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Examination of impact of Di(2-Ethylhexyl) Phthalate and Dibutyl Phthalate on Rat internal organs by scanning acoustic microscopy and inductively coupled plasma optical emission spectroscopy

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ABSTRACT

Objective: Phthalates, despite their endocrine-disrupting effects, are widely used as plastifiants. Environmental exposure of phthalates was demonstrated to cause fetal death and reproductive toxicity in human beings, as well as in laboratory animals. However, underlying mechanisms are not clear.

Material and Methods: Here, we examine the impact of di(2-Ethylhexyl) phthalate (DEHP) and dibutyl phthalate (DBP) on rat lungs, brain, and heart by scanning acoustic microscopy (SAM) and inductively coupled plasma optical emission spectroscopy (ICP-OES). First, we evaluate tissues of mother rats and we show that the acoustic impedance values of tissues of DEHP and DBP delivered rats differ from those of tissues of the control rat. Then, element level analyses within these tissues are done and element levels within tissues of DEHP and DBP delivered pregnant rats are found to be higher than those within tissues of the control pregnant rat. We then evaluate the tissues of offspring female rats.

Results: It is shown that acoustic impedance values of tissues of offspring rats of DEHP and DBP delivered mother rats are higher than those of tissues of the control offspring rats of the control mother rat. Besides, element analysis reveals higher element levels in the tissues of offspring rats of DEHP and DBP delivered mother rats.

Conclusion: Therefore, we can conclude that phthalates cause structural and functional changes within rat internal organs such as lungs, brain, and heart. In summary, both modalities are confirmatory in a way that tissues of DEHP and DBP exposed pregnant rats and their offspring rats are differentiated by different acoustic impedance values obtained by SAM and higher element levels specified by ICP-OES.

Keywords: scanning acoustic microscopy, inductively coupled plasma optical emission spectroscopy, phthalate exposure

Research Article

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INTRODUCTION

Advanced technology provoked the invention of new chemicals, which are used predominantly for the enhancement of agricultural production and also as ingredients in various products. Major types of these chemicals include herbicides, fungicides, insecticides, plastifiants, polychlorinated biphenyls and alkylphenolic compounds. Most of these chemicals are mainly released into the environment as a consequence of industrial production. These substances may mimic natural hormones or present antagonistic effects (**1, 2, 3, 4**).

Therefore, they are named as endocrine disruptors. Bisphenol A (BPA), whose endocrine-disrupting effect has been confirmed, is used in the production of agricultural, industrial and house detergents, pesticide and herbicide formulations, dyes and plastics, industrial products and antioxidant and drug agents. European Union has banned the use of BPA in the production of toys for children under three years old, since high doses of BPA have been linked to infertility and other health problems. However, these substances are still in use in toys for children over three years of age. Other chemicals with proven endocrine-disrupting effects include natural and synthetic hormones, phytoestrogens, and many industrial chemicals such as pesticides and plastifiants. Plastifiants are used for making rigid polyvinyl chloride (PVC) more elastic (**5**). Phthalates, phthalate esters or phthalic acid esters are usually added to plastics to increase flexibility. Phthalate plastifiants are produced industrially and used in PVC applications of artificial leather, electric cables, shoe bases, ground tiles and tubes (such as dialysis tubes), syringes, and gloves used in hospitals. Phthalates are released over time, since they do not tightly bound to PVC and humans get exposed to phthalates via inhalation, ingestion, dermal route or during medical procedures (**6, 7, 8**). Non-PVC applications of phthalate plastifiants include dyes, rubber, glues and baby milk, cheese, margarine, and chips packages. The most studied and most prevalent form of phthalates is di(2-ethylhexyl) phthalate (DEHP) and dibutyl phthalate (DBP). Animal studies in recent years revealed that accumulation of DEHP in tissues leads to long-term toxicity, causing embryo death, fetal toxicity, and teratogenicity at high doses (1000 mg/kg/day) (**6, 9**). In animals, body weight loss and testicular weight loss were observed and the decrease in testicular weight caused reduced zinc concentrations in the testis, apoptosis, and mass destruction of spermatogenic cells (**10**). Perinatal exposure of DBP was examined and found to induce neurotoxicity in neonatal and immature rats (**11**). Similarly, it was demonstrated that gestational or postnatal DEHP exposure induced adverse effects on rat brain development and function (**12**). Exposure to bisphenol A is directly associated with inflammation, which causes initiation and progression of cardiovascular disease (**13**). Many of the population-based human studies confirm the relationship with phthalates, specifically DEHP, with the increased risk of developing wheeze, asthma, and allergy (**14**). Therefore, examination of the phthalate exposure and the dose at which disruptions occur in living organisms is crucial.

