CONSTRUCTING THE MODE OF ACTION FOR DI-(2-ETHYLHEXYL) PHTHALATE-INDUCED OVARIAN TOXICITY

Kristina Pogrmic-Majkic, Dragana Samardzija Nenadov, Biljana Tesic, Svetlana Fa, Dunja Kokai, Bojana Stanic, Nebojsa Andric

University of Novi Sad, Faculty of Sciences, Department of Biology and Ecology, Laboratory for Endocrine Disrupters and Signaling (ENDOS), Serbia

nebojsa.andric@dbe.uns.ac.rs

INTRODUCTION AND OBJECTIVES

di-(2-ethylhexyl) phthalate (DEHP)

is a widely used synthetic chemical of high concern, categorized in Group I – Evidence of endocrine disrupting activity. The adverse effects of DEHP on female reproductive system are well described. Data from animal studies indicate that DEHP can affect steroidogenesis in the ovary and interfere with oocyte maturation and ovulation. The mechanism of DEHP action in the ovaries involves oxidative stress, expression of peroxisome proliferator-activated receptors, DNA damage, and apoptosis. However, identification of the sequential series of molecular and biological events in DEHP-induced ovarian toxicity remains to be established. The mode of action (MOA)/adverse outcome pathway is a useful tool in linking key molecular and biological events with adverse outcomes of chemical exposure. Identification of MOA could also be valuable in risk assessment of DEHP exposure.

Here, we aimed to assess the potential MOA for DEHP-induced ovarian toxicity by using the Comparative Toxicogenomic Database (CTD)-based bioinformatics analysis and the qualitative and quantitative weight of evidence (QWOE) analysis.

METHODS AND DESIGN

Literature search

→ CTD (http://ctdbase.org/), May 2021; the word "*DEHP*" was an input in the "Chemicals" search box.

→ Only experimental research papers on DEHP-induced toxicity were selected for the study.

→ The exclusion criteria were: 1) co-exposure; 2) epidemiological studies; 3) without available full text; 4) non-relevant to DEHP-induced toxicity; and 5) environmental monitoring.

Bioinformatics analysis

→ All genes retrieved from the literature search were manually inspected, deduplicated, and pooled.

→ The genes were used as an input for bioinformatics analysis in the Database for Annotation, Visualization and Integrated Discovery (DAVID v6.8).

→ The functional annotation pathways of the Kyoto Encyclopedia of Genes and Genomes (KEGG) and the enriched Gene Ontology (GO) term "*Biological Processes*" (BP) were extracted.

→ The data were presented using the "*ggplot2*" package in the R software (version 3.6.0).

QWOE analysis

→ Five steps: 1) MOA hypothesis; 2) qualitative evaluation of the evidence for each key event (KE); 3) quantitative rating of each KE by using the Bradford Hill causal considerations; 4) composite score derivation for each KE; and 5) causality evidence integration for the MOA.

RESULTS

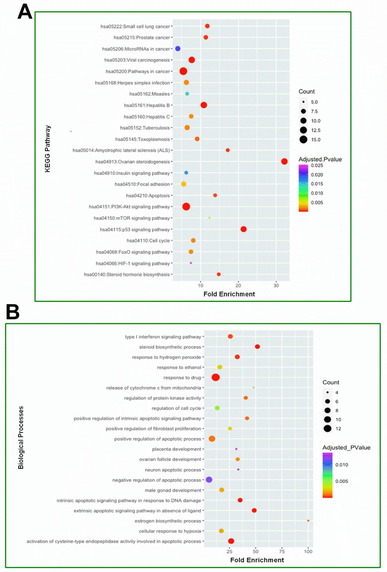

FIGURE 1. **(A)** Functional annotation pathways of KEGG and **(B)** biological processes associated with the DEHP-affected genes in the ovaries.

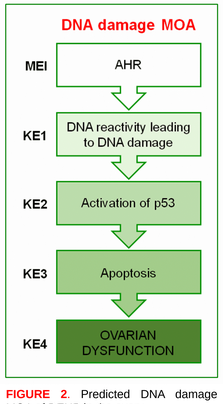

FIGURE 2. Predicted DNA damage MOA of DEHP in the ovary.

TABLE 2. Quantitative rate of each KE for DEHP acting via DNA damage MOA.

