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Effects of mono(2-ethylhexyl)phthalate on cytoplasmic maturation of oocytes – The bovine model

D. Kalo, Z. Roth*

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

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ABSTRACT

Phthalates are known reproductive toxicants, but their intracellular disruptive effects on oocyte maturation competence are less known. We studied the potential risk associated with acute exposure of oocytes to mono(2-ethylhexyl)phthalate (MEHP). First, bovine oocytes were matured in vitro with or without 50 μM MEHP and examined for mitochondrial features associated with DNA fragmentation. MEHP increased reactive oxygen species levels and reduced the proportion of highly polarized mitochondria along with alterations in genes associated with mitochondrial oxidative phosphorylation (*CYC1*, *MT-CO1* and *ATP5B*). In a second set of experiments, we associated the effects of MEHP on meiotic progression with those on cytoplasmic maturation. MEHP impaired reorganization of cytoplasmic organelles in matured oocytes reflected by reductions in category I mitochondria, type III cortical granules and class I endoplasmic reticulum. These alterations are associated with the previously reported reduced developmental competence of MEHP-treated bovine oocytes, and reveal the risk associated with acute exposure.

1. Introduction

Phthalates are used as plasticizers in plastic and PVC products [1], and are ubiquitous in the environment [2]. They are known as endocrine-disruptors (EDs) that deleteriously affect developmental and reproductive function in both males and females [2–5]. As EDs, they can interfere with the endocrine system; however, whether their effects on oocytes are adverse or adaptive (i.e., non-adverse) is less well understood [6].

Humans and animals can potentially be exposed to DEHP via contaminated food, the air indoors or household items [2]. However, exposure to relatively high doses of DEHP is mostly due to intensive use of PVC-based devices for medical procedures [2], such as blood transfusions and hemodialysis in infants [7]. DEHP concentrations in blood bags range from 1.8 to 83.2 μg/ml [8]. In addition, MEHP levels in red blood cells packed for storage have been found to increase 20-fold – from 3.7 μM on day 1 to 74 μM on day 42 [9] – the latter level similar to that used in the current study. The most abundantly used phthalates, di(2-ethylhexyl)phthalate (DEHP) and its primary metabolite mono(2-ethylhexyl)phthalate (MEHP), have been detected in human serum (6.74 ng/ml), seminal plasma (0.45 ng/ml) [10], cord blood serum (0.52 μg/ml) [11], urine (4.49–9.18 ng/ml) [10–13], and peritoneal (0.37 μg/m) [14] and amniotic fluids (2.8 ng/ml) [15].

In vitro studies in mouse have shown that exposure to a wide range of DEHP doses (10 and 100 μM) has a negative impact on primordial follicle assembly [16], and on follicular growth and estradiol production in antral follicles (100 μg/ml) [17]. Other studies have shown a deleterious effect on meiotic progression in ovines (0.12 μM) [18], and on fertilization ability and embryonic development in bovines (50 μM) [19] and in mice (1 μg/ml) [20]. Lenie and Smitz [21] showed that exposure to 10–200 μM MEHP has only a minor effect on steroidogenesis in mouse follicles, with no effect on oocyte meiotic progression. In contrast, 45–180 μM MEHP induced an oxidative response in human placental cells [22], and inhibited follicular development in rats at doses higher than 0.036 nM [23]. MEHP at 10 μg/ml reduced CCND2, CDK4 and aromatase mRNA expression in the mouse antral follicle [17], and at 250 and 500 μM, it altered mouse oocyte viability in association with SOD1 and ND1 mRNA expression [24].

The oocyte reaches competence through the lengthy processes of folliculogenesis and oocyte growth, which involve both nuclear and cytoplasmic maturation. Growth of mammalian oocytes is arrested in the ovarian follicle at the diplotene stage of the first meiotic prophase, termed the germinal vesicle (GV) stage. As maturation progresses, the oocyte resumes meiosis and progresses to

* Corresponding author. Tel.: +972 8 9489103; fax: +972 8 9465763.
E-mail address: roth@agri.huji.ac.il (Z. Roth).

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form the second metaphase (MII) plate accompanied by the first polar body extrusion [25]. In bovines, concentrations of MEHP ranging between 10 and 250 μM have been shown to impair oocyte nuclear maturation in a dose-responsive manner. According to Beker van Woudenberg et al. [26], MEHP at a concentration 217 μM is potent enough to impair oocyte meiotic progression. In our previous study, we reported that exposure of cumulus oocyte complexes (COCs) to 50 μM MEHP for 22 h impairs nuclear maturation, whereas 25 or 100 μM only increased the number of TUNEL-positive oocytes [19]. On the other hand, Anas et al. [27] reported that exposure of bovine COCs to 25 μM MEHP impairs nuclear maturation.

Less is known about the effects of phthalates on oocyte cytoplasmic maturation. Studies have shown that cytoplasmic maturation consists of multiple events that are essential for fertilization and early embryonic development [29,30]. These include reorganization of the cytoskeletal filaments and redistribution of cytoplasmic organelles, including the mitochondria, cortical granules (CGs) and endoplasmic reticulum (ER), through the actions of cytoskeletal microfilaments and microtubules [31,32]. In bovines, the mitochondria relocate from the restricted peripheral region at the GV stage into a diffuse pattern throughout the cytoplasm center at the MII stage [33,34]. Studies in rodents [35–37], humans [38] and bovines [39] have shown a similar pattern of ER relocation, from a fine network spread throughout the interior cytoplasm at the GV stage to an accumulation of bright cortical clusters in the cortex at the MII stage. Studies in bovines [40,41], and mice [42] have shown that CG distribution changes from a dispersed pattern throughout the cytoplasm at the GV stage to an even dispersion in a thin layer lining the oolemma at the MII stage.

In our previous study, in vitro maturation (IVM) with 50 μM MEHP impaired the developmental competence of bovine oocytes [19], reflected by a reduced proportion of oocytes that showed nuclear maturation, reached the MII-stage, were fertilized, underwent first cleavages and developed to the blastocyst stage [19]. Given that oocyte maturation is a prerequisite for successful fertilization and is highly correlated with embryonic development [28,43], the aim of the current study was to examine the potential risk posed by phthalate exposure to oocyte cytoplasmic maturation. We utilized bovine oocytes and an IVM procedure as research tools to examine the effects of relatively high dose (50 μM MEHP), similar to the level found in PVC-based red blood cell bags [9], on cytoplasmic organelle reorganization and function, including mitochondrial distribution and function, CG reorganization and ER relocation.

2. Materials and methods

All chemicals were purchased from Sigma (Rehovot, Israel) unless otherwise indicated.

2.1. Oocyte collection and IVM

Bovine ovaries were obtained from a local abattoir and transported to the laboratory within 60–90 min in physiological saline solution (0.9%, w/v NaCl at 38.5 °C with 50 μg/ml penicillin–streptomycin). COCs were aspirated from 3- to 8-mm follicles with an 18-gauge needle attached to a 10-ml syringe. They were collected into Hepes–Tyrode's lactate (TL) prepared in our laboratory [44], supplemented with 0.3% (w/v) bovine serum albumin (BSA), 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.

Selected COCs were washed three times in Hepes–TALP and groups of 10 were transferred into 50-μl droplets of oocyte maturation medium (OMM) made up of TCM-199 and Earle's salts supplemented with 10% (v/v) heat-inactivated fetal calf serum (Promega, Madison, WI, USA), 0.2 mM sodium pyruvate, 50 μg/μl gentamicin, 2.2 g/l sodium bicarbonate, 2 μg/ml 17-β estradiol and 1.32 μg/ml follicle-stimulating hormone (Folltropin-V; Bioniche Animal Health, Belleville, Ontario, Canada). The COC-containing droplets were overlaid with mineral oil and incubated in humidified air with 5% CO_2 for 22 h at 38.5 °C.

2.2. Classification of oocyte meiotic status

The meiotic status of each oocyte was determined using the cell-permeant DNA dye 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI). Oocytes were fixed in 2% (v/v) paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) in Dulbecco's phosphate buffered saline (PBS; Promega) for 15 min at room temperature and stored in PBS supplemented with 1 mg/ml polyvinylpyrrolidone (PBS–PVP) at 4 °C. Before staining, oocytes were washed three times in PBS–PVP and labeled with 10 μg/ml DAPI in PBS–PVP for 15 min at room temperature. Stained oocytes were classified into germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase I (MI), anaphase I (AI) and telophase I (TI) stages, or MII stage with an extruded first polar body (PB). Oocytes at the GV, GVBD, MI, AI and TI stages and those with abnormal chromosomal organization were classified as immature oocytes; MII-stage oocytes were classified as mature.

2.3. Assessment of mitochondrial features

2.3.1. Measurements of reactive oxygen species (ROS)

Oocytes were stained with 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) as previously described for bovine oocytes [45–47] with some modifications. H_2DCFDA is a cell-permeant fluorogenic reagent. In the cell, it is deacetylated to H_2DCF, which can then be oxidized by ROS to the highly fluorescent compound 2′,7′-dichlorofluorescein (DCF), thus reflecting ROS levels. Briefly, oocytes were incubated in 100 μM H_2DCFDA at 38.5 °C and 5% CO_2 for 30 min, then washed in Hepes–TALP and immediately examined under an inverted fluorescence microscope using Nis Elements software (Nikon, Tokyo, Japan). Intracellular fluorescence of DCF was measured (excitation at 450–490 nm and emission at 515–565 nm) in oocytes (in groups of 5) over 30 s at three time points: 0–10 s, 11–20 s and 21–30 s. The wavelengths and exposure times were held constant for all oocytes during the time of measurement. The intensity of the fluorescent signal was quantified using ImageJ software version 1.4 (National Institutes of Health, Bethesda, MD, USA) by measuring brightness for each oocyte. A circle was drawn around each measured oocyte and the fluorescence intensity was recorded. Measurement of a blank circled area was recorded for normalization.

2.3.2. Mitochondrial membrane polarity

Mitochondrial membrane polarity was evaluated with the Mitocapture mitochondrial apoptosis detection fluorometric kit (Biovision, Milpitas, CA, USA) as previously performed in our laboratory [48]. Briefly, live denuded oocytes were incubated in Mitocapture diluted in prewarmed incubation buffer (1:10, v/v) at 38.5 °C for 20 min. Oocytes were then examined under an inverted fluorescence microscope using Nis Elements software. The kit's cationic dye exhibits potential-dependent accumulation in mitochondria, indicated by a fluorescence-emission shift from green to red (low to highly polarized potential). Each individual oocyte was visualized under the inverted fluorescence microscope using the FITC channel for green monomers (excitation at 450–490 nm and emission at 515–565 nm) and the PI channel for red aggregates (excitation at 488 nm and emission at 590 nm). The