EFFECT OF Di-(2-ETHYHLHEXYL) PHTHALATE (DEHP) EXPOSURE ON MICROARCHITECTURE OF FEMORAL BONE IN MALE LABORATORY MOUSE: PRELIMINARY RESULTS

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ABSTRACT

Di-2-ethylhexyl phthalate (DEHP) is a toxic and hazardous endocrine disruptor with adverse effects on animal and human health. However, its impact on bone tissue has not been sufficiently investigated. Therefore, the purpose of our preliminary study was to examine the effects of DEHP on compact bone structure in two 57-days-old male mice. Daily oral administration of DEHP (4.5 mg/kg body weight dissolved in 500 μl of peanut oil per 15 days) was studied, compared to a control. We observed a significant effect of DEHP exposure on macroscopic bone characteristics. Similarly, we identified differences in qualitative characteristics, such as the presence of resorption lacunae and absence of non-vascular and primary vascular radial bone tissue near the endosteal border, compared to the control. On the contrary, quantitative analysis showed no demonstrable alterations in morphometric parameters. Our preliminary findings support the hypothesis about the negative impact of DEHP on bone tissue. However, further investigation is needed to understand this issue better and more precisely.

Keywords: di-(2-ethylhexyl) phthalate; endocrine disruptor; compact bone; mouse; behavior

INTRODUCTION

Phthalates are added in the polyvinyl chloride (PVC) manufacture and act as plasticizers to increase the elasticity, persistence, and longevity of plastic products including cosmetics, medical equipment, food packaging, children’s toys, and clothing (Richardson et al., 2018). These compounds are man-made chemicals and work as endocrine disruptors due to their similar structure with steroid hormones (Hlinskaková et al., 2020). Among them, the most commonly used member, di-(2-ethylhexyl) phthalate (DEHP) (Rowdhval and Chen, 2018) represents a serious threat to humans health and the environment (Dong et al., 2020). Tripathi et al. (2019) state that its production increases yearly by 5% due to growing production demand worldwide. DEHP is compound non-covalently bound to PVC, therefore it can easily release into the environment and cause ambient air, food, water and soil pollution (Dong et al., 2020; Ernst et al., 2020). Organisms can absorb DEHP mainly through ingestion and also through skin contact and inhalation (Rusyn et al., 2016). Tolerable daily intake was set by European union on 37μg/kg of body weight (Lo et al., 2014) and estimated daily human exposure is 3–30 μg/kg/day (Hannon et al., 2016). Health-related effect is associated with endocrine toxicity, hepatotoxicity, testicular toxicity, neurotoxicity, cardiotoxicity, reproductive toxicity, teratogenicity of DEHP (Kamijo et al., 2007; Rowdhval and Chen, 2018) and also in adipogenesis where acts as an obesogen (Schaeidich et al., 2018).

The toxic potential of DEHP is large due to the metabolic transformation into more toxic metabolites by hydrolysis (mono-(2-ethylhexyl) phthalate (MEHP) and subsequent oxidation reactions (mono-(2-ethyl)-5-hydroxyhexyl) phthalate (5-OH-MEHP), mono-(2-ethyl-5-oxo-hexyl) phthalate (5-oxo-MEHP), mono-(2-ethyl-5-carboxypentyl) phthalate (5-cx-MEHP)). Secondary oxidized DEHP metabolites (5-OH-MEHP, 5-oxo-MEHP, 5-cx-MEPP and mono-[2-(carboxymethyl) hexyl] phthalate (2-cx-MMHP)) are most valuable biomarkers of DEHP exposure (Koch et al., 2006; Eljezi et al., 2017). Despite the fact DEHP and its metabolites are rapidly excreted from the body (Barakat et al., 2017), their widespread use and therefore the constant presence in the environment increase the concern about numerous adverse effects on organisms (Herrero et al., 2017).

The studies in rodents have established that estrogens and androgens are locally activated or catabolized within target tissues such as bone (Vandenput et al., 2016). These hormones contribute to the maintenance of bone mass during adulthood, primarily by slowing bone remodeling rate. They also attenuate the apoptosis of osteocytes, and estrogen or androgen deficiency increases the prevalence of osteocyte apoptosis in both cancellous and cortical bone in animals and humans (Tomkinson et al., 1997; Kousteni et al., 2002; Almeida et al., 2017).