Alterations in tissues with certain limitations can be identified with imaging modalities such as magnetic resonance imaging (MRI), micro-optical coherence tomography (micro-OCT), or positron emission tomography (PET), which are either very expensive or involve ionizing radiation (**15**). On the other hand, ultrasound (US) imaging, which is a very common modality for the observation of soft tissue, has high axial and lateral resolutions of approximately 20-100 µm, a good penetration depth of approximately 5 mm. Although it can only provide morphological information, US imaging has a low cost. Scanning acoustic microscopy (SAM) can give information about the morphology and elastic properties of biological tissues simultaneously at microscopic levels with focused high-frequency ultrasound. Major advantages of SAM are its speed in obtaining the two-dimensional images and immediate installation of the specimen without special preparation and staining. SAM has two modes calculating either the speed of sound (SOS) through tissues (**16,17,18,19,20,21,22,23,24,25**) or acoustic impedance of samples (**26,27**). Spectrometric techniques are largely used for obtaining chemical information about biological samples. Inductively coupled plasma optical emission spectroscopy (ICP-OES) is one of them, which is widely used for element analysis due to its high sensitivity, versatility, high speed, and acceptable cost (**28,29,30**). ICP-OES is an emission spectroscopy that determines elemental composition in a biological material by analyzing emitted electromagnetic radiation characteristic to a particular element. Combining ICP-OES with SAM will enable morphological and chemical information simultaneously with a less expensive system. In this study, we want to investigate the effects of DEHP and DBP exposure during the gestation period by examining internal organs of DEHP and DBP exposed pregnant rats and their offspring female rats. For this purpose, two doses of 61 µg/kg/day and 61 mg/kg/day of DEHP and DBP, which are established according to the observed human exposure, are given to the rats with a gavage. Lung, brain, and heart tissues are characterized by scanning acoustic microscopy (SAM) and inductively coupled plasma optical emission spectroscopy (ICP-OES). While SAM provides micrometer resolution structural and mechanical information regarding the internal organs, ICP-OES provides information about the elements within these organs. Elements examined within all tissues are copper (Cu), iron (Fe), magnesium (Mg), manganese (Mn), selenium (Se), and zinc (Zn). Acoustic impedance values of tissues of DBP and DEHP delivered pregnant rats and their offspring rats differ from those of tissues of control pregnant and their offspring rats. Similarly, element levels in tissues of DBP and DEHP delivered pregnant rats and their offspring rats are higher compared to those in tissues of control pregnant and their offspring rats. Finally, we can conclude that the toxic agent-induced structural and functional deviations are observed and confirmed by these techniques.

MATERIAL and METHODS

Specimens: The study was approved by the Ethical Committee of Istanbul University (Number: 2013/138). Healthy adult male and female Sprague Dawley rats were provided by Laboratory Animal Center in Istanbul University and housed in one cage in a controlled environment at 22 ± 3 °C and relative humidity level of 50-60 % with a 12/12 h light/dark cycle. 4 groups, composed of pregnant rats of control, low dose prenatal (given a low dose of DEHP and DBP, dissolved in corn oil) and high dose prenatal (given a high dose of DEHP, dissolved in corn oil), together with their female offspring rats, were studied. In the control group, animals received edible corn oil by intragastric administration. Low and high intragastric doses of 61 µg/kg/day and 61 mg/kg/day, respectively, were administered to the low and high dose prenatal groups at the same time of each day from the sixth to the nineteenth day of the pregnancy. The mother rats were anesthetized using ether and cervical dislocation was performed in the twentieth day of pregnancy. The lung, brain, and heart tissues were carefully removed, cleaned and fixed in 4 % paraformaldehyde (PFA). There were 2 female offspring rats of the control mother. There were 3 female offspring rats of DBP (Low) exposed

mothers. There were 6 female offspring rats of DEHP (Low) exposed mothers. There were 3 female offspring rats of DEHP (High) exposed mother.

Scanning Acoustic Microscopy: The methods were carried out in accordance with the Institutional Ethics Committee for the Local Use of Animals in Experiments in Bogazici University. The internal organs of the rats were characterized by scanning acoustic microscope (AMS-50SI) developed by Honda Electronics (Toyohashi, Japan). SAM setup and the principle of SAM, in acoustic impedance mode, can be found in our previous study (**31**). SAM is composed of a transducer with quartz lens, a pulser/receiver, an oscilloscope, a computer, and a display monitor. 80 MHz transducer is used as a generator and a receiver of ultrasonic signals, therefore, it acts as a pulser/receiver. It has a spot size of 17 µm and a focal length of 1.5 mm. Single pulses of a width of 5 ns with a repetition rate of 10 kHz are transmitted to the specimen and the reflected signals are collected. Water is the coupling medium between the quartz lens and the substrate. X-Y stage controlled by the computer scans the transducer. The reflected signals from the reference and the target material are analyzed by the oscilloscope. Finally, intensity and acoustic impedance maps of the region of interest with 300 x 300 sampling points are constructed with a lateral resolution of approximately 20 µm. The target is the tissue under investigation and distilled water is the reference material. The target signal is

$$S_{target} = \frac{Z_{target} - Z_{sub}}{Z_{target} + Z_{sub}} S_0$$

where S0 is the signal generated by the 80 MHz transducer, Ztarget is the acoustic impedance of the tissue and Zsub is the acoustic impedance of the polystyrene substrate (2.37 MRayl). The tissue's acoustic impedance is calculated by comparing the reflected signal from the tissue with the one from the reference. The reflected signal from the reference is

$$S_{ref} = \frac{Z_{ref} - Z_{sub}}{Z_{ref} + Z_{sub}} S_0$$

where Zref is the reference's acoustic impedance (1.50 MRayl). Consequently, the target's acoustic impedance can be written as with a constant generated signal S0 (**26**).

$$Z_{target} = \frac{1 + \frac{S_{target}}{S_0}}{1 - \frac{S_{target}}{S_0}} Z_{sub}$$

