Asian Journal of Medicine and Health Sciences Vol 7 Issue 2, November 2024

ORIGINAL ARTICLE

Zinc and Iron Concentrations in Blood and Organs of Di-(2-ethylhexyl)phthalate (DEHP)-treated Rats.

Shigeru Suna[1]* **and Fumihiko Jitsunari**[2].

[1]*Private Health Research Laboratory, Kagawa, Japan.*
[2]*Professor Emeritus, Kagawa University, Kagawa, Japan.*

Corresponding Author
Shigeru Suna
Private Health Research Laboratory
14-22 Shinkita-machi, Takamatsu-shi,Kagawa 760-0001, Japan.
Email: jpsuna0218@yahoo.co.jp

Submitted: 05/06/2024. Revised edition: 17/07/2024. Accepted: 14/09/2024. Published online: 01/11/2024.

Abstract

Oral administration of di(2-ethylhexyl)phthalate (DEHP) to rats is known to induce testicular atrophy and hepatomegaly. This is thought to be the result of increased oxidative stress due to induction of peroxisomal enzymes by mono(2-ethylhexyl)phthalate (MEHP), a metabolite of DEHP. Therefore, changes in the metal content of organs are predicted. Therefore, an experiment was conducted in which rats were fed a diet containing DEHP for two weeks. Metal concentrations in blood and organs were measured, with a focus on zinc and iron. Results are as follows: DEHP treated rats had lower body weight and zinc concentrations in blood, liver, kidney, and testicular tissue, and higher iron concentrations in the liver compared to controls. These changes also correlated with plasma MEHP concentrations, suggesting MEHP-mediated oxidative stress-induced tissue damage and thyroid hormone disruption.

Keywords: *di-(2-ethylhexyl)phthalate (DEHP), iron, oxidative stress, zinc.*

Introduction

Di(2-ethylhexyl)phthalate (DEHP) is a common plasticiser for polyvinyl chloride (PVC), which is used in an extremely wide range of applications, including construction materials, water pipes, household goods, toys, medical devices, blood transfusion tubes, and bags. These PVC products often contain high concentrations of DEHP. Consequently, DEHP is one of the endocrine-disrupting chemicals that people of all ages, from young children to the elderly, are regularly exposed to, raising concerns about contamination and health risks.

It has been reported that feeding rats a diet containing high concentrations of DEHP causes testicular atrophy and hepatomegaly. Testicular atrophy is attributed to the metabolite of DEHP, mono(2-ethylhexyl) phthalate (MEHP), which damages Sertoli cells and induces apoptosis in germ cells [1-7]. Hepatomegaly, on the other hand is caused by MEHP and 2-ethyl hexanol, which cooperate with peroxisome proliferator-activated receptors (PPARs) to activate their peroxisome proliferative effects [8-11], resulting in induction of β-oxidase and cytochrome P450 enzymes [12- 15]. These findings suggest that the toxic effects of DEHP may be due to increased oxidative stress caused by elevated levels of oxidative enzymes, such as heme enzymes, in organs. This oxidative stress could lead to tissue damage via reactive oxygen species (ROS) and alterations in metal levels within tissues due to enzyme induction. Therefore, metal levels in blood and organs were investigated following DEHP administration, focusing on zinc (Zn) and iron (Fe).

Materials and methods

Animal experiment

Male Sprague-Dawley rats were purchased from Charles River (Kanagawa, Japan) and fed a CE-2 diet (Clea, Tokyo, Japan) containing 2 w/w% DEHP for the oral administration experiment. The feed was prepared by Oriental Yeast Industry (Chiba, Japan). For the analysis of MEHP by high-performance liquid chromatography (HPLC), MEHP (purity: 90% or higher) from Tokyo Kasei Kogyo and acetonitrile (HPLC grade) from Wako Pure Chemical Industries were utilised, along with other special grade commercial reagents.

The animal experiment was conducted in accordance with protocols approved by the Kagawa University Animal Committee (No. 123). The rats were housed under the following conditions: a room temperature of 22-24°C, relative humidity of 55-60%, and a light/dark cycle of 12 hours in the animal experiment facility. Five-week-old rats weighing 168.3 ± 9.0 g were divided into two groups, a control group and a treatment group, each consisting of six rats. The treatment group was fed a diet containing 2% (w/w) DEHP for two weeks. At the end of the experiment, rats were sacrificed using ether anesthesia. The testes, liver, and kidneys were removed and weighed. Cardiac blood samples were collected in heparinized tubes and plasma was separated from whole blood by centrifugation at 1500 g. Plasma and organs were frozen at -40 °C until MEHP measurement.

