

In vitro exposure to di-(2-ethylhexyl) phthalate (DEHP) stimulates spontaneous feline uterine contractions

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Received: 08.04.2020 • Accepted/Published Online: 28.06.2020 • Final Version: 18.08.2020

Abstract: Di-(2-ethylhexyl) phthalate (DEHP) is widely used as a plasticizer in various products such as PVC-derived plastics, toys, packaging materials, cosmetics, and pharmaceuticals. This study aimed to investigate the effect of DEHP on spontaneous contractions of the feline uterus in vitro. Tubal 1-cm uterine samples prepared from 10, 9, and 12 uteri obtained from adult cats in estrus (n = 5), diestrus (n = 5), and interestrus (n = 5), respectively, after ovariectomy were suspended in an isolated organ bath in aerated Krebs solution at 39 ± 1 °C, and an initial 1 g tension was given. After 1 h equilibration of tissues, the spontaneous contractions were recorded for 10 min as control. The effects of solvent and DEHP (0.001–100 µM) on contractions were then evaluated in terms of frequency and mean amplitude parameters. It was observed that DEHP had no effect on uterine contractions of cats in interestrus. However, DEHP significantly increased the mean amplitude of uterine contractions during the estrus and diestrus periods at concentrations of 1 µM and 10 µM, respectively, depending on the dose (P < 0.05). Decreases in the frequency of the contractions in the estrus and diestrus periods were not statistically significant (P > 0.05). This study, carried out for the first time in cats, showed that DEHP has a stimulatory effect on uterine contractions. We concluded that disruption of the uterine contractions, which are essential for physiological reproductive processes such as regular estrous cycles, sperm and zygote transport, implantation and continuation of pregnancy, by DEHP exposure may cause many reproductive problems.

Key words: DEHP, feline, uterus, spontaneous contraction, reproduction

1. Introduction

Phthalate esters are synthetic organic chemicals derived from phthalic acid which are used as a plasticizer to make rigid plastics such as polyvinyl chloride which have elasticity, transparency, pliability, and durability [1]. Di(2-ethylhexyl) phthalate (DEHP) is one of the most widely utilized among the number of phthalates of which 18 billion tons per year are produced throughout the world, and which are found in approximately 40% of plastics [2]. Bottles, containers, food packaging materials, medical devices, cosmetics, and building materials are commonly used products which contain DEHP in their structure [3]. Since these chemicals are bound to plastics noncovalently, they are leached easily and rapidly out of the products into the environment. Contaminated air, soil, food, and water are the main potential exposure sources for human and animals [2,3]. Pets are also exposed to phthalates through training devices, toys, and veterinary medicine equipment [4,5]. Their exposure to these chemicals is greater than humans' because they are closer to contaminated sources.

According to the report of a study conducted with cats and dogs, the average levels of many chemicals in blood and urine were higher in pets than in humans [5]. Serum DEHP concentrations of cats living in the Paris area were reported to be 32.9 ng/L [6], and urinary levels of phthalic acids were measured with a median of 630 ng/mL in pets [7]. Additionally, pets develop and age faster than humans, and their health problems develop more rapidly [5].

As it is a widespread environmental toxic chemical, DEHP leads to many adverse effects on metabolic [8], nervous [9], cardiovascular [10], and reproductive systems as well [11] in living organisms. Mariana et al. [10] have demonstrated that DEHP at higher doses can inhibit L-type calcium (Ca²⁺) channels and cause relaxation on potassium chloride (KCl)-induced contractions of the rat aorta. Similarly, the number of dilated blood vessels in the uterine wall were enhanced in DEHP-exposed mice [12]. On the other hand, DEHP increased the blood pressure of about 20% of mice offspring following maternal exposure [13], and increased contraction-related gene expression