Inductively Coupled Plasma Optical Emission Spectroscopy

The element levels in lung, brain, and heart tissues were measured using an inductive coupled plasma optical emission spectrophotometer (iCAP 6000 series, Thermo Fischer Scientific Inc., Istanbul, Turkey) at Trace Element Analysis Laboratory in Department of Biophysics in Cerrahpasa Faculty of Medicine. Tissue samples were placed into tared glassware tubes and the weight of the samples was recorded. The tissue samples were subjected to ashing with 2 mL of nitric acid (65 %) and 1 mL of perchloric acid (70 %) in a drying furnace at 150 °C. The digested samples were left to cool at room temperature and diluted with distilled water then vortexed to get ready to conduct elemental analysis in an ICP-OES device. The analyses of copper (Cu), iron (Fe), magnesium (Mg), manganese (Mn), selenium (Se), and zinc (Zn) levels were performed with ICP-OES Thermo iCAP 6000 series at Trace Element Analysis Laboratory of Biophysics Department of Cerrahpasa Medical Faculty in Istanbul, Turkey. The wavelengths of 324.754 nm, 259.940 nm, 285.213 nm, 257.610 nm, 196.090 nm, and 206.200 nm were used for the detection of Cu, Fe, Mg, Mn, Se and Zn, respectively, in the device. Measurement of each element level was carried out three times and averaged. The ICP-OES system was operated with incident power of 1.3 kW, 15 L/min plasma argon flow rate, 1.5 L/min auxiliary argon flow rate, 0.7 L/min nebulizer argon flow rate in this study. Transport lines were obtained using 1.25 mm internal diameter polytetrafluoroethylene tubing. The standard concentrations for standard graph calibration were prepared from standard stock solutions of 1000 µg/ml for each analyzed trace element. Trace element levels in serum samples were expressed in micrograms per milliliter (µg/ml).

Statistical Analysis

Statistical analysis was performed using SPSS 21 statistical software for Windows. Results were presented as means ± standard deviation (SD). For comparison of parameters between the 4 groups, ANOVA parametric test was used. The mean and median values were presented along with their 95% confidence interval. All analyses were assumed statistically significant at $p < 0.05$.

RESULTS

Scanning Acoustic Microscopy Results

The lung, brain, and heart tissues, received from pregnant control mother rats and mother rats exposed to DEHP and DBP and their female offspring rats were sliced cross-sectional for SAM studies. **Table1** presents acoustic impedance values within all tissues of the mother rats examined. **Table2** presents average acoustic impedance values within all tissues of the offspring rats examined. The average acoustic impedance values and their standard deviations were calculated considering 2 female offspring rats of the control mother, 3 female offspring rats of DBP (Low) exposed mother, 6 female offspring rats of DEHP (Low) exposed mother and 3 female offspring rats of DEHP (High) exposed mother. In **Table1** and **Table2**, low is for a concentration of 61 µg/kg/day, and high is for a concentration of 61 mg/kg/day, applied to mother rats from the sixth to the nineteenth day of pregnancy. Tissue samples, which belong to the rats exposed to DEHP and DBP, are easily distinguishable with higher acoustic impedance values, since, they are quite stiff when compared to tissues with smaller acoustic impedance values. In other words, the tissues have distinctive acoustic impedance values due to the discrepancy in elastic properties. The SAM images, obtained using the acoustic impedance mode of SAM, were constructed by comparing the reflections of ultrasound signals from the reference and front surfaces of the slices. **Figure1** shows the acoustic impedance distribution of a lung sample obtained from an offspring rat of the control mother rat. **Figure2** shows the acoustic impedance distribution of a lung sample obtained from an offspring rat of DEHP (Low) exposed mother rat, with higher acoustic impedance values

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when compared with the one of the control offspring rat, shown in **Figure1**. In **Figure2** an offspring rat of DEHP (Low) exposed mother rat is chosen since the acoustic impedance value of that rat's lung tissue is significantly higher than that of the lung tissue of the control offspring rat.

Inductively Coupled Plasma Optical Emission Spectroscopy Results

We determined Cu, Fe, Mg, Mn, Se, and Zn levels in tissue samples. **Table3** presents element levels within lung tissues of the pregnant rats. Similarly, **Table4** and **Table5** present element levels within brain and heart tissues of pregnant rats, respectively. As can be observed in **Table3**, Cu, Fe, Mg, Mn, Se, and Zn levels are the highest in the lung tissue of DEHP (Low) exposed pregnant rat, when compared to element levels in tissues of control, DBP (Low) and DEHP (High) exposed pregnant rats. Besides, Cu, Mg and Se levels in lung tissue of DBP (Low) exposed pregnant rats are apparently lowered when compared to those in lung tissue of control mother rats. As can be seen in **Table4** and **Table5** element levels within brain and heart tissues of DEHP exposed pregnant rats are higher than those within tissues of DBP exposed mother rats. **Table6** presents average element levels within lung tissues of female offspring rats.

Similarly, **Table 7** and **Table 8** present average element levels within brain and heart tissues of female offspring rats, respectively. The average values and their standard deviations were calculated considering 2 female offspring rats of the control mother, 3 female offspring rats of DBP (Low) exposed mother, 6 female offspring rats of DEHP (Low) exposed mother and 3 female offspring rats of DEHP (High) exposed mother. As can be observed in **Table 6**, Cu, Fe, Mg, Mn, Se, and Zn levels are the highest in the lung tissues of DEHP (Low) exposed offspring rats, when compared to element levels in tissues of control, DBP (Low) and DEHP (High) exposed pregnant rats. Besides, Cu and Se levels in lung tissues of offspring rats of DBP (Low) exposed pregnant rat are apparently lowered when compared to those in lung tissues of offspring rats of control mother rat. As can be seen in **Table7**, the average Mg level within brain tissues of offspring rats of DBP exposed pregnant rats reaches the highest value while, average Fe level within brain tissues of offspring rats of DBP exposed pregnant rats reaches the highest value as can be seen in **Table8**. Besides, levels of most of the elements examined the increase in offspring rats of DBP and DEHP exposed rats when compared with offspring rats of the control mother, together with a higher variance due to variable structural and functional changes in offspring rats of DEHP and DBP exposed mother rats.