Blood hemoglobin (Hb) Analysis

Haemoglobin (Hb) concentration was determined using the colorimetric cyanomethemoglobin method with a kit from Wako Pure Chemical Industries, Ltd.

Metal analysis in blood and organs

Approximately, 200 µl of whole blood and 200 mg of organs were taken and placed in a Teflon jar, and 0.5 to 1 ml of mixed acid (nitric acid 50:60% perchloric acid 50: sulfuric acid 1) was added. The mixture was wet ashed at 100-140°C on a hot plate, then heated to 200°C and concentrated. The mixture was evaporated to dryness until white smoke was generated, cooled, diluted with 0.1N hydrochloric acid, and metal analysis was performed by flame atomic absorption spectrometry (1-drop method). The analytical instrument was a Seiko-SAS 7500 model equipped with deuterium background correction (Seiko, Tokyo, Japan).

Zn and Fe content in the animal diets were also analyzed in the same manner. The diets contained 70-80 ppm Zn and 270-310 ppm Fe, with no significant differences in Zn and Fe concentrations between the diets.

MEHP analysis in blood and testis

MEHP in blood and testes was extracted and analyzed by HPLC according to the analysis method previously reported [16].

Statistical analysis

The results were expressed as means ± standard deviations (SD). Statistical analyses were performed using a T-test to detect differences between the groups. Differences were regarded as significant at $P < 0.05$.

Results

Body weights and organ weights

Table 1 presents the body weights and organ weights for each treatment group. Despite the DEHP group consuming more food than the control group (Figure 1), their weight gain was less than the control group. Figure 2 illustrates a significant negative correlation between plasma MEHP concentrations and final body weight. The DEHP group also exhibited a clear increase in liver weight and a decrease in testicular weight. There was no significant difference in kidney weight. The estimated dose of DEHP (in grams per kilogram per day) based on food intake and average body weight during the treatment period was 1.5 grams per kilogram per day for the DEHP group.

Blood metal concentrations

The blood concentrations of zinc, iron, calcium, and hemoglobin in the DEHP group were found to be slightly lower than in the control group (Table 2). These concentrations demonstrated a statistically insignificant but negative correlation with plasma MEHP concentrations (Figure 3).

Organ metal concentrations

Organ tissue concentrations and organ content of Zn and Fe in liver, kidney and testis are shown in Table 3, Table 4, and Table 5. In the liver, a decrease in Zn concentration and an increase in Fe concentration were observed in the DEHP group. On the other hand, in terms of metal content per organ, there was a clear increase in Fe, whereas Zn did not differ from the control group. In the kidneys, Zn concentrations in the DEHP group were significantly lower than in the control group and the amount of Zn per organ was also lower.

In the testes, Zn concentrations were reduced and Fe concentrations increased in the DEHP-treated group. In terms of metal content per organ, Zn showed a clear decrease, whereas Fe did not differ from the control group.

As shown in Figure 4, the concentration of Fe in liver tissue showed a statistically significant negative correlation with the concentration of MEHP in plasma. Zn concentrations in liver tissue showed a negative, although not statistically significant, correlation with MEHP concentrations in plasma.

As shown in Figure 5, Zn concentrations in kidney tissue showed a statistically significant negative correlation with plasma MEHP concentrations, whereas Fe concentrations in kidney tissue showed no correlation with plasma MEHP concentrations.

Testicular tissue Zn concentrations showed a statistically significant negative correlation with plasma MEHP concentrations, whereas testicular tissue Fe concentrations showed no correlation with plasma MEHP concentrations (Figure 6). However, the concentration of Fe in testicular tissue showed a statistically significant positive correlation with the concentration of MEHP in testicular tissue (Figure 7).

Discussion

MEHP, a metabolite of DEHP, is a potent oxidative stressor, damaging thyroid tissue and lowering thyroid hormones that play an important

role in the process of skeletal muscle formation [19-23]. The suppression of body weight gain and reductions in blood metal and Hb concentrations in the DEHP-treated group observed in this experiment all correlate with plasma MEHP concentrations, implying a disturbance of thyroid function by MEHP.

Furthermore, Zn concentrations in the liver, kidneys and testes of the DEHP-treated group were lower than in the control group and negatively correlated with plasma MEHP concentrations. As nearly 90% of thyroid hormones in the circulating blood are present as the prohormone thyroxine (T4), zinc essential deiodinases in various organs convert this to the biologically active triiodothyronine (T3) [24, 25]. Therefore, there is concern that reduced zinc levels in organs may reduce their ability to activate thyroid hormones, leading to impaired organ function.

