Di (2-ethylhexyl) phthalate exposure impairs meiotic progression and DNA damage repair in fetal mouse oocytes in vitro

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Di (2-ethylhexyl) phthalate (DEHP), is the most common member of the class of phthalates that are used as plasticizers and have become common environmental contaminants. A number of studies have shown that DEHP exposure impacts reproductive health in both male and female mammals by acting as an estrogen analog. Here, we investigated the effects of DEHP on meiotic progression of fetal mouse oocytes by using an in vitro model of ovarian tissue culture. The results showed that 10 or 100 μM DEHP exposure inhibited the progression of oocytes throughout meiotic prophase I, specifically from the pachytene to diplotene stages. DEHP possibly impairs the ability to repair DNA double-strand breaks induced by meiotic recombination and as a consequence activates a pachytene check point. At later stages, such defects led to an increased number of oocytes showing apoptotic markers (TUNEL staining, expression of pro-apoptotic genes), resulting in reduced oocyte survival, gap junctions, and follicle assembly in the ovarian tissues. Microarray analysis of ovarian tissues exposed to DEHP showed altered expression of several genes including some involved in apoptosis and gonad development. The expression changes of some genes clustered in cell-cell communication and signal transduction, along with plasma membrane, extracellular matrix and ion channel function classes, were dependent on the DEHP concentration. Together, these results bring new support to the notion that exposure to DEHP during gestation might exert deleterious effects on ovary development, perturbing germ cell meiosis and the expression of genes involved in a wide range of biological processes including ovary development.

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Di (2-ethylhexyl) phthalate (DEHP), is a compound largely used in plasticizing polyvinyl chloride resin (PVC) products worldwide.1 More than 10 million tons of DEHP is estimated to be used in the production of plastics and plastic-based products every year.2 DEHP, due to its weak non covalent link to plastic components, can be easily released into the environment and humans and animals are exposed through oral ingestion, inhalation and skin contact, after which it enters into the blood circulation.3

Previous studies have indicated that DEHP exposure is associated with testicular, liver, kidney and ovary tissue disease.3–7 The modes of action of DEHP are not well understood. Mechanisms may include activation of peroxisome proliferator-activated receptors (PPARs), estrogen receptors (ERs) and to a lesser extent androgen receptors (ARs). PPARs are a group of nuclear receptor proteins that function as transcription factors for genes involved in a variety of cell activities including steroiogenesis and antioxidative actions. ERs are receptors that are activated by estrogens, in particular 17β-estradiol (E2). Two classes of ERs have been described: nuclear/cytoplasmic estrogen receptors ERα and ERβ, and membrane estrogen receptors (mERs). Although the expression and role of PPARs in the mammalian ovary before birth are not known, ERα is known to be expressed by both primordial germ cells (PGCs)6 and somatic cells9 within sexually undifferentiated mouse gonads. DEHP effects on early postnatal and adult ovaries appear to be a consequence of activation of PPAR and/or ER-dependent pathways. It is apparent that DEHP effects on oogenesis are strictly dependent on the period of exposure, both in vivo and in vitro. Effects are seen during several developmental waypoints including during the formation and development of PGCs, entrance and progression of oocytes into meiotic prophase I, germ cell cyst breakdown during the prenatal or early postnatal period, along with primordial follicle activation and development in the adult ovary.10,11

In the present paper, we utilized an in vitro ovarian tissue culture system, developed in our lab, to study the effects of DEHP on prenatal or early postnatal oogenesis.12 While it has been reported that DEHP effects folliculogenesis through impairment of oocyte meiosis, survival, and follicle assembly,10 the mechanisms involved remain largely unknown. Using our in vitro system we investigated the mechanisms of such effects and identified the gene expression profile induced by DEHP in the fetal ovary.

