±0.8	219.7±0.1	217.9±0.1
Final body weight (g)	330.9±1.2	331.02±0.8	332.01±0.3	323.2±0.1	330.4±0.4	350.7±0.1
Weight gain %	50.02±0.2	50.09±0.7	51.07±0.8	50.09±0.8	54.01±0.2	55.06±0.3
Liver						
Absolute weight (g)	10.5±1.0	11.5±0.1	12.4±0.3	**17.4±0.9**[a,b]	**17.9±0.1**[a,b]	**18.44±0.1**[a,b,c]
Relative weight (mg/g)	34.8±1.2	33.3±1.3	33.1±1.2	32.8±0.7	33.9±0.4	34.1±1.1
Kidney						
Absolute weight (g)	1.07±0.1	1.09±1.1	1.10±1.2	1.09±0.9	1.10±0.1	1.11±0.1
Relative weight (mg/g)	3.2±0.1	3.1±0.1	3.2±0.3	3.1±0.2	3.1±0.1	3.1±0.2
Pancreas						
Absolute weight (g)	0.253±0.1	0.245±0.4	0.240±0.3	0.220±0.2	0.210±0.3	**0.201±0.1**[a,b]
Relative weight (mg/g)	0.74±0.1	0.73±0.1	0.73±0.1	0.71±0.2	**0.68±0.3**[a,b,c,d]	**0.66±0.5**[a,b,c,d]

Data are expressed as means ± standard deviations (n=6). The level of significance was set to $p<0.05$; [a] significantly different from control group, [b] significantly different from the group receiving MBP dose of 25 mg/kg bw a day, [c] significantly different from the group receiving MBP dose of 50 mg/kg bw a day, [d] significantly different from the group receiving MBP dose of 100 mg/kg bw a day

Table 5 Biochemical markers measured in the oil control and experimental groups treated with mono(2-ethylhexyl) phthalate (MEHP)

Parameters	Control	MEHP (mg/kg bw a day)				
		25	50	100	200	400
ALT (IU/L)	45.65±17.41	50.3±0.22	55.4±0.11	**65.25±15.99**[a,b]	**85.63±10.72**[a,b,c]	**96.91±7.82**[a,b,c,d]
AST (IU/L)	110.1±101.6	115.2±15.12	**120.45±22.12**[d,e,f]	**139.8±88.9**[a]	**182.6±36.9**[a,b]	**268.3±110.6**[a,b,c]
Triglycerides (mg/dL)	945.8±704.6	1050.27±5.43	**1210.12±3.54**[a,d]	**1450.8±294.5**[a]	**1650.3±250**[a,b]	**1702.6±266.4**[a,b,c]
Glucose (IU/L)	191.5±61	201.27±13.07	205.17±0.6	**211.1±33.5**[a,e,f]	**251.1±19.3**[a,b]	**301.6±43.5**[a,b,c]
Total protein (g/dL)	8.6±0.8	8.3±2.34	8.22±3.34	8.5±2.6	**6.8±0.3**[a,b,c,d]	**6.6±0.4**[a,b,c,d]
Albumin (g/dL)	32.35±2.73	29.8±4.44	29.8±1.14	27.93±1.34	**21.03±1.88**[a,b,c,d]	**18.66±1.53**[a,b,c,d]
Creatinine (mg/dL)	0.46±0.22	0.47±8.91	0.46±6.45	0.49±0.25	0.48±0.16	0.45±0.15
Urea (mg/dL)	14.8±3.3	14.7±4.12	13.09±2.44	13.7±1.8	12.3±8.3	**11.5±2**[a,b,c]

Data are expressed as means ± standard deviations (n=6). The level of significance was set to $p<0.05$; [a] significantly different from control group, [b] significantly different from the group receiving MEHP dose of 25 mg/kg bw a day,, [c] significantly different from the group receiving MEHP dose of 50 mg/kg bw a day, [d] significantly different from the group receiving MEHP dose of 100 mg/kg bw a day, [e] significantly different from the group receiving MEHP dose of 200 mg/kg bw a day, [f] significantly different from the group receiving MEHP dose of different from 400 mg/kg bw a day

Table 6 Biochemical markers measured in the oil control and experimental groups treated with monobutyl phthalate (MBP)

