Effect of DEHP (Di-2-Ethyl Hexyl-Phthalate) on Resumption of Meiosis and *in-vitro* Maturation of Mouse Oocytes and Development of Resulting Embryos

Hossein Eimani, Ph.D.[☆★♦], Azam Dalman, M.Sc.[☆☆], Hoori Sepehri, Ph.D.[☆]
Saeed Kazemi, Ph.D.[☆], Fatemeh Hassani, M.Sc.[☆☆], Mojtaba Rezazadeh, Ph.D.[☆★]
Hossein Baharvand, Ph.D.[☆], Abdolhossein Shahverdi, M.Sc.[☆], Reza Omani Samani, M.D.[☆]

☆ Embryology Department, Royan Institute
★ Biology Department, Medical School, Tehran University
★ Anatomy Department, Faculty of Medicine, Baghyatallah University
★ Anatomy Department, Faculty of Medicine, Tarbiat Modarres University

♦ *Corresponding Address: P.O. Box: 19395-4644, Embryology Department, Royan Institute, Tehran, Iran*

Email: info@royaninstitute.org

Abstract

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Introduction: DEHP [di(2-ethylhexyl) phthalate)] is widely used in plastic industry and some reproductive toxicity has been shown with it. So, this study was designed to evaluate DEHP effects on resumption of meiosis and *in vitro* maturation of mouse oocytes as well as development of embryos resulted from them.

Material and Methods: Mice of 4-6 weeks old were administered daily doses of 50, 100, 200 µl of 2.56 µM DEHP solution for 12 days. Immature mouse oocytes were recovered from all experimental groups and matured in MEM-α medium containing 5% FCS with and without 7.5 IU hCG and 100 mIU rFSH. IVF was performed T6 medium.

Results: Resumption of meiosis and *in vitro* maturation were significantly lower in all experimental groups in culture media without hormones compared to controls. Fertilization and embryo development were also significantly decreased in both culture media (with and without hormones).

Conclusions: This study showed the adverse effects of DEHP on *in vitro* maturation and embryo development in a dose dependent manner.

Keywords: Di-2(Ethylhexyl) Phthalate (DEHP), *in vitro* Maturation, Immature Mouse Oocyte, Embryo Development

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Introduction

The possible exposure of human and animals to many industrial chemicals and pesticides has been a growing concern over the last decade for both scientific community and general public. Several studies have suggested that environmental contaminants, including a group of chemicals known as phthalates, could adversely affect reproductive functions in a variety of vertebrates (1-5). Phthalates are widely used in plastic industry, and are widespread in human environment, from infant toys to food packaging materials. Globally, more than 8 billion kilograms of phthalates are used in industry each year (6). Di(2-ethylhexyl) phthalate (DEHP) is one of the most commonly used phthalates (7) and has been shown to induce developmental and reproductive toxicity, testicular toxicity, and adverse effects on sex hormone release in rodents (8, 9, 10, 11). The principal metabolite of di(2-ethylhexyl) phthalate (DEHP) is monoethylhexyl phthalate (MEHP) (12, 13), which also has been shown to have toxic effects on male reproductive system and may be more

effective than DEHP (14-16). So, it seems that MEHP is responsible for the toxic effects of DEHP, as it is produced in the body from DEHP (14, 15) and no toxic effect has been seen in other metabolites of DEHP.

There are some reports about the mechanism of DEHP toxicity in female reproductive tract of the rodents. It has been shown that it can suppress estradiol secretion and ovulation in rats (9, 17), and as pre-ovulatory follicle cells are responsible for estradiol secretion, it seems that these cells are the target for DEHP. Morphometric analysis of preovulatory follicles determined that granulosa cells in DEHP-treated rats were smaller than controls; this evidence could confirm ovaries as a target for DEHP (9, 18). MEHP also decreases the estradiol production by suppressing aromatase transcript levels (19).

In oocyte maturation, there is a report indicating that MEHP could negatively modulate bovine oocyte meiotic maturation *in vitro* (20), and also it has been shown that DEHP can decrease oocyte development in fish (21). So, this study was aimed to investigate the effects of DEHP on resumption of meiosis and *in vitro* maturation of mouse oocytes, as well as evaluation of the embryos resulted from DEHP-treated oocytes to study its effect on embryo development.

Material and Methods

Immature oocyte retrieval: Four to six weeks old NMRI mice were obtained from Razi Institute, Iran. The mice were orally administered 50, 100, and 200 µl of 2.56 µM DEHP solution for 12 days in four groups. They were then sacrificed by spine dislocation and dissected under sterile conditions, and their ovaries were collected and preserved in 500 µl drops of MEM-α culture media containing 5% FCS. The ovaries were dissected using insulin syringe, and the adipose tissue was removed and immature oocytes in addition to surrounding germinal vesicles and granulosa cells were collected. Then, the granulosa cells were removed by pipetting, and immature germinal oocytes with clear cytoplasms and homogenous zona pellucida in addition to adequate perivitelline space were selected from each group.

