Effects of Dimethyl Phthalate (DMP) on Serum Sex Hormone Levels and Apoptosis in C57 Female Mice

Yue Mei¹, Ma Rongshuang¹, Zhang Ruizhi¹, Huang Hongyuan¹, Tan Qiyue¹ and Zhao Shuhua¹,*

¹Jilin University School of Public Health, Changchun, China
*Corresponding author: Jilin University School of Public Health, Changchun, China. Tel: +86-13596077054, Email: yuemei16@mails.jlu.edu

Received 2018 August 03; Revised 2019 January 15; Accepted 2019 February 16.

Abstract

Background: The effects of dimethyl phthalate (DMP) on the reproductive system of mammal females are unclear because no studies have been conducted on this topic.

Methods: In this study, 40 C57 female mice were used as experimental subjects and evenly divided into 8 groups, which were fed with mixed DMP (0, 0.5, 1, and 2 g/kg bw/day) and corn oil. After 20 days and 40 days of gavage, the mice were weighed and their individual ovary organ coefficients measured.

Results: Changes were discovered on progesterone, estradiol, follicle-stimulating hormone and luteinizing hormone in mouse serum, and on the apoptosis rate of ovarian granulosa cells.

Conclusions: Prolonged exposure to DMP led to decreased secretion of FSH hormones and increased secretion of E2 and LH hormones. Furthermore, DMP interfered with the pituitary-ovary axis and increased the apoptosis rate of ovarian granulosa cells. Therefore, prolonged exposure to DMP is likely to have negative effects on reproduction and development.

Keywords: Dimethyl Phthalate, Sex Hormone, Apoptosis, Endocrine Disruptors

1. Background

Phthalates (PAEs) are a kind of organic compound of low water solubility that are widely used as plasticizers and abundant in toys, food packaging, medical materials, and consumer products (1). There are 6.0 million metric tons of PAEs produced per year worldwide (2) and they are difficult to degrade by traditional biological methods (3). China National Environmental Monitoring Center and the U.S. Environmental Protection Agency have added dimethyl phthalate (DMP), di-n-butyl phthalate (DBP) and di-n-octyl phthalate (DOP) to the list of major pollutants (1). In recent years, there have been many studies on the toxicity of DEHP, DOP and DBP to the reproductive system, and great progresses have been made. In Taiwan, an epidemiological study shows that phthalate exposure has an effect on the level of female reproductive hormones (4). Di (2-ethylhexyl) phthalate (DEHP) causes developmental and functional impairment of reproductive organs in female mice, resulting in abnormal serum progesterone levels (5). Prenatal exposure to phthalates can lead to changes in uterine weight, anogenital distance and body weight, and induce cystic ovary of mice’s offspring (6). Therefore, it is crucial to study the impact of phthalates on human health.

DMP is the most leached PAEs in water coolers and mineral waters (7). The DMP content in the solvent or fixative of 47 brands of perfumes is higher than the limit of 0.1 ppm, and micronucleus test has shown that it may cause DNA damage (8). Its exposure to humans is found to cause stimulating irritation to the sensitive organs such as eyes, nose and throat (9). DMP has a fatal effect on frog embryos. The DMP level increase leads to a significant rise in the malformation rate from 22.8% to 97.4% (10). At present, the influence and mechanism of DMP on the reproductive system is not clear, as no studies have been conducted on the effects of DMP exposure to the reproductive system. DMP also affects the health of future generations. The sex hormone can regulate the function of the reproductive system.

2. Objectives

The objective of this study is to investigate the effects of DMP exposure on serum sex hormone levels and apoptosis of ovarian cells, and to explore the correlation between changes in serum sex hormone levels and apoptosis, as DMP for female mice. This study explores the role of DMP in reproductive endocrine disruption, giving rise to a better understanding of the adverse effects of DMP on humans.
and animals, and also providing a scientific basis for a comprehensive assessment of the toxicological effects of DMP and the potential hazards of human endocrine disruption.