Previous studies have reported that testicular atrophy occurs after oral administration of DEHP and that Zn concentrations in testicular tissue are reduced [26-28]. Testicular atrophy also occurs with reduced Zn concentrations in the testis due to Zn deficiency [29, 30]. However, in the case of DEHP-induced testicular atrophy, apoptosis of testicular tissue appears before the Zn concentration is reduced, and Zn administration cannot prevent testicular atrophy [26, 27]. The decrease in Zn concentration is therefore considered to be a secondary phenomenon. In the present experiment, Zn in the testes of the DEHP-treated group was clearly reduced, but the decrease in blood Zn concentration was minor, suggesting that at least DEHP does not affect Zn absorption. Testicular atrophy, on the other hand, occurs when MEHP, a metabolite of DEHP, damages Sertoli cells, which activate the Fas/Fas ligand system and induce germ cell apoptosis [2-5], although the involvement of reactive oxygen species in the Sertoli cell damage process has been suggested [3-7]. In testes with progressive atrophy due to apoptosis induction, a histological picture of loss of sperm, germ cells and other cells is observed (Reference figure). In addition, high concentrations of Zn have been found to be localised in normal seminiferous tubular tissue [30]. Taken together, the loss of sperm, germ cells, etc. appears to be the major cause of the reduced Zn concentration in atrophied testicular tissue.

There was an increase in the concentration of Fe in the liver and testicular tissue in DEHP-treated group. On the other hand, when converted to Fe content per organ, there was a clear increase in Fe content in the liver, whereas there was no increase in testicular Fe content. Hepatic hypertrophy is based on the coordination of MEHP and 2-ethylhexanol to peroxisome proliferator-activated receptors (PPARs) and activation of their peroxisome proliferative effects [8-15]. This results in induction of β-oxidase and CYP4A [12]. Therefore, it is possible that the large amount of heme enzymes induced in the liver by DEHP administration may partly contribute to the increase in hepatic Fe content, but it is also possible that the heme oxygenase induced by oxidative stress may also promote iron deposition [16-18].

Tissue iron concentrations showed a significant positive correlation with testicular MEHP, even though testicular iron levels in the DEHP-treated group did not differ from those in the control group. This may indicate that iron is unevenly distributed in non-germline supporting tissues and that the relative iron concentration in testicular tissue increases as germline apoptosis progresses.

Conclusion

This study showed that DEHP-treated rats had lower body weight and zinc concentrations in blood, liver, kidney and testicular tissue, and higher iron concentrations in the liver compared to controls. These changes were also correlated with plasma MEHP concentrations, suggesting MEHP-mediated oxidative stress-induced tissue damage and thyroid hormone disruption. due to MEHP-mediated oxidative stress.

Acknowledgment
This work was conducted at Kagawa University. The author appreciates the help of colleagues in Kagawa University.

Disclosure of conflict of interest
Authors declare that there is no conflict of interests.

Authors contribution
Both authors supervised the laboratory research, analysed results, writing and editing of the manuscript.

Table 1. Body and organ weights

Group	n	Body (g)	Liver (g)	Kidneys (g)	Testes (g)
Control	6	286 ± 9	14.1 ± 0.8	2.49 ± 0.18	2.49 ± 0.19
DEHP	6	248 ± 26 *	22.1 ± 4.6 ***	2.28 ± 0.38	1.43 ± 0.42 **

*p<0.05, **p<0.01, ***p<0.001, as compared to control.

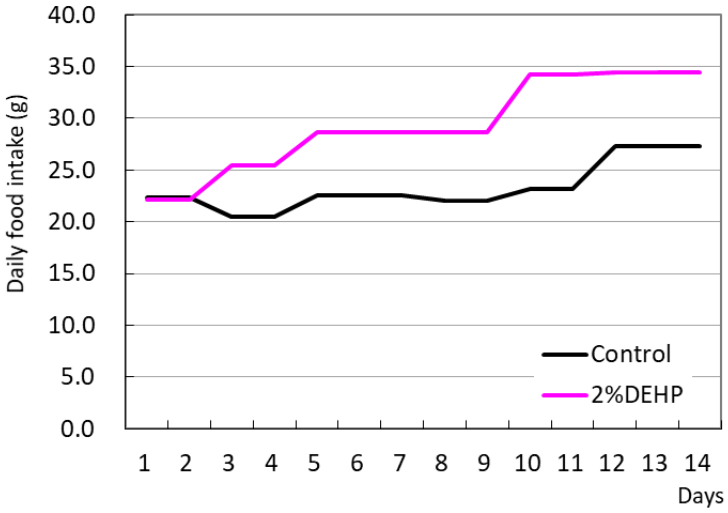


Figure 1. Daily food intake of experimental rats.