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in rat cardiomyocytes [14]. In an epidemiological study, positive correlations between phthalate levels (including DEHP) in cord blood and preterm delivery in women who had no history of occupational exposure to phthalates were shown [15]. DEHP also leads to disruption in endometrial receptivity and a reduction in the number of implanted embryos in mice [16]. The implantation of embryos and endometrial receptivity are critical aspects of pregnancy which are under the control of reproductive steroids estrogen (E2) and progesterone (P4) [17]. These steroids also regulate the contractions of the uterus, which change during the estrous cycle and support fertilization and gestation [18]. However, previous studies have shown that DEHP can interfere with steroidogenesis and/or receptor functions via its endocrine-disrupting effect [19–22]. The uterus has steroid receptors and is one of the main target organs for ovarian hormones. In vivo exposure of rats [23] or mice [12] to DEHP for 30 days resulted in alterations in uterus diameter, the number of uterine glands, serum steroid concentrations, and steroid receptors in the uterus. Lactational exposure to DEHP entails a reduction in the thickness of the myometrial layer of the rat uterus [24]; DEHP has also been suggested to induce uterine leiomyoma [25] or breast cancer [26] in women by increasing cell proliferation. These adverse effects may originate from the estrogenic effect of DEHP [27]. Although the reprotoxic effects of DEHP have already been shown in many studies in human and rodents, we still have lack of knowledge on the effects of DEHP on uterine contractions, especially in cats. Therefore, in this study reported for the first time, we aimed to investigate how DEHP affects in vitro spontaneous contractions of the feline uterus at various stages of the estrous cycle.

2. Materials and methods

2.1. Animals

Uterine tissues used in this study were collected from 15 healthy, mixed breed, 1–2-year-old queens following ovariectomy in the Small Animal Clinics of Kirikkale University Faculty of Veterinary Medicine. The queens did not have any reproductive diseases, had not been medicated (in terms of contraceptives), nor had previous pregnancies. All procedures of the experiments were conducted with approval of the Local Ethical Committee of Kirikkale University, Turkey (2019/3-26).

2.2. Determination of the estrous phase

The queens (n = 15) from which we obtained uterine tissues were classified into 3 groups according to their phase of the estrous cycle: estrus (n = 5), diestrus (n = 5), and interestrus (n = 5). Classification of estrous cycle phases was performed according to behavioral observations, follicle/corpus luteum existence in ovaries, and serum estradiol and progesterone levels. It was assumed that cats

showing heat signs such as lordosis, rolling, and vocalizing as well as having follicles on their ovaries were in estrus. Cats without heat signs and having corpus luteum on their ovaries were assumed to be in diestrus [28,29]. Serum E2 and P4 concentrations were measured from blood samples taken from the vena cephalica antebrachia of queens prior to ovariectomy. Once the blood clotted and was centrifuged at 1000 g for 10 min at 4 °C, the collected sera were frozen at –20 °C until hormone analysis was completed. The concentrations of E2 and P4 of the sera were assessed by electrochemiluminescence immunoassay kits (Roche Diagnostic, Indianapolis, IN, USA) with the Roche Cobas E601 analyzer according to the kit manufacturer's recommendations. In addition to estrus behavioral signs and morphological examinations of the ovaries, with the assessment of steroid levels, uterine tissues taken from cats with E2 levels higher than 20 pg/mL were classified as in estrus. The uterine tissues taken from cats with P4 levels higher than 1.5 ng/mL were classified as in diestrus. The uterine tissues taken from cats with E2 and P4 levels lower than 20 pg/mL and 1.5 ng/mL, respectively, were classified as in interestrus. The proestrus phase was omitted because it is difficult to distinguish from estrus [30].

2.3. Isolated feline uterine samples

Once collected, the uterine tissues were placed in Krebs solution at pH 7.4 containing the following: NaCl 118 mM, KCl 4.69 mM, MgSO₄ 0.6 mM, NaHCO₃ 25 mM, CaCl₂ 2.5 mM, and glucose 11.1 mM (Merck KGaA, Darmstadt, Germany) dissolved in 1 L deionized water, and transported to the laboratory within 2–5 min at 20–22 °C. All procedures in the experiments were performed according to the protocols of our previous study [31]. Briefly, the harvested uterine tissues were freed from blood vessels and the surrounding adipose tissues, and 10–12-mm tubal segments were separated out from the ovarian side of each horn in Petri dishes containing Krebs solution. The uterine samples were mounted vertically in a tissue bath (IOBS 99 Isolated Tissue Bath Stand Set; Commat, Ankara, Turkey), in which one end (ovarian side) was attached to force transducers and the other end was attached to a glass holder prefilled with 10 mL Krebs solution bubbled with an atmosphere of 95% O₂ and 5% CO₂ at 39 ± 1 °C.