3. Methods

3.1. Animal Care, Diets, and DMP Exposure

This research protocol was approved by the Animal Experiment Ethics Committee of Jilin University (2018 joint trial no. 2018-04-06). All experimental animals were treated humanely with alleviation of suffering. Mice were fed individually under controlled light and temperature (12 h light/dark cycle, 22 ± 1°C, relative humidity of approximately 50% - 60%) with free access to food and water. We randomly divided 40 mature female C57 mice (purchased from Liaoning Changsheng Biological Technology Co. Ltd. China) weighing 18 - 22 g into 8 groups (each group has 5 mice) and gave them three days to acclimate to the lab conditions before the start of the experiments. Mice were treated with (0, 0.5, 1, and 2 g/kg bw/day) DMP (J&K Scientific LTD, minimum purity 99.5%) in corn oil for 20 days and 40 days by gavage at a dose of 0.1 mL/10 g bw.

3.2. Weight Measurement and Sample Collection

Female mice were sacrificed by cervical dislocation after weighing between 8:00 and 10:00 A.M. Mouse ovaries were removed and weighed to calculate organ coefficients. Orbital sinus blood samples were collected and allowed to clot on an ice bath (4°C) for 2 h. Serum was collected after centrifugation and stored at -20°C before it was analyzed. According to the manufacturer’s instructions, ELISA (R&D Systems, USA) was used to measure the level of serum progesterone (P), estradiol (E2), follicle stimulating hormone (FSH) and luteinizing hormone (LH). Samples and standards were analyzed in duplicate and mean values of each sample were used in the analysis. Intra-assay and inter-assay coefficient of variation for all assays was 15%. The sensitivities were 0.1 µmol/L, 1.0 pmol/L, 1.0 mIU/mL, and 1.0 pg/mL for P, E2, FSH and LH, respectively. No samples were below the limits of detection.

3.3. Cell Apoptosis Analysis

Propidium iodide (PI) can stain necrotic cells or cells that lost cell membrane integrity at the late stage of apoptosis, and can make them show red fluorescence. For necrotic cells, due to loss of integrity of the cell membrane, Annexin V-FITC can enter the cytoplasm and be bound to the phosphatidylyserine located on the inner side of the cell membrane, thereby rendering the necrotic cells with green fluorescence. The mouse ovaries were triturated on a glass slide and transferred to 0.1 mL of phosphate buffered saline (PBS). The cells were centrifuged at 1000 rpm for 5 minutes, the pellets gently resuspended with PBS and count. Approximately 1 × 10⁶ resuspended cells were centrifuged at 1000 rpm for 5 minutes and the pellets were resuspended in 500 µL of binding solution. Annexin V-FITC and PI were then added and the cell suspension was incubated in the dark for 10 minutes at room temperature (20 - 25°C). The proportion of apoptotic cells were analyzed by FACS Calibur FCM (BD, USA) (11).

3.4. Statistical Analysis

Each experiment was repeated a minimum three times. The data were analyzed by using the Statistical Package for Social Sciences version 21.0 (SPSS, USA). Results are displayed by means ± SD (S). Statistical significance among groups was determined by one-way analysis of variance (ANOVA). Significant ANOVA was followed by pairwise multiple comparisons of group mean scores using either the least significant difference (LSD) test (for homogeneous variances). We used a significance level of P < 0.05 (two-tailed tests).

4. Results

The initial and final body weights of C57 female mice exposed to DMP respectively for 20 and 40 days are showed in Table 1. There are no statistical differences of these groups (P > 0.05).

Table 2 shows the ovarian organ coefficient of C57 female mice exposed for 20 days and 40 days. Statistically significant difference (P < 0.05) of the mouse visceral organ coefficients were found between the treatment group and the control group in DMP exposure for 20 days. In the results of DMP exposure for 40 days, compared with the control group, the groups exposed to DMP 0.5 (0.167 ± 0.014) and 2 (0.125 ± 0.003) g/kg/d were both statistically significant (P < 0.05). Statistical significances (P < 0.05) can be observed comparing the 0.5 g/kg group to the 1 g/kg group and the 2 g/kg group respectively. The 1 g/kg group was also significantly (P < 0.05) different from the 2 g/kg group.