Various animal experiments found possible interference of phthalates also with the bone remodeling process and metabolism. Phthalates have been observed to have an inhibitory effect on osteoblasts in mice, thereby affecting bone mineral density (BMD), along with weak estrogenic, antiestrogenic, and anti-androgenic activity (Agas et al., 2013; DeFlorio-Barker and Turyk, 2016; Fan et al., 2020).

Gestational exposure to phthalate esters causes a sequence of abnormalities in rat pups including elongated and fused ribs (bilateral and unilateral), absence of tail bones, abnormal or incomplete skull bones, and incomplete or missing leg bones (US Environmental Protection Agency, 2021). Chiu et al. (2018) present that the oral administration of DEHP may affect the homeostasis between osteoblastogenesis and adipogenesis and further alter the BMD and microstructure, which is mainly contributed by its primary metabolite MEHP. DEHP, the so-called environmental estrogen (Kanno et al., 2004), can mimic the structure of the endogenous ligand, interfering with the action of the hormone or affects the activities of both androgen and estrogen receptors (Park et al., 2019). Although bones are the target organs of great significance for estrogen, the effects of DEHP (as an endocrine disruptor) on bone histomorphometry has not been sufficiently described yet. In this study, we evaluated the changes in femoral bone microarchitecture in the adult male after 15-days of oral administration of DEHP. Secondary, possible changes in the behavior of experimental animal due to DEHP exposure were observed.

MATERIAL AND METHODS

The experiment was carried out on two 21-days old male Swiss mice from one litter. After 3 weeks of quarantine, the mice were divided into two groups. Mouse...
from the experimental group was orally administrated DEHP (Sigma-Aldrich, USA) at 4.5 mg/kg body weight (b.w.) dissolved in 500 µl of peanut oil (Biopurus, Germany) per 15 days. The dose of DEHP was chosen as the dose in which no statistically significant adverse effect on the body was observed in other studies compared to the control group (NOAEL DEHP = 4.5 mg/kg b.w.) estimated by ATSDR (EFSA, 2019). The DEHP solution was made every day and calculated based on the equation:

$$V_{DEHP} = \frac{dose \times m}{\rho} \times 1000 \text{ [µl]}$$

where the dose is 4.5 mg/kg b.w., $\rho = 0.981 \text{ g/cm}^3$ at 25 °C and $m$ is the daily weight of the mouse (Haynes, 2014). Mouse from the control group was orally administered on sponge cake (adjusted according to Degroote et al. (2014)) at the same time by 500 µl/animal-vehicle (peanut oil). Animals were housed in individual cage (800 cm³) and under specific pathogen-free conditions at environmental temperature 21 ± 3°C and 55 ± 10% relative humidity (datalogger UNI-T UT330B USB (UNI-T, Guangdong, China)) with a constant photoperiod of 12 h daylight. Mice were administered with water and diet on ad libitum base. Experimental breathing, handling and conditions were in accordance with Slovak republic Regulation 377/2012 (laying down requirements for the protection of animals used for scientific or educational purposes), with Regulation 432/2012 (laying down requirements for the protection of animals at the time of killing). The study received approval of the Institutional Review Board of Constantine the Philosopher University in Nitra. Behavior of both mice before starting the experiment and throughout its realization (feeding, drinking, grooming, movement and potential presence of pain) were observed and compared according to study Langford et al. (2010).

The experiment ended after 15 days (57 days of age) by cervical dislocation of both mice. Right femoral bones were weighed by analytical scales; length was measured by a digital caliper with precision to 0.01 mm. Analyzed bones were macerated, degreased, and embedded in epoxy resin Biodur (Günter von Hagens, Germany). Thin sections (70-80 µm) from femurs were prepared according to the methodology of Martiniaková et al. (2006). Histological sections of all bones were observed and photographed using a polarizing microscope (Leica DM 2000+, Germany) at 50x and 100x magnifications. The qualitative characteristics of the compact bone tissue were determined according to the classification system (Enlow and Brown, 1956; Ricgles et al., 1991).

The quantitative parameters of the compact bone tissue were assessed using the software Motic Images Plus 2.0 ML (Motic China Group Co., Ltd., Xiamen, China). We measured area, perimeter, maximum and minimum diameter of Haversian canals and osteons in all views (i.e., cranial, caudal, medial, and lateral) of thin sections to minimize statistical differences in the individual.

