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Ascorbic acid alleviates reproductive toxicity of di-(2-ethyl hexyl) phthalate in female Wistar rats

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ABSTRACT

Objective: To investigate the potential of ascorbic acid in mitigating reproductive toxicity induced by di-(2-ethyl hexyl) phthalate (DEHP) in female Wistar rats, focusing on oxidative stress, hormone levels, and gonadotropin receptors expression.

Methods: Forty female Wistar rats [30 days old, weighing (60±10)g] were randomly divided into five groups (n=8 per group). Group 1 received corn oil (control). Groups 2 and 3 were administered DEHP at 10 and 100 mg/kg body weight (b.wt.), respectively. Groups 4 and 5 received DEHP at 10 and 100 mg/kg b.wt., respectively, plus ascorbic acid 100 mg/kg b.wt.. All treatments were given orally for 30 days. Blood and ovarian tissues were collected to assess serum reproductive hormones, gonadotropin receptor gene expression, oxidative stress markers, and apoptosis.

Results: DEHP, particularly at the higher dose, significantly decreased hormone levels (follicle-stimulating hormone, luteinizing hormone, estradiol) and gonadotropin receptor gene expression (*FSHR*, *LHR*), while increasing oxidative stress and apoptosis. Co-treatment with ascorbic acid significantly improved these parameters, reducing oxidative stress and apoptosis, and restoring hormone levels and gonadotropin receptor expression. Histopathology revealed fewer atretic follicles and less disruption in ovarian structure in DEHP and ascorbic acid-treated groups compared to those treated with DEHP alone.

Conclusions: Ascorbic acid demonstrates protective effects against DEHP-induced reproductive toxicity in female rats, likely through mitigating oxidative stress and normalizing hormone levels and ovarian function.

KEYWORDS: Di-(2-ethylhexyl) phthalate; Ascorbic acid; Oxidative stress; Apoptosis; Ovarian morphology

Key Points

Question: Does ascorbic acid effectively mitigate reproductive toxicity in female rats caused by di-(2-ethyl hexyl) phthalate (DEHP)?

Findings: This study found that ascorbic acid restored serum reproductive hormone levels and normalized gonadotropin receptor expression affected by DEHP toxicity. Additionally, ascorbic acid reduced apoptosis in ovarian tissue.

Meaning: The study findings have potential implications for developing therapeutic strategies to combat reproductive dysfunction induced by environmental toxins like DEHP in humans.

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1. Introduction

Endocrine-disrupting chemicals (EDCs), termed so due to their interference with hormone production, storage, metabolism, and action are recognized as environmentally toxic compounds that have a detrimental impact on reproductive health[1]. Phthalates, recognized as EDCs, play a crucial role in imparting plasticity to materials. Their non-covalent bonding nature enables them to gradually leach into the environment, resulting in their persistent release as significant environmental contaminants[2]. Di-2-ethylhexyl phthalate (DEHP) stands out as the most prevalent member among the various classes of phthalates. DEHP continues to be the primary component in food wraps, medical devices, and cosmetic merchandise. Globally, around 18 million pounds of phthalates are utilized, mainly for improving the flexibility of polyvinyl chloride (PVC), with well over 2 million tons of DEHP produced annually[3]. The initial stage in the absorption and metabolic process of DEHP involves its hydrolysis by lipase enzymes, resulting in the breakdown into mono-2-ethyl hexyl phthalate (MEHP) and 2-ethyl hexanol. These metabolites are potentially responsible for the toxicological effects associated with DEHP[4]. Several studies have reported that *in vivo* exposure to DEHP leads to disruption in ovarian folliculogenesis[5] and alters ovarian hormonal and receptor levels[6]. MEHP impedes antral follicle growth, interferes with steroidogenesis, and triggers atresia in mouse ovaries[6,7].

Liu *et al* showed that DEHP treatment in lactating mice decreases estradiol levels, increases oxidative stress (OS) markers, damages oocytes, and leads to decreased proliferation and increased apoptosis[8]. OS is increased due to an imbalance between antioxidants and pro-oxidants. Pro-oxidants such as reactive oxygen species (ROS) and reactive nitrogen species (RNOS) greatly impact ovulation, steroidogenesis, and folliculogenesis, thus leading to hormonal imbalance[9].