Serum sex hormone levels in female mice exposed to DMP for 20 and 40 days are displayed in Tables 3 and 4. In mice exposed to DMP for 40 days, there was significant increase in estradiol levels in the treated group (P < 0.05), among which the highest increase was 0.5 g/kg (210.14 ± 3.56 pmol/L). The serum FSH concentration in mice treated by 2 g/kg (126.35 ± 8.46 mIU/mL) DMP was significantly lower compared with others (P < 0.05). The serum LH exposed to 2 g/kg (5192.87 ± 362.02 pg/mL) DMP had a significant increase compared to the other groups (P < 0.05).
Table 1. The Effects of DMP Exposure on the Weights of Female C57 Micea

| Treatment | 0 Days | 20 Days | 0 Days | 40 Days |
|-----------|--------|---------|--------|---------|
| Control   | 16.74 ± 1.09 | 19.52 ± 1.22 | 16.84 ± 1.19 | 20.64 ± 0.66 |
| DMP, g/kg |        |         |        |         |
| 0.5       | 17.46 ± 0.39 | 19.50 ± 0.32 | 17.52 ± 0.38 | 19.84 ± 0.53 |
| 1         | 16.98 ± 1.04 | 20.16 ± 1.05 | 16.96 ± 0.78 | 20.52 ± 0.99 |
| 2         | 17.86 ± 0.65 | 19.04 ± 1.21 | 17.82 ± 0.49 | 20.30 ± 1.12 |

a The data are presented as mean ± SD; N = 5 for each group. The effect of DMP was analyzed by one-way analysis of variance (ANOVA) and post hoc testing by the Games-Howell test (for heterogeneous group variances).

Table 2. The Effects of DMP Exposure on the Ovarian Organ Coefficient of Female C57 Mice

| Treatment | Ovarian Organ Coefficient |
|-----------|---------------------------|
|           | 20 Days | 40 Days |
| Control   | 0.051 ± 0.007 | 0.144 ± 0.013 |
| DMP, g/kg |        |        |
| 0.5       | 0.125 ± 0.013b | 0.167 ± 0.014b |
| 1         | 0.068 ± 0.003b,c | 0.145 ± 0.004c |
| 2         | 0.144 ± 0.01b,c,d | 0.125 ± 0.003b,c,d |

b Significantly different from control (P < 0.05).

Table 3. The Effects of DMP Exposure on the Ovarian Cell Apoptosis of Female C57 Mice

| Treatment | Ovarian Cell Apoptosis |
|-----------|-----------------------|
|           | 20 Days | 40 Days |
| Control   | 1.43 ± 0.05 | 1.60 ± 0.17 |
| DMP, g/kg |        |        |
| 0.5       | 1.57 ± 0.05 | 1.60 ± 0.17 |
| 1         | 1.60 ± 0.17 | 1.60 ± 0.17 |
| 2         | 1.60 ± 0.17 | 1.60 ± 0.17 |

5. Discussion

It is known that DEHP, DOP, DBP and DEP have adverse effects on mammalian reproduction (11-14), but in recent studies, it is not known what effect DMP has on the animal’s reproductive system. We designed the experiment, which involves exposure of C57 female mice to DMP for 20 and 40 days respectively to demonstrate changes in serum sex hormone levels and ovarian cell apoptosis in mice.

In this study, sexual maturation of C57 female mice exposed to DMP for a period of time including gonadal development, sexual maturation, and oogenesis was evaluated. The reproductive cycle of female mammals is regulated by the endocrine effects of the hypothalamic-pituitary-ovarian axis. Through the cAMP-protein kinase system, cholesterol is converted to the androgen testosterone (T). T is transported to granule cells acting on the rate-limiting enzyme aromatase P450 via FSH, converting T into E2. Small changes in sex hormones may have a lasting effect on reproductive system development (15).
Table 3. The Effects of DMP Exposure on the Serum Progesterone and Estradiol in Female C57 Mice