Differences in quantitative parameters were tested for statistical significance by T-test using SPSS 17.0 software (SPSS Inc., Chicago, Illinois, USA). The normality of data was tested using the Shapiro-Wilk test. Levene’s test was used for homogeneity of variances testing. All data were expressed as mean ± standard deviation and were considered statistically significant when $p<0.05.$

**RESULTS**

Our study brings pilot results from the pre-research phase, which was affected by the pandemic situation of Covid-19. Repetition of further analysis on a larger animal group size, therefore, could not be realized. Because of this, we present a preliminary real analysis of individuals.

Based on macroscopic analysis, we observed increase of femoral length in both analyzed mice. The non-exposed individual (16.75 mm) comparing to DEHP exposed sample (16.19 mm). Based on macroscopic analysis, we observed increase of femoral length in the exposed sample (16.19 mm).

On the other hand, the femoral weight of control mouse (18.8 ± 1.3 µm) was observed by refusing food. This had resulted in the decrease in the weight. Average weight had reached statistically significantly lower value ($p=0.0001$) in experimental animal ($m = 30.373 ± 1.608 \text{ g}$) in comparison to the control ($m = 33.026 ± 1.616 \text{ g}$) (Figure 3).

Figure 1: Microstructure of compact bone tissue of control mouse: 1 - non-vascular bone tissue, 2 - primary vascular radial bone tissue, 3 - Haversian bone tissue, 4 - intact osteons

Figure 2: Microstructure of compact bone tissue of exposed mouse: 1 - non-vascular bone tissue, 2 - primary vascular radial bone tissue, 3 - Haversian bone tissue, 4 - intact osteons, 5 - resorption lacunae

In total, Haversian canals ($n=40$) and osteons ($n=40$) underwent a metric analysis. In quantitative analysis we observed non-significant effect of DEHP exposure in all measured parameters (area, perimeter, maximal and minimal diameter) of Haversian canals and osteons between control and experimental sample. The results are summarized in Tables 1 and 2.

The overall average of Haversian canals for the control sample was 6.6 ± 0.3 µm and for the exposed sample 6.6 ± 0.4 µm. According to the classification of Haversian canals (Rämsh and Zerndt, 1963; Gladuhsew, 1964), the osteons of both analyzed samples belong to the category of very narrow Haversian canals.

Overall average of osteons acquired the value of 19.2 ± 1.4 µm in the control and 18.8 ± 1.3 µm in the exposed sample.

In the present study, we also observed the side finding – the potential effect of DEHP exposure on mouse behavior. Comparing the behavior of animals, we had noticed a change of food intake after exposure to DEHP in experimental animal, manifested by refusing food. This had resulted in the decrease in the weight. Average weight had reached statistically significantly lower value ($p=0.0001$) in experimental animal ($m = 30.373 ± 1.608 \text{ g}$) in comparison to the control ($m = 33.026 ± 1.616 \text{ g}$) (Figure 3).

Figure 1: Microstructure of compact bone tissue of control mouse: 1 - non-vascular bone tissue, 2 - primary vascular radial bone tissue, 3 - Haversian bone tissue, 4 - intact osteons

Figure 2: Microstructure of compact bone tissue of exposed mouse: 1 - non-vascular bone tissue, 2 - primary vascular radial bone tissue, 3 - Haversian bone tissue, 4 - intact osteons, 5 - resorption lacunae
Table 1 Data of Haversian canals (mean ± standard deviation)

<table>
<thead>
<tr>
<th>Observed files</th>
<th>sample</th>
<th>n</th>
<th>area (µm²)</th>
<th>perimeter (µm)</th>
<th>max. diameter (µm)</th>
<th>min. diameter (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cranial</td>
<td>control 5</td>
<td>34.3 ± 6.0</td>
<td>21.1 ± 1.8</td>
<td>7.6 ± 0.3</td>
<td>5.8 ± 0.3</td>
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</tr>
<tr>
<td></td>
<td>exposed 5</td>
<td>36.7 ± 3.8</td>
<td>22.4 ± 1.1</td>
<td>8.4 ± 0.3</td>
<td>5.6 ± 0.4</td>
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<tr>
<td>lateral</td>
<td>control 5</td>
<td>35.7 ± 3.5</td>
<td>21.2 ± 0.9</td>
<td>7.0 ± 0.3</td>
<td>6.6 ± 0.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>exposed 5</td>
<td>34.0 ± 6.2</td>
<td>20.8 ± 1.9</td>
<td>7.2 ± 0.5</td>
<td>6.0 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>medial</td>
<td>control 5</td>
<td>31.5 ± 5.9</td>
<td>20.3 ± 1.8</td>
<td>7.4 ± 0.3</td>
<td>5.4 ± 0.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>exposed 5</td>
<td>30.6 ± 1.2</td>
<td>20.6 ± 0.4</td>
<td>7.8 ± 0.3</td>
<td>5.0 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>caudal</td>
<td>control 5</td>
<td>31.5 ± 5.9</td>
<td>20.4 ± 1.8</td>
<td>7.4 ± 0.4</td>
<td>5.4 ± 0.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>exposed 5</td>
<td>27.4 ± 4.5</td>
<td>18.8 ± 1.5</td>
<td>6.6 ± 0.3</td>
<td>5.4 ± 0.3</td>
<td></td>
</tr>
</tbody>
</table>