Parameters	Control	MBP (mg/kg bw a day)				
		25	50	100	200	400
ALT (IU/L)	45.65±17.41	47.26±0.22	50.62±0.11	**55.25±14.99**[a]	**76.63±19.72**[a,b,c]	**82.91±6.82**[a,b,c,d]
AST (IU/L)	110.1±101.6	120.9±15.12	135.66±22.12	**159.8±65.9**[a]	**191.6±36.9**[a,b]	**276.3±90.6**[a,b,c,d]
Triglycerides (mg/dL)	945.8±704.6	960.8±5.43	1100.9±3.54	**1550.8±294**[a,b]	**1850.3±250**[a,b,c]	**1820.4±116.4**[a,b,c]
Glucose (IU/L)	191.5±61	220.27±13.07	251.17±0.6	**291.1±22.5**[a]	**351.1±18.3**[a,b]	**401.6±41.5**[a,b,c]
Total Protein (g/dL)	8.6± 0.8	8.3±2.34	8.2±3.34	8.1±2.6	**7.1±0.3**[ab]	**6.4±0.4**[a,b,c]
Albumin (g/dL)	32.35±2.73	31.9±4.44	31.2±1.14	29.92±1.32	**22.03±1.98**[a,b,c]	**21.33±1.43**[a,b,c]
Creatinine (mg/dL)	0.46±0.22	0.47±8.91	0.48±6.45	0.48±0.15	0.38±0.17	**0.35±0.14**[a,b,c]
Urea (mg/dL)	14.8±3.3	14.5±4.12	14.3±2.44	14.8±1.8	13.2±8.3	**11.3±2**[a,b,d]

Data are expressed as means ± standard deviation (n=6). The level of significance was set to $p<0.05$; [a] significantly different from control group, [b] significantly different from the group receiving MBP dose of 25 mg/kg bw a day, [c] significantly different from the group receiving MBP dose of 50 mg/kg bw a day, [d] significantly different from the group receiving MBP dose of 100 mg/kg bw a day

Table 7 Haematological parameters measured in the oil control and experimental groups treated with mono(2-ethylhexyl) phthalate (MEHP)

Parameters	Control	MEHP (mg/kg bw a day)				
		25	50	100	200	400
Leukocytes (mm^3)	2.55±1.43	2.50±0.22	2.53±0.11	2.98±2.34	2.63±1.11	**1.19±0.17**[a,b,c,d,e]
Lymphocytes (%)	68.50±16.17	68.50±15.12	69.45±22.12	70.83±21.34	65.42±8.39	78.41±16.90
Monocytes (%)	11.17±5.40	10.27±5.43	9.12±3.54	11.40±8.42	10.30±1.97	**4.40±0.11**[a,b,c,d,e]
Neutrophils (%)	19.27±11.06	18.27±13.07	20.17±0.6	17.73±13.12	24.28±6.89	17.48±12.79
Erythrocytes (mm^3)	13.22±3.53	14.23±2.34	14.22±3.34	11.6±2.68	13.3±1.76	8.26±0.68
MCV (fL)	27.8±5.34	29.8±4.44	28.8±1.14	29.41±1.13	25.78±3.89	**53.12±2.89**[a,b,c,d,e]
Haematocrit (%)	35.27±8.18	33.17±8.91	34.56±6.45	33.6±6.06	27.1±6.29	39.27±1.62
MCH (pg)	7.87±4.52	8.77±4.12	9.01±2.44	8.83±3.38	11.6±11.24	14.9±1.24
MCHC (g/dL)	26.75±8.78	25.15±11.22	28.65±7.55	29.8±9.16	23.05±6.05	28.07±1.06
Haemoglobin (g/dL)	8.92±1.26	8.92±0.1	8.44±1.77	8.50±1.16	8.05±0.5	**6.33±0.22**[a,b,c,d,e]
Thrombocytes (mm^3)	3287.83±1276.73	3367.83±1131.02	3266.83±1144.03	3299.33±2483.02	2265±1567.18	2764.17±1876.21
PCT (%)	3.92±1.45	4.62±1.76	4.12±1.22	4.5±2.31	3.96±1.72	4.01±1.42

Data are expressed as means ± standard deviation (n=6). The level of significance was set to $p<0.05$; [a] significantly different from control group, [b] significantly different from the group receiving MEHP dose of 25 mg/kg bw a day, [c] significantly different from the group receiving MEHP dose of 50 mg/kg bw a day, [d] significantly different from the group receiving MEHP dose of 100 mg/kg bw a day, [e] significantly different from the group receiving MEHP dose of 200 mg/kg bw a day. MCH – mean corpuscular haemoglobin; MCHC – mean corpuscular haemoglobin concentration; MCV – mean corpuscular volume; PCT - volume occupied by platelets in the blood (platelet crit)

Table 8 Haematological parameters measured in the oil control and experimental groups treated with monobutyl phthalate (MBP)