Table 1: Acoustic impedance values of lung, brain, and heart tissues of the pregnant rats exposed to different concentrations of phthalates of DEHP and DBP. Low is for a concentration of 61 µg/kg/day and high is for a concentration of 61 mg/kg/day, applied to these rats from the sixth to the nineteenth day of pregnancy.

Tissue	Control Acoustic Impedance (MRayl)	DBP (Low) Acoustic Impedance (MRayl)	DEHP (Low) Acoustic Impedance (MRayl)	DEHP (High) Acoustic Impedance (MRayl)
Lung	1.61 ± 0.19	1.38 ± 0.03	1.74 ± 0.21	1.60 ± 0.04
Brain	1.55 ± 0.04	1.48 ± 0.03	1.83 ± 0.08	1.73 ± 0.10
Heart	1.41 ± 0.07	1.60 ± 0.07	1.66 ± 0.12	1.78 ± 0.18

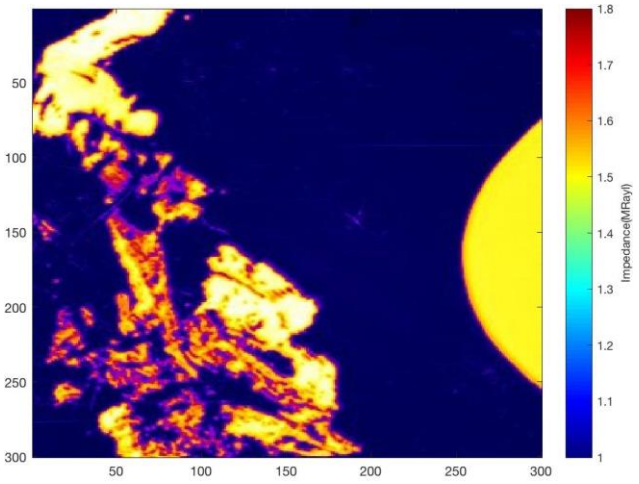
Data are shown as the means ± SD ($p < 0.05$)

Table 2: Average acoustic impedance values of lung, brain and heart tissues of offspring rats of the pregnant rats, which were exposed to different concentrations of phthalates of DEHP and DBP. Low is for a concentration of 61 µg/kg/day and high is for a concentration of 61 mg/kg/day, applied to mother rats from the sixth to the nineteenth day of pregnancy.

Tissue	Control Acoustic Impedance (MRayl)	DBP (Low) Acoustic Impedance (MRayl)	DEHP (Low) Acoustic Impedance (MRayl)	DEHP (High) Acoustic Impedance (MRayl)
Lung	1.49 ± 0.17	1.56 ± 0.04	1.68 ± 0.06	1.59 ± 0.06
Brain	1.51 ± 0.06	1.56 ± 0.03	1.63 ± 0.03	1.75 ± 0.06
Heart	1.51 ± 0.07	1.60 ± 0.06	1.69 ± 0.07	1.56 ± 0.05

Data are shown as the means ± SD ($p < 0.05$)

Figure 1. SAM image of the lung tissue of an offspring rat of the control mother rat. The image is obtained by comparing reflected ultrasound signals from both surfaces of water and the tissue. Scanning area is 4.8 mm x 4.8 mm.



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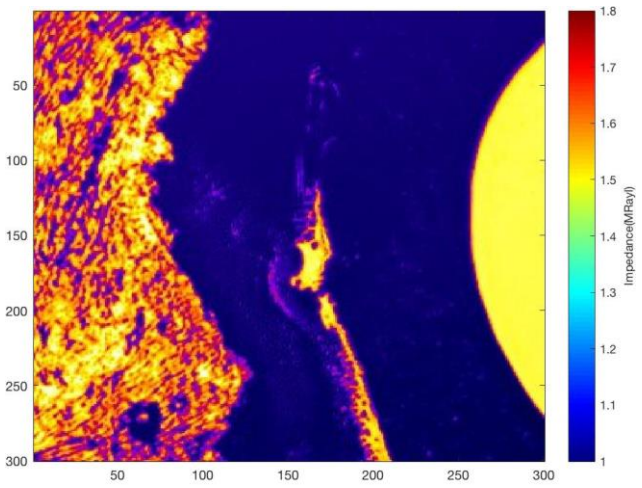

Figure 2. SAM image of the lung tissue of an offspring rat of DEHP (Low) exposed mother rat. The image is obtained by comparing reflected ultrasound signals from both surfaces of water and the tissue. Scanning area is 4.8 mm x 4.8 mm.

Table 3: Element levels within the lung tissues of the pregnant rats exposed to different concentrations of phthalates of DEHP and DBP. Low is for a concentration of 61 µg/kg/day and high is for a concentration of 61 mg/kg/day, applied to these rats from the sixth to the nineteenth day of pregnancy.

