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Low level of mono(2-ethylhexyl) phthalate reduces oocyte developmental competence in association with impaired gene expression

D. Kalo[a,b], Z. Roth[a,b,*]

[a] Department of Animal Sciences, Robert H. Smith Faculty of Agriculture, Food and Environment, The Hebrew University, Rehovot 76100, Israel
[b] Center of Excellence in Agriculture and Environmental Health, The Hebrew University, Rehovot 76100, Israel

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ABSTRACT

Di-(2-ethylhexyl) phthalate (DEHP) and its metabolite, mono-(2-ethylhexyl) phthalate (MEHP), are reproductive toxicants. However, disruptive effects of MEHP at low concentrations on the oocyte and developing blastocyst are unknown. Previously, we detected low levels of MEHP in follicular fluid aspirated from DEHP-treated cows associated with reduced estradiol levels. Moreover, the MEHP concentrations found were similar to those reported for follicular fluid aspirated from women who have undergone IVF cycles. In the current study, we used an *in vitro* embryo production model to examine the effect of MEHP at low levels on oocyte developmental competence. We set up several experiments to mimic the follicular fluid content, *i.e.*, low MEHP level and low estradiol. For all experiments, cumulus oocyte complexes (COCs) were aspirated from bovine ovaries, then matured *in vitro* in standard oocyte maturation medium (OMM) supplemented with: MEHP at a range levels (20–1000 nM) or with estradiol at a range levels (0–2000 ng/ml). Then, oocytes were fertilized and cultured for an additional 7 days to allow blastocyst development. Findings revealed that MEHP at low levels impairs oocyte developmental competence in a dose-dependent manner ($P < 0.05$) and that estradiol by itself does not impair it. Accordingly, in another set of experiments, COCs were matured *in vitro* with MEHP at two choosen concentrations (20 or 1000 nM) with or without estradiol, fertilized and cultured for 7 days. Samples of mature oocytes and their derived blastocysts were subjected to quantitative real-time PCR to examine the profiles of selected genes (*CYC1, MT-CO1, ATP5B, POU5F1, SOX2* and *DNMT3b*). Maturation of COCs with MEHP (20 or 1000 nM) affected gene expression in the mature oocyte. Maturation of COCs with MEHP (20 or 1000 nM) in the absence of estradiol reduced oocyte developmental competence ($P < 0.05$). A differential carryover effect on transcript abundance was recorded in blastocysts developed from MEHP-treated oocytes. In the presence of estradiol, increased expression was recorded for *CYC1, ATP5B, SOX2* and *DNMT3b*. In the absence of estradiol, decreased expression was recorded, with a significant effect for 1000 nM MEHP ($P < 0.05$). Taken together, the findings suggest that low levels of phthalate must be taken into consideration in risk assessments.

1. Introduction

Several studies have recently demonstrated a strong association between reproductive health and endocrine-disrupting compounds (EDCs). EDCs are a group of synthetic chemicals that have the ability to disrupt the normal function of endogenous hormones, thereby potentially inducing adverse developmental, reproductive, neurological and/or immune effects in the organism (Crain et al., 2008; Diamanti-Kandarakis et al., 2009). However, while most of these studies have focused on male reproduction, less attention has been paid to the effect of EDCs, and in particular phthalates, on female reproduction. Phthalate esters are plasticizers that are commonly used in polyvinyl chloride products (Kavlock et al., 2006; Schettler, 2006). Among the 18 important commercial phthalates, di(2-ethylhexyl) phthalate (DEHP) is predominant and considered a reproductive toxicant (Balabanič et al., 2011; Johnson et al., 2012; Kay et al., 2013).

* Corresponding author at: Department of Animal Sciences, Robert H. Smith Faculty of Agriculture, Food and Environment, The Hebrew University, Rehovot 76100, Israel.
E-mail address: roth@agri.huji.ac.il (Z. Roth).

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Human exposure to DEHP occurs mainly through ingestion of contaminated food, indoor air and household items, dermal application of personal care and cosmetic products, and parenteral exposure through medical products (Wormuth et al., 2006; Wittassek et al., 2011). When DEHP enters the body, it is hydrolyzed to mono(2-ethylhexyl) phthalate (MEHP) and 2-ethylhexanol (2-EH). Data from the United States and Canada have indicated that more than 95% of their populations have detectable levels of phthalate metabolites in their urine (Saravanabhavan et al., 2013; Zota et al., 2014). Moreover, exposure to phthalate is associated with reduced reproductive performance and fertility (Cobellis et al., 2003; Marsee et al., 2006; Lambrot et al., 2009; Weuve et al., 2010; Jurewicz et al., 2013). Hauser et al. (2016) reported on DEHP metabolites [MEHP, mono (2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), mono-(2-ethyl-5-oxohexyl) phthalate (MEOHP) and mono(2-ethyl-5-carboxypentyl) phthalate (MECPP)] in urine samples collected from 256 women who had undergone *in vitro* fertilization (IVF) cycles. These findings were associated with a reduced number of aspirated oocytes and metaphase II (MII)-stage oocytes at retrieval, which might be involved in lower IVF outcome (Sunkara et al., 2011; Ji et al., 2013). Accordingly, Messerlian et al. (2016) linked DEHP metabolites in the urine and the ovarian pool of growing follicles: they found a decrease in the number of antral follicles associated with higher levels of DEHP metabolites in urine samples of women seeking infertility care. Nevertheless, the effects of DEHP or MEHP on the follicle-enclosed oocytes are less known.