In-vitro maturation

Experimental group I: 248 oocytes were retrieved from mice treated with 50 µl DEHP in 8 consecutive procedures. The oocytes were then divided into two groups; 131 oocytes were placed in MEM-α medium containing 5% FCS (group A) and 117 oocytes in MEM-α containing 100 mIU rFSH, 7.5IU HCG, and 5% FCS (group B). Experimental group II: 436 oocytes were retrieved from mice treated with 100 µl DEHP in 8 consecutive procedures. The oocytes were then divided into two groups; 240 oocytes were placed in MEM-α medium containing 5% FCS (group C) and 196 oocytes in MEM-α containing 100 mIU rFSH, 7.5IU HCG, and 5% FCS (group D).

Experimental group III: 248 oocytes were retrieved from mice treated with 200 µl DEHP in 8 consecutive procedures. The oocytes were then divided into two groups; 138 oocytes were placed in MEM-α medium containing 5% FCS (group E) and 110 oocytes in MEM-α containing 100 mIU rFSH, 7.5IU HCG, and 5% FCS (group F).

Control group: 446 oocytes were retrieved from untreated normal mice. These oocytes also were divided into two groups; 209 oocytes were placed in MEM-α medium containing 5% FCS (MEM control group) and 237 oocytes in MEM-α containing 100 mIU rFSH, 7.5IU HCG, and 5% FCS (group g).

All groups were placed in 37ºC incubator containing 5% CO_2 for 24 hours and then *in vitro* maturation stages were determined by inverted microscope.

Oocytes without any change in their nuclei were considered as GV (Germinal Vesicle) or immature, those with nuclear breakdown considered as GVB (Germinal Vesicle Breakdown), and those with meiotic signs and polar bodies as mature or MII (Metaphase II) oocytes.

IVF and development of the matured oocytes

After sacrificing NMRI mice by spinal dislocation, their epididymal tails were dissected and placed into 500 µl drops of

T6 media containing 5 mg/ml BSA (bovine serum albumin). After preserving in 37ºC incubator containing 5% CO_2 for 1.5 hour, active and normal sperms were collected from drop margins (10^5 sperms/ml) and transferred along with matured oocytes to drops of T6 media containing 16 mg/ml BSA. After 4-6 hours, the oocytes were transferred to a new medium containing T6 in addition to 5 mg/ml BSA. The oocytes were evaluated after 24, 48, 72, and 96 hours for embryo formation by inverted microscope.

Statistical analysis

Chi-square test was performed to compare the experimental and control groups. SPSS software was used for data analysis.

Results

In our study, 893 oocytes were treated using various dosages of DEHP and compared to 446 normal oocytes. In experimental group I (treated with 50 µl DEHP), meiosis resumption was found in 66% of the oocytes in group A (MEM + FCS) and 74% of those in group B (MEM + HCG + rFSH + FCS).

The rate of nucleus breakdown (GVB) was 22% and 19% in groups A and B, respectively, while the rate of MII oocytes was 43% and 54%, respectively. There were significant maturational differences between group A and the control group (p=0.001). After insemination of 60 and 63 mature oocytes from groups A and B respectively, the fertilization rate in group A (40%) was significantly lower than controls (50%) (p=0.02).

In experimental group II (treated with 100 µl DEHP), meiosis resumption was found in 64% of the oocytes in group C (MEM+FCS) and 77% of those in group D (MEM + HCG + rFSH + FCS). The rate of nucleus breakdown (GVB) was 13% and 18% in groups C and D, respectively, while the rate of MII was 50% and 59%, respectively. The meiosis resumption was found to be significantly different in group C compared to control group (p0.03). Upon insemination of 131 and 116 mature oocytes from groups C and D, the fertilization rates of both groups were significantly lower than controls after 96 hours (p0.001 and p0.01, respectively).