| Treatment | Progesterone, µmol/L | Estradiol, pmol/L |
|-----------|----------------------|------------------|
|           | 20 Days | 40 Days | 20 Days | 40 Days |
| Control   |         |         |         |         |
|           | 38.15 ± 2.89 | 33.82 ± 2.35 | 109.49 ± 7.29 | 181.60 ± 7.48 |
| DMP, g/kg |         |         |         |         |
| 0.5       | 42.84 ± 3.31 | 33.03 ± 1.48 | 103.11 ± 7.43 | 201.34 ± 3.56b |
| 1         | 37.56 ± 3.07 | 34.06 ± 3.16 | 103.97 ± 9.15 | 215.48 ± 10.23b, c |
| 2         | 38.57 ± 2.74 | 36.08 ± 2.67 | 94.47 ± 7.80 | 196.59 ± 8.58b, c |

a The data are presented as mean ± SD; n = 5 for each group. The effect of DMP was analyzed by one-way analysis of variance (ANOVA) and post hoc testing by the Games-Howell test (for heterogeneous group variances).

b Significantly different from control (P < 0.05).
c Significantly different from 0.5 g/kg/d (P < 0.05).

d The study did not find out whether the weight changes of the mice in each group were related to the increase in the exposure time, and there was no statistically significant difference compared with the control group. This may be due to the fact that the exposure dose does not reach the range of dose changes. The change of organ coefficient suggests that the organ may be the target organ for granulosa cells and oocyte-derived microvilli and loss of condensation of mitochondria and iliac crest (18). Apoptosis in pre-ovulatory follicles was the major cause of atresia during follicular development (19, 20). After DMP exposure for 20 and 40 days, the apoptosis rate of ovarian cells in the low-dose group (0.5 g/kg bw) was lower than that in the control group, which was speculated to be related to the interference of low-dose DMP with the normal apoptosis process of ovarian cells. The results need further study. Larger dose exposure to DMP can induce apoptosis in ovarian granulosa cells. DMP may directly or indirectly cause apoptosis or necrosis by disrupting the granule cell mitochondria. Moreover, decreased secretion of FSH may also lead to a large number of follicles undergoing apoptosis and cause atresia (18).

Table 4. Effects of DMP Exposure on Serum FSH and LH in Female C57 Mice

| Treatment | FSH, mIU/ml | LH, mIU/ml |
|-----------|-------------|------------|
|           | 20 Days | 40 Days | 20 Days | 40 Days |
| Control   |         |         |         |         |
|           | 214.92 ± 17.67 | 162.73 ± 9.39 | 4339 ± 290 | 4164 ± 184 |
| DMP, g/kg |         |         |         |         |
| 0.5       | 215.03 ± 11.80 | 158.93 ± 9.21 | 3885 ± 295 | 4115 ± 155 |
| 1         | 204.97 ± 5.74 | 153.16 ± 11.61 | 4109 ± 407 | 4447 ± 304 |
| 2         | 207.99 ± 16.78 | 152.35 ± 8.46b, c, d | 3857 ± 309 | 5192 ± 162b, c, d |

a The data are presented as mean ± SD; N = 5 for each group. The effect of DMP was analyzed by one-way analysis of variance (ANOVA) and post hoc testing by the Games-Howell test (for heterogeneous group variances).

b Significantly different from control (P < 0.05).
c Significantly different from 0.5 g/kg/d (P < 0.05).
d Significantly different from 1 g/kg/d (P < 0.05).

evidence has shown that in mammals, early morphological changes in occluded oocytes include retraction of
toxic effects (21). The increase or decrease of visceral organ coefficient reflects that the organ has enlarged or shrunk, such as congestion, edema, atrophy and others. When the ovarian organs of mice in the 0.5 g/kg dose group were removed, hyperemia of the ovaries could be observed in individual mice.

Previous studies have confirmed that the efficacy of phthalates is related to the length of the alkyl chain, and the most potent phthalate has a chain length of 4 to 6 carbon atoms (22, 23). DMP is a phthalate diester with the shortest alkyl chain length and therefore is considered less toxic than other phthalates. In the only one study found, it was discovered that the dose not exceeding 2 mL/kg of DMP in the abdominal cavity of Sprague-Dawley rats in early pregnancy didn’t have the toxic effect of DMP (24). Nevertheless, our experiment was conducted by intragastric administration, also the exposure time is different. We found a similar negative effect on the endocrine of the pituitary-ovarian axis of female mice of DEMP and DBP. In other phthalate-like studies, DEHP doses were as high as 1.5 - 2 g/kg/d (16, 17), DEP doses as high as 1 - 1.375 g/kg/d (13), and DBP doses as high as 1.25 - 1.5 g/kg/d (25). In human exposure studies, the detection rate of DMP in food was 37%, which is 6% more than DBP (26). These indicated that the DMP safety problem is severe.