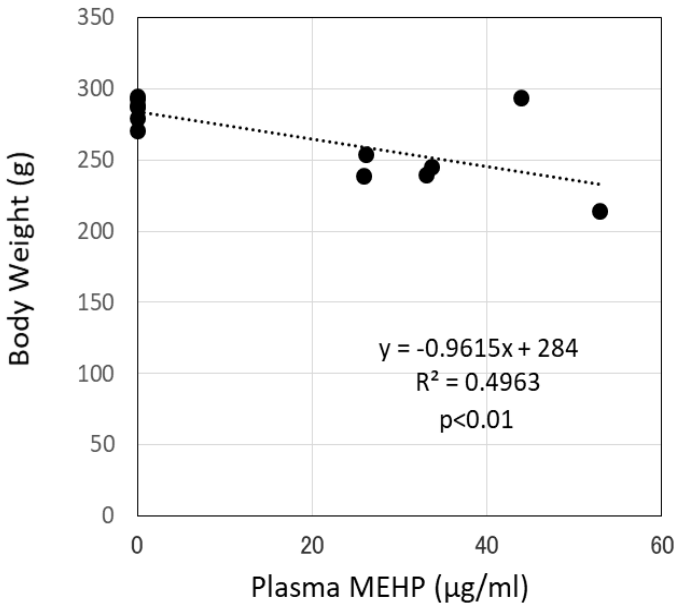


Figure 2. Relation between plasma MEHP concentrations and final body weight

Table 2. Blood zinc, iron, calcium and hemoglobin concentrations

Group	n	Zn (ppm)	Fe (ppm)	Ca (ppm)	Hb (g／dl)
Control	6	4.76 ± 0.60	456 ± 57	44.6 ± 10.2	13.5 ± 0.9
DEHP	6	4.33 ± 0.43	421 ± 39	40.3 ± 10.3	12.7 ± 0.8

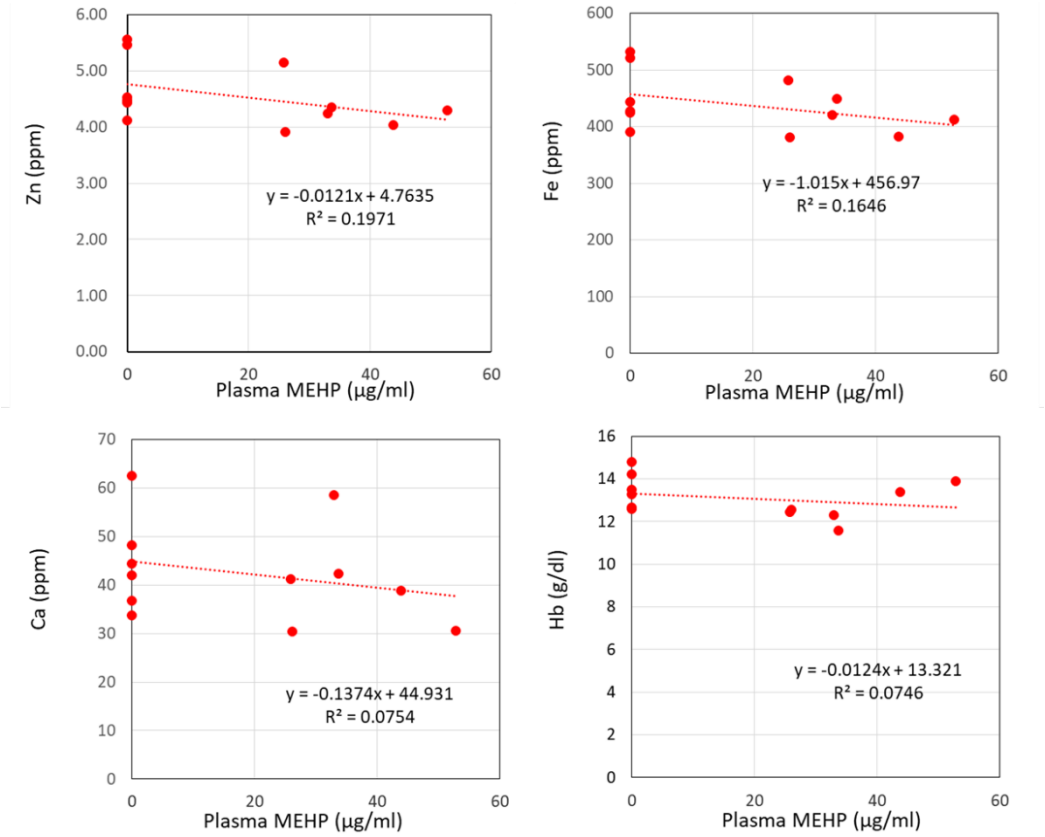


Figure 3. Relation between plasma MEHP concentrations and blood metal and hemoglobin concentrations