2.4. Recording and treatments

An initial tension of 1 g was given to tissues and the samples were allowed to equilibrate for 1 h by rinsing with fresh Krebs every 15 min before taking the control recordings. During the experiments, the spontaneous uterine contractions were measured with a force-displacement transducer (FDT 05 MAY, Commat) and recorded using an MP35 Biopac system (Biopac Systems, Goleta, CA, USA). Tissue samples which were not seen to have optimal spontaneous contractions until the end of

equilibration were excluded from the study. Therefore, the contractions were monitored from 10, 9, and 12 uterine preparations of feline in estrus, diestrus, and interestrus, respectively.

After the tissue stabilization period, spontaneous contractions were recorded for 10 min as control values while the tissues were continually perfused with Krebs solution. Each uterine sample served as its own control. Subsequently, uterine samples were exposed to the vehicle (0.1% dimethyl sulfoxide: DMSO) and different cumulative concentrations of DEHP, and contraction responses were recorded for 10 min to compare with the control value. The DEHP (Sigma-Aldrich Corp., St. Louis, MO, USA) was dissolved in DMSO and diluted in Krebs solution. The tissues were exposed to a concentration of DEHP for 10 min, and then the next higher concentration of DEHP was added to the bath. The final doses of DEHP in the bath were 0.001, 0.01, 0.1, 1, 10, and 100 μ M. The doses of DEHP used in this study were based on previous *in vitro* toxicological studies [10,11] and our preliminary experiments with feline uterine tissue.

2.5. Data and statistical analysis

The frequency (number of contractions) and the mean amplitude of uterine contractions were calculated manually from 10-min recordings of the control and all treatment groups. These parameters obtained from recordings are presented as percentages of control contractions, i.e. the control was 100% and the other groups were determined by comparing the results to the control. All the data were expressed as the mean \pm standard error mean (SEM), and statistically analyzed with GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA) by comparing the control with each group. The analyses were performed by using a one-way ANOVA followed by Dunnett's test for post-hoc comparison. The value considered statistically significant was $P < 0.05$.

3. Results

3.1. Serum steroid hormone levels

The E2 and P4 concentrations analyzed from the sera are shown in Table 1. These values supported the classification

of stages in the estrous cycle performed according to clinical observation and macroscopic examination of the ovaries of cats. Therefore, the serum E2 and P4 levels were as follows: >20 pg/mL and <1.5 ng/mL for cats in estrus; <20 pg/mL and >1.5 ng/mL for cats in diestrus; <20 pg/mL and <1.5 ng/mL for cats in interestrus, respectively.

3.2. Spontaneous uterine contractions

The frequencies and mean amplitudes of spontaneous contractions measured from uterine segments of felines in estrus, diestrus, and interestrus during the 10-min control period are given in Table 2. The findings obtained from control groups were considered to be 100%, and the percentages for the other treatment groups were calculated by comparing the results to the control. The effects of the vehicle DMSO on the frequencies and mean amplitudes of spontaneous contractions were not significant compare to the control in any phases of the estrous cycle ($P > 0.05$). However, different concentrations of DEHP (0.001–100 μ M) treatment of the uterine samples significantly affected the amplitudes of spontaneous contractions dose-dependently and based on the phases of the estrous cycle ($P < 0.05$).

3.3. Effects of DEHP on *in vitro* spontaneous feline uterine contractions in estrus

The representative tracings in Figure 1A demonstrate the details for the responses of the tissues to the control conditions and treatment with different doses of DEHP (0.001–100 μ M) on feline uterine contractions in the estrus phase of the reproductive cycle. Although administration of different doses of DEHP to the uterine samples caused a reduction in the frequency of spontaneous isometric contractions (Figure 1B), the measured data were not statistically different from those of the control ($P > 0.05$).