Within the ovary, during the development stages that precede ovulation of the dominant follicle, the oocyte undergoes capacitation, which is required for fertilization competence and further embryonic development (Assey et al., 1994; Hyttel et al., 1997). The follicular fluid, *i.e.*, the microenvironment of the enclosed oocyte, plays a pivotal role in these processes. As follicular fluid is essentially filtered plasma that contains follicle-secreted compounds (Hennet and Combelles, 2012), incorporation of toxicants such as MEHP from the circulation into the follicular fluid might have an adverse effect on follicular steroidogenesis and oocyte competence (Hunt and Hassold, 2008). Krotz et al. (2012) reported that follicular fluid aspirated from women undergoing IVF cycles contains relatively low concentrations of MEHP, ranging from 5.96 to 14.1 ng/ml (21.5–50.7 nM). Similarly, Du et al. (2016) reported that follicular fluid aspirated from 112 women attending infertility clinics contained a mean of 2.8 ng/ml (10 nM) MEHP with no correlation to IVF parameters. We recently found that follicular fluid aspirated from cows treated with DEHP contains 20 nM MEHP and lower levels of estradiol than in non-treated cows, 26 days after the exposure. Moreover, *in vitro* maturation (IVM) of bovine oocytes in the aspirated follicular fluid resulted in a decreased proportion of oocytes maturing and reaching the MII stage (Kalo et al., 2015).

Here we examined the effect of MEHP at low concentrations on oocyte developmental competence. Concentrations used in this study were similar to those previously found in the follicular fluid of human and animal origin. Because a toxicity study cannot be performed on women's oocytes, we established an *in vitro* bovine model to mimic the oocyte microenvironment in the follicular fluid following exposure to DEHP, *i.e.*, a low concentration of MEHP and reduced concentration of estradiol (Kalo et al., 2015). In particular, we examined whether MEHP has a direct effect on oocyte developmental competence or acts indirectly by reducing estradiol production, or both. Although it was the oocyte that was treated, we assessed whether exposure to MEHP during maturation can be carried over, resulting in impaired embryonic development and altered expression of genes in 7-day blastocysts.

2. Materials and methods

All chemicals were purchased from Sigma (Rehovot, Israel) unless otherwise indicated. MEHP was purchased from AccuStandard Inc. (New Haven, CT, USA) and dissolved in dimethyl sulfoxide (DMSO) at a maximum concentration of 0.01% (v/v).

2.1. Oocyte collection

Bovine ovaries were collected at a local abattoir from multiparous Holstein cows, and transported to the laboratory within 60 to 90 min in physiological saline solution (0.9% w/v NaCl at 38.5 °C with 50 μg/ml penicillin–streptomycin). Cumulus oocyte complexes (COCs) were aspirated from 3- to 8-mm follicles with an 18-gauge needle attached to a 10-ml syringe. COCs were collected into Hepes–Tyrode's lactate supplemented with 0.3% (w/v) bovine serum albumin, 0.2 mM sodium pyruvate and 0.75 mg/ml gentamicin at 38.5 °C (Hepes–TALP). At the end of the collection, COCs with at least three layers of cumulus surrounding a homogeneous cytoplasm were selected for IVM.

2.2. In-vitro *maturation, fertilization and culture*

In vitro production (IVP) of bovine embryos was performed according to the lab routine described by Kalo and Roth (2015). Briefly, selected COCs were washed three times in Hepes–TALP and transferred in groups of 30 to a 4-well dish (30 COCs per well). Each well contained 500 μl oocyte maturation medium (OMM) made up of TCM-199 and Earle's salts supplemented with 10% (v/v) heat-inactivated fetal calf serum to prevent COC attachment to the bottom of the well (Promega, Madison, WI, USA), 0.2 mM sodium pyruvate, 50 μg/μl gentamicin, 2.2 g/l sodium bicarbonate, 2000 ng/ml 17-ß estradiol and 1.32 μg/ml follicle-stimulating hormone isolated from ovine pituitary extract (Ovagen, ICP Bio, Auckland, New Zealand). COCs were incubated in humidified air with 5% CO_2 for 22 h at 38.5 °C.

At the end of maturation, COCs were washed three times in Hepes–TALP and transferred in groups of 30 to another 4-well dish (30 COCs per well) containing 600 μl *in vitro* fertilization (IVF)–TALP and 25 μl PHE (0.5 mM penicillamine, 0.25 mM hypotaurine, and 25 μM epinephrine in 0.9% NaCl) per well. For IVF, COCs were co-incubated for 18 h at 38.5 °C in a humidified atmosphere (5% CO_2) with spermatozoa from the same bull purified by swim-up technique ($\sim 1 \times 10^6$; 'Sion', Hafetz-Haim, Israel). After fertilization, putative zygotes were denuded of cumulus cells by gentle vortexing in Hepes–TALP containing 1000 U/ml hyaluronidase, and placed in groups of 10 in 25-μl droplets of potassium simplex optimized medium (KSOM) containing 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 polyvinyl alcohol, 10 μl/ml essential amino acids and 5 μl/ml non-essential amino acids, 100 U/ml penicillin-G and 0.1 mg/ml streptomycin. All embryo droplets were overlaid with mineral oil and cultured for 7 days at 38.5 °C in an atmosphere of humidified air with 5% CO_2 and 5% O_2. Oocyte developmental competence was evaluated as the proportion of oocytes that cleaved to 2- to 4-cell-stage embryos 42–44 h postfertilization, and the proportion of embryos that developed to blastocysts 7 days postfertilization.

2.3. Quantitative real-time PCR (qPCR) assay

Oocytes and blastocysts were collected for real-time assays at the end of 22 h maturation and on day 7, respectively. Each oocyte