Elements	Control ($\mu g\ gr^{-1}$)	DBP (Low) ($\mu g\ gr^{-1}$)	DEHP (Low) ($\mu g\ gr^{-1}$)	DEHP (High) ($\mu g\ gr^{-1}$)
Cu	4.48 ± 0.96	2.38 ± 0.02	23.64 ± 0.62	7.21 ± 0.60
Fe	21.66 ± 0.21	35.05 ± 0.72	409.91 ± 1.50	118.50 ± 1.77
Mg	99.07 ± 0.34	53.34 ± 0.54	574.09 ± 2.33	172.99 ± 2.83
Mn	0.37 ± 0.08	0.33 ± 0.09	4.09 ± 0.13	0.95 ± 0.15
Se	3.24 ± 0.86	0.00 ± 0.00	18.83 ± 0.23	10.27 ± 0.88
Zn	14.31 ± 0.74	13.12 ± 0.34	194.93 ± 0.79	36.12 ± 0.95

Cu, copper; Fe, iron; Mg, magnesium; Mn, manganese; Se, selenium; Zn, zinc,
Data are shown as the means ± SD ($p < 0.05$)

Table 4: Element levels within the brain tissues of the pregnant rats exposed to different concentrations of phthalates of DEHP and DBP. Low is for a concentration of 61 µg/kg/day and high is for a concentration of 61 mg/kg/day, applied to these rats from the sixth to the nineteenth day of pregnancy.

Elements	Control ($\mu g\ gr^{-1}$)	DBP (Low) ($\mu g\ gr^{-1}$)	DEHP (Low) ($\mu g\ gr^{-1}$)	DEHP (High) ($\mu g\ gr^{-1}$)
Cu	15.28 ± 1.23	14.66 ± 2.10	44.72 ± 2.59	29.40 ± 1.38
Fe	104.44 ± 2.84	153.38 ± 2.56	158.978 ± 2.95	187.67 ± 2.59
Mg	120.06 ± 2.22	191.05 ± 3.29	244.49 ± 3.45	324.88 ± 3.34
Mn	0.69 ± 0.07	1.07 ± 0.12	2.99 ± 0.44	5.41 ± 0.75
Se	3.27 ± 0.76	2.07 ± 0.55	13.39 ± 0.83	5.62 ± 0.81
Zn	25.84 ± 1.69	54.94 ± 1.46	112.76 ± 2.13	69.75 ± 1.74

Cu, copper; Fe, iron; Mg, magnesium; Mn, manganese; Se, selenium; Zn, zinc,
Data are shown as the means ± SD ($p < 0.05$)

Table 5: Element levels within the heart tissues of the pregnant rats exposed to different concentrations of phthalates of DEHP and DBP. Low is for a concentration of 61 µg/kg/day and high is for a concentration of 61 mg/kg/day, applied to these rats from the sixth to the nineteenth day of pregnancy.

Elements	Control ($\mu g\ gr^{-1}$)	DBP (Low) ($\mu g\ gr^{-1}$)	DEHP (Low) ($\mu g\ gr^{-1}$)	DEHP (High) ($\mu g\ gr^{-1}$)
Cu	8.00 ± 0.78	5.44 ± 0.95	43.80 ± 1.49	7.53 ± 0.68
Fe	108.93 ± 1.88	221.86 ± 1.79	284.00 ± 2.09	501.83 ± 2.55
Mg	41.44 ± 1.58	50.59 ± 1.66	123.60 ± 2.32	131.77 ± 2.41
Mn	0.34 ± 0.06	0.51 ± 0.05	5.84 ± 0.76	1.08 ± 0.06
Se	3.09 ± 0.67	1.70 ± 0.85	10.36 ± 1.15	8.28 ± 1.09
Zn	23.28 ± 1.59	23.53 ± 1.23	65.68 ± 2.05	28.10 ± 1.85

Cu, copper; Fe, iron; Mg, magnesium; Mn, manganese; Se, selenium; Zn, zinc,
Data are shown as the means ± SD ($p < 0.05$)

Table 6: Average element levels within the lung tissues of offspring rats exposed to different concentrations of phthalates of DEHP and DBP. Low is for a concentration of 61 µg/kg/day and high is for a concentration of 61 mg/kg/day, applied to mother rats from the sixth to the nineteenth day of pregnancy.

Elements	Control ($\mu g\ gr^{-1}$)	DBP (Low) ($\mu g\ gr^{-1}$)	DEHP (Low) ($\mu g\ gr^{-1}$)	DEHP (High) ($\mu g\ gr^{-1}$)
Cu	5.02 ± 0.98	3.75 ± 0.46	13.98 ± 3.45	5.87 ± 1.69
Fe	18.11 ± 1.70	19.27 ± 4.10	247.48 ± 94.71	65.55 ± 23.73
Mg	61.14 ± 7.66	86.82 ± 20.7	215.07 ± 92.01	81.91 ± 14.22
Mn	0.12 ± 0.08	0.72 ± 0.20	2.21 ± 0.83	0.49 ± 0.16
Se	1.73 ± 0.79	0.79 ± 0.16	10.19 ± 4.69	3.68 ± 0.43
Zn	14.48 ± 2.92	19.54 ± 6.98	52.44 ± 15.62	24.24 ± 7.17

Cu, copper; Fe, iron; Mg, magnesium; Mn, manganese; Se, selenium; Zn, zinc
Data are shown as the means ± SD ($p < 0.05$)

Table 7: Element levels within the brain tissues of offspring rats exposed to different concentrations of phthalates of DEHP and DBP. Low is for a concentration of 61 µg/kg/day and high is for a concentration of 61 mg/kg/day, applied to mother rats from the sixth to the nineteenth day of pregnancy.