As shown in Figure 1B, all of the concentrations of DEHP used in this study increased the mean amplitude of contractions dose-dependently. When the stimulatory effect of DEHP on the contractions' amplitude was compared to the control, effects of DEHP at the doses of 0.001–0.1 μ M were not statistically remarkable. However, 1 μ M DEHP significantly enhanced the force of contractions ($P < 0.05$). The next higher 2 concentrations of DEHP (10

Table 1. The serum estradiol and progesterone concentrations of cats in different phases of estrous cycle.

Phases of estrous cycle	Serum steroid concentrations	
	Estradiol (pg/mL)	Progesterone (ng/mL)
Estrus (n = 5)	29.46 \pm 5.38	0.63 \pm 0.08
Diestrus (n = 5)	10.18 \pm 2.47	22.22 \pm 4.04
Interestrus (n = 5)	11.92 \pm 2.28	0.92 \pm 0.26

Table 2. The frequencies and mean amplitudes of spontaneous contractions of feline uterine segments in different phases of estrous cycle.

Phases of estrous cycle	Uterine contractions of 10-min	
	Mean amplitude (g)	Frequency
Estrus (n = 10)	3.1 ± 0.5	14.8 ± 1.2
Diestrus (n = 9)	3.9 ± 0.7	9.04 ± 1.0
Interestrus (n = 12)	3.8 ± 0.6	12.4 ± 0.8

and 100 µM) also dramatically increased the contractile activity of uterine in terms of the mean amplitude compared to the control (P < 0.01).

3.4. Effects of DEHP on in vitro spontaneous feline uterine contractions in diestrus

The details for the responses of the feline uterine contractions with diestrus in the control and different DEHP (0.001–100 µM) concentrations for the treated groups are demonstrated in the representative tracings in Figure 2A. The calculated frequency values of the DEHP

treatment groups were lower than those of the control (Figure 2B). Nevertheless, this difference did not have significance according to statistical analyses (P > 0.05).

It can be seen in Figure 2B that the application of DEHP at doses of 0.001–1 µM enhanced the mean peak amplitude of contractions but was not significant (P > 0.05), while the responses of the spontaneous contractions in terms of amplitude were significantly increased by the highest 2 DEHP concentrations (10 and 100 µM) compared to the control (P < 0.05).

3.5. Effects of DEHP on in vitro spontaneous feline uterine contractions in interestrus

In vitro exposure of uterine samples to DEHP concentrations did not alter either the mean amplitude or the frequency of contractions (Figures 3A and 3B). Although there was a slight increase in the forces of contractions at doses of DEHP of 0.01 µM and above, it was not statistically significant (P > 0.05).

4. Discussion

During the last few decades, adverse effects of endocrine disrupting chemicals (EDCs) on physiological systems of living organisms have caused increasing public concern.