Table 3. Tissue concentrations and organ content of Zn and Fe in liver

Group	n	Zn		Fe	
		ppm	μg／organ	ppm	μg／organ
Control	6	22.7 ± 2.7	319 ± 34	54.1 ± 9.5	757 ± 120
DEHP	6	16.9 ± 1.1***	377 ± 105	71.9 ± 12.1	1561 ± 262**

*p<0.05, **p<0.01, ***p<0.001, as compared to control.

Table 4. Tissue concentrations and organ content of Zn and Fe in kidney

Group	n	Zn		Fe	
		ppm	μg／organ	ppm	μg／organ
Control	6	17.2 ± 5.5	43.4 ± 16.5	57.6 ± 19.4	144 ± 53
DEHP	6	12.2 ± 2.9*	28.6 ± 11.1	58.3 ± 13.3	134 ± 39

*p<0.05, **p<0.01, ***p<0.001, as compared to control.

Table 5. Tissue concentrations and organ content of Zn and Fe in testis

Group	n	Zn		Fe	
		ppm	μg／organ	ppm	μg／organ
Control	4	20.0 ± 1.3	49.9 ± 7.0	9.9 ± 1.2	24.6 ± 4.2
DEHP	4	14.3 ± 4.7	21.6 ± 11.2*	17.6 ± 6.0	24.1 ± 7.2

*p<0.05, **p<0.01, ***p<0.001, as compared to control.

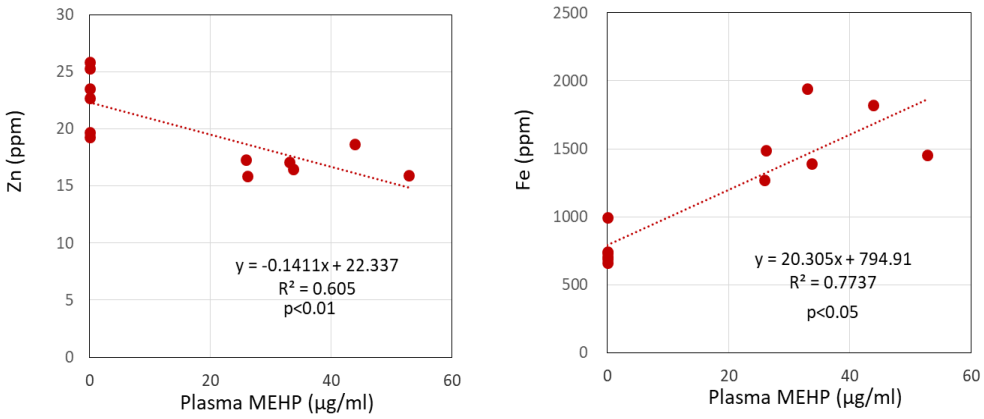


Figure 4. Relation between plasma MEHP concentrations and Zn, Fe concentrations in liver tissue

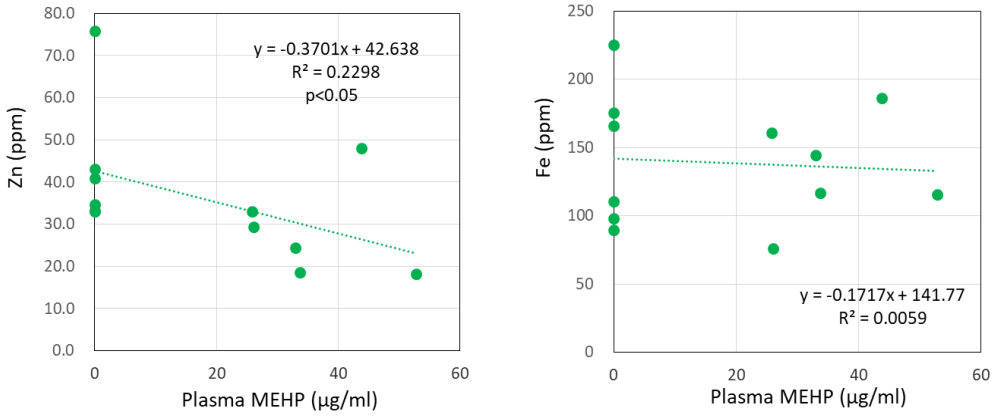


Figure 5. Relation between plasma MEHP concentrations and Zn, Fe concentrations in kidney tissue