Elements	Control ($\mu g\ gr^{-1}$)	DBP (Low) ($\mu g\ gr^{-1}$)	DEHP (Low) ($\mu g\ gr^{-1}$)	DEHP (High) ($\mu g\ gr^{-1}$)
Cu	10.14 ± 0.82	31.58 ± 17.42	21.60 ± 13.51	24.43 ± 12.02
Fe	73.43 ± 7.54	161.41 ± 41.17	181.28 ± 62.23	153.33 ± 23.74
Mg	69.68 ± 6.88	280.43 ± 97.47	204.31 ± 94.58	173.22 ± 27.11
Mn	0.42 ± 0.03	3.97 ± 1.04	2.07 ± 0.88	1.56 ± 0.93
Se	2.92 ± 0.73	2.38 ± 0.95	6.67 ± 1.28	1.54 ± 0.53
Zn	13.84 ± 0.16	64.92 ± 27.74	60.89 ± 18.89	77.52 ± 25.44

Cu, copper; Fe, iron; Mg, magnesium; Mn, manganese; Se, selenium; Zn, zinc
Data are shown as the means ± SD ($p < 0.05$)

Table 8: Element levels within the heart tissues of offspring rats exposed to different concentrations of phthalates of DEHP and DBP. Low is for a concentration of 61 µg/kg/day and high is for a concentration of 61 mg/kg/day, applied to mother rats from the sixth to the nineteenth day of pregnancy.

Elements	Control ($\mu g\ gr^{-1}$)	DBP (Low) ($\mu g\ gr^{-1}$)	DEHP (Low) ($\mu g\ gr^{-1}$)	DEHP (High) ($\mu g\ gr^{-1}$)
Cu	8.70 ± 1.84	5.23 ± 0.55	16.33 ± 7.50	7.95 ± 2.84
Fe	111.54 ± 3.21	435.91 ± 99.67	288.62 ± 43.94	235.22 ± 93.64
Mg	83.17 ± 68.41	147.66 ± 53.80	154.71 ± 27.71	93.59 ± 31.88
Mn	0.67 ± 0.44	1.34 ± 0.21	1.68 ± 0.54	0.81 ± 0.36
Se	2.37 ± 0.16	2.02 ± 0.33	4.47 ± 0.75	4.14 ± 0.26
Zn	18.29 ± 1.29	18.54 ± 3.03	47.26 ± 23.05	29.87 ± 6.46

Cu, copper; Fe, iron; Mg, magnesium; Mn, manganese; Se, selenium; Zn, zinc
Data are shown as the means ± SD ($p < 0.05$)

DISCUSSION

Even though it has been revealed that accumulation of phthalates in animals and also in humans leads to damages in reproductive organs with high dose exposure, literature lacks the dose at which these disruptions start to occur. It has been reported that an average DEHP exposure dose is between 3 to 30 µg/kg/day for an adult whereas multiple times higher for a baby or an infant (**32**). Average DBP exposure of an adult was determined to be 7 µg/kg/day (**33**).

These doses would be even higher during a medical intervention due to the use of plastic blood and serum bags. In this study, phthalate exposure doses were determined to be 61 µg/kg/day and a much higher value of 61 mg/kg/day for comparison, considering the differences in the metabolisms and sizes of humans and rats. Only female offspring rats are taken into account together with mother rats to avoid parameters depending on the sex.

Examination of lung, brain, and heart tissues of pregnant rats and their offspring female rats by SAM is done for the first time in literature and also supported by ICP-OES in our study.

SAM is successful in determining the alterations within tissue samples, by calculating acoustic impedance values in tissues of mother and offspring rats. In **Table 1** acoustic impedance values of lung and brain tissues of DBP exposed pregnant rat were lower than those of lung and brain tissues of the control mother rat. Besides, acoustic impedance values of lung and brain tissues of DEHP (Low) exposed mother rats were higher than those of lung and brain tissues of DEHP (High) exposed mother rats. The decrease in acoustic impedance value of lung tissue of DBP exposed mother rat can be a result of noticeable Cu, Mg, and Se level decrease as can be seen in **Table3**.

Besides, the highest acoustic impedance value observed in lung tissue of DEHP (Low) exposed mother rat can be a result of the observation of the highest element levels in that tissue as can be seen in **Table 3**. Similarly, the decrease in acoustic impedance value of brain tissue of DBP exposed mother rat can be a result of Cu and Se level decrease as can be seen in **Table4**. In **Table1**, the acoustic impedance value of heart tissue of DEHP (High) exposed mother rat is the highest, confirmed with the elevated element levels in that tissue (**Table 5**).

Table 2 presents average acoustic impedance values within all tissues of the offspring rats examined. Each value in Table 2 is the average of multiple female offspring rats of the same mother. Tissues of offspring rats of DBP and DEHP exposed mother rats have higher acoustic impedance values due to increased element levels. The highest acoustic impedance value of lung tissue is observed in offspring rats of DEHP (Low) exposed mother rat, which may be a result of the apparent increase in Fe, Mg, Mn, Se, and Zn levels as can be seen in Table 6, also, the highest acoustic impedance value of brain tissue, observed in offspring rats of DEHP (High) exposed mother rat, maybe a result of the apparent increase in Zn level as can be seen in **Table 7**. Similarly, the highest acoustic impedance value of heart tissue, observed in offspring rats of DEHP (Low) exposed mother rat, maybe a result of the apparent increase in Cu, Mg, Mn, Se and Zn levels as can be seen in **Table 8**.

Inducing oxidative stress and, therefore, making the oxidant and/or antioxidant mechanisms inactive, is one of the important toxicity mechanisms of phthalate esters and this has been investigated both in in vivo and in vitro studies (**34,35,36,37,38,39**). Phthalates have been shown to cause oxidative stress targeting the endocrine system, and reproductive anomalies by decreasing the levels of steroidogenic enzymes. The levels of essential elements and minerals play an active role in enzyme expression and synthesis. DEHP exposure has been found to affect trace element or mineral levels due to changes in antioxidant enzyme levels (**40,41,42,43,44**). Cell culture studies have also been reported to state that DEHP causes changes in antioxidant enzyme levels and results in intracellular reactive oxygen species (ROS) formation and deoxyribonucleic acid (DNA) damage (**34,35,44**). In our study, we determined Cu, Fe, Mg, Mn, Se, and Zn levels by ICP-OES. Even though element levels in tissues of DEHP exposed rats are increased when compared to those of the tissues of the control group, some element levels, as seen in tissues of DBP exposed rats, are decreased when compared to those of the tissues of the control group. The reason for this discrepancy may be due to the fact that DBP and DEHP esters cause changes in the levels of different proteins.