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Exposure of the ovary to DEHP delays oocyte progression from pachytene to diplotene. In order to explore the effects of DEHP exposure on the progression of meiotic prophase I in female germ cells, 12.5 dpc fetal mouse ovarian tissues were cultured in vitro and exposed to DEHP for 6 days as the first meiotic prophase I accomplished at 18.5 dpc in vivo, relative to day 6 in vitro (Supplementary Figure S1). Cytospread immunofluorescence staining of meiotic chromosomes for SCP3 revealed a clear delay in meiotic progression from the pachytene to the diplotene stage in oocytes cultured in the presence of 10 μM or 100 μM DEHP (Figures 1a and b).

Western blot and RT-qPCR analysis showed that after 2 days of culture the expression of germ cell and meiotic genes such as Dazl and Mvh or Stra8, Scp1, Scp3 and Rec8, respectively, were significantly decreased at both the protein and/or mRNA level (Figures 1c and d).

DEHP exposure affects the patterns of γH2AX and causes DNA damage in oocytes. Since the DEHP-induced meiotic delay reported above resembles the meiotic arrest occurring in a variety of species as a consequence of the pachytene checkpoint triggered by defects in DNA double-strand break (DSB) repair,13 we decided to investigate the status of DNA breaks and repair in DEHP treated oocytes.

As shown in Figure 2a, we observed that in both the control and DEHP treated oocytes the staining pattern of γH2AX (a marker of DNA breaks) could be classified into three main categories: negative (none or rare barely detectable foci), weak (a few number of small foci) and strong (numerous small

Results

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and large foci). As shown in Figure 2b following 6 days of culture, the percent of γH2AX-positive oocytes (including weak and strong staining) was higher in DEHP treated oocytes in comparison to the control, although significantly different only in the 100 μM DEHP treatment group. However, comparing negative and weak oocytes against strong γH2AX stained oocytes, the difference was significant at both DEHP concentrations (Figure 2c). This difference was evident also when the analysis was restricted to pachytene and diplotene oocytes, in which the frequency of strong γH2AX staining was much higher both in control and treated oocytes (Figure 2d). The presence of large γH2AX foci in the DEHP exposed oocytes potentially reflects abnormal chromatin modifications during late prophase I or alternatively, a failure to resolve DSBs, and therefore a defect in DNA repair.

Evidence of increased number of oocytes with DNA damage following DEHP exposure, were also obtained by performing chromosome staining for the proteins BRCA1, usually recruited with γH2AX to sites of DNA breaks (Figure 3), and RAD51, involved in DNA repair by homologous recombination (Figure 4). Since RAD51 foci, indicative of DNA damage repair, normally disappears at the pachytene/diplotene stage oocytes at these stages still showed positive RAD51 staining both as discrete or lined dots, can be considered defective. On the other hand, the chromosome staining for the cross over protein MLH1 was not significantly different between the control and the experimental groups (Supplementary Figure S2).

Western blot results of γH2AX depicted in Supplementary Figure S3A show that exposure to 10 μM and 100 μM DEHP for 6 days caused increased expression of γH2AX in the ovarian tissue (Figure 2). Moreover, RT-qPCR analysis showed that exposure to 10 μM and 100 μM DEHP for 6 days caused increased expression of Rad51, and Brca1 transcripts.
along with Mlh1 and Spo11 genes, the latter encoding a protein involved in the creation of DSB in the DNA at the preleptotene/leptotene stage (Supplementary Figure S3C). Finally the level of mRNA of the Atm gene encoding a protein that is recruited and activated by DNA breaks was not significantly altered while the expression of the p53 gene, a gene essential for chromosome stability, was decreased both at the mRNA and protein level (Supplementary Figure S3B–C), further indicating that DEHP exposed cells accumulated DNA damage.