96 hours after insemination						72 hours after insemination			48 hours after insemination			24 hours after insemination		Number of mature oocytes	24 hours after IVM			Administered dos of	Number of immature	Experimental groups
T	2-cell	4-cell	8-cell	morula	early B	2-cell	4-cell	8-cell	2-cell	4-cell	8-cell	2-cell	4-cell		GV	GVB	MII			
24*d 40%	16 27%	7 12%	1 2%			20 33%	8 13%	5 8%	25 42%	7 12%	4 7%	27 45%	7 12%	60	44 33 %	30 22%	57*b 43%	50	131	Group A
33 52%	22 35%	9 14%	1 1%	1 1%		25 40%	13 21%	2 3%	29 46%	12 19%	1 1%	34 54%	4 6%	63	29 23 %	13 10%	82 66%	50	124	Group B
48*b 37%	28 21%	17 13%	3 2%			38 29%	26 20%	2 1%	38 29%	29 22%		53 40%	10 8%	131	86 35 %	33 13%	121*c 50%	100	240	Group C
48*c 41%	28 24%	17 15%	3 2%			38 33%	26 22%	2 2%	48 41%	23 20%		54 46%	12 10%	116	45 23 %	35 18%	116 59%	100	196	Group D
14*c 35%	9 22%	3 7%	1 2%	1 2%		13 32%	6 15%	1 2%	17 42%	3 7%		16 40%	1 2%	40	74 53 %	18 13%	46*a 33%	200	138	Group E
16*c 25%	14 31%	2 4%				18 40%	3 7%		20 44%	5 11%		18 40%	4 9%	45	19 17 %	33 30%	58 53%	200	110	Group F
35 50%	21 30%	9 13%	3 4%	2 3%		25 36%	19 27%	3 4%	35 50%	15 21%		33 47%	10 14%	70	54 22 %	43 18%	140 59%	0	237	Group G
69 58%	30 25%	13 11%	7 6%	15 13%	4 3%	35 29%	20 17%	12 10%	49 41%	27 23%	9 7%	63 53%	11 9%	119	55 26 %	24 11%	130 62%	0	209	Control group

Table 1. Maturational and developmental stages of mouse oocytes in different experimental and control groups. GV, Germinal Vesicle; GVB, Germinal Vesicle Breakdown; MII, Metaphase II; T, Total; early B, early Blastocyst.
a, p = 0.000; b, p = 0.001; c, p = 0.01; d, p = 0.02.

In experimental group III (treated with 200 µl DEHP), meiosis resumption was found in 46% of the oocytes in group E (MEM + FCS) and 83% of those in group F (MEM + HCG + rFSH + FCS) after 24 hours. The rate of nucleus breakdown (GVB) was 13% and 30% in groups E and F respectively, while the rate of MII oocytes was 33% and 53%, respectively.

There was a significant difference in meiosis resumption between group E and the controls ($p<0.001$). Upon insemination of 40 and 50 mature oocytes from groups E and F, the fertilization rate in both groups were significantly lower than controls after 96 hours ($p=0.01$).

The rates of meiosis resumption, GVB, and MII oocytes were not significantly different among 237 untreated oocytes maintained in MEM + rFSH + HCG + FCS medium and 209 untreated oocytes maintained in MEM + FCS medium. Also, we could not see any significant differences between mentioned groups regarding the fertilization rate upon insemination of mature oocytes.

Discussion

There are few reports about the effects of environmental pollutants on IVM. We showed here that DEHP adversely affected oocyte maturation. GV stage was found to be significantly higher in the oocytes treated with 200 µl DEHP and a lower maturation was found in the oocytes treated with 50 and 100 µl DEHP. The DEHP adversely affected the oocyte maturation in a dose-dependent manner. As mentioned before, the lowest rate of MII oocytes was seen in 200 µl group, followed by 100 and 50 µl groups. The fertilization and embryo development were also adversely affected by DEHP. The mechanism of this action is not completely clear. It was reported that MEHP (a DEHP metabolite) inhibited FSH-stimulated cAMP accumulation in cultured Sertoli cells (22) and FSH-stimulated cAMP production in cultured rat granulosa cells (23). It has also been shown that intracellular build up of cAMP is necessary for optimum developmental competence (24, 25). Impaired responsiveness to FSH has been shown in Sertoli cells (22) and if it occurs in oocytes and cumulus cells, it can be considered as a likely mechanism. DEHP/MEHP probably mimic the effects of

fatty acids on granulosa cells (18), because they are ligands for fatty acid binding proteins (26), and it has been shown that fatty acids adversely affect *in vitro* maturation (27, 28).

There are many reports indicating that lower estradiol secretion from granulosa cells is responsible for impaired oocyte maturation, either directly or through aromatase inhibition. Hu et al. debated this by showing that aromatase inhibition and lower amounts of estradiol can not impair *in vitro* maturation of the oocyte (29). Moreover, several reports have focused on granulosa cells and various changes inside them as possible toxic effects of DEHP/MEHP. One report on MEHP showed higher negative effect on denuded oocytes compared to intact cumulus oocyte complex (20) and so it seems that this agent may directly affect oocyte itself. Further studies are needed for complete knowledge of the mechanism of action of DEHP and MEHP on oocyte maturation. As we could not measure the MEHP level in oocytes, we are not sure about the effects of intracellular MEHP on *in vitro* maturation.

Conclusion

Oral DEHP adversely affects meiosis resumption and *in vitro* maturation of mouse oocytes as well as the development of embryos resulted from them, most likely through its metabolite, MEHP.

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