5.1. Conclusions

In this study, prolonged exposure to DMP reduced the secretion of FSH hormones and increased the secretion of E2 and LH hormones. It may lead to ovarian enlargement or atrophy, and cellular DNA content decrease. The apoptotic rate is increased, and changes in the pituitary-ovarian axis may have adverse effects on human development and reproductive health. These are similar to a large number of experimental studies of the effects of DEHP on mammalian hormone levels, and it can be speculated that the mechanism of action is the same. However, further experiments are needed to confirm this hypothesis.

Footnotes

Authors’ Contribution: Zhao Shuhua and Yue Mei conceived and designed the experiments; Yue Mei, Zhang Ruizhi, Huang Hongyuan, Tan Qiyue, and Ma Rongshuang performed the experiments; Yue Mei and Zhang Ruizhi analyzed the data; Zhao Shuhua contributed reagents, materials and analysis tools.

Conflict of Interests: There is no conflict of interests.

Ethical Approval: This research protocol was approved by The Animal Experiment Ethics Committee of Jilin University (2018 joint trial no. 2018-04-06).

Funding/Support: This experiment is self-funded.

References

1. Wang J, Liu P, Qian Y. Biodegradation of phthalic acid esters by immobilized microbial cells. Environ Int. 1997;23(6):775-82. doi: 10.1016/s0160-4120(97)00089-5.
2. Jia W, Chu X, Ling Y, Huang J, Chang J. Analysis of phthalates in milk and milk products by liquid chromatography coupled to quadrupole Orbitrap high-resolution mass spectrometry. J Chromatogr. 2014;1362:110-8. doi: 10.1016/j.chroma.2014.08.030. [PubMed: 25155064].
3. Jianlong W, Lujun C, Hanchang S, Yi Q. Microbial degradation of phthalic acid esters under anaerobic digestion of sludge. Chemosphere. 2000;40(8):13245-8. [PubMed: 10902554].
4. Wen HJ, Chen CC, Wu MT, Chen ML, Sun CW, Wu WC, et al. Phthalate exposure and reproductive hormones and sex-hormone binding globulin before puberty: Phthalate contaminated foodstuff episode in Taiwan. PLoS One. 2012;7(4): e0117536. doi: 10.1371/journal.pone.0117536. [PubMed: 20844141]. [PubMed Central: PMC3199494].
5. Rattan S, Brehm E, Gao I, Niermann S, Flaws JA. Prenatal exposure to (2-ethylhexyl) phthalate disrupts ovarian function in a trans-generational manner in female mice. Biol Reprod. 2018;98(1):130-45. doi: 10.1093/biolre/ioy154. [PubMed: 29165555]. [PubMed Central: PMC5803793].
6. Zhou C, Gao L, Flaws JA. Exposure to an environmentally relevant phthalate mixture causes trans-generational effects on female reproduction in mice. Endocrinology. 2017;158(5):1739-54. doi: 10.1210/endo.2017-00000. [PubMed: 28168545]. [PubMed Central: PMC5460945].
7. Surhio MA, Talpur FN, Nizamani SM, Talpur MK, Afridi HI, Khaskheli AA, et al. Leaching of phthalate esters from different drinking stuffs and their subsequent biodegradation. Environ Sci Pollut Res Int. 2017;24(22):18663-71. doi: 10.1007/s11356-017-9470-y. [PubMed: 26847882].
8. Al-Saleh I, Al-Rajudi T, Al-Qudaihi G, Manogaran P. Evaluating the potential genotoxicity of phthalates esters (PAEs) in perfumes using in vitro assays. Environ Sci Pollut Res Int. 2017;24(30):23903-14. doi: 10.1007/s11356-017-9978-1. [PubMed: 28875446].
9. Benson R. Hazard to the developing male reproductive system from cumulative exposure to phthalate esters—dibutyl phthalate, diisobutyl phthalate, butylbenzyl phthalate, diethylhexyl phthalate, dipentyl phthalate, and diisononyl phthalate. Regul Toxicol Pharmacol. 2009;53(2):190-101. doi: 10.1016/j.yrtph.2008.11.005. [PubMed: 19100224].
10. Mathieu-Denoncourt J, Martyniuk CJ, Loughery JR, Yargeau V, de Solla AA, et al. Leaching of phthalate esters from different drinking stuffs and their subsequent biodegradation. Environ Int. 2008;33(6):775–82. doi: 10.1016/j.chemosphere.2008.03.009. [PubMed: 18641546].
15. Booker SM. NTP center reports on phthalate concerns. *Environ Health Perspect*. 2000;109(6):A260-1. doi: 10.1289/ehp.109-a260. [PubMed: 11445530]. [PubMed Central: PMC1240353].