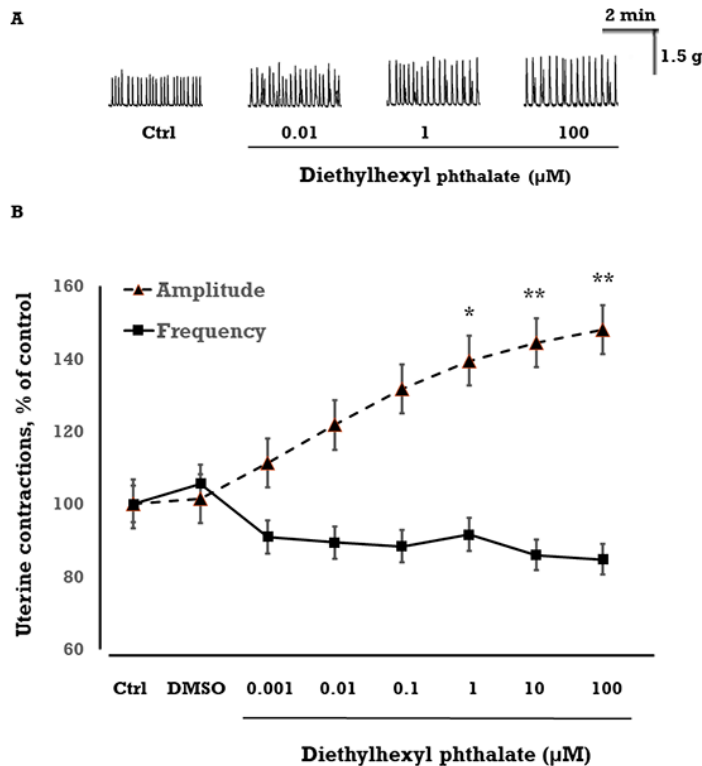


Figure 1. Effects of vehicle (DMSO) and cumulative concentrations of DEHP (0.001–100 µM) on the frequency and mean amplitude of feline uterine contractions in estrus phases. The upper panel (A) demonstrates the representative tracings of responses of uterine contractions to control condition and DEHP exposure for 10 min. the lower panel (B) presents the graph of data expressed as the mean ±SEM (n = 10). The responses in DEHP-treated groups were significantly different from control (*: P < 0.05 and **: P < 0.01). Ctrl: Control, DMSO: Demthyl sulphoxide, DEHP: Diethylhexyl phthalate.

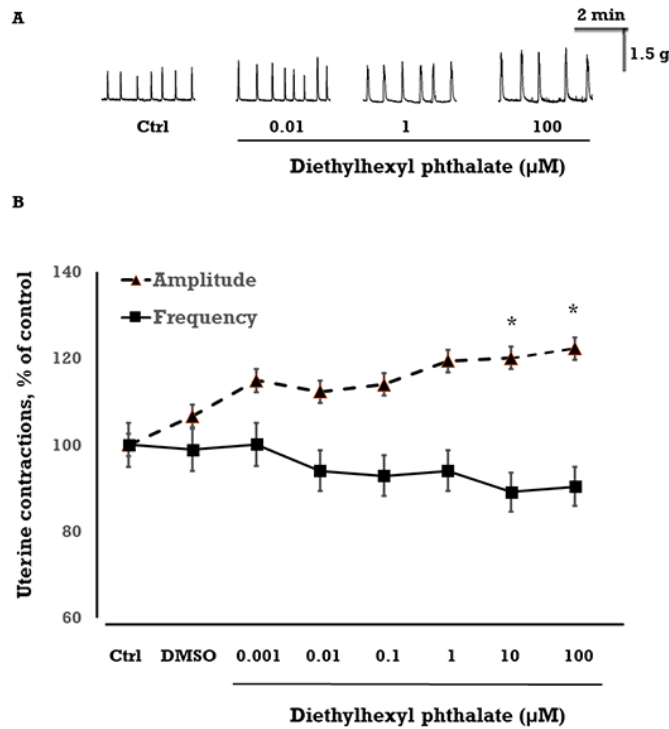


Figure 2. Effects of vehicle (DMSO) and cumulative concentrations of DEHP (0.001–100 µM) on the frequency and mean amplitude of feline uterine contractions in diestrus phases. The upper panel (A) demonstrates the representative tracings of responses of uterine contractions to control condition and DEHP exposure for 10 min. the lower panel (B) presents the graph of data expressed as the mean \pm SEM (n = 9). The responses in DEHP-treated groups were significantly different from control (*: P < 0.05). Ctrl: Control, DMSO: Demthyl sulphoxide, DEHP: Diethylhexyl phthalate.

Researchers are particularly interested in their detrimental effects on reproductive systems [16]. DEHP is one of the most widely used phthalates among EDCs. Although the reprotoxic effects of DEHP have been reported in earlier studies [11,20,32,33], little attention has been given to its effects on the contractile activity of the uterus. In the present study, we investigated how DEHP, in a range of 0.001–100 µM concentrations, affects feline uterine contractions in various reproductive phases. Our observations first showed that DEHP stimulated spontaneous uterine activity of female cats depending on the estrous cycle. In a few recent reports, phthalates including DEHP have been detected in urine [7] and blood [6] of cats, and it is suggested that pets have been exposed to these chemicals via pet toys, training devices, and veterinary medicine equipment [4,5], as well as different environmental sources. EDCs do not have monotonic dose-response curves since they have more potent effects at low/high doses than high/low doses [34]. Hence, studying a wide range of concentrations gives more accurate and reliable results than a narrow range of concentrations [11,34]. Therefore, we chose the doses of 0.001–100 µM for DEHP, as in previous studies [10,35,36].