DEHP inhibits follicle growth through OS, leading to follicular atresia and disruption of ovarian processes[10]. Antioxidants such as catalase or superoxide dismutase (SOD) antioxidants, ascorbic acid are potent antioxidants capable of scavenging free radicals. This property enables ascorbic acid to protect the oocyte from oxidative damage and aid in the production of ovarian hormones through the hydroxylation process in steroidogenesis[11]. Indeed, an intriguing clinical study found that supplementation with vitamins C and E in patients with endometriosis led to a decrease in malondialdehyde (MDA) and lipid peroxide levels. This reduction contributed to an improvement in reproductive function[12].

Several experimental studies have investigated the effects of vitamin C or ascorbic acid as antioxidants on DEHP-induced male reproductive toxicity in animals, yet there remains a gap in understanding the impact of ascorbic acid on female reproductive toxicity induced by DEHP[13,14]. There remains a scarcity of literature focusing on natural antioxidants like ascorbic acid to mitigate DEHP-induced toxicity, particularly about female reproductive health.

Liu *et al* demonstrated the protective role of ascorbic acid against MEHP-induced dysregulated folliculogenesis in cultured neonatal mouse ovaries[15]. While promising, the use of cultured ovaries instead of an *in vivo* rodent model limits the generalizability of these findings. Nonetheless, the study underscores the need for further research on ascorbic acid's role in DEHP-induced female reproductive toxicity in animal models.

The present study extends our previous research, in which we exposed adult female Wistar rats through oral DEHP doses of 1, 10, and 100 mg/kg body weight. We observed a dose-dependent effect on uterine histology, with the 100 mg/kg dose having the most pronounced impact[16]. The 10 mg dose is relevant to routine exposure levels, while the 100 mg dose is relevant to occupational exposure levels[17]. In a two-generational toxicity assay, the single lowest observed adverse effect level showed that DEHP at 141 mg/kg body weight per day decreased fertility rates in breeding pairs and reduced the number of live pups per litter[18].

The current research focused on evaluating the protective effects of ascorbic acid against DEHP-induced reproductive toxicity in pubertal Wistar rats, specifically its impact on reproductive hormones, OS, and related ovarian health parameters. In addition to analyzing hormone levels and OS markers, we investigated the molecular mechanisms of DEHP's effects on ovarian function by examining gonadotropin receptor expression and apoptosis. Furthermore, ovarian morphology changes were assessed to provide a comprehensive understanding of ascorbic acid's potential in mitigating DEHP-induced reproductive damage.

2. Materials and methods

2.1. Experimental animals

Female Wistar rats aged 30 days, weighing (60±10) g were obtained from the Central Animal Research Facility. Polypropylene cages were used for housing the animals (3 animals per cage). The animals were maintained under definite temperature [(22±2) °C], humidity [(55±5)%] and a consistent 12-h light/12-h dark schedule. Animals were fed a standard pellet diet with *ad libitum* access to fresh drinking water.

Forty female Wistar rats aged postnatal day (PND) 35 were randomly assigned to five groups. The oral gavage treatments administered were as follows: Group 1 received corn oil (vehicle), Group 2 received DEHP at a dose of 10 mg/kg body weight/day, Group 3 received DEHP at a dose of 100 mg/kg body weight/day, Group 4 received DEHP at a dose of 10 mg/kg body weight/day along with ascorbic acid at a dose of 100 mg/kg body weight/day, and group 5 received DEHP at a dose of 100 mg/kg body weight/day along with ascorbic acid at a dose of 100 mg/kg body weight/day. Treatments were administered according to the respective body weights of the rats for a duration of 30 consecutive days. Since DEHP is lipid soluble substance, we have used corn oil as used in

earlier experiments from the past work by the researchers.

Ascorbic acid is water soluble and can be administered orally by dispersing it in water at a dose of 100 mg/kg body weight per day to prevent the OS induced by DEHP. Ascorbic acid dispersed in distilled water, was orally administered to the rats. Estrous cycles were monitored from approximately PND 45 to PND 50, starting five days after vaginal opening, until PND 65, with treatment cessation at the diestrus phase (30-32 days after the completion of treatment). The animals falling in the diestrus phase proceeded further for blood collection and were euthanized using Pentobarbital IP.