Parameters	Control	MBP (mg/kg bw a day)				
		25	50	100	200	400
Leukocytes (mm^3)	2.55±1.43	2.30±2.14	2.55±0.84	2.58±2.34	2.83±1.11	2.19±0.39
Lymphocytes (%)	68.50±16.17	67.51±17.17	70.60±26.01	71.83±21.34	72.42±8.39	73.41±16.90
Monocytes (%)	11.17±5.40	12.17±4.11	11.21±4.39	12.20±9.41	11.30±1.97	9.33±0.10
Neutrophils (%)	19.27±11.06	18.11±9.87	18.44±8.77	18.73±14.22	19.19±6.89	18.48±12.79
Erythrocytes (mm^3)	13.22±3.53	12.12±3.21	13.01±1.21	12.6±2.68	13.3±1.76	12.26±0.68
MCV (fL)	27.8±5.34	28.9±4.53	28.8±1.01	28.42±2.13	29.78±3.89	**62.11±1.89**[a,b,c,d,e]
Haematocrit (%)	35.27±8.18	34.10±1.21	35.09±5.23	34.7±6.06	37.1±6.29	38.27±3.65
MCH (pg)	7.87±4.52	8.17±2.33	8.41±1.43	7.93±3.39	9.6±11.24	8.9±1.24
MCHC (g/dL)	26.75±8.78	27.15±5.56	27.31±2.56	27.2±9.16	25.05±6.05	26.07±1.06
Haemoglobin (g/dL)	8.92±1.26	9.02±0.26	9.98±0.26	9.5±1.16	9.02±1.6	**6.01±0.93**[a,b,c,d,e]
Thrombocytes (mm^3)	3287.83±1276.7	3311.83±1222.01	3317.02±1143.3	3309.33±2483.0	3266±1598.1	3765.17±1659.2
PCT (%)	3.92±1.45	3.81±1.11	4.01±0.22	4.7±3.34	3.87±1.89	3.51±1.42

Data are expressed as means ± standard deviation (n=6). The level of significance was set to $p<0.05$; [a] significantly different from control group, [b] significantly different from the group receiving MBP dose of25 mg/kg bw a day, [c] significantly different from the group receiving MBP dose of 50 mg/kg bw a day, [d] significantly different from the group receiving MBP dose of 100 mg/kg bw a day, [e] significantly different from the group receiving MBP dose of 200 mg/kg bw a day. MCH – mean corpuscular haemoglobin; MCHC – mean corpuscular haemoglobin concentration; MCV – mean corpuscular volume; PCT – volume occupied by platelets in the blood (platelet crit)

Table 9 Insulin levels measured in oil control and experimental groups treated with mono(2-ethylhexyl) phthalate (MEHP)

Groups	Control	MEHP (mg/kg bw a day)				
		25	50	100	200	400
Insulin (ng/mL)	1.43±0.48	1.35±0.12	1.32±0.1	**1.24±1.03**[a]	**0.77± 0.82**[a,b,c,d]	**0.69±0.61**[a,b,c,d]

Data are expressed as means ± standard deviation (n=6). The level of significance was set to $p<0.05$; [a] significantly different from control group, [b] significantly different from the group receiving MEHP dose of 25 mg/kg bw a day, [c] significantly different from the group receiving MEHP dose of 50 mg/kg bw a day, [d] significantly different from the group receiving MEHP dose of 100 mg/kg bw a day

Table 10 Insulin levels measured in oil control and experimental groups treated with monobutyl phthalate (MBP)

Groups	Control	MBP (mg/kg bw a day)				
		25	50	100	200	400
Insulin (ng/mL)	1.43±0.48	1.40±0.70	1.38±0.60	1.33±1.01	**0.81±0.71**[a,b,c,d]	**0.80±0.51**[a,b,c,d]

Data are expressed as means ± standard deviation (n=6). The level of significance was set to $p<0.05$; [a] significantly different from control group, [b] significantly different from the group receiving MBP dose of25 mg/kg bw a day, [c] significantly different from the group receiving MBP dose of 50 mg/kg bw a day, [d] significantly different from the group receiving MBP dose of 100 mg/kg bw a day

Table 11 Antibody levels measured in oil control and experimental groups treated with mono(2-ethylhexyl) phthalate (MEHP) and monobutyl phthalate (MBP)

Groups	Islet cell antibody (ICA)	Insulin antibody (IAA)
Control	(-)	(-)
MEHP (mg/kg bw a day)		
25	(-)	(-)
50	(-)	(-)
100	(-)	(-)
200	(-)	(-)
400	(-)	(-)
MBP (mg/kg bw a day)		
25	(-)	(-)
50	(-)	(-)
100	(-)	(-)
200	(-)	(-)
400	(-)	(-)