DEHP promotes the expression of ERs and decreases the expression of PPARα. In order to examine the expression of ERs and PPARα, two possible DEHP targets, we analyzed ovarian tissues by RT-qPCR. The analysis showed that the exposure to 10 μM and 100 μM DEHP for 6 days caused significantly increased mRNA expression of ERα and ERβ, and decreased the expression of PPARα. Such effects were partly abolished by the contemporaneous addition to the culture of 1 μM tamoxifen, an ER antagonist (Figure 5a). Western blot results confirmed the increased expression of the ERα protein after exposure to 10 μM and 100 μM DEHP, and the inhibitory effect by 1 μM tamoxifen (Figure 5b). Furthermore, the percentage of cells showing PPARα positive staining was significantly decreased by treatment with DEHP in comparison to controls (Figure 5c).

DEHP exposure induces apoptosis in ovarian cells. Since a major consequence of unrepaired DNA damage is cell apoptosis, we analyzed ovarian tissues incubated in the presence of DEHP for 6 days for apoptotic markers. The number of cells showing TUNEL-positive staining was significantly increased by DEHP treatment when compared to the control (Figure 6a; Supplementary Figure S4). Moreover, as shown in Figure 6b, the ratio of the Bax/Bcl-2 transcripts and the level of mRNA for the Caspase3 gene were significantly increased in the DEHP exposed ovaries. Western blot analyses of the ovarian tissue confirmed the increased ratio of Bax/Bcl-2 at protein level (Figure 6c), and, in addition, showed an apparent paradoxically increased expression of the anti-apoptotic protein MCL-1 after DEHP treatment in comparison to control (Figures 6d and e; Supplementary Figure S3D).

TEM images displayed some apoptotic characteristics in oocytes following DEHP treatment. While control oocytes showed a regular nuclear membrane with homogenously dispersed chromatin and normal cytoplasmic structures, oocytes in DEHP treated groups showed condensed chromatin regions (Supplementary Figure S5A). Furthermore, numerous myelin bodies and dark homogenous osmophilic lipid droplets with distinct lamellae were observed in the 100 μM DEHP treated oocytes (Supplementary Figure S5B).
DEHP exposure reduces the number of oocytes, decreases the expression of gap junction proteins and inhibits cyst breakdown. By prolonging the culture up to 10 days we observed additional effects of DEHP exposure on the ovarian tissues. DEHP exposure caused a significant reduction in the number of oocytes (Control $= 258.67 \pm 31.5\%$; $10\;\mu\text{M DEHP} = 202.33 \pm 49.5\%$ and $100\;\mu\text{M DEHP} = 124.83 \pm 28.86\%$, Figures 7a and b).

Furthermore, a survey of the distribution of gap junctions using an antibody against Cx43, known to form channels mostly among granulosa cells, showed a decreased number of gap junctional plaques in the ovarian fragments incubated in the presence of DEHP when compared to the control unexposed fragments (Control $= 94.50 \pm 20$; $10\;\mu\text{M DEHP} = 54.25 \pm 8.26$; $100\;\mu\text{M DEHP} = 34.75 \pm 8.14$; Figures 7d and e). RT-qPCR analyses showed a DEHP dependent reduction not only of Cx43 expression but also of the Cx37 gene, encoding a connexin forming gap junction channels between granulosa cells and the oocyte (Figure 7f).

Finally, numerous germ cell nests and rare follicles were detected within the ovarian tissues in the presence of DEHP (Figure 7c). Follicle counts confirmed a marked reduction in the follicle number and higher percentage of oocytes in nests in ovarian tissues cultured in the presence of DEHP (Control =

![Figure 4](https://example.com/fig4.png)

**Figure 4** Exposure to DEHP impairs DNA repair in oocytes. (a) Immunolabeling of the oocyte chromosomes with anti-SYCP3 (red) and anti-RAD51 (green) antibodies. (b) Percentage of oocytes showing none, RAD51 staining after six days of culture with or without DEHP. (c) Percentage of oocytes showing none, point or line RAD51 staining after six days of culture with or without DEHP. (d) Percentage of oocytes at the pachytene and diplotene stages showing none, point or line RAD51 staining after six days of culture with or without DEHP (*$P<0.05$; **$P<0.01$)
RNA microarray analysis of 12.5 dpc ovarian tissues exposed to DEHP. To gain a better understanding of the biological effects of DEHP on embryonic ovaries, we performed an RNA microarray analysis on 12.5 dpc ovarian tissues cultured for two days in the presence of DEHP. DEGs between the control group and DEHP exposed tissues were screened. We found a total of 206 upregulated and 54 downregulated genes following 10 μM DEHP treatment, and 1529 upregulated and 1421 genes downregulated following 100 μM DEHP treatment (Figures 8a and b; Supplementary Table S2). Compared to the 10 μM DEHP, 1474 genes were significantly upregulated and 1505 genes were significantly downregulated in the 100 μM DEHP group (Figures 8a and b; Supplementary Table S2). As a whole, 225 genes were significantly upregulated and 85 genes were significantly downregulated between control and DEHP exposed groups (Figures 8a and b; Supplementary Table S2).

Gene ontology (GO) analysis of the DEGs showed that DEHP exposure mostly perturbed genes involved in apoptosis and gonadal development (Figure 8c). As shown in Supplementary Table S2 and 3, apoptotic and gonad development genes altered after DEHP exposure included Foxl2, Gm13237, Nkx2-1, Lhx9, and Peg10, Opa1, Krt18, Rock1, 2610018G03RIK, Id1, Rhob, Psmem3, Pmaip1, Ii24, Phlda1, and Elm01.

Moreover, we found DEGs of DNA binding factors in both the 10 μM DEHP treatment and control groups (Supplementary Figure S5A). The effects of DEHP exposure on the gene
expression showed considerable dose-dependent effects. In particular, by comparing DEGs between the 10 μM and 100 μM DEHP groups, we found that the higher treatment mostly clustered in cell-cell communication and signal transduction, plasma membrane and extracellular matrix and ion channel functions (Supplementary Figure S5B).

**Figure 6** DEHP exposure promotes apoptosis in the ovary. (a) (Left) TUNEL-staining of the ovarian tissues after 6 days of culture with or without DEHP; (Right) Quantitative analyses of the number of TUNEL-positive cells. (b) Representative qRT-PCR for the apoptosis related genes Bax, Bcl-2 and Casp3 in ovarian tissues cultured for 6 days with or without DEHP. (c) (Left) representative WB of BAX and BCL-2; (Right) Quantitative analysis of BAX/BCL2 expression. (d) (Left) MCL-1-staining of the ovarian tissues after 6 days of culture with or without DEHP; (Right) Percentages of MCL-1 positive area. (e) (Up) Representative WB of MCL-1; (Down) Quantitative analysis of MCL-1 expression. The percentage of each group is presented as mean ± SD. All experiments were repeated at least three times. (*P < 0.05; **P < 0.01)
Discussion

Several studies have reported that DEHP exposure can affect the correct development and functions of the ovary. Depending on the stage of development and exposure concentration and time, both in vivo and in vitro, DEHP has been shown to alter germ cell formation and development, meiotic initiation and progression, and primordial follicle assembly. The concentrations of 10–100 μM DEHP used in our study are in the range used by us and others in order to investigate the effect of this compound in vitro on some processes of oogenesis. They correspond to about 4 to 40 μg/ml, a range reported in the blood of patients having a long-term exposure to DEHP-containing devices about 70 to 80 μg/ml, or after neonatal exposure to DEHP following exchange transfusion with PVC catheters, between 13.2 to 84.9 μg/ml.

We report here that in vitro exposure of the mouse fetal ovaries to DEHP inhibited the progression of oocytes throughout meiosis prophase I, specifically, the transition from the pachytene to the diplotene stage. Moreover, we found that oocytes exposed to DEHP showed increased DNA damage as evaluated by γH2AX and BRCA1 staining and the perdurability of RAD51 foci. As a consequence, defective oocytes could be prone to undergo apoptosis which is consistent with what we found in ovaries cultured in the presence of DEHP for six days.

Besides some morphological evidence in oocytes, we obtained indication of apoptosis in the ovarian cells following DEHP exposure from the increased Bax/Bcl-2 ratio, Caspase 3 and MCL-1 expression. As a matter of fact, although MCL-1 is considered an anti-apoptotic protein, its expression has been reported to increase upon exposure to various types of apoptotic stimuli including DNA damage, likely as an attempt by cells to preserve their viability.

Activation of a quality check point, at the pachytene stage, which is able to eliminate oocytes carrying DNA defects has been proposed as one of the causes of oocyte depletion occurring during prenatal oogenesis in mammals. Increased apoptosis explains the reduced number of oocytes scored by us after a prolonged culture period. Likewise, in vitro exposure of mouse fetal ovaries to DEHP impaired oogenesis.

Figure 7  DEHP exposure causes a reduction of oocyte number and of cyst breakdown (a) Control and DEHP exposed ovarian tissue stained for MVH (green specific for oocytes) after 10 days DEHP exposure, nuclei red; bar is 20 μM. (b) Number of oocytes in control and DEHP exposed groups. (c) Percentage of oocytes in cysts and primordial follicles in control and DEHP exposed groups. DEHP impairs the expression of connexins. (d) Control and DEHP exposed ovarian tissues stained for Cx43 (red), nuclei blue; bar is 20 μM. (e) Number of Cx43 positive gap junction plaques in control and DEHP exposed ovarian tissues. (f) qRT-PCR for Cx43 and Cx37. Relative fold changes are presented as mean ± SD. All experiments were repeated at least three times. (* P<0.05; ** P<0.01)
Figure 8  Microarray analyses of gene expression in 12.5 dpc ovary cultured for 2 days in Control (Ctr) in the presence of DEHP. (a) Scatter plots of differentially expressed genes between Ctr and 10 μM DEHP, control and 100 μM DEHP, 10 μM and 100 μM DEHP groups. (b) Heatmap of differentially expressed genes between control and DEHP treated groups. (c) Gene ontology (GO) enrichment analysis of differentially expressed genes between Control (Ctr) and DEHP groups.
Since DEHP can interact with a subset of PPARs, a group of nuclear receptors that function as transcription factors for genes encoding enzymes involved in oxidative stress, it can be postulated that the increased DNA damage detected in cultured oocytes exposed to DEHP results from reduced activity of antioxidant enzymes. Our finding that ovarian tissues express PPARs and that such expression decreased in culture confirmed ER expression in the ovarian tissues regardless of whether in somatic cells, oocytes or both and report an increased expression of both receptors in culture in the presence of DEHP. It is of note that in a previous study, we reported that the meiotic progression of fetal oocytes was delayed following the exposure of the pregnant mice to DEHP. This effect, resembling the present results, was associated to a reduction of the mRNA and protein expression of the meiosis-specific gene Stra8 while the DNA methylation level of the gene was increased. The capability of DEHP to perturb methylation of meiotic genes such as Stra8 and other genes in oocytes of neonatal mouse ovaries such as Lhx8 and of the maternal imprinted genes Igf2r and Peg3 in a transgenerational way, can be seen in the context of the contribution by ERs signaling to epigenetic changes. Several acetylases/deacetylases and methylases/demethylases can interact directly or indirectly with ERs and cause histone modifications. In fact, in previous studies, using the same culture system, we found that Notch signaling dependent Stra8 methylation was necessary for correct meiotic progression. Moreover, ablation of G9a, a major mammalian H3K9 methyltransferase, showed that epigenetic gene silencing was crucial for proper meiotic progression beyond the pachytene stage. Interestingly, ERα and ERβ are present also in the human fetal ovary were they are present until the 20th week of gestation, when the expression of ERs substantially increases.

It is to be mentioned here that exposure of mice from midgestation until birth to daily doses of bisphenol (BPA), a compound that like DEHP is considered an estrogenic endocrine disruptor, resulted in chromosome synaptic breakdown and also in expression of several genes involved beside in apoptosis, in a number of process guiding prenatal gonadal development.

Materials and methods
Reagents. Unless otherwise indicated, all chemicals were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Stock solutions of DEHP were prepared using dimethylsulfoxide (DMSO) as the solvent at concentrations of 0.127 or 1.27 mM allowing an equal volume to be added to culture medium for each experimental groups. Tamoxifen, an ER antagonist, was dissolved in 50% ethanol and was added to the culture medium at the final concentrations of 1 μM, and only 0.05% ethanol was present in the medium.

Animals. The procedures involving animals followed the regulations of the Ethics Committee of Qingdao Agriculture University, Shandong, China. Mice used in this experiment were of CD1 strain purchased from Vital River Laboratory Animal Technology Co. Ltd (Beijing, China). Mice were housed in 21-22 °C, 12 h dark/12 h light cycles (lights on at 7:30) with free access to food and water. Female and male mice were paired together and checked for the presence of a vaginal plug the next morning. The day when the vaginal plug was detected was considered 0.5 day post coitum (d.p.c.).

Isolation and culture of the ovarian tissues. Isolation and culture of the ovarian tissues from 12.5 dpc embryos were performed as previously described. In particular, after isolation, the ovaries were dissected in half and each piece was cultured in 600 μl of α-minimal essential medium (α-MEM; HyClone, SH30265.01B, Beijing, China), supplemented with 10% fetal bovine serum (FBS; Gibco, 10099-141, USA), 0.23 mM sodium pyruvate (HyClone, SH40003-12), 100 IU/mL of penicillin G, and 100 mg/ml of streptomycin sulfate, 10 µIU/ml follicle stimulating hormone (FSH; RD, 5925-FS, MN, USA). In each well, 300 μl of medium was changed every two days and the culture carried out in a humidified incubator in 5% CO2 in air at 37 °C for the indicated time (Supplementary Figure S1).

According to United States Food and Drug Administration (FDA), tolerable intake values for DEHP are 0.6 mg/kg bodyweight/day for parental exposure and 0.04 mg/kg bodyweight/day for oral exposure, mouse ovaries were cultured in vitro and exposed to DEHP (Sigma, 36735-1G) at dose of 10 μM or 100 μM DEHP was dissolved in 0.1% DMSO and 0.1% DMSO alone as a vehicle control, respectively. After DEHP treatment, the ovaries were collected and kept for further analysis.

Staining of meiotic chromosomes. Ovarian tissues were incubated in Hypo extraction buffer (HEB; 0.6 M Tris, 0.5 M Sucrose, 0.17 M trisodium citrate dehydrate, 0.5 M EDTA, 0.5 M DTT, 0.1 M PMSF) for 1.5 h at room temperature. Tissues were then mechanically dispersed and cells transferred onto a slide fixed with 1% PFA overnight. Blocking was performed by bumping the slides in antibody dilution buffer (ABD; 0.3% BSA, 10% normal goat serum and 0.005% Triton-X-100 in TBS) for 30 min before incubation in primary antibody, anti-SCCP at a dilution of 1:150 (Abcam, ab26762), anti-RAD51 at a dilution of 1:150 (Abcam, ab26350), anti-RAD51 at a dilution of 1:150 (Abcam, ab33534), BRCa1 at a dilution of 1:100 (Boster, PB9015, Wuhan, China), or anti-MLH1 at a dilution of 1:150 (BD Pharmingen, 551091, Franklin Lakes, NJ, USA) for 3 h at 37 °C. The slides were further fixed with ABD overnight at 4 °C, washed three times with TBS, before incubation in secondary antibody (Beyotime, A0521 or A0516, Nantong, China; or Sigma, F3082 or F9006).
at a dilution of 1:150 dilution in ADB for 3 h at 37 °C. Hoechst 33342 was used to stain nuclei and the slides were mounted with Vectashield (Vector, H-1000, Shanghai, China). Images were taken under a fluorescence microscope (BX51; Olympus, Tokyo, Japan).

**Immunofluorescence.** Ovaries tissues were fixed in 4% paraformaldehyde (Solarbio, Beijing, China) for 12 h and processed for standard 5 μM thick histological sections. Before staining, slides were incubated in 0.01 M sodium citrate at 96 °C for 10 min and blocked with BDT (3% BSA, 10% normal goat serum in TBS) for 45 min. and incubated with primary antibodies, rabbit anti-MVH antibody (1:150; Abcam, ab13840), rabbit anti-CX43 antibody (1:150) (Abcam, ab11370), rabbit anti-PPAR (Sangon, D161086, Shanghai, China), overnight at 4 °C. After careful washing with PBS, sections were incubated with Cy3-conjugated goat anti-rabbit secondary antibody for 1:150 dilution (Beyotime, A0516, Nantong, China) at 37 °C for 30 min. Vectashield (Vector) was used to mount the slides. Images were taken under a fluorescence microscope (BX51; Olympus, Tokyo, Japan).

**Immunohistochemistry.** MCL-1 proteins were localization using immunohistochemistry (IHC). Put the slides in 0.01 M sodium citrate at 96 °C for 10 min, then in 3% H2O2 for 10 min and blocked with BDT (3% BSA, 10% normal goat serum in TBS) for 45 min. Rehydrated sections were incubated with MCL-1 (Sangon, D261457) primary antibodies overnight at 4 °C, then reacted with biotin-labeled secondary antibodies for 40 min at room temperature and finally stained using DAB peroxidase substrate and counterstained with hematoxylin. Image Pro Plus software 6.0 (Media Cybernetics, Rockville, MD, USA) was used to count the positive cells. In each ovary, five non-redundant sections were selected for counting and at least three ovaries per experimental group was analyzed. The data were collected from at least nine mice per treatment, and calculated the DAB peroxidase stained (positive) region, hematoxylin stained (negative) region and defined the collected from at least nine mice per treatment, and calculated the DAB peroxidase stained (positive) region, hematoxylin stained (negative) region and defined the collected from at least three times independent replicates. The PCR conditions were as follows: 10 min at 95 °C, followed by 45 cycles of 95 °C for 10 s, 60 °C for 30 s and 72 °C for 20 s. Gene expression levels were calculated using β-actin for normalization. Relative transcript abundance was calculated using the 2^ΔΔCT method. Data were expressed as mean ± SD and calculated from at least three times independent replicates.

**RNA microarray.** The protocols of total RNA microarray hybridization were consistent with our previous study. Briefly, the integrity and concentration of total RNA were measured by the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). We used 6 μg of high-quality RNA labeled with Cy5, and hybridized to a mouse oligo microarray (Phalanx Mouse Whole Genome One Array; Phalanx Biotech Group, Palo Alto, CA, USA). Each array contained 26423 DNA oligonucleotide probes of the sense strand. After hybridization, the fluorescent signals on the array were scanned using an Axon 4000 (Molecular Devices, Sunnyvale, CA). Data analysis was performed based on the manufacturer’s instructions. The Differential Expression Genes (DEGs) were defined as log2 fold change > 1 (absolute fold change > 2) and P < 0.05. For Gene Ontology enrichment analysis, we used the DAVID software (https://david.ncifcrf.gov/). Each array was performed using tissues from 16 ovaries for every experimental groups, and the analysis repeated at least two times.

**Statistical analysis.** For each set of results, independent trials were repeated at least three times; data were represented as mean ± SD. Differences among groups were statistically tested by Student’s t-test or one-way analysis of variance (ANOVA) multiple comparisons by using Graph-Pad Prism analysis software. Comparisons were considered significant at *P < 0.05* and highly significant at **P < 0.01.**

**Conflict of Interest**

The authors declare no conflict of interest.

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DEHP impair early oogenesis in vitro

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