Evolved Bradford Hill causal consideration	Assigned weight	Quality rating: DNA damage (KE1)	p53 pathway (KE2)	Apoptosis (KE4)	Ovarian dysfunction (KE5)
Biological Plausibility		Evidence suggests that DEHP can induce DNA damage, thereby causing apoptosis and ovarian dysfunction			
Essentiality	0.4	+ (1)	No evidence (0)	++ (2)	++ (2)
Empirical Support					
Dose and Incidence Concordance	0.2	+++ (3)	No evidence (0)	+++ (3)	+++ (3)
Temporal Concordance	0.2	+++ (3)	+++ (3)	+++ (3)	+++ (3)
Consistency Across Test Systems	0.1	++ (2)	No evidence (0)	+++ (3)	+++ (3)
Analogy	0.1	+++ (3)	No evidence (0)	+++ (3)	+++ (3)
KE Score		2.1	0.6	2.3	2.3

MOA score = 100x (actual KE score/total possible score) = 100x (7.1/12); MOA score = **61**

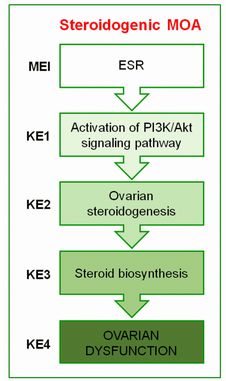

FIGURE 3. Predicted steroidogenic MOA of DEHP in the ovary.

TABLE 4. Quantitative rate of each KE for DEHP acting via steroidogenic MOA.

Evolved Bradford Hill causal consideration	Assigned weight	Quality rating: Activation of PI3/AKT (KE1)	Ovarian steroidogenesis (KE2)	Steroid biosynthesis (KE3)	Ovarian dysfunction (KE4)
Biological Plausibility		Some evidence suggests that DEHP can affect steroidogenesis through PI3/AKT signaling pathway, thereby leading to ovarian dysfunction			
Essentiality	0.4	No evidence (0)	No evidence (0)	No evidence (0)	No evidence (0)
Empirical Support					
Dose and Incidence Concordance	0.2	+ (1)	++ (2)	+++ (2)	+++ (3)
Temporal Concordance	0.2	++ (3)	+++ (3)	+++ (3)	+++ (3)
Consistency Across Test Systems	0.1	No evidence (0)	+++ (3)	+++ (3)	+++ (3)
Analogy	0.1	+ (1)	+++ (3)	+++ (3)	+++ (3)
KE Score		0.9	1.5	1.5	1.7

MOA score = 100x (actual KE score/total possible score) = 100x (5.6/12); MOA score = **46.6**

TABLE 1. Qualitative evaluation of the WOE for DEHP acting via DNA damage MOA.

MEI: Activation of AHR
Supporting Data: DEHP induces expression of AHR in human granulosa cells (Ernst et al., 2014). Indirect evidence: MEHP, a major metabolite of DEHP, increases mRNA expression of AHR in granulosa cells (Lovekamp-Swan et al., 2003).
Potentially inconsistent data: N/A

KE1: DNA damage
Supporting Data: DEHP exposure induced DNA damage, apoptosis, and reduces oocyte number in *C. elegans* (Yin et al., 2018). DEHP causes DNA damage and apoptosis leading to reduced survival (Liu et al., 2017) and fertilization capabilities (Lu et al., 2019) of murine oocytes. In DEHP-treated mice, an increased level of the ovarian DNA damage marker γH2AX, apoptosis and abnormal oocyte maturation were observed (Liu et al., 2021). Melatonin prevents DEHP-induced abnormalities in DNA repair, apoptosis, and meiotic defect in fetal murine ovaries (Sun et al., 2018)
Potentially inconsistent data: DEHP did not cause DNA damage (Douglas et al., 1986); however, MEHP caused genotoxicity in Chinese hamster ovary cells (Chang et al., 2017).

KE3: p53 signaling pathway
Supporting Data: N/A

KE4: Apoptosis
Supporting Data: DEHP exposure induces apoptosis and reduces oocyte number in *C. elegans* (Yin et al., 2018). DEHP causes apoptosis leading to reduced survival (Liu et al., 2017) and fertilization capabilities (Lu et al., 2019) of mouse oocytes. DEHP causes significant increase in the rates of late and total apoptosis, thereby prolonging the estrous cycle and altering the ovarian morphology in rats (Li et al., 2020). Melatonin reduces the number of apoptotic cells alleviating DEHP-induced reduction of primordial follicle assembly (Liu et al., 2019). Melatonin suppresses apoptosis in the DEHP-exposed fetal ovaries (Sun et al., 2018).
Potentially inconsistent data: DEHP did not affect the relative proliferation of KGN granulosa cells (Ernst et al., 2014); however, the apoptotic process was not examined. Therefore, these data cannot be accounted for as potential inconsistent data.

KE5: Ovarian dysfunction
Supporting Data: DEHP causes primary follicle damage and reduces fertilization capabilities in mice (Lu et al., 2019). DEHP delayes oocyte progression to meiotic prophase I in mice (Liu et al., 2017). DEHP alters the estrous cycle and causes pathological changes of the rat ovarian tissue (Li et al., 2020). DEHP causes morphological changes in the oocytes (Tripathi et al., 2019b). NAC protects granulosa cells against DEHP-induced apoptosis and cellular toxicity (Tripathi et al., 2019a). Melatonin reduces the deleterious effect of DEHP on the oocyte cyst breakdown and follicle assembly (Liu et al., 2019) and alleviates meiotic defects in fetal murine oocytes (Sun et al., 2018).
Potentially inconsistent data: The number of oocytes scored in DEHP-treated ovaries was lower but not significantly different from the control. However, DEHP impairs primordial follicle assembly (Liu et al., 2019).

TABLE 3. Qualitative evaluation of the WOE for DEHP acting via steroidogenic MOA.

MEI: Activation of estrogen receptor
Supporting Data: ICI 182,780, an efficient antagonist of ER, reverses DEHP-induced cyst break down (Mu et al., 2015).

KE1: Activation of PI3/AKT signaling pathway
Supporting Data: DEHP increases phosphorylation of AKT in murine ovaries; however, steroidogenesis was not examined (Hannon et al., 2014). *Indirect evidence*: DEHP increases expression of the PI3K factors in murine ovaries and alters expression of steroidogenic genes; steroids were not examined (Rattan et al., 2019). MEHP increases AKT phosphorylation, alters the expression of steroidogenic genes, the production of steroids, and accelerates early folliculogenesis in murine ovaries (Hannon et al., 2015).
Potentially inconsistent data: The study of Zhang et al. showed a decrease in AKT phosphorylation in murine ovaries following DEHP exposure (Zhang et al., 2019), whereas the study of Hannon et al. showed that DEHP did not affect phosphorylation of AKT in murine ovaries (Hannon et al., 2015).

KE2: Expression of steroidogenic genes
Supporting Data: DEHP leads to decrease in steroidogenesis-related enzymes and estradiol production. The reduction in estradiol inhibits proliferation of granulosa cells and limits the growth and development of antral follicles (Liu et al., 2021). DEHP significantly decreases expression of steroidogenic genes, steroid production, and causes ultrastructural changes in murine ovaries (Lai et al., 2017). DEHP decreases expression of steroidogenic responsive genes, lowers steroid hormone production, thereby leading to induction of apoptosis in rat granulosa cells (Tripathi et al., 2019a).
Potentially inconsistent data: In porcine ovaries, DEHP affects the expression of STAR (200 mg DEHP), but the production of progesterone and pregnenolone was affected at 20 mg DEHP. DEHP did not affect folliculogenesis (ovarian dysfunction) (Lee et al., 2021).

KE3: Steroid biosynthesis
Supporting Data: DEHP exposure of suckling mice causes decrease in estradiol production, inhibits proliferation of granulosa cells, and limits the growth and development of antral follicles (Liu et al., 2021). DEHP exposure reduces the concentrations of steroid hormones and changes the ultrastructural characterization of murine ovaries (Lai et al., 2017). DEHP decreases progesterone concentrations in the media of human granulosa cells in culture (Guerra et al., 2016). Exposure of 20-day-old female rats to 500 mg DEHP by oral gavage once daily for 10 days reduces serum levels of progesterone and estradiol. The effect on the weight of ovaries was not noticed (Svechnikova et al., 2007). *Indirect evidence*: MEHP, a DEHP metabolite, increases plasma testosterone and 17β-estradiol (E2) in zebrafish females (Zhu et al., 2016).
Potentially inconsistent data: DEHP does not change estradiol production but changes progesterone in the culture of murine follicles. No effects on follicular development or survival were noted in the culture systems (Guerra et al., 2016).

KE4: Ovarian dysfunction
Supporting Data: Theca cells show altered structure of the nuclear envelope, fewer mitochondria, and mitochondria with a reduced number of cristae (Lai et al., 2017). *Indirect evidence*: MEHP, a DEHP metabolite, decreases egg production in zebrafish females (Zhu et al., 2016).
Potentially inconsistent data: No effects of DEHP on follicular development or survival were noted in the culture systems (Guerra et al., 2016).

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CONCLUSIONS

Based on the CTD-based bioinformatics analysis and QWOE evaluation, we have constructed two MOAs for DEHP-induced ovarian toxicity. QWOE showed a moderate confidence for both MOAs, suggesting that the data gap still exists in some KEs. Additional studies are needed to improve the MOA and risk assessment for DEHP-induced ovarian toxicity.

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