Legend: n – number of measurements; NS – non-significant differences

Table 2 Data of osteons (mean ± standard deviation)

<table>
<thead>
<tr>
<th>Observed files</th>
<th>sample</th>
<th>n</th>
<th>area (µm²)</th>
<th>perimeter (µm)</th>
<th>max. diameter (µm)</th>
<th>min. diameter (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cranial</td>
<td>control 5</td>
<td>232.9 ± 71.6</td>
<td>54.5 ± 7.7</td>
<td>18.8 ± 1.2</td>
<td>15.6 ± 1.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>exposed 5</td>
<td>343.4 ± 84.6</td>
<td>65.5 ± 8.4</td>
<td>22.2 ± 1.3</td>
<td>19.6 ± 1.5</td>
<td></td>
</tr>
<tr>
<td>lateral</td>
<td>control 5</td>
<td>377.1 ± 95.6</td>
<td>68.4 ± 10.1</td>
<td>22.4 ± 2.0</td>
<td>21.2 ± 1.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>exposed 5</td>
<td>263.4 ± 91.5</td>
<td>57.0 ± 9.0</td>
<td>18.4 ± 1.5</td>
<td>17.8 ± 1.4</td>
<td></td>
</tr>
<tr>
<td>medial</td>
<td>control 5</td>
<td>305.8 ± 57.8</td>
<td>62.5 ± 6.7</td>
<td>21.4 ± 1.8</td>
<td>18.2 ± 0.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>exposed 5</td>
<td>204.7 ± 27.6</td>
<td>51.7 ± 3.4</td>
<td>18.6 ± 0.6</td>
<td>14.0 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>caudal</td>
<td>control 5</td>
<td>249.2 ± 72.0</td>
<td>55.7 ± 8.3</td>
<td>18.6 ± 1.4</td>
<td>16.8 ± 1.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>exposed 5</td>
<td>309.7 ± 96.0</td>
<td>61.9 ± 8.3</td>
<td>20.6 ± 2.0</td>
<td>18.8 ± 1.6</td>
<td></td>
</tr>
</tbody>
</table>