In the present study, DEHP exhibited different effects on feline uterine contractions which were grouped in terms

of estrous cycle according to behavioral observations, follicle/corpus luteum existence in ovaries, and serum E2 and P4 levels as compatible with earlier studies [28–30]. Our findings showed that DEHP significantly (P < 0.05) increased the mean amplitude of uterine contractions at higher doses from 1 µM to 10 µM in estrus and diestrus, respectively. However, the force of contractions was not altered with exposure to DEHP in the interestrus phase. Serum E2 levels are high during the estrus phase of the reproductive cycle and E2 upregulates oxytocin receptors in the uterus, resulting in increased contractions [37]. In an in vivo study, it has been shown that uterine responses of cats to oxytocics were elevated with a 1-mg estrogen injection [38]. Wang et al. [39] have reported that 0.1–10 ng/mL DEHP stimulated secretions of prostaglandin F2 α and oxytocin in bovine ovarian and endometrial cells, but inhibited prostaglandin E2. The authors suggested that these changes may increase the contractile activity of myometrium. Additionally, the estrogenic activity of DEHP has previously been described; it induced the proliferation of breast cancer cells in vitro even at low doses (10 nM) [26,27]. In vivo exposure of rats to DEHP caused uterine cell proliferation [23], and gradually decreased the embryo implantation number in mice depending on the

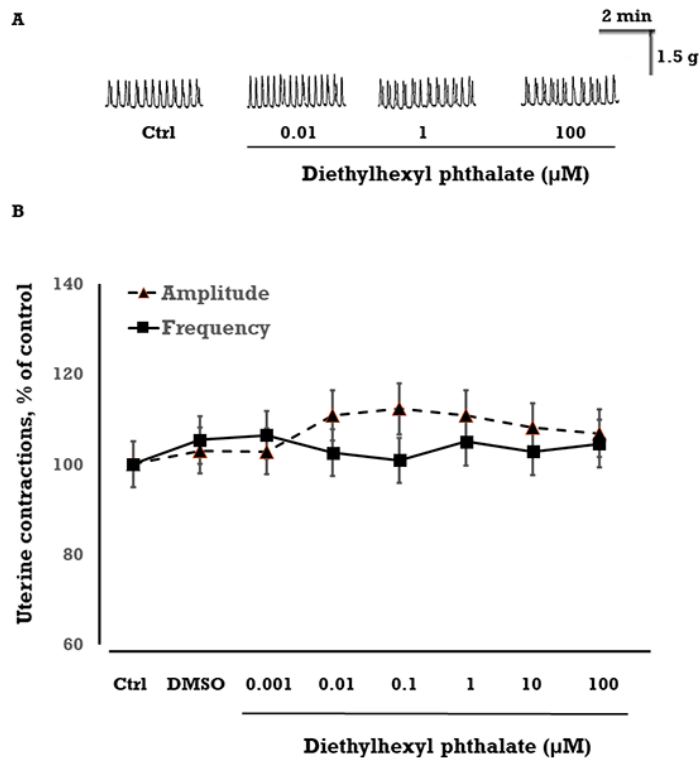


Figure 3. Effects of vehicle (DMSO) and cumulative concentrations of DEHP (0.001–100 µM) on the frequency and mean amplitude of feline uterine contractions in interestrus phases. The upper panel (A) demonstrates the representative tracings of responses of uterine contractions to control condition and DEHP exposure for 10 min. The lower panel (B) presents the graph of data expressed as the mean \pm SEM (n = 12). The responses in DEHP-treated groups were not significantly different from control ($P > 0.05$). Ctrl: Control, DMSO: Demthyl sulphoxide, DEHP: Diethylhexyl phthalate.