(-) negative

Table 12 Incidence of exposure-related histopathological lesions observed in oil control and experimental groups treated with mono(2-ethylhexyl) phthalate (MEHP)

Parameters / Groups	Control	MEHP (mg/kg bw a day)				
		25	50	100	200	400
Liver						
Sinusoidal degeneration	0/6	0/6	1/6	**4/6***	**5/6***	**6/6***
Congestion	0/6	1/6	1/6	**5/6***	3/6	**5/6***
Cytoplasmic dissolution	0/6	1/6	1/6	1/6	**4/6***	2/6
Mononuclear cell infiltration	0/6	0/6	1/6	**4/6***	**5/6***	**5/6***
Kidney						
Glomerulus degeneration	0/6	0/6	1/6	3/6	4/6	**6/6***
Congestion	1/6	2/6	2/6	**4/6***	3/6	**5/6***
Cytoplasmic dissolution	0/6	1/6	1/6	3/6	3/6	3/6
Mononuclear cell infiltration	1/6	0/6	2/6	1/6	2/6	1/6
Pancreas						
Degeneration of pancreatic cells	0/6	0/6	1/6	**4/6***	**4/6***	**6/6***
Congestion	1/6	1/6	2/6	**4/6***	3/6	**5/6***

Data are expressed as number of rats with the lesion / number of rats examined. * significantly different from control group ($p<0.05$; Fisher's exact test)

Table 13 Incidence of exposure-related histopathologic lesions observed in oil control and experimental groups treated with monobutyl phthalate (MBP)

Parameters / Groups	Control	MBP (mg/kg bw a day)				
		25	50	100	200	400
Liver						
Sinusoidal degeneration	0/6	0/6	1/6	**4/6***	**4/6***	**6/6***
Congestion	1/6	2/6	2/6	**4/6***	3/6	**5/6***
Cytoplasmic dissolution	0/6	1/6	1/6	3/6	**3/6***	3/6
Mononuclear cell infiltration	1/6	0/6	2/6	2/6	**5/6***	**5/6***
Kidney						
Glomerulus degeneration	0/6	0/6	1/6	3/6	**4/6***	**5/6***
Congestion	1/6	2/6	2/6	**4/6***	3/6	**6/6***
Cytoplasmic dissolution	0/6	1/6	1/6	1/6	1/6	2/6
Mononuclear cell infiltration	1/6	0/6	2/6	1/6	1/6	1/6
Pancreas						
Degeneration of pancreatic cells	0/6	0/6	1/6	2/6	**5/6***	**6/6***
Congestion	1/6	1/6	1/6	2/6	2/6	**5/6***

Data are expressed as number of rats with the lesion / number of rats examined. * significantly different from control group ($p<0.05$; Fisher's exact test)

Histopathological findings

Figures 1–3 show respective typical histological changes in the liver, kidney, and pancreas of rats receiving either MEHP or MBP. Liver changes included mononuclear cell infiltration and minimal congestion (Figure 1B, E, and F), sinusoidal degeneration (Figure 1C), cytoplasmic dissolution (Figure 1D), and a necrotic region (1G). Kidney changes included separation in parietal and visceral leaves in glomerulus and minimal congestion (Figure 2B and C) and congestion with atrophic glomerulus and tubular degeneration (Figure 2D and E). Changes in the pancreas included congestion and extensive destruction (Figure 3B) and degeneration and vacuolar cytoplasm in the islets of Langerhans (Figure 3C, D, and E).

Lesions in all organs are visible in more than a half of treated animals per group receiving the MEHP dose of 100 mg/kg a day or higher (Table 12). Similar are the findings for MBP, save for the pancreas, where more than a half of the animals were affected at the two highest doses (Table 13).

DISCUSSION

This is one of the first comprehensive *in vivo* studies of MEHP and MBP effects in a pubertal male rat model. As expected, our results confirm the hepatotoxic effects of both. Starting with the doses of 100, 200, and 400 mg/kg bw a day MEHP increased absolute liver weight significantly, which may be related to lipid accumulation, considering similar reports from previous studies (13–15). As reported by Zhang et al. (16), accumulation of lipids in hepatocytes increases susceptibility to liver damage, which could lead to non-alcoholic fatty liver disease (NAFLD).

MBP also increased absolute liver weights starting with the doses of 100, 200, and 400 mg/kg bw a day. Such toxicity was expected, considering the effects of its parent compound DBP as a peroxisome enhancer, which causes liver growth due to hepatocyte proliferation, reduces blood triglyceride levels, can lead to weight gain, and stimulates many enzymes and enzymatic pathways (17). It also confirms the results of a study in Sprague Dawley rats (18) reporting an increase in liver weight after a brief exposure to phthalate diesters and monoesters.

As for haematological findings, the highest MEHP dose (400 mg/kg bw a day) led to a significant increase in leukocyte count, which points to the activation of the defence system. Some researchers (19) report a positive association between urinary phthalate metabolites and serum biomarkers of inflammation and oxidative stress. The significant decrease in monocyte count with the highest MEHP or MBP dose, in turn, indicates a decrease in immune resistance, whereas higher MCV indicates the presence of large-volume red blood cells, which usually occurs due to vitamin B12 and folic acid deficiency, lung diseases, some liver diseases, or heavy alcohol use (20, 21). Higher doses of MEHP and MBP also lowered the haemoglobin levels, which points to an increase in glycated haemoglobin, called Hba1C, associated with diabetes (22). This finding may also point to oxidative stress that may have accelerated eryptosis and removal of red blood cells from the circulation, as reported by Sicińska (23).

A part of our research was focused on insulin, because insulin deficiency in rodents is associated with pancreatic β-cell dysfunction (24) and because phthalate exposure may disrupt adipose tissue signalling and trigger insulin resistance (glucose intolerance) through antagonistic effects on the thyroid receptor and subsequent thyroid hormone suppression (25) usually owed to oxidative stress (26). Our findings confirm lower insulin secretion in rats exposed to higher doses of MEHP (from 100 mg/kg bw a day up) and MBP (from 200 mg/kg bw a day up), which is in line with earlier reports (27, 28). Considering that insulin deficiency may be owed to autoimmune processes that damage insulin-producing beta cells, we checked for the presence of autoantibodies against insulin and pancreatic cells (29, 30), which have been reported in 70–90 % of type 1 diabetes patients (31). However, antibody tests were negative in all groups, which suggests that the lower insulin levels were not related to the damage of the islets of Langerhans by autoantibodies created by the immune system.

In line with insulin findings, we also found an increase in glucose levels in rats exposed to MEHP (starting with the dose of 100 mg/kg bw a day) and MBP (starting with 50 mg/kg bw a day).

Previous studies (32, 33) suggest that both MEHP and MBP increase oxidative stress and fatty acid oxidation. Yang et al. (34) also found that antioxidant 6-gingerol could prevent oxidative stress induced by MEHP. Hyperglycaemia is well known to lead to excessive production of free radicals and consequently to affect normal cell function, that of pancreatic β-cells in particular (29), by making them vulnerable to oxidative stress (35). Excessive levels of free oxygen radicals induce β-cell apoptosis, accelerate cellular aging, and eventually lead to diabetes (36). This is why we believe that our findings could be related to β-cell damage caused by insulin deficiency and hyperglycaemia. That lipid metabolism is compromised by the two phthalate metabolites has also been confirmed by the increased triglyceride levels, starting with the daily MEHP dose of 50 mg/kg bw and the MBP dose of 100 mg/kg bw, which is in line with earlier reports for their parent compounds, such as that by Bastos Sales et al. (37). As we know, insulin participates in fat metabolism by lowering plasma fatty acid levels as it stimulates cellular uptake of triglycerides in the adipose and muscle tissues (38). Insulin resistance, however, results in high plasma free fatty acid level, caspase-3 activation, DNA fragmentation, and cytochrome c release, leading to β-cell apoptosis (39). In the development of type 2 diabetes, glucose tolerance decreases in the first phase of beta cell function loss, and insulin secretion decreases in the second phase (40). Taking into account the above literature data, we believe that higher triglyceride levels in our study are closely related to the decrease in insulin levels.

Our results on biochemical markers ALT and AST, often associated with liver damage and fatty liver further confirm adverse effects of MEHP and MBP at daily doses starting from 100 mg/kg bw.

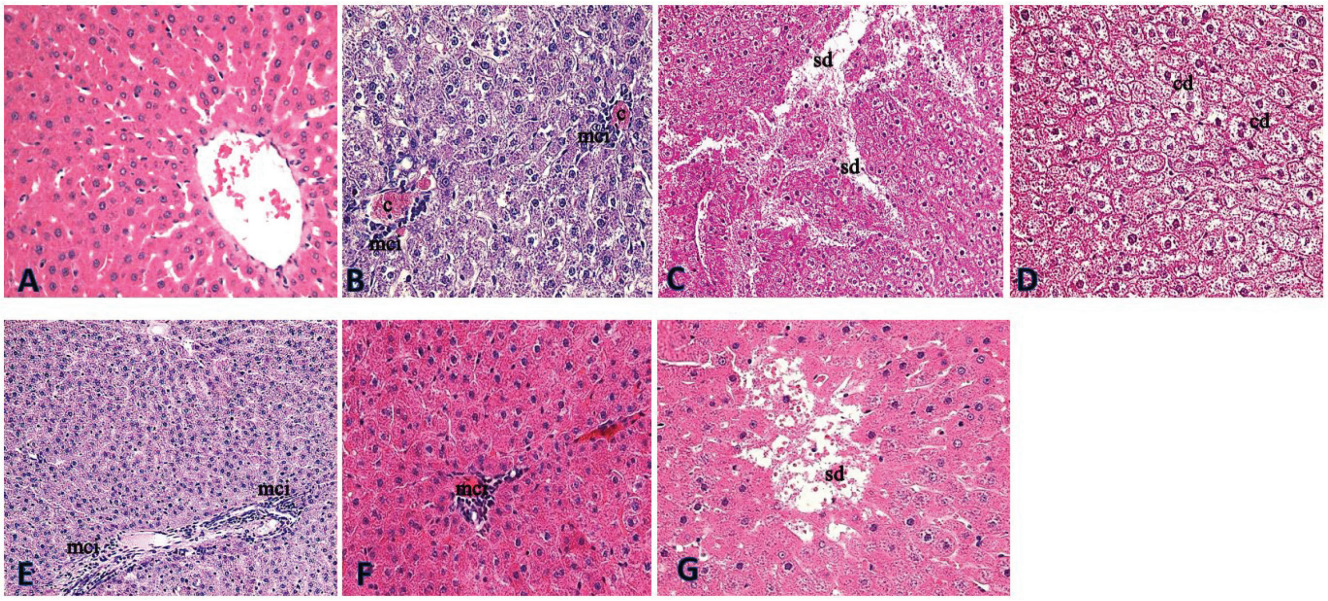

Figure 1 Liver tissue photomicrographs in A) – the oil control group (×400 magnification); B and C – the group receiving MEHP dose of 100 mg/kg bw a day (×400 and ×200 magnification, respectively); D – the group receiving MEHP dose of 200 mg/kg bw a day (×200 magnification); E – the group receiving MEHP dose of 400 mg/kg bw a day (×200 magnification): (F) 200 mg/kg bw a day of monobutyl phthalate (MBP) (×400), (G) 400 mg/kg bw a day of MBP (×400). c – congestion; cd – cytoplasmic dissolution; mci – mononuclear cell infiltration; sd – sinusoidal degeneration

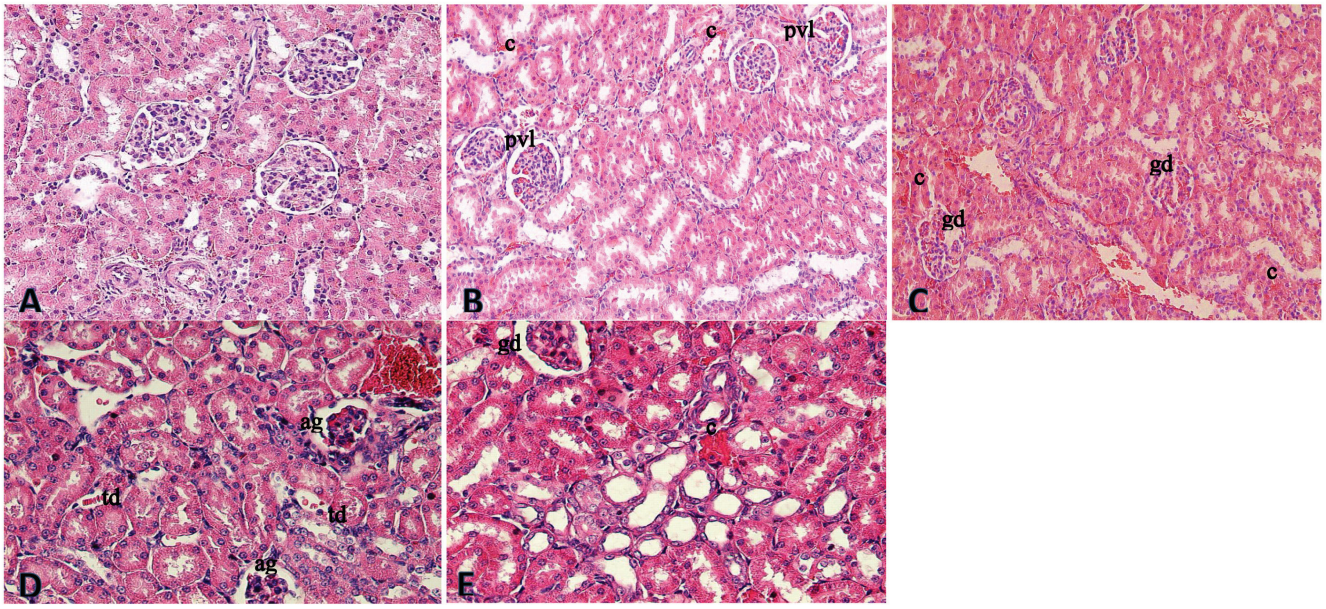

Figure 2 Kidney tissue photomicrographs in A – the oil control group (×400 magnification); B – the group receiving MEHP dose of 100 mg/kg bw a day (×200 magnification); C – the group receiving MEHP dose of 200 mg/kg bw a day (×200 magnification); D – the group receiving MBP dose of 200 mg/kg bw a day (×400 magnification); E – the group receiving MBP dose of 400 mg/kg bw a day (×400 magnification). ag – athropic glomerulus; c – congestion; gd – glomerular degeneration; pvl – parietal and visceral leaves; td – tubular degeneration

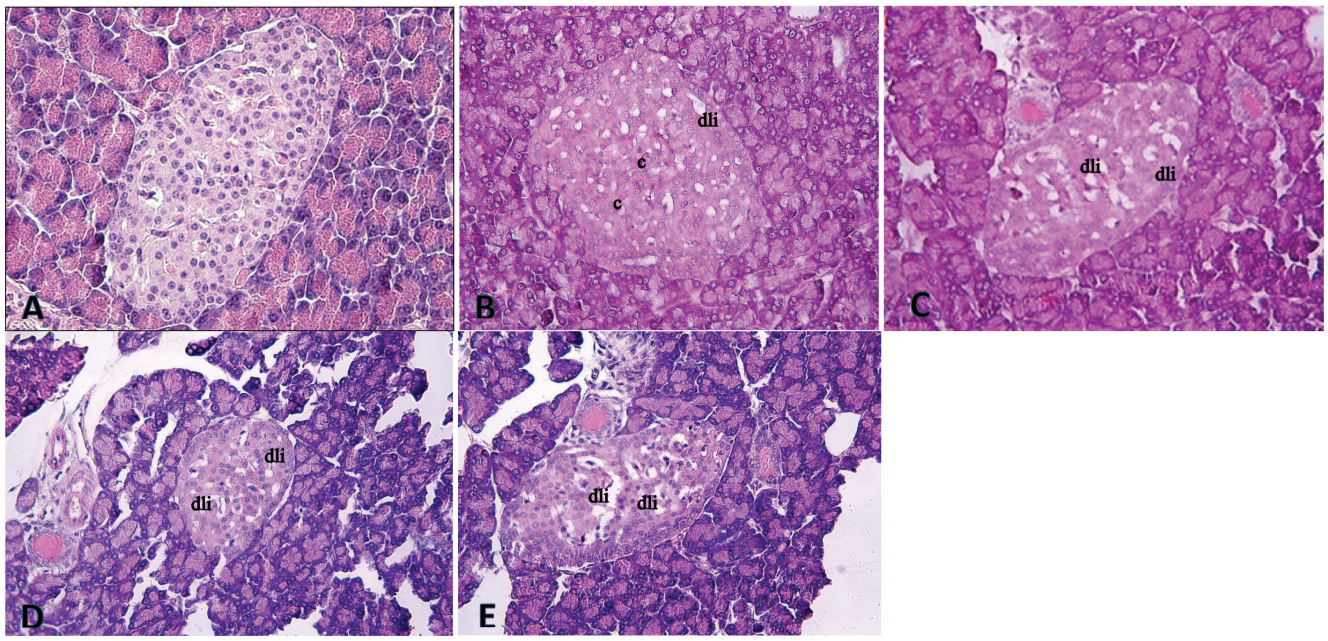

Figure 3 Pancreas tissue photomicrographs in A – the oil control group (×400 magnification); B – the group receiving MEHP dose of 200 mg/kg bw a day (×400 magnification); C – the group receiving MEHP dose of 400 mg/kg bw a day (×400 magnification); D – the group receiving MBP dose of 200 mg/kg bw a day (×200 magnification); E – the group receiving MBP dose of 400 mg/kg bw a day (×200 magnification). c – congestion; dli – degeneration of Langerhans islets

Considering that serum albumin is used as a marker of liver dysfunction (41), liver damage is also indicated by significantly lower total protein and albumin levels in our rats exposed to the highest MEHP and MBP doses. All these results agree well with our histopathological findings in the liver.

High blood creatinine levels, in turn, indicate kidney damage. However, creatinine levels in MEHP and MBP-exposed rats mostly did not deviate from control, save for the highest MBP dose, which lowered it significantly. This calls for further investigation in the context of kidney damage. Similar to creatinine, urea as another indicator of kidney damage (42) did not significantly deviate control, save for treatments with the highest MEHP and MBP doses, which resulted in their significant decrease compared to all other groups. While urea levels are expected to increase as a result of kidney tissue damage, the opposite finding in our study suggests that the tested doses of MEHP and MBP did not significantly impair kidney function. However, what happens after prolonged exposure? Namely, kidney is considered another target of phthalate toxicity, as urinary excretion is the major elimination route of DEHP metabolites (43). Previous studies (44, 45) show that DEHP accumulates mostly in the kidney and liver after oral administration, and that DEHP and its metabolites induce the expression of genes associated with oxidative stress. Since we did not investigate oxidative stress biomarkers, we cannot establish the connection between MEHP and MBP treatment and ROS-mediated organ damage.

Even so, our extensive histopathological examination reveals various histopathological changes in the liver, kidney, and pancreas of MEHP- and MBP-treated rats that agree well and complement our biochemical marker results and immunochemical insulin measurements.

CONCLUSION

Our study has yielded several important novel findings that contribute to the existing knowledge of the toxicity of phthalate metabolites MEHP and MBP, including the exposure-related decline in blood insulin levels and histopathological findings of damage in the liver, kidney, and pancreas tissues. All these findings point to oxidative stress, but this association needs to be confirmed by future research, such as the one confirming a relationship between oxidative stress and urinary concentrations of phthalate metabolites (46).

Future research should also investigate whether these metabolites would produce different effects in chronic exposure and should focus on cellular mechanisms and measurements *in vivo*.

Conflicts of interest

None to declare.

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Endokrini štetni učinci mono(2-etilheksil) ftalata i monobutil ftalata u mužjaka štakora u pubertetu

Budući da su ograničene spoznaje o štetnim učincima mono(2-etilheksil) ftalata i monobutil ftalata, dvaju ključnih metabolita najčešćih ftalata koji se rabe u izradi plastike u različitim proizvodima za svakodnevnu primjenu, cilj je našeg istraživanja bio dobiti potpuniju sliku o njima s obzirom na organske sustave, uključujući krv, jetru, bubreg i gušteraču u 66 mužjaka štakora u pubertetu, nasumce raspoređenih u jedanaest skupina po 6 životinja, od kojih su neke gavažom primale jedan od tih dvaju metabolita 28 dana u dozama od 25, 50, 100, 200 ili 400 mg/kg tjelesne mase na dan. Kontrolna je skupina primala samo kukuruzno ulje, koje je u drugim skupinama služilo kao otapalo za metabolite. Na kraju pokusa prikupljeni su uzorci krvi za biokemijske, hematološke i imunološke pretrage. Uzorci bubrega, jetre i gušterače uzeti su disekcijom za histopatološku analizu. Izloženost bilo kojemu od tih dvaju metabolita dovela je do povećanja mase jetre i smanjenja mase gušterače, posebice pri najvišim dozama. Izloženi štakori imali su povišene vrijednosti ALT, AST, glukoze i triglicerida te snižene vrijednosti ukupnih proteina i albumina u krvi. Oba su metabolita dovela do povećanja prosječnog volumena eritrocita (MCV) i pada hemoglobina u usporedbi s kontrolnom skupinom. Premda su doveli i do pada razina inzulina u krvi, nalazi na antitijela na stanice Langerhansovih otočića i inzulin u izloženih štakora bili su negativni, baš kao i u kontrolnoj skupini. Histopatološke promjene povezane s izloženosti obuhvaćale su sinusoidnu degeneraciju u jetri, glomerulsku degeneraciju u bubregu te degeneraciju otočića gušterače. Naši rezultati potvrđuju toksično djelovanje MEHP-a i MBP-a na endokrine organe mužjaka štakora u pubertetu, ali isto tako upućuju na potrebu za daljnjim istraživanjima ne bi li se bolje razumjeli mehanizmi koji stoje iza štetnih događaja, naročito pri kroničnoj izloženosti ftalatima.

KLJUČNE RIJEČI: dijabetes; endokrini disruptori; histopatološke promjene; metaboliti; toksičnost