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

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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 phosphatidylserine 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 1×10^6 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 Mice[a]

Treatment	Weight, g			
	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[a]

Treatment	Ovarian Organ Coefficient	
	20 Days	40 Days
Control	0.051 ± 0.007	0.148 ± 0.013
DMP, g/kg		
0.5	0.125 ± 0.017[b]	0.167 ± 0.014[b]
1	0.068 ± 0.005[b,c]	0.145 ± 0.004[c]
2	0.144 ± 0.013[b,c,d]	0.125 ± 0.003[b,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).

The apoptosis of ovarian cells of female C57 mice exposed to DMP for 20 and 40 days is showed in Table 5. Compared to the control group, the apoptosis rates of ovarian cells 1 and 2 g/kg dose groups exposed to DMP for both 20 days significantly increased (P < 0.05), which was respectively (1.57 ± 0.05)% and (1.60 ± 0.17)%, however, that of 0.5 g/kg (1.43 ± 0.05)% was observed remarkably lower (P < 0.05). As for the ovarian cells 1 and 2 g/kg dose groups exposed to DMP for 40 days, the apoptosis rates were also of enormous growth (P < 0.05), which were (17.31 ± 1.61)% and (16.18 ± 0.81)%, and 0.5 g/kg (14.45 ± 0.89)% also dramatically decreased (P < 0.05), in comparison with the control group.

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).

Furthermore, we found no significant changes in progesterone hormone levels in mice serum. FSH levels decreased significantly after 2 g/kg/d intragastric administration of DMP for 40 days, while E2 and LH hormone levels increased remarkably. Svechnikova et al. (16) studied on 20-day-old female rats exposed to 500 mg of DEHP by oral gavage once daily for 10 days and the same results were obtained. They stimulated gonadotropin-releasing hormone in primary cultures of granulosa cells and pituitary cells were isolated from rats exposed to DEHP to reduce the transport of endogenous cholesterol to mitochondria in granular cells. The ability of the pituitary cells to produce and secrete LH is greatly enhanced. This shows that DEHP exerts a dual-effect on the pituitary-ovarian axis, stimulating the production of pituitary LH and inhibiting steroid production in granular cells. Based on this, it is reasonable to speculate that DMP also has a dual-effect on the pituitary-ovarian axis, and that prolonged exposure to DMP promotes serum estradiol levels, resulting in a secondary reduction in FSH levels. Research by Davis et al. (17) agrees with our conjecture. They also found that adult female rats exposed to 2 g/kg DEHP for 8 days suffered significant ovarian cell estradiol production inhabitation before ovulation, resulting in a secondary increase in FSH levels and failure in stimulating the LH surge necessary for ovu-

Table 3. The Effects of DMP Exposure on the Serum Progesterone and Estradiol in Female C57 Mice[a]

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	183.60 ± 7.48
DMP, g/kg				
0.5	42.84 ± 3.31	33.03 ± 1.48	103.11 ± 7.43	210.14 ± 3.56[b]
1	37.56 ± 3.07	34.06 ± 3.16	103.97 ± 9.15	195.48 ± 10.23[b,c]
2	38.57 ± 2.74	36.08 ± 2.67	94.47 ± 7.80	196.59 ± 8.58[b,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).

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

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	126.35 ± 8.46[b,c,d]	3857 ± 309	5192 ± 362[b,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).

Table 5. Effects of DMP Exposure on Cell Apoptosis of Ovarian Cells in Female C57 Mice[a]

Treatment	Apoptosis, %	
	20 Days	40 Days
Control	1.43 ± 0.05	14.45 ± 0.89
DMP, g/kg		
0.5	0.88 ± 0.07[b]	12.67 ± 1.06[b]
1	1.57 ± 0.05[b,c]	17.31 ± 1.61[b,c]
2	1.60 ± 0.17[b,c]	16.18 ± 0.81[b,c]

[a] Data 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). Cell apoptosis was measured with PI nuclear staining and flow cytometry.
[b] Significantly different from control (P < 0.05).
[c] Significantly different from 0.5 g/kg/d (P <0.05).

lation and ultimately no ovulation in rats. In addition, the increase E2 level increase resulted in a significant decrease in steroids, which also echoes the results of a significant increase in apoptosis in the dose group in this experiment.

Evidence has shown that in mammals, early morphological changes in occluded oocytes include retraction of 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).

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

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).

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