Limitations: The small number of the samples is the major limitation due to incorporating only the female rats into this study and the loss of some of the offspring rats of DEHP and DBP exposed pregnant rats through resorption. Besides, the number of pregnant rats kept at the minimum level, since it is the priority in an animal study. Therefore, another correlation study, between exposure dose and element levels within tissues, will be conducted in a larger cohort and presented later on.

CONCLUSION

There is a limited number of researches about the impact of DBP and DEHP on levels of elements within lung, brain, and heart tissues. In this study, we tried to evaluate the impact of DEHP and DBP on lung, brain, and heart tissues of both mother rats and their offspring female rats, by SAM and ICP-OES. Effects of high dose (1000 mg/kg/day) exposure are studied and determined in animal reproductive systems. However, the dose at which disruptions start to occur is not known. Imaging of the tissues with a micrometer resolution by obtaining acoustic impedance distributions, predict structural and functional changes, induced due to DBP and DEHP exposure of doses of 61 µg/kg/day and 61 mg/kg/day. ICP-OES confirms this prediction by expressing higher element levels within tissues of DEHP and DBP exposed rats. Consequently, we can state that even with these DBP and DEHP exposure doses, toxic agent-induced deviations within lung, brain, and heart tissues of mother and offspring rats can be observed by these techniques.

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REFERENCES

1. Neubert D. Vulnerability of the Endocrine System to Xenobiotic Influence. Regul Toxicol Pharmacol. 1997; 26: 9–29.

2. Sharpe RM. Natural and anthropogenic environmental oestrogens: the scientific basis for risk assessment; environmental oestrogens and male ınfertility. Pure Appl Chem. 1998; 70(9): 1685–1701.

3. Pryor JL, Hughes C, Foster W, et al. Critical windows of exposure for children's health: the reproductive system in animals and humans. Environ Heal Perspectives 2000; 108: 491–503.

4. Sweeney T. Is exposure to endocrine distrupting compounds during fetal/postnatal development affecting the reproductive potential of farm animals? Domest Animal Endocrinol. 2002; 23: 203–9.

5. Latini G, Felice CD, Verrott A. 2004. Plasticizers, infant nutrition and reproductive health. Reproductive Toxicol. 2004; 19: 27–33.

6. Latini G. The potential hazards of exposure to di-(2-ethylhexyl)-phthalate in babies: A review. Biol Neonate. 2000; 78: 269–76.

7. Saiko I, Onuki A, Seto H. Determination of organic phosphate triesters in indoor and outdoor air. Aerosol Res. 2001; 16: 209–16.

8. Okubo T, Suzuki T, Yokoyama Y, et al. 2003. Estimation of estrogenic and anti-estrogenic activites of some phthalate diesters and monoesters by MCF-7 cell proliferation assay in vitro. Biol Pharm Bull. 2003; 26: 1219–24.

9. Hellwig J, Freudenberger H, Jackh R. Differential prenatal toxicity of branched phthalate esters in rats. Food Chem Toxicol. 1997; 35: 501–12.

10. Park JD, Habeebu SSM, Klaassen CD. Testicular toxicity of di-(2-ethylhexyl)phthalate in young sprague-dawley rats. Toxicology 2002; 171: 105–15 .

11. Li XJ, Jiang L, Chen L, et al. Neurotoxicity of dibutyl phthalate in brain development following perinatal exposure: A study in rats. Environ Toxicol Pharmacol. 2013; 36: 392–402.

12. Lin H, Yuan K, Li L, et al. In utero exposure to diethylhexyl phthalate affects rat brain development: A behavioral and genomic approach. Int J Environ Res Public Heal. 2015 12(11): 392–402.

13. Choi YJ, Ha KH, Kim DJ. Exposure to bisphenol a is directly associated with inflammation in healthy korean adults. Environ Sci Pollut Res 2016; 24(1): 284–90.

14. Robinson L, Miller R. The impact of bisphenol a and phthalates on allergy, asthma, and immune function: a review of latest findings. Curr Environ Heal Reports 2015; 2(4): 379–87 .

15. Nam SY, Ricles LM, Suggs LJ, et al. Imaging strategies for tissue engineering applications. Tissue Eng. 2015; 21(1): 88–102.

16. Saijo Y, Ohashi T, Sasaki H, et al. Application of scanning acoustic microscopy for assessing stress distribution in atherosclerotic plaque. Annals Biomed Eng. 2001; 29: 1048–53.

17. Saijo Y, Miyakawa T, Sasaki H, et al. Acoustic properties of aortic aneurysm obtained with scanning acoustic microscopy. Ultrasonics 2004; 42: 695–98.

18. Saijo Y, Filho ES, Sasaki H, et al. Ultrasonic tissue characterization of atherosclerosis by a speed-of-sound microscanning system. IEEE Transactions on Ultrason. Ferroelectr Freq Control 2007; 54(8):1571–77.

19. Miura K, Yamamoto S. Pulmonary imaging with a scanning acoustic microscope discriminates speed-of-sound and shows structural characteristics of disease. Lab Investig. 2012; 92: 1760–65.