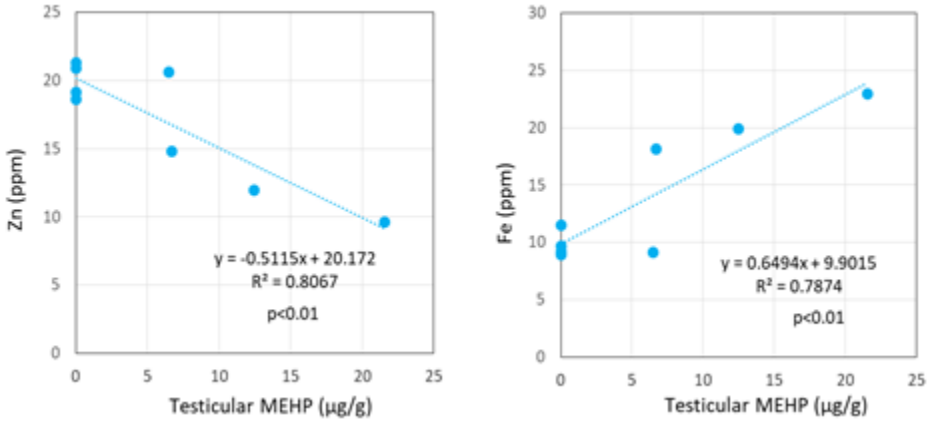


Figure 6. Relation between plasma MEHP concentrations and Zn, Fe concentrations in testicular tissue

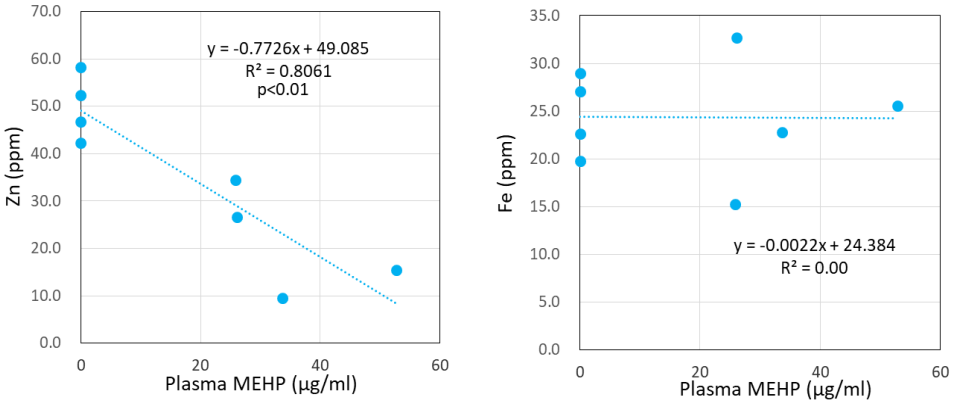
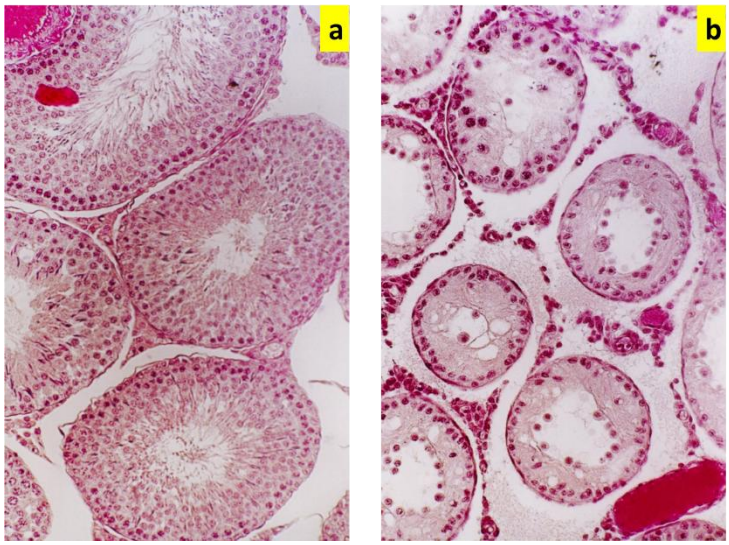


Figure 7. Relation between testicular MEHP concentrations and Zn, Fe concentrations in testicular tissue



Reference figure. Seminiferous tubules of control and DEHP-treated rats (stained with hematoxylin–eosin). a: control, b: DEHP.