Legend: n – number of measurements; NS – non-significant differences

Figure 3 Average weight of control (a) and DEHP-exposed (b) mouse

Coprophagy had been observed only in DEHP exposed individual. We also had observed a noticeable increase in water intake and grooming, apathy and lethargy, tightening, rapid breathing, observed files only hypothesize to differences due to exposure to DEHP. In the study of Bielanowicz et al. (2016), reduced growth of femur of DBP (di-n-butyl phthalate)-exposed mice was indicated by a lower volume of bone marrow (DBP dose 100 and 250 mg/kg/day). Shortened femoral bones in this study were observed at a dose of 500 mg/kg/day of DBP. Bastos Sales et al. (2018) recorded higher weight of right femurs of female mice exposed to DEHP (0.018–555.6 mg/kg/day). These results correspond to the findings obtained in our study. The results of the qualitative analysis of the control sample are in accordance with several studies (Enlow and Brown, 1956; Treuting and Dintzis, 2012; Šarocká et al., 2016) however, different chemicals have been studied. On the other hand, in our study the exposure to DEHP leads to absence of non-vascular bone tissue and presence of resorption lacunae in endosteal surface (pars lateralis). The bone as a dynamic tissue is constantly reorganized by osteoclasts and osteoblasts due to various stress stimuli (Rodan, 1992). Hormones (e.g., testosterone, estrogen and cortisol) affect the bone synthesis and resorption and thus osteoblasts and osteoclasts functions. Due to this fact, estrogen-like endocrine disruptors (phthalates included) could be possibly related to the lower bone mass or affect the process of bone remodeling or formation (Age, 2013). Simultaneously, the increased osteocyte apoptosis caused by loss of estrogens is regional, rather than uniform. In the bone cortex, the apoptotic osteocytces' location is tightly correlated with the areas where endocortical resorption is subsequently activated. Only a few studies showed that the increased osteocyte apoptosis caused by loss of estrogens is regional, rather than uniform. In the bone cortex, the apoptotic osteocytces' location is tightly correlated with the areas where endocortical resorption is subsequently activated. A large number of studies have been conducted on the topic of estrogen effects on bone health. Estrogen levels have been shown to influence bone density, mineralization, and remodeling processes. However, more research is needed to fully understand the complex interplay between estrogen and bone health. Further studies are required to investigate the role of estrogen in bone health and to explore potential therapeutic strategies for managing osteoporosis. Only a few studies showed that the increased osteocyte apoptosis caused by loss of estrogens is regional, rather than uniform. In the bone cortex, the apoptotic osteocytces' location is tightly correlated with the areas where endocortical resorption is subsequently activated. A large number of studies have been conducted on the topic of estrogen effects on bone health. Estrogen levels have been shown to influence bone density, mineralization, and remodeling processes. However, more research is needed to fully understand the complex interplay between estrogen and bone health. Further studies are required to investigate the role of estrogen in bone health and to explore potential therapeutic strategies for managing osteoporosis.
Analyses conducted in our study did not record significant differences in the size of Haversian canals and osteons between the femoral bones of control and DEHP exposed animal. Blood vessels passing through each Haversian canal provide the necessary supplements to the bone cells. These blood vessels can react to any functional change and adapt their structure (vascular remodeling) (Pries et al. 2005). VEGF (vascular endothelial growth factor) is important cytokine regulating angiogenesis in various physiological and pathological processes (Li et al., 2018). Some studies hypothesize on the disrupting effect of phthalates during angiogenesis. However, their data are focused mainly on tumor angiogenesis (Romani et al., 2014). Of course, in our study, the small sample size could affect the reliability of a survey’s results because it leads to a higher variability. 

According to studies (Hanahan, 1997; Thurston, 2002), the angiogenesis-promoting factors (include VEGF, bFGF (basic fibroblast growth factor), ANG (angiotropin), PDGF (platelet-derived growth factor), TGF-β (transforming growth factor-beta)) increase the vascularization of bone tissue due to vitamin B12 avitaminosis that is necessary for Haversian canals and osteons of both individuals. We assume that this behavior appears mostly due to vitamin B12 avitaminosis that is necessary for vitamin B12 mineralization of rat calvarial osteoblasts in vitro. "ça se passe jambon", 1937, 56, 2562-2574.

METHODS AND SCENARIOS MENTIONED IN OUR STUDY OFFER HYPOTHESES THAT HAVE NOT BEEN ELUCIDATED YET, AS THE EFFECTS OF DEHP ON ANGIOGENESIS AND TUMOR GROWTH HAVE NOT BEEN EXAMINED.

REFERENCES


Methods and scenarios mentioned in our study offer hypotheses that have not been researched yet and which are necessary to be verified by the larger animal groups. This step was affected by the pandemic situation. Further study is needed to elucidate our findings of bone health parameters. Our study supports the hypothesis about the negative impact of the DEHP exposure on bone tissue. Oral administration of DEHP at sublethal dose for 15 days caused significant changes in macroscopic characteristics of the compact bone tissue in a male mouse. Conducting microscopic qualitative analysis, we observed the presence of resorption lacunae and the absence of non-vascular and primary vascular radial bone tissue of the experimental mouse. This result supports the hypothesis of the interfering effect of DEHP on osteoblastogenesis and angiogenesis. The microscopic quantitative analysis did not show any significant differences in measured parameters of Haversian canals and osteons of both individuals. We assume, the effect of DEHP on bone tissue depends on the dose and duration of phthalate exposure. Our study provides only initial information related to DEHP’s impact on femoral bone microstructure and behavior in an animal model. A larger research sample, systematic approach, testing of different concentrations, and different phthalate diesters may provide more accurate results and more insights into bone microstructural changes after the application of various endocrine disruptors.