dose [16]. Therefore, the stimulatory effect of DEHP on the force of spontaneous uterine contractions may have resulted from its estrogenic activity. The findings that the amplitude of contractions did not differ from the control in the interestrus phase support our suggestion. In the reproductive season, unovulated felines go through estrus and interestrus phases in turn in which the E2 level is higher and lower, respectively [29]. Thus, estrogen-related effects of DEHP may disappear in this phase of the estrous cycle. On the other hand, *in vitro* DEHP (0.001–100 µM) exposition of the rat aorta led to relaxation of KCl-induced contractions [10]. Azevedo et al. [40] also demonstrated that similar DEHP concentrations reduced the histamine and KCl-induced contractions of the human umbilical artery, while 5-hydroxytryptamine(5-HT)-induced contractions increased. Mariana et al. [10] have suggested that the vasorelaxant effect of DEHP on rat aorta may result in inhibiting L-type Ca²⁺ channels, but Azevedo et al. [40] did not agree with them, due to different results with KCl- and 5-HT-induced contractions of the human umbilical artery. Posnack et al. [14] demonstrated that DEHP treatment increased the expression of some genes associated with Ca²⁺ handling, including voltage-gated

L-type Ca²⁺ channels. In addition, we also found that DEHP increased uterine contractions, while in a previous study, DEHP had no effect on contractions of the gastric muscle [41]. Dissimilar effects of DEHP on smooth muscles belonging to different tissues denote that further studies are required.

There are 2 mechanisms that stimulate uterine contractions: electrochemical and pharmacomechanical coupling. In the first, the action potential that initiates contractions is formed by the electrochemical mechanism which changes Ca²⁺ permeability by stimulating the voltage-gated Ca²⁺ channels [42]. However, as shown in the results of previous studies [10,40] the effects of DEHP on the voltage-gated L-type Ca²⁺ channels are still uncertain. In the pharmacomechanical coupling mechanism, secondary messengers are activated by the binding of membrane receptors and agonists, which leads to the increased influx of Ca²⁺ ions into the cell [42]. Liu and Lin [43] have reported that phthalates including DEHP may modulate the activity of membrane ion channels, and have also suggested that among receptor-gated Ca²⁺ channels, nicotinic acetylcholine receptors are more sensitive to phthalates than voltage-gated channels.

Thus, DEHP may enhance the amplitude of contractions by altering the activity of ligand-gated channels.

Progesterone released in high levels during diestrus physiologically reduces uterine activity, calming the uterus, while estrogens induce contractility and excitability [44]. Spontaneous activity of the uterus must be kept under control by ovarian steroids for the success and maintenance of many reproductive processes [45]. Although serum E2 level is lower in the diestrus phase, DEHP also stimulated the mean amplitude of contractions in that phase. It was shown in earlier studies that DEHP intervened in steroidogenesis in ovarian cells by inhibiting synthesis or secretion of reproductive steroids including progesterone [19,20,46]. Therefore, the stimulatory effects of DEHP on the force of uterine contractions in diestrus may be related to its inhibitory effects on P4 secretion, which suppress the gene expression of contraction-associated proteins in order to relax the myometrium [47]. However, more comprehensive studies will be needed to explain the underlying mechanisms.

According to our observations, treatment of feline uterine samples with various DEHP concentrations (0.001–100 µM) did not alter the frequency of spontaneous contractions in any phases of the estrous cycle, unlike the mean amplitude. Similarly, different effects were observed on the frequency and amplitude of contractions with the exposure of rat or cat uterus to bisphenol A, another commonly used plasticizer/EDC, which has been associated with the control of frequency and amplitude by different mechanisms [31,48]. Although myometrial

activity is stimulated by a single action potential, more coordinated depolarizations are required for forceful and continual contractions which could be affected by the number of action potentials [44,49]. Therefore, the amplitude of contractions may be more sensitive to DEHP than are frequencies.

In conclusion, many reproductive processes such as transport of sperm and oocytes, fertilization and embryo implantation, and continuation of pregnancy are related to suitable uterine contractions. Our results indicated that DEHP induced feline uterine contractions in different degrees depending on the estrous cycle periods. Because DEHP may have modulatory effects on membrane receptors and channels, especially Ca²⁺ channels [43], it may have stimulated contractions by increasing intracellular Ca²⁺ concentrations or estrogenic activity. The lack of studies on the effects of DEHP on smooth muscle, including myometrium, has caused some difficulties for comparing our results with the findings of other researchers. Nevertheless, we could conclude that DEHP may cause reproductive problems in felines by disrupting the balance of uterine activity.

Acknowledgments/Disclaimers

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflict of interest

No conflict of interest was declared by authors.

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