Ovarian tissues were promptly excised, with adjacent fatty tissue removed and stored for subsequent analysis. Blood samples collected in red vacutainers were allowed to clot at room temperature for 10 min before being centrifuged at 2000 *g* for 5 min. The right ovary was fixed in 10% neutral buffered formalin, while the left ovary was stored at -80 °C for further analysis of gene expression and OS markers.

2.2. Chemicals and reagents

Diethyl phthalate (D201154), corn oil (C8267), and ascorbic acid/L-ascorbic acid (A92902) were obtained from Sigma-Aldrich (St. Louis, MO). Follicle-stimulating hormone (FSH) (ITLK01315) and luteinizing hormone (LH) (ITLK02367) kits were sourced from Immunotag, USA. Estradiol (E₂) (CSB-E05110r), progesterone (CSB-E07282r), and testosterone (CSB-E05100r) were acquired from Cusabio, USA. RNA iso plus (Cat No. 9108), PrimeScriptTM RT reagent kit (Cat No. RR037A), TB green premix Ex TaqTM II kit (RR820A), and in situ apoptosis detection kit (MK505) were purchased from Takara. Proteinase K (Cat No. TC687) was obtained from Himedia. Chemicals required for other procedures were sourced from Sigma, MP Biomedicals, and Sisco Research Laboratories, India.

2.3. Hormonal assay

FSH, LH, estradiol, progesterone, and testosterone levels in rat serum samples were measured following the manufacturer's protocol and guidelines for each respective assay kit. Enzyme linked immunosorbent assay (ELISA) plates were read using a plate reader, and samples were analyzed in duplicate to ensure the accuracy and reliability of the values obtained.

2.4. Gene expression analysis

The gene expression analysis for the follicle stimulating hormone receptor (*FSHR*) and luteinizing hormone receptor (*LHR*) was conducted in rat ovaries. Total RNA was extracted from the

Table 1. List of primer sequences.

Gene	Accession number	Primer sequence
β -actin	NM_031144.3	Forward 5'-CGTCCACCCGCGAGTACAACC-3' Reverse 5'-TCCATGGCGAACTGGTGGCG-3'
<i>FSHR</i>	NM_199237.1	Forward 5'-TGGCATTCTTGGGCACGGGA-3' Reverse 5'-TTCATGGCGTTCCGGGGGA-3'
<i>LHR</i>	NM_012978.1	Forward 5'-ACGCTCATCGCCCTGTCTTCT-3' Reverse 5'-ATTCTGAAGGCGCAGCAGTGG-3'

FSHR: follicle stimulating hormone receptor; LHR: luteinizing hormone receptor.

ovaries using Takara RNA iso plus. The extracted RNA underwent quality and integrity checks *via* agarose gel electrophoresis and quantification using a Qubit 4 fluorometer system.

Subsequently, the total ovarian RNA was reverse transcribed into complementary DNA (cDNA) using the PrimeScriptTM RT reagent kit from Takara. The generated cDNA, along with specific forward and reverse primers (Table 1), was utilized with the TB Green Premix Ex TaqTM II kit for qRT-PCR analysis. Reactions were conducted in triplicate using a Real-Time PCR system (Applied Biosystems-Quant studio 5) under defined PCR conditions.

Primer design was executed using the NCBI BLAST tool. The comparative threshold cycle (CT) was quantified and calculated for relative expression employing the $2^{-\Delta\Delta CT}$ method. To ascertain the normalized expression level for each gene, individual data points were normalized to the housekeeping gene β -actin.

2.5. OS markers

The assessment of OS markers was conducted using 50 mg of ovarian tissue homogenized in 2 mL of phosphate buffered saline (PBS) (pH 7.4). The ovarian tissue homogenate was employed for the estimation of glutathione (GSH) and lipid peroxidation (LPO), while catalase and SOD activities were determined in the ovarian post-mitochondrial fraction. The total protein content for both the ovarian cytoplasmic tissue homogenate and the mitochondrial fraction was quantified to express the status of OS markers accurately[19].

Glutathione (GSH) was assessed in the ovarian tissue homogenate, which was prepared in ice-cold PBS (pH 7.4) using a homogenizer at 3600 *g* for 5 min. The homogenate was mixed with 25% trichloroacetic acid and centrifuged at 1600 *g* for 15 min at 4 °C. After separating the supernatant, 0.2 M phosphate buffer (pH 8.0) and 0.6 mM DTNB solution were added. The samples were incubