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ORIGINAL RESEARCH

Effect of di-(2-ethylhexyl) phthalate and mono-(2-ethylhexyl) phthalate on in vitro developmental competence of bovine oocytes

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Abstract In the last decade, potential exposure of humans and animals to industrial chemicals and pesticides has been a growing concern. In the present study, di-(2-ethylhexyl) phthalate (DEHP) and mono-(2-ethylhexyl) phthalate (MEHP) were used to model the effects of endocrine-disrupting compounds and their risk in relation to early embryonic losses. Exposure of cumulus oocyte complexes during maturation to 50 μM MEHP reduced the proportion of oocytes that underwent nuclear maturation ($p<0.05$) and increased the proportion of apoptotic oocytes ($p<0.05$). Furthermore, phthalates reduced cleavage rate in the MEHP-treated group ($p<0.05$) and the proportion of embryos developing to the blastocyst stage in both DEHP- and MEHP-treated groups ($p<0.05$). The total cell count for blastocysts developing from MEHP-treated oocytes was lower than in controls ($p<0.05$). Exposure of oocytes to MEHP during maturation reduced ($p<0.05$) the expression of *ASAH1* (an anti-apoptotic factor), *CCNA2* (involved in cell cycle control), and *POU5F1* (responsible for pluripotency) in matured oocytes. Furthermore, the reduced mRNA expression of *POU5F1* and *ASAH1* lasted into two-cell stage embryos ($p<0.05$). Phthalate-induced alterations in *POU5F1*, *ASAH1*, and *CCNA2* expression might explain in part the reduced developmental competence of MEHP-treated oocytes.

Keywords Endocrine-disrupting compound · Oocyte competence · Pre-implantation embryo · Phthalate

Introduction

Potential exposure of humans and animals to industrial chemicals and pesticides has been of growing concern in the last decade, concomitant with industrial development and the release of synthetic chemicals into the environment. Studies have suggested that environmental contamination compromises reproduction and is contributing to a gradual decline in fertility in humans and farm animals. Among the responsible chemicals are phthalates (Colborn et al. 1993; Rhind 2005), a class of water-insoluble, synthetic organic chemicals that are widely used as plasticizers in a variety of industrial applications (e.g., perfumes, lotions, cosmetics, toys, and medical devices) to confer flexibility and durability to polyvinyl chloride (PVC)-based plastics. Phthalates are not chemically bound to the PVC product, and can therefore leak into the environment, where they are widely distributed in the air, soil, natural water, wastewater, and agricultural lands (Liang et al. 2008). As a consequence, humans and livestock are potentially exposed to these compounds on a daily basis, through ingestion, inhalation, and dermal exposure.

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Phthalates are diesters of *o*-phthalic acid which are rapidly hydrolyzed by esterases in the gut, liver, and blood into monoesters—the biologically active form (Lyche et al. 2009) and the ultimate toxicants for both male and female reproductive tracts (Lovekamp-Swan and Davis 2003; Hauser and Calafat 2005; Koch et al. 2006). Exposure of female rats to di-(2-ethylhexyl) phthalate (DEHP) and mono-(2-ethylhexyl) phthalate (MEHP) resulted in ovulation failure of the pre-ovulatory follicles, apparently due to decreased estradiol levels and a low LH surge (Davis et al. 1994). Oral administration of DEHP to female mice during pregnancy and lactation dramatically increased post-implantation losses and impaired semen characteristics of pups (Pocar et al. 2011). In that respect, Berman et al. (2009) recently detected nine monoester forms in 95 % of pregnant women's urine samples.

Despite the evidence for environmental phthalate contamination and its potential negative effects on the reproductive system, to date, a relative small number of studies have examined the effects of phthalates on the ovary and its pool of oocytes, considered a potential target site for phthalates. MEHP has been shown to decrease aromatase mRNA and protein levels in a dose-dependent manner in rat granulosa cells (Lovekamp and Davis 2001). Eimani et al. (2005) reported an inhibitory effect on meiotic maturation in mice following oral administration of DEHP. A negative effect of DEHP on nuclear maturation was recently found in equine oocytes matured in vitro (Ambruosi et al. 2011). In zebrafish, DEHP impairs the expression of molecular biomarkers associated with oocyte growth, maturation and ovulation (Carnevali et al. 2010). Whereas DEHP did not affect meiosis in porcine oocytes (Mlynarcíková et al. 2009), MEHP inhibited meiotic maturation in a dose-dependent manner in bovine (Anas et al. 2003) and mouse (Dalman et al. 2008) oocytes. Nevertheless, the effect of phthalates on oocyte meiotic maturation is still not fully understood.

In mammals, the oocyte accumulates maternal factors during folliculogenesis up until embryonic genome activation (Gandolfi and Gandolfi 2001). Therefore, oocyte developmental competence is encoded in the expression levels of selected genes (Dode et al. 2006). Bonilla and Del Mazo (2010) recently reported that MEHP alters the expression of two genes involved in mitochondrial function and affects fetal mouse oocyte viability. Thus, examining the effects of phthalates on the oocyte and on mRNA expression patterns in oocytes and pre-implantation embryos is of interest. In the present study, DEHP, the most abundant phthalate in the environment (Lorz et al. 2002), and its primary metabolite MEHP were used to model the effects of endocrine-disrupting compounds (EDCs) and their risk in terms of early embryonic loss, using a system for in vitro bovine embryo production. The mRNA expression of *CCNA2*, *CCNB1*, *POU5F1*, *ASAH1*, and *Zar1*, genes that are essential for various cell processes (metabolism, protection, development, and proliferation), was examined in matured oocytes exposed to MEHP and the two-cell stage embryos developed from these oocytes.

Materials and methods

Chemicals and media

All chemicals, unless otherwise stated, were purchased from Sigma (Rehovot, Israel). Follicle-stimulating hormone (FSH) isolated from ovine pituitary extract (Ovagen) was from ICP Bio (Auckland, New Zealand). Double-distilled water was from Merck (Darmstadt, Germany). Dulbecco's phosphate-buffered saline (PBS), fetal calf serum (FCS), and RQ1 RNase-free DNase I were from Promega (Madison, WI, USA). Diethlpyrocarbonate (DEPC)-treated water was from Biological Industries (Beit Haemek, Israel). Paraformaldehyde (16 %) was from Electron Microscopy Sciences (Hatfield, PA, USA). Superscript II reverse transcriptase, Dynabeads mRNA DIRECT Kit, non-essential amino acids and essential amino acids were from Invitrogen (Carlsbad, CA, USA). DyNAmo ColorFlash SYBR® Green qPCR Kit was from Finnzymes (Espoo, Finland). In situ cell death detection kit was from Roche (Indianapolis, IN, USA). Fluoromount was from Diagnostic Biosystems (Pleasanton, CA, USA). Mono-(2-ethylhexyl) phthalate was from Wako Chemicals (Neuss, Germany). The culture media Hepes–Tyrode's lactate (TL), sperm (SP)–TL, and in vitro fertilization (IVF)–TL were prepared in our laboratory: Hepes-TL was supplemented with 0.3 % (*w*/*v*) bovine serum albumin (BSA), 0.2 mM sodium pyruvate, and 0.75 mg/mL gentamicin (Hepes–Tyrode's albumin lactate pyruvate (TALP)); SP–TL was supplemented with 0.6 % BSA, 1 mM sodium pyruvate and 0.2 mg/mL gentamicin (SP–TALP); IVF-TL was supplemented with 0.6 % (*w*/*v*) essential fatty acid-free BSA, 0.2 mM sodium pyruvate, 0.05 mg/mL gentamicin, and 0.01 mg/mL heparin (IVF–TALP) as described by Parrish et al.

(1986). Oocyte maturation medium (OMM) was made up of TCM-199 and Earle's salts supplemented with 10 % (*v*/*v*) heat-inactivated FCS (Bio-Lab, Jerusalem, Israel), 0.2 mM sodium pyruvate, 50 μg/μL gentamicin, 2.2 g/L sodium bicarbonate, 2 μg/mL 17-ß estradiol, and 1.32 μg/mL FSH. Potassium simplex optimized medium (KSOM) contained 95 mM NaCl, 2.5 mM KCl, 0.35 mM KH_2PO_4, 0.2 mM $MgSO_4 \cdot 7H_2O$, 0.8 % (*v*/*v*) sodium lactate, 0.2 mM sodium pyruvate, 0.2 mM D(+)-glucose, 25 mM $NaHCO_3$, 0.01 mM phenol red, 1 mM L-glutamine and 0.01 mM ethylenediaminetetraacetic acid (EDTA) supplemented with 1.7 mM $CaCl_2 \cdot 2H_2O$, 0.1 mg/mL polyvinylalcohol, 10 μL/mL essential amino acids and 5 μL/mL non-essential amino acids, and 100 U/mL penicillin-G and 0.1 mg/mL streptomycin.

Oocyte recovery

Bovine ovaries were obtained from a local abattoir from multiparous Holstein cows and transported to the laboratory within 60–90 min in physiological saline solution (0.9 % *w*/*v* NaCl at 37 °C with 50 μg/mL penicillin–streptomycin). In the laboratory, ovaries were washed with fresh saline, cut through the center, and placed over a transillumination stand for easy visualization of follicles (Arav 2001). Cumulus oocyte complexes (COCs) were aspirated from 3- to 8-mm follicles with an 18-gauge needle attached to a 10-mL syringe. COCs with at least three layers of cumulus surrounding a homogeneous cytoplasm were selected for further processing.

In vitro production of embryos

In vitro production of embryos was performed as previously described by Gendelman et al. (2010). Briefly, COCs were washed three times in Hepes–TALP and groups of 10 oocytes were transferred to 50-μL droplets of OMM overlaid with mineral oil. The COC-containing droplets were incubated in humidified air with 5 % CO_2 for 22 h at 38.5 °C. Matured COCs were washed three times in Hepes–TALP and transferred in groups of 30 oocytes to four-well plates containing 600 μL IVF-TALP and 25 μL PHE per well (0.5 mM penicillamine, 0.25 mM hypotaurine, and 25 μM epinephrine in 0.9 % NaCl). Frozen semen from "Sion" (Hafetz-Haim, Israel), used for the in vitro fertilization, was from the same bull (I.D. PAZIL 3421) and was evaluated for motility (92.7 %), velocity (73 μm/s), and concentration (57.8 million/mL). Semen quality parameters were evaluated in an automated sperm quality analyzer (SQA-Vb) from Medical Electronic Systems (Caesarea, Israel) as previously described by Orgal et al. (2012). Percoll-purified spermatozoa ($\sim 1 \times 10^6$) from frozen–thawed semen were used for fertilization. Spermatozoa were co-incubated with COCs for 18 h at 38.5 °C in a humidified atmosphere with 5 % CO_2. After fertilization, putative zygotes were removed from the fertilization wells, denuded of cumulus cells by gentle vortexing in Hepes-TALP containing 1,000 U/mL hyaluronidase, and randomly placed in groups of 10 in 25-μL droplets of KSOM. All embryo droplets were overlaid with mineral oil and cultured for 8 days at 38.5 °C in an atmosphere of humidified air with 5 % CO_2, 5 % O_2.

Nuclear staining to determine nuclear maturation

At the end of maturation, oocytes were denuded of cumulus cells, fixed in 4 % (*v*/*v*) paraformaldehyde in PBS for 15 min at room temperature and stored in PBS with 1 mg/mL polyvinylpyrrolidone (PBS–PVP) at 4 °C. Before staining, oocytes were washed three times in PBS–PVP and stained with 10 μg/mL 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) in PBS–PVP for 15 min at room temperature, washed three times in PBS–PVP, placed in drops of Fluoromount and examined under an inverted fluorescence microscope (Nikon, Tokyo, Japan) using Nis Elements software (Nikon). Only oocytes with a metaphase II (MII) plate and first polar body were classified as MII-stage oocytes.

Detection of DNA fragmentation by TUNEL assay

The terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was used to detect DNA fragmentation in both matured oocytes and 8-day embryos as previously performed in our laboratory (Kalo and Roth 2011). In this method, free 3′OH ends of DNA are labeled with fluorescein isothiocyanate (FITC)-conjugated dUTP by means of the enzyme terminal deoxynucleotidyl transferase (TdT) Briefly, matured oocytes and embryos were collected at the end of 22 h maturation and on day 8, respectively. The samples were washed three times in PBS–PVP, fixed in 4 % paraformaldehyde in PBS for 15 min at room temperature, and stored in PBS–PVP at 4 °C. During

the assay, samples were washed three times in PBS–PVP and placed in permeabilization solution containing PBS with 1 mg/mL PVP, 0.3 % (*v*/*v*) Triton X-100 and 0.1 % (*w*/*v*) sodium citrate for 20 min at room temperature in a humidified box. For positive and negative controls, samples were incubated in 50-μL drops of 50 U/mL RNase-free DNase at 37 °C for 1 h in the dark. After RNase-free DNase treatment, TUNEL assay was performed using the in situ cell death detection kit following the manufacturer's instructions (Roche). Samples were incubated in 50-μL droplets of TUNEL reaction mixture (containing FITC-conjugated dUTP and TdT) for 1 h at 37 °C in the dark. The negative control was incubated under the same conditions, but without TdT. Finally, samples were stained with 1 μg/mL DAPI (oocytes) or Hoechst 33342 (embryos) in PBS–PVP for 15 min at room temperature, washed three times in PBS–PVP, and placed in drops of Fluoromount. TUNEL labeling was examined under an inverted fluorescence microscope using Nis Elements software. The apoptotic cell ratio for each blastocyst was determined by calculating the number of TUNEL-positive blastomeres out of total blastocyst cell number.

Gene quantification

Sample collection Putative MII stage oocytes were collected after 22 h of in vitro maturation and denuded of cumulus cells by gentle vortexing in Hepes–TALP containing 1,000 U/mL hyaluronidase as described above. Three to four replicates (n=20 oocytes per sample) were taken from different in vitro production runs. Due to transcript level variations between early- and late-cleaved embryos (Gendelman et al. 2010), two-cell stage embryos (n=10 embryos per sample) were collected at 27 h postfertilization (i.e., early-cleaved embryos) from four to six different in vitro production runs. All collected samples were washed in PBS, snap frozen in liquid nitrogen, and stored at −80 °C until RNA extraction.

Real-time PCR Poly(A) RNA was isolated using Dynabeads mRNA DIRECT Kit according to the manufacturer's instructions (Invitrogen) as previously described by Gendelman et al. (2010). In brief, oocytes and embryos were lysed by adding 100 μL lysis-binding buffer to each sample. Prewashed oligo $(dT)_{25}$ Dynabeads (20 μL) were added to each tube and mixed for 5 min at room temperature to allow binding of poly(A) to the beads. The samples were put into a magnetic separator to remove the lysis buffer while retaining the Dynabeads. The Dynabeads were washed twice with 100 μL washing buffer A (100 mM Tris–HCl pH7.5, 500 mM LiCl, 10 mM EDTA, pH8, 5 mM dithiothreitol), twice with 100 μL washing buffer B (10 mM Tris–HCl pH7.5, 0.15 M LiCl, 1 mM EDTA) and once with 100 μL 10 mM Tris–HCl. After removal of HCl, 8 μL sterile DEPC water was added and the samples were immediately subjected to reverse transcription.

Reverse transcription was performed in a total volume of 20 μL. The first step was incubation at 70 °C with 8 μL RNA sample, 1 μL oligo$(dT)_{12–18}$ (500 μg/mL), 1 μL RNAseout, 1 μL dNTPs (10 mM each), and 1 μL (50 ng) random primer, followed by 50-min incubation at 42 °C and 5 min at 70 °C with RT mix containing 4 μL 5× reverse transcriptase buffer, 200 U of Superscript II reverse transcriptase, 2 μL 0.1 M dithiothreitol and DEPC water. The samples were transferred to −20 °C until use.

Quantitative reverse transcription was carried out with primers for *Zar1*, *CCNA2*, *CCNB1*, *ASAH1* and *POU5F1*, using *YWHAZ* as a reference gene. The primers were derived from bovine sequences found in Genbank and designed using Primer Express software (Table 1). Briefly, real-time PCR was conducted on an Mx3000p cycler (Stratagene, La Jolla, CA, USA) using SYBR green in a final volume of 20 μL containing ultra pure water, 500 nM of each primer, and 3 μL diluted cDNA. The reaction efficiency ranged between 90 and 110 % with R^2>0.995. The amplification program included preincubation at 95 °C for 7 min to activate *taq* polymerase, followed by 40 amplification cycles of denaturation at 95 °C for 10 s and annealing elongation at 60 °C for 15 s. All samples were run in duplicate in 96-well plates. A melting curve analysis was performed at the end of the amplification to confirm single-gene specificity. Fluorescence was recorded to determine the threshold cycle during the log-linear phase of the reaction at which fluorescence rises above background. Gene expression was quantified and analyzed by MxPRO QPCR software for Mx3000p and Mx3005p QPCR ver. 3 and the $\Delta\Delta C_t$ method was used to calculate the relative expression of each gene.

Experimental design

The in vitro production of embryos was performed as described above. Selection of phthalate concentration was based on previous reports (Kim et al. 2002; Anas et al. 2003; Wan et al. 2010) and on preliminary dose–response examinations for the present study. Since phthalates were dissolved in dimethyl sulfoxide (DMSO), the effect of DMSO on oocyte developmental competence was examined as well (see further on).

In the first set of experiments, oocytes were collected and matured in OMM supplemented with DMSO at a final concentration of 0.1 % (*v*/*v*; control) or in OMM with 50 μM DEHP dissolved in 0.1 % (*v*/*v*) DMSO (treatment), then fertilized and cultured for 8 days. The percentage of oocytes that cleaved to two- to four-cell stages was assessed at 42–44 h postfertilization. The average number of embryos that further developed to the blastocyst stage was assessed on days 7–8 postfertilization and their percentage was calculated (1) out of the total number matured oocytes (reflecting treatment effects on fertilization and development) and (2) out of the number of cleaved oocytes (referring to embryonic developmental competence).

In the second set of experiment, oocytes were collected and matured in OMM supplemented with DMSO at a final concentration of 0.16 % (*v*/*v*; control) or in OMM with 50 μM MEHP dissolved in 0.16 % (*v*/*v*) DMSO (treatment), then fertilized and cultured for 8 days. The percentage of oocytes that were fertilized and cleaved to the two- to four-cell stage at 42–44 h postfertilization was calculated. The average number of embryos that further developed to the blastocyst stage was assessed on days 7–8 postfertilization and their percentage was calculated (1) out of the total number matured oocytes and (2) out of the number of cleaved oocytes.

The distribution of embryos to the different developmental stages (early blastocyst, blastocyst, expanded blastocyst) was recorded. From each experimental group, subgroups were collected at the end of 22 h maturation to examine the meiotic stage of the nucleus and its apoptotic status. In addition, subgroups of MII-stage oocytes and two-cell stage embryos were collected for mRNA quantification. mRNA was isolated and real-time PCR was carried out with primers for *Zar1*, *CCNA2*, *CCNB1*, *ASAH1*, and *POU5F1*, using *YWHAZ* as the reference gene (Table 1). At the end of culture, 8-day embryos were taken for TUNEL assay to determine apoptotic index (i.e., proportion of TUNEL-positive cells out of total cell number).

We performed a preliminary study to examine the effect of the solvent DMSO on oocyte maturation and further embryonic development, as previously described by Kalo and Roth (2011). COCs were matured in OMM with or without 0.16 % DMSO (the maximum concentration used in this study) for 22 h at 38.5 °C. COCs were then fertilized and cultured as described above. The cleavage rates did not differ between the experimental groups and were 86 vs. 85 % for OMM and OMM with DMSO, respectively. The proportion of developed blastocysts on days 7–8 postfertilization did not differ between the groups and were 17.14 vs. 17.16 % for OMM and OMM with DMSO, respectively.

Table 1 Primers used for real-time PCR

Genes	Primer	Accession number	Sequence (5′→3′)	Size (bp)
Zar1	Forward Reverse	NM001076203	CCACCGTCAAGATTTGTGTG TGGGACCTCAGTTTTCTTGG	123
CCNA1	Forward Reverse	NM001075123	GCAGCAGCCTTCATTTAGC CTGGTGAAGGTCCAGGAGAC	120
ASAH1	Forward Reverse	NM001075459	GACCCTCCAGGGAATAAAGC TCAGAGCCAGGAGGAAAAGA	143
POU5F1	Forward Reverse	AY490804	ATATACCCAGGCCGATGTGG TGCACAAGGGTCTCTGCCTT	201
CCNB1	Forward Reverse	NM001045872	TGGTGCACTTTCCTCCTTCT CCAGGTGTTGCATAACAACG	144
YWHAZ	Forward Reverse	NM00174814	GCATCCCACAGACTATTTCC GCAAAGACAATGACAGACCA	124

Statistical analysis

Data were tested by one-way ANOVA followed by a one-way variance analysis (*t* test). Before analysis, data were arcsine-transformed. Variables were proportion of MII-stage oocytes, TUNEL-positive oocytes, cleavage and blastocyst rate, total cell number per blastocyst, and mean number of TUNEL-positive cells per embryo. Embryo distribution into developmental stages (i.e., early blastocyst, blastocyst, and expanded blastocyst) were tested by chi-square using JMP 8 (SAS Institute, Cary, NC, USA). Gene expression levels were analyzed by one-way ANOVA followed by Student's *t* test. Data are presented as means±SEM. For all analyses, $p<0.05$ was considered significant, and *p* values between 0.05 and 0.1 were also reported as trends that may be real and worthy of note.

Results

Effect of DEHP on oocyte developmental competence

The experiment included 50–57 oocytes per experimental group per in vitro production run ($n=3$). Exposing oocytes throughout maturation to 50 μM DEHP reduced, albeit not significantly, the proportion of oocytes that underwent cleavage 44 h postfertilization (Fig. 1). In addition, DEHP had a deleterious carryover effect on oocyte developmental competence, reflected by a threefold decrease in the percentage of developing blastocysts on days 7–8 postfertilization relative to controls ($p<0.05$, Fig. 1). Further analysis in which the percentage of developed blastocysts out of number of cleaved embryos was calculated revealed a decrease ($p<0.03$) toward reduced developmental competence.

Effect of MEHP on oocyte nuclear maturation and DNA fragmentation

The experiment included 505 COCs from at least three replicates. COCs were exposed to 0, 25, 50, or 100 μM MEHP throughout maturation and examined for nuclear maturation and apoptosis. Exposing COCs throughout maturation to 25 μM MEHP did not affect the proportion of oocytes that resumed meiosis and progressed to the MII stage compared with the control group (80.0 vs. 76.9 %, respectively). Exposing oocytes to 50 μM MEHP reduced the proportion of oocytes that resumed meiosis and progressed to the MII stage ($p<0.05$, Fig. 2a–c). Surprisingly, exposure to 100 μM MEHP did not affect meiosis progression compared with the control group (73.2 vs. 76.9 %, respectively). In addition, the proportion of TUNEL-positive oocytes (i.e., DNA fragmentation) was elevated in a dose-dependent manner. Oocyte maturation with 25 or 50 μM MEHP increased the proportion of TUNEL-positive oocytes compared with the control (27.4, 21.2 vs. 0 %, respectively; $p<0.05$, Fig. 2d–j). The most dominant effect was observed with 100 μM MEHP (53.7 %, $p<0.05$). Based on these results, 50 μM MEHP was used for subsequent experiments.

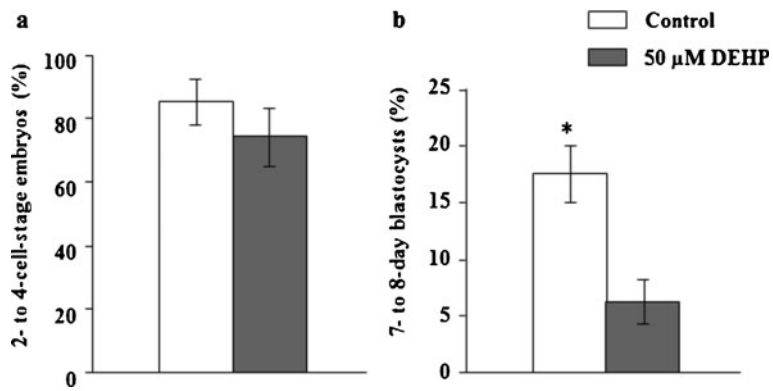

Fig. 1 Carryover effect of DEHP on developmental competence of bovine oocytes. Presented is the proportion of oocytes cleaved to the two- to four-cell stage, 42–44 h postfertilization (**a**), and the proportion of embryos that developed to the blastocyst stage on days 7–8 postfertilization (**b**). Data are presented as means±SEM; treatment effect within embryonic stages, *$p<0.05$

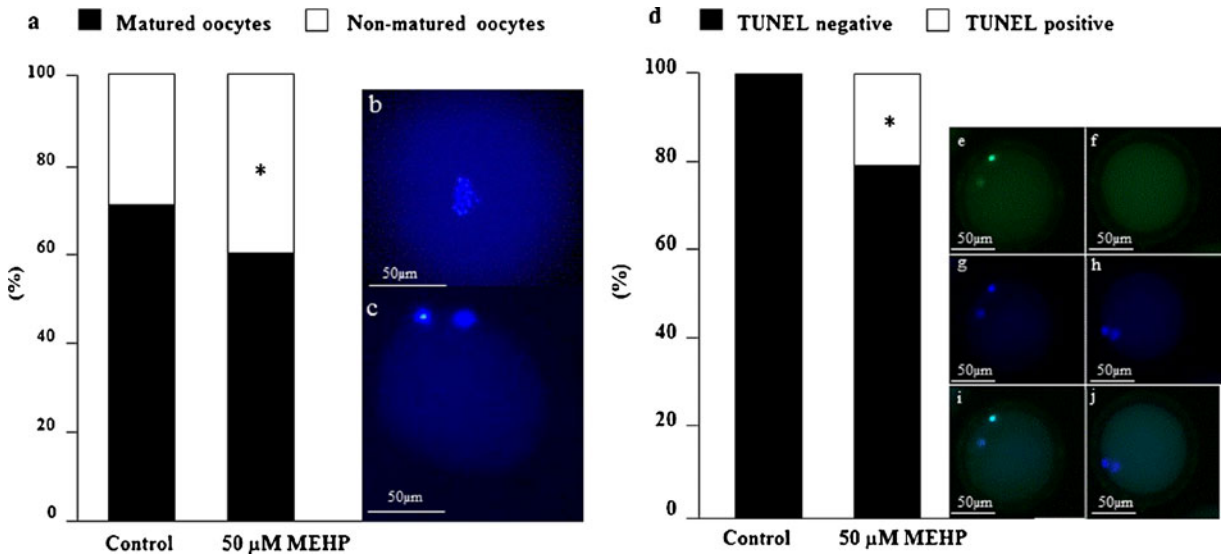

Fig. 2 Effect of MEHP on nuclear maturation and apoptosis in bovine oocytes. Oocytes were matured in standard oocyte maturation medium without (control) or with 50 μM MEHP. The proportion of MII stage oocytes is presented as means; treatment effect within nuclear stages, $*p<0.05$ (**a**). At the end of 22 h maturation, oocytes were fixed and stained to determine the meiotic (DAPI) and apoptotic (TUNEL) status of the nucleus. Shown are representative images of oocytes classified as nonmatured oocytes (**b**), and matured oocytes with a MII-plate and first polar body (**c**). The proportion of TUNEL-positive oocytes is presented as means; treatment effect within TUNEL labeling, $*p<0.05$ (**d**). Pictures (**e**–**j**) are representative images of TUNEL-positive oocytes with green nuclei (**e**), TUNEL-negative oocytes (**f**), oocytes counterstained with DAPI (**g**, **h**), and merged pictures of TUNEL and DAPI labeling (**i**, **j**)

Effect of MEHP on oocyte developmental competence

This experiment included 70 oocytes per experimental group per in vitro production run ($n=3$). Exposing oocytes throughout maturation to 50 μM MEHP reduced the proportion of oocytes that underwent cleavage ($p<0.05$, Fig. 3), reflected by a 10 % decrease in embryos developed to the two- to four-cell stage, 44 h postfertilization. Moreover, MEHP had a carryover effect on further stages of embryonic development, expressed by a reduced percentage of developing blastocysts on days 7–8 postfertilization ($p<0.05$, Fig. 3). Further analysis in which the percentage of developed blastocysts out of number of cleaved embryos was calculated revealed a tendency ($p<0.08$) toward reduced developmental competence. The distribution of embryos among the developmental stages (i.e., early blastocyst, blastocyst, and expanded blastocyst) on days 7 and 8 postfertilization did not differ between MEHP-treated and control groups ($p>0.3$, Table 2).

About 10 blastocysts per experimental group, from three different in vitro production runs, were taken for the TUNEL procedure. The total cell number per embryo was significantly lower for blastocysts developing from MEHP-treated oocytes compared to controls ($p<0.05$, Fig. 4). The proportion of TUNEL-positive (apoptotic) cells per embryo tended to be lower in the MEHP-treated embryos relative to controls ($p<0.08$, Fig. 4).

Effect of MEHP on the expression of maternal mRNA

The examination included 20 matured oocytes and 10 embryos at the two-cell stage for each experimental group, collected from at least three different in vitro production runs. Real-time PCR revealed altered mRNA expression patterns in both matured oocytes and two-cell stage embryos from oocytes which had been exposed to 50 μM MEHP throughout maturation. Whereas MEHP did not affect *CCNB1* and *Zar1* expression in matured oocytes, it reduced the expression of *CCNA2*, *POU5F1*, and *ASAH1* mRNA ($p<0.05$, Fig. 5). An impaired mRNA pattern was further observed for *POU5F1* and *ASAH1* in two-cell stage embryos from the MEHP-treated group compared to the control ($p<0.05$, Fig. 6). Similar to the matured oocytes, MEHP did not affect mRNA expression of *Zar1* or *CCNB1*.

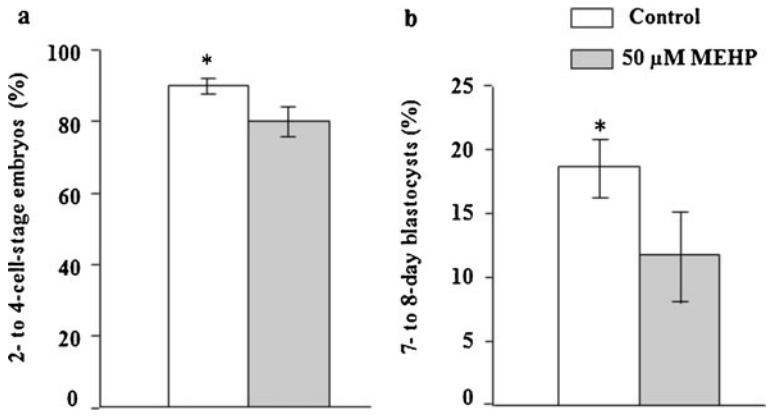

Fig. 3 Carryover effect of MEHP on developmental competence of bovine oocytes. Presented is the proportion of oocytes cleaved to the two- to four-cell stage, 42–44 h postfertilization (**a**) and the proportion of embryos that developed to the blastocyst stage on days 7–8 postfertilization (**b**). Data are presented as means ± SEM; treatment effect within embryonic stages, *$p<0.05$

Discussion

The widespread distribution of phthalates in the environment (Liang et al. 2008) makes them a suggested risk factor for ovarian function. The current study provides evidence that exposure of oocytes to phthalates during maturation impairs their meiotic and developmental competence and subsequently reduces, to some extent, the quality of the developing embryo, expressed by impaired gene expression in two-cell stage embryos and reduced total cell number in blastocysts.

Phthalates can cross the placental barrier, pass into breast milk (Dostal et al. 1987), and reach the embryo (Lindgren et al. 1982; Parkhie et al. 1982). Exposure of female rodents to DEHP and MEHP has been shown to result in ovulation failure of the preovulatory follicle (Davis et al. 1994). However, almost no accurate data are currently available on phthalate concentration in the follicular fluid in vivo, although a high correlation between follicular fluid and serum concentrations has been reported for other environmental contaminants (Pauwels et al. 1999; Younglai et al. 2002). For example, chlorinated biphenyl and p,p′-DDE (dichlorodiphenyldichloroethylene) in human plasma or follicular fluid is associated with reduced embryonic development (Petro et al. 2012). As the molecular mass of phthalates (270 Da) is lower than the follicle–blood barrier threshold in the follicular basal lamina (Clarke et al. 2006), phthalates can potentially pass from the peripheral blood system into the follicular fluid. Taken together, it is suggested that the presence of phthalates (DEHP or its metabolite MEHP) in the oocyte micro-environment in vivo (i.e., follicular fluid) negatively affects COCs. Using an in vitro model, the current study provides evidence that not only the follicle, but also its enclosed oocyte are target sites for phthalates. Lovekamp and Davis (2001) reported that MEHP decreases aromatase mRNA and protein levels in a dose-dependent manner in rat granulosa cells. Here, we show that exposure of COCs during maturation to 50 μM DEHP or 50 μM MEHP, levels similar to those found in the blood of patients following a transfusion (Rael et al. 2009), impaired their developmental competence. MEHP increased the proportion of TUNEL-positive oocytes and reduced the proportion of oocytes that were fertilized and cleaved, indicating effect on maturation and fertilization. Both DEHP and MEHP reduced the proportion of oocytes that developed to the blastocyst stage. In addition, DEHP did not affect cleavage rate but reduced the proportion of cleaved embryos that developed to the blastocyst stage, indicating effect on the embryo developmental competence. It should be noted, however, that one of the limitations of the in vitro maturation model used in the current study is

Table 2 Embryo distribution into three different developmental stages

Embryonic developmental stages					
Days PF	Exp. groups	Embryos (#)	Early blastocyst (%)	Blastocyst (%)	Expanded blastocyst (%)
Day 7	Control	35	52.6	40.1	7.2
	MEHP	19	56.6	42.2	6.6
Day 8	Control	44	29.3	45.6	24.6
	MEHP	30	35.6	32.2	31.2

COCs were matured in vitro in maturation medium (OMM, control) or in OMM with 50 μM MEHP for 22 h. Thereafter, matured oocytes were fertilized in vitro (18 h) and cultured in vitro for 7–8 days postfertilization (PF). Distribution of embryos in embryonic stages was recorded on days 7 and 8 PF; treatment effect within embryonic stages, $p<0.05$

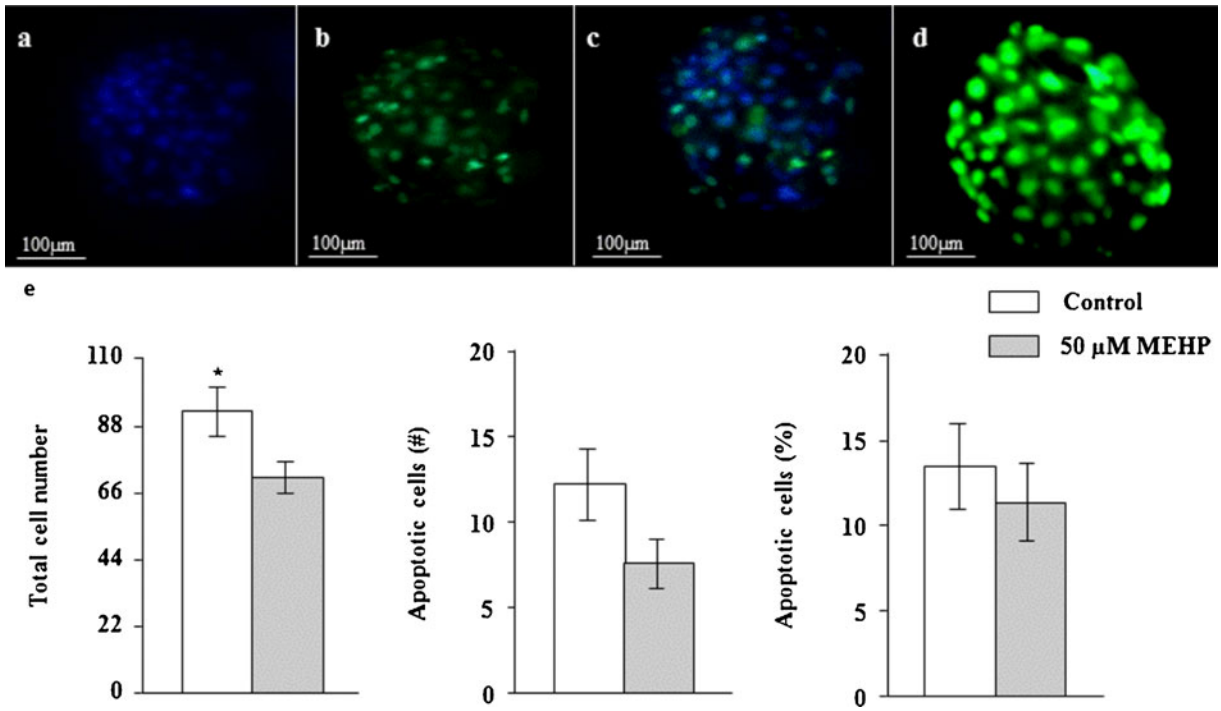

Fig. 4 Effect of MEHP on 8-day embryo characteristics. Representative images of 8-day blastocyst stained with Hoechst 33342 (**a**, *blue* nuclei), TUNEL (**b**, *green* nuclei), merged pictures (**c**), and TUNEL-positive control (**d**). The total cell number, and the apoptotic index (**e**) are presented as means±SEM; treatment effect within embryonic characteristic, *$p<0.05$

that after being aspirated from the follicle, oocytes undergo germinal vesicle (GV) breakdown and resume meiosis, i.e., spontaneous maturation (Rodriguez and Farin 2004). Thus, the effect of phthalates on the GV stage oocyte remains unclear.

Oocyte developmental competence is acquired in a progressive manner throughout follicle development, and includes a variety of molecular and cellular modifications that are required for the oocyte to complete meiosis, successful fertilization, maternal zygote transition, and further pre- and post-implantation development (Coticchio et al. 2004). The findings of the current study support the view that exposing oocytes to phthalates impairs their meiotic competence since exposure of oocytes to 50 μM MEHP throughout maturation impaired meiotic progression to the MII stage. Similarly, other in vitro studies have shown a deleterious dose-responsive effect of phthalates on oocyte developmental competence. Culturing of bovine COCs in maturation medium with MEHP (10, 25, 50, 75, 100 μM for 24 h)

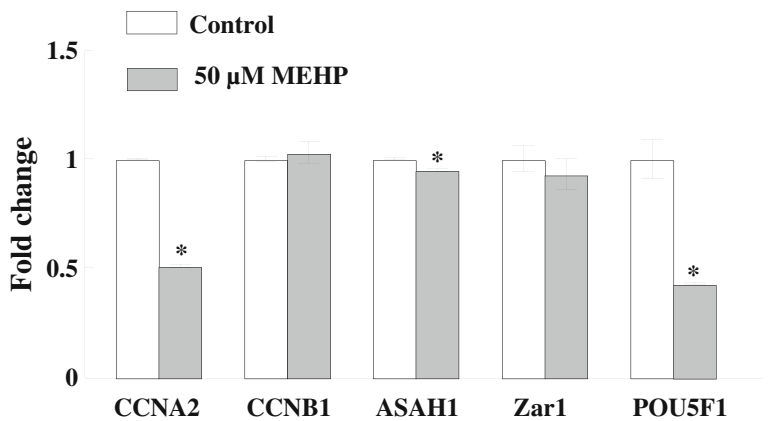

Fig. 5 Effect of MEHP on maternal mRNA levels in MII-stage bovine oocytes. Presented are transcript levels of *Zar1*, *CCNA2*, *CCNB1*, *ASAH1*, and *POU5F1* in MII-stage oocytes collected at the end of 22 h maturation from control and 50 μM MEHP-treated groups. Data from real-time PCR, presented as means±SEM; treatment effect within genes, *$p<0.05$

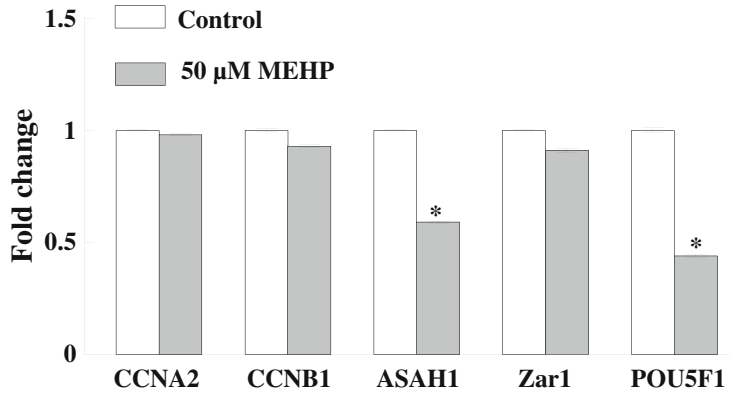

Fig. 6 Effect of MEHP on maternal mRNA levels in bovine oocytes in two-cell stage embryos. Presented are transcript levels of *Zar1*, *CCNA2*, *CCNB1*, *ASAH1*, and *POU5F1* in two-cell stage embryos collected 27 h postfertilization from control and 50 μM MEHP-treated groups. Data from real-time PCR, presented as means±SEM; treatment effect within genes, *$p<0.05$

decreased the proportion of oocytes progressing to the MII stage, and when they were cultured with 50, 75, or 100 μM MEHP, none of them reached the MII stage (Anas et al. 2003). A study in mice revealed that oocyte maturation with doses higher than 50 μM MEHP (i.e., 100, 200, and 400 μM) significantly decreases the proportion of oocytes undergoing nuclear maturation (Dalman et al. 2008). Similarly, Eimani et al. (2005) showed that DEHP adversely affects oocyte maturation in mice. In addition, Ambruosi et al. (2011) reported that DEHP at a low dose (0.12 μM) induces chromatin fragmentation in cumulus cells and reduces meiosis progression in equine oocytes. Upon entering the body, DEHP is hydrolyzed to MEHP, which is known for its high biological activity (Hauser and Calafat 2005). It is therefore possible that the effects seen in DEHP-treated cells result from MEHP. Taken together, these findings suggest that exposure to phthalates disrupts meiotic progression and impairs oocyte maturation, which might explain in part the reduced developmental competence of phthalate-treated oocytes.

Developmental competence of the embryo depends on the quality of the oocyte from which it originates (Sirard et al. 2006). Here, we show that exposing COCs to phthalates during maturation not only affects the proportion of oocytes undergoing successful maturation but also affects their competence for fertilization, cleavage and further development into embryos. MEHP reduced the proportion of oocytes that cleaved to two- and four cell-stage embryos 42–44 h postfertilization. Furthermore, both DEHP and MEHP reduced the proportion of embryos that developed to blastocysts, suggesting a carryover effect of phthalates on oocyte developmental competence. MEHP was found to significantly reduce the proportion of blastocysts developed on days 7–8, rather than slowing down the pace of embryonic development. Accordingly, the distribution into different embryonic developmental stages (early blastocyst, blastocyst, and expanded blastocyst) did not differ between the control and MEHP-treated groups on either days 7 or 8 postfertilization. Further supporting this finding, the proportion of cleaved embryos that developed to the blastocyst stage tended to be lower in the MEHP-treated group.

In mammals, oocyte growth during folliculogenesis is characterized by high transcription levels and accumulation of maternal mRNA and proteins, which are required for postfertilization events prior to embryonic genome activation; in particular, the first mitotic divisions (Sirard 2001). It is therefore accepted that oocyte developmental competence can be encoded in the expression levels of selected genes' maternal mRNA (Dode et al. 2006). In the current study, the mRNA levels of *POU5F1*, *CCNA2*, *CCNB1*, *ASAH1*, and *Zar1* were examined for a quality assessment of oocyte and embryo pre-embryonic genome activation, which in bovines occurs upon division from eight to 16 cells (Fair et al. 2004). While the expression of *Zar1*, an oocyte-specific maternal gene that is involved in the oocyte-to-embryo transition (Wu et al. 2003), was not affected, MEHP reduced the expression of *CCNA2*, *ASAH1*, and *POU5F1* mRNA in matured oocytes, with a further reduction in the expression of *ASAH1* and *POU5F1* mRNA in two-cell stage embryos.

CCNA2 encodes cyclin A2, involved in cell cycle control and essential for further embryonic development (Winston 2001). *CCNA2* is detected in GV- and MII-stage oocytes in mice, and in early two-cell stage embryos (Winston et al. 2000) and immature and mature oocytes (Lonergan et al. 2003) in bovines. Cyclin A2 deficiency results in embryonic lethality around the peri-implantation stage (Murphy et al. 1997). Thus reduced *CCNA2* expression in MEHP-treated oocytes seems to explain the reduced percentage of oocytes undergoing nuclear maturation, fertilization, and cleavage to the two- and four-cell stage. In support of this assumption, daily oral administration of 500 and 750 mg/kg DEHP downregulated cell cycle-related regulator proteins (pRB, cyclin D, CDK2, cyclin E, and CDK4) in association with apoptosis in rat testicular cells (Ryu et al. 2007). In contrast, Kudo et al. (2004) reported that exposure of murine neural stem cells to phthalates does not affect the level of cyclin A. On the other hand, exposure to 4-nonylphenol, another endocrine disruptor, downregulated the levels of both cyclin A and cyclin B1, indicating potential disruption of their genes. Cyclin B is encoded by *CCNB1*. It is involved in resumption of meiosis in oocytes (Lévesque and Sirard 1996) by regulating the activity of maturation promoting factor (MPF; Gautier et al. 1990). In bovines, cyclin B1 is not expressed in the GV-stage oocyte but its expression increases during maturation (Lévesque and Sirard 1996). In the present study, MEHP did not affect *CCNB1* transcription level in either matured oocytes or two-cell stage embryos. Therefore, the reduced proportion of oocytes resuming meiosis and arriving to the MII stage is mostly related to impairment in the expression of cyclin A2 rather than cyclin B1. Nevertheless, since both cyclin A and B bind to Cdc2 protein kinase to form the MPF complex (Draetta et al. 1989; Roy et al. 1991), MEHP-induced alteration in MPF activation is suggested.

POU5F1 (also known as OCT4) is a POU family transcription factor expressed in totipotent or pluripotent embryonic cells. POU5F1 maintains cell pluripotency (Kellner and Kikyo 2010), thereby ensuring proper embryonic development. A critical amount of POU5F1 is necessary to maintain self-renewal of stem cells (Niwa et al. 2000). In bovines, *POU5F1* transcription is highly expressed in immature oocytes up to the four-cell stage, downregulated in the eight-cell stage embryo until the morula, and relatively high at the blastocyst stage (Nganvongpanit et al. 2006). Various factors acting upstream or downstream of *POU5F1* activation are candidate players in the acquisition of oocyte developmental competence (Zuccotti et al. 2009). Thus, the low level of *POU5F1* found in MEHP-matured MII oocytes might indicate alterations in the oocyte's mRNA and reduced developmental competence. Supporting these findings is the reduced expression of *POU5F1* in two-cell stage embryos developed from MEHP-matured oocytes, suggesting that impairments in the maternal mRNA at the maturation stage carry over and are expressed in the first two cleavages (i.e., before embryonic genome activation). In murines, the level of *POU5F1* governs the embryo's fate since a decrease in *POU5F1* expression in embryonic stem cells induces loss of pluripotency, resulting in de-differentiation to trophectoderm rather than inner cell mass (Niwa et al. 2000). Moreover, deletion of *POU5F1* in primordial germ cells leads to apoptosis (Kehler et al. 2004). Recently, we provided evidence for thermal stress-reduced transcription of *POU5F1* mRNA before embryonic genome activation being further expressed at the blastocyst stage, i.e., following embryonic genome activation (Gendelman and Roth 2012). Although not examined in the current study, it is possible that the impaired expression of *POU5F1* reported for the two-cell stage embryos developed from MEHP-treated oocytes might also be further expressed in later developmental stages, thus underlying the reduced percentage of embryos that developed to the blastocyst stage (i.e., developmental competence) and of number of cells per embryo (i.e., embryo quality).

ASAH1 mRNA encodes acid ceramidase, an enzyme that is essential for embryo survival since it removes the pro-apoptotic factor, ceramide, thus inhibiting the default sphingomyelin apoptotic pathway (Haimovitz-Friedman et al. 1997). $ASAH^{-/-}$ mouse embryos undergo apoptotic death at the two-cell stage, due to increased ceramide (Eliyahu et al. 2007). Here, we report MEHP-induced reduction in *ASAH1* transcription levels in MII-stage oocytes. This was associated with an increased number of TUNEL-positive oocytes, presumably due to an increase in ceramide levels. Moreover, the reduced transcription level of *ASAH1* mRNA carried over to the two-cell stage embryo, which might explain in part the reduced percentage of cleaved embryos that further developed to the blastocyst stage. Given the reduced expression of *ASAH1* in the two-cell stage embryos, one might expect an increased proportion of apoptotic cells in the blastocysts. Nevertheless, based on

TUNEL assay, the apoptotic index did not differ between blastocysts developed from MEHP-treated and nontreated oocytes. On the other hand, embryos that developed from MEHP-treated oocytes showed a reduced number of cells, likely due to impairment in the cell cycle machinery (i.e., reduced *CCNA2* expression) rather than alterations in the apoptotic machinery (i.e., reduced *ASAH1* expression). A previous study suggested that DEHP induces the apoptosis cascade through activation of the peroxisome proliferator-activated receptor (PPAR) and ERK1/2 pathway (Ryu et al. 2007). Moreover, epidemiological and experimental evidence suggest that PPARs are mediators of phthalate-induced alterations in the male and female reproductive tract (Latini et al. 2008); therefore, PPAR activation cannot be ruled out.

In summary, our in vitro model indicates that phthalates have a deleterious carryover effect on oocyte developmental competence, reflected by a reduced proportion of oocytes undergoing nuclear maturation, fertilization, cleavage, and further development to the blastocyst stage. The reduced developmental competence of MEHP-treated oocytes was strongly associated with alterations in the levels of *CCNA2*, *ASAH1*, and *POU5F1* mRNA expression. Whether exposure to environmental concentrations of phthalate affect the ovarian pool of oocytes similarly in vivo, and whether these effects persist into the post-implantation period, remains unclear. However, phthalate-induced alterations in oocyte maternal mRNA may highlight the risk associated with exposure of humans and animals to environmental contamination and its potential to compromise fertility.

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