16. Svechnikova I, Svechnikov K, Soder O. The influence of di-(2-ethylhexyl) phthalate on steroidogenesis by the ovarian granulosa cells of immature female rats. *J Endocrinol*. 2007;194(3):603-9. doi: 10.1677/JOE-07-0238. [PubMed: 17760899].

17. Davis BJ, Maronpot RR, Heindel JJ. Di-(2-ethylhexyl) phthalate suppresses estradiol and ovulation in cycling rats. *Toxicol Appl Pharmacol*. 1994;128(2):216-23. doi: 10.1006/taap.1994.1200. [PubMed: 7940536].

18. Hussein MR. Apoptosis in the ovary: Molecular mechanisms. *Hum Reprod Update*. 2005;11(2):62-77. doi: 10.1093/humupd/dmi001. [PubMed: 15705959].

19. Hughes FM Jr, Gorospe WC. Biochemical identification of apoptosis (programmed cell death) in granulosa cells: Evidence for a potential mechanism underlying follicular atresia. *Endocrinology*. 1991;129(5):2445-22. doi: 10.1210/endo-129-5-2445. [PubMed: 1935775].

20. Tilly JL, Kowalski KL, Johnson AL, Hsueh AJ. Involvement of apoptosis in ovarian follicular atresia and postovulatory regression. *Endocrinology*. 1991;129(5):2799-801. doi: 10.1210/endo-129-5-2799. [PubMed: 1718732].

21. Kleinsasser NH, Wallner BC, Kastenbauer ER, Weisscher H, Harreus UA. Genotoxicity of di-n-butyl-phthalate and di-iso-butyl-phthalate in human lymphocytes and mucosal cells. *Teratog Carcinog Mutagen*. 2001;21(3):389-96. [PubMed: 11301413].

22. Heindel JJ, Gulati DK, Mounce RC, Russell SR, Lamb J. Reproductive toxicity of three phthalic acid esters in a continuous breeding protocol. *Fundam Appl Toxicol*. 1989;12(3):504-18. [PubMed: 2736651].

23. Gray LE Jr, Ozby J, Furr J, Price M, Veeramachaneni DN, Parks L. Perinatal exposure to the phthalates DEHP, BBE, and DINP, but not DEP, DMP, or DOTP, alters sexual differentiation of the male rat. *Toxicol Sci*. 2000;58(2):350-65. [PubMed: 11099647].

24. Peters JW, Cook RM. Effect of phthalate esters on reproduction in rats. *Environ Health Perspect*. 1973;3:91-4. doi: 10.1289/ehp.730391. [PubMed: 4704574]. [PubMed Central: PMC1474910].

25. Ema M, Miyawaki E, Kawashima K. Effects of dibutyl phthalate on reproductive function in pregnant and pseudopregnant rats. *Reprod Toxicol*. 2000;14(2):121-9. [PubMed: 10699999].

26. Schecter A, Lorber M, Guo Y, Wu Q, Yun SH, Kannan K, et al. Phthalate concentrations and dietary exposure from food purchased in New York State. *Environ Health Perspect*. 2013;121(4):473-94. doi: 10.1289/ehp.1206367. [PubMed: 23461894]. [PubMed Central: PMC3620091].