20. Miura K, Nasu H, Yamamoto S. Scanning acoustic microscopy for characterization of neoplastic and inflammatory lesions of lymph nodes. Sci Reports 2013; 3: 1255.

21. Brewin MP, Srodon PD, Greenwald SE, et al. Carotid atherosclerotic plaque characterization by measurement of ultrasound sound speed in vitro at high frequency, 20 MHz. Ultrasonics 2014; 54: 428–41.

22. Miura K, Egawa Y, Moriki T, et al. Microscopic observation of chemical modification in sections using scanning acoustic microscopy. Pathol Int. 2015; 65 (7): 355–66.

23. Akhtar R, Cruickshank JK, Zhao X, et al. A pilot study of scanning acoustic microscopy as a tool for measuring arterial stiffness in aortic biopsies. Artery Res. 2016; 13: 1–5.

24. Saijo Y, Hozumi N, Lee C, et al. Ultrasonic speed microscopy for imaging of coronary artery. Ultrasonics 2016; 44: e51–e55.

25. Miura K, Katoh H. Structural and histochemical alterations in the aortic valves of elderly patients: a comparative study of aortic stenosis, aortic regurgitation, and normal valves. Biomed Res Int. 2016; 6125204.

26. Kobayashi K, Yoshida S, Saijo Y, et al. Acoustic impedance microscopy for biological tissue characterization. Ultrasonics 2014; 54: 1922–28.

27. Hatori K, Saijo Y, Hagiwara Y, et al. Acoustic diagnosis device for dentistry. Interface Oral Heal Sci. 2016; 181–201.

28. Gomez MR, Cerutti S, Sombre LL, et al. Determination of heavy metals for the quality control in argentinian herbal medicines by ETAAS and ICP-OES. Food Chem Toxicol. 2007; 45(6): 1060–64.

29. Junior AFS, Matos R, Andrade EM, et al. Multielement determination of macro and micro contents in medicinal plants and phytomedicines from brazil by ICP OES. J Braz Chem Soc. 2017; 28(2): 376–84.

30. Karıs D, Tarhan D, Boyacıoglu K, et al. The comparison of zinc, copper and iron levels in serum, aorta and left internal mammarian artery tissues in coronary by-pass graft surgery patients. J Trace Elem Medicine Biol. 2019; 51: 86–90.

31. Bilen B, Gokbulut B, Kafa U, et al. Scanning acoustic microscopy and time-resolved fluorescence spectroscopy for characterization of atherosclerotic plaques. Sci Reports 2018; 8: 14378 .

32. Kavlock R, Barr D, Boekelhaıde K, et al. NTP-CERHR expert panel update on the reproductive and developmental toxicity of di(2-ethylhexyl) phthalate. Reproductive Toxicol. 2006; 22: 291–399.

33. Kavlock R, Boekelhaide K, Chapin R, et al. Ntp center for evaluation risks to human reproduction: phthalates expert panel report on the reproductive and developmental toxicity of di-n-butyl phthalate. Reproductive Toxicol. 2002; 16: 489–527.

34. Prasanth GK, Divya LM, Sadasivan CN. Effects of mono and di(nbutyl) phthalate on superoxide dismutase. Toxicology, 2009; 262(1): 38–42.

35. Tetz LM, Cheng AA, Korte CS, et al. Mono-2-ethylhexyl phthalate induces oxidative stress responses in human placental cells in vitro. Toxicol Appl Pharmacol. 2013; 268(1): 47–54.

36. Sedha S, Kumar S, Shukla S. Role of oxidative stress in male reproductive dysfunctions with reference to phthalate compounds. Urol Journal. 2015; 12(5): 2304–16.

37. Sobarzo CM, Nde MR, Livia L. Et al. Mono-(2-ethylhexyl) phthalate (MEHP) affects intercellular junctions of sertoli cell: A potential role of oxidative stress. Reproductive Toxicol. 2015; 58: 203–12.

38. Cho YJ, Park SB, Han MN. Di-(2-ethylhexyl)-phthalate induces oxidative stress in human endometrial stromal cells in vitro. Mol Cell Endocrinol. 2015; 407: 9–17.

39. Asghari MH, Saeidnia S, Abdollahi M. A review on the biochemical and molecular mechanisms of phthalate-induced toxicity in various organs with a focus on the reproductive system. Int J Pharmacol. 2015; 11(2): 95–105.

40. Lingren A, Lindquıst NG, Lyden A., et al. A whole body autoradiographic study on the distribution of 14C-labelled di-(2-ethylhexyl)phthalate in mice. Toxicology 1982; 23: 149–158.

41. Garberg P, Hogberg J. Selenium metabolism in isolated hepatocytes: inhibition of incorporation in proteins by mono(2-ethylhexyl)phthalate, a metabolite of the peroxisome proliferator di(2-ethylhexyl)phthalate. Carcinogenesis 1991; 12(1): 7–12.

42. Erkekoglu P, Rachidi W, Yuzugullu OG, et al. Induction of ROS, p53, p21 in DEHP- and MEHPexposed LNCaP cells-protection by selenium compounds. Food Chem Toxicol. 2011; 49(7): 1565–71.

43. Aydemir D, Karabulut G, Franci GS, et al. Impact of the di(2-ethylhexyl) phthalate administration on trace element and mineral levels in relation of kidney and liver damage in rats. Biol Trace Elem Res. 2018; 186(2): 474–88.

44. Aydemir D, Karabulut G, Gok M, et al. Data the DEHP induced changes on the trace element and mineral levels in the brain and testis tissues of rats. Data Brief. 2019; 26: 104526.