References

[1] Gray TJ, Butterworth KR. Testicular atrophy produced by phthalate esters. Arch Toxicol. 1980; 4: 452-455. doi: 10.1007/978-3-642-67729-8_106.

[2] Albro PW and Thomas RO. Enzymatic hydrolysis of di(2-ethylhexyl)phthalate by lipases Biochem Biophys Acta. 1973; 306: 380-390. doi: 10.1016/0005-2760(73)90176-8.

[3] Lee J, Richburg JH, Younkin SC, Boekelheide K, 1997. The Fas system is a key regulator of germ cell apoptosis in the testis. Endocrinology. 1997; 138: 2081-2088. doi: 10.1210/endo.138.5.5110.

[4] Lee J, Richburg JH, Shipp EB, Meistrich ML, Boekelheide K. The Fas system, a regulator of testicular germ cell apoptosis, is differentially up-regulated in Sertoli cell versus germ cell injury of the testis. Endocrinology1999; 140, 852-858. doi: 10.1210/endo.140.2.6479.

[5] Richburg JH, Johnson KJ, Schoenfeld HA, Meistrich ML, Dix DJ. Defining the cellular and molecular mechanisms of toxicant action in the testis. Toxicol Lett. 2002; 135: 167-183. doi: 10.1016/s0378-4274(02)00254-0.

[6] Poon R, Lecavalier P, Mueller R, Valli V E. Subchronic oral toxicity of di-n-octyl phthalate and di(2-ethylhexyl) phthalate in the rat. Food and Chemical Toxicology. 1997; 35: 225–239. doi: 10.1016/s0278-6915(96)00064-6.

[7] W.E.I. Yi, Tang Xiang-Liang, Zhou Yu, Liu Bin, Shen Lian-Ju, Long Chun-lan, L.I.N. Tao, H.E. Da-wei, W.U. Sheng-de, W.E.I. Guang-hui. DEHP exposure destroys blood-testis barrier (BTB) integrity of immature testes through excessive ROS-mediated autophagy. Genes & Diseases. 2018; 5(3): 263-274. doi: 10.1016/j.gendis.2018.06.004.

[8] Gray TJ, Beamand JA, Lake BG, Foster JR, Gangolli SD. Peroxisome proliferation in cultured rat hepatocytes produced by clofibrate and phthalate ester metabolites. Toxicol Lett, 1982; 10: 273-279. doi: 10.1016/0378-4274(82)90087-x.

[9] Bell DR, Elcombe CR. Induction of acyl-CoA oxidase and cytochrome P450IVA1 RNA in rat primary hepatocyte culture by peroxisome proliferators. Biochem J. 1991; 280: 249-253. doi: 10.1042/bj2800249

[10] Maloney EK, Waxman DJ. trans-Activation of PPARalpha and PPARgamma by structurally diverse environmental chemicals. Toxicol Appl Pharmacol. 1999; 161: 209-218. doi: 10.1006/taap.1999.8809.

[11] Hurst CH, Waxman DJ.Activation of PPARalpha and PPARgamma by environmental phthalate monoesters. Toxicol Sci, 2003; 74: 297-308. doi: 10.1093/toxsci/kfg145.

[12] R T Okita, J R Okita. Characterization of a cytochrome P450 from di(2-ethylhexyl) phthalate-treated rats which hydroxylates fatty acids. Archives of Biochemistry and Biophysics. 1992; 294 (2): 475-481. doi: 10.1016/0003-9861(92)90714-8.

[13] Oguro T, Kaneko E, Numazawa S, Imaoka S, Funae Y, Yoshida T. Induction of hepatic heme oxygenase and changes in cytochrome P-450s in response to oxidative stress produced by stilbenes and stilbene oxides in rats. J Pharmacol Exp Ther. 1997; 280: 1455-1462.

[14] Bell DR, Elcombe CR. Induction of acyl-CoA oxidase and cytochrome P450IVA1 RNA in rat primary hepatocyte culture by peroxisome proliferators. Biochem J. 1991 ;280 :249-253. doi: 10.1042/bj2800249

[15] Maloney EK, Waxman DJ. trans-Activation of PPARalpha and PPARgamma by structurally diverse environmental chemicals. Toxicol Appl Pharmacol. 1999 ; 161: 209-218. doi: 10.1006/taap.1999.8809.

[16] Ishizaka N, Aizawa T, Yamazaki I, Usui S, Mori I, Kurokawa K, Tang SS, Ingelfinger JR, Ohno M, Nagai R. Abnormal iron deposition in renal cells in the rat with chronic angiotensin II administration. Lab Invest. 2002; 82(1):87-96. doi: 10.1038/labinvest.3780398..

[17] Mehindate K, Sahlas DJ, Frankel D, Mawal Y, Liberman A, Corcos J, Dion S, Schipper HM. Proinflammatory cytokines promote glial heme oxygenase-1 expression and

mitochondrial iron deposition: implications for multiple sclerosis. J Neurochem. 2001; 77:1386-1395. doi: 10.1046/j.1471-4159.2001.00354.x.

[18] Shigeru Suna and Fumihiko Jitsunari. Blood and organ distribution of mono (2-ethylhexyl) phthalate (MEHP) in rats exposed to di (2-ethylhexyl) phthalate (DEHP) via diet. World Journal of Biology Pharmacy and Health Sciences. 2023; 13(3): 19–25. doi: 10.30574/wjbphs.2023.13.3.0116

[19] Antonio Mancini, Chantal Di Segni, Sebastiano Raimondo, Giulio Olivieri, Andrea Silvestrini, Elisabetta Meucci and Diego Currò. Thyroid Hormones, Oxidative Stress, and Inflammation. Mediators of Inflammation Volume 2016, Article ID 6757154, 12 pages http://dx.doi.org/10.1155/2016/6757154

[20] Joanna Kochman Karolina Jakubczyk Piotr Bargie and Katarzyna Janda-MilczarekThe Influence of Oxidative Stress on Thyroid Diseases. Antioxidants (Basel). 2021; 10(9): 1442. doi: 10.3390/antiox10091442

[21] Min Joo Kim, Hwan Hee Kim, Young Shin Song, Ok-Hee Kim, Kyungho Choi. Sujin Kim,5,6 Byung-Chul Oh,4 and Young Joo Park. DEHP down-regulates Tshr gene expression in rat thyroid tissues and FRTL-5 rat thyrocytes: A potential mechanism of Thyroid Disruption. Endocrinol Metab (Seoul). 2021; 36(2): 447–454. doi: 10.3803/EnM.2020.920.

[22] Xueting Zhang, Wen Qi, Qi Xu, Xu Li, Liting Zhou, Lin Ye. Di(2-ethylhexyl) phthalate (DEHP) and thyroid: biological mechanisms of interference and possible clinical implications. Environ Sci Pollut Res Int. 2022; 29(2):1634-1644. doi: 10.1007/s11356-021-17027-y.

[23] Flavia F Bloise 1, Aline Cordeiro 2, Tania Maria Ortiga-Carvalho. Role of thyroid hormone in skeletal muscle physiology. J Endocrinol. 2018 ; 236(1):R57-R68. doi: 10.1530/JOE-16-0611.

[24] Yoko Narasaki, Peter Sohn and Connie M. Rhee. Semin Nephrol. The Interplay between thyroid dysfunction and kidney disease. 2021; 41(2): 133–143.

[25] P R Larsen. Thyroid-pituitary interaction: feedback regulation of thyrotropin secretion by thyroid hormones. N Engl J Med. 1982 306(1):23-32. doi: 10.1056/NEJM198201073060107.

[26] Oishi S, Hiraga K. Testicular atrophy induced by di-2-ethylhexyl phthalate: effect of zinc supplement. Toxicol Appl Pharmacol. 1983; 70(1):43-48. doi: 10.1016/0041-008x(83)90177-1.

[27] Park JD, Habeebu SS, Klaassen CD. Testicular toxicity of di-(2-ethylhexyl) phthalate in young Sprague-Dawley rats. Toxicology. 2002; 171: 105-115. doi: 10.1016/s0300-483x(01)00567-4.

[28] Nodera M, Yanagisawa H, Wada O. Increased apoptosis in a variety of tissues of zinc-deficient rats. Life Sci 2001; 69:1639-1649.

[29] Rahman AS, Kimura M, Itokawa Y. Testicular atrophy, zinc concentration, and angiotensin-converting enzyme activity in the testes of vitamin A-deficient rats. Biol Trace Elem Res. 1999 ; 67: 29-36. doi: 10.1007/BF02784272.

[30] Fujimori O, Tsukise A, Yamada K. Histochemical demonstration of zinc in rat epididymis using a sulphide-silver method.Histochemistry. 1988; 88: 469-473. doi: 10.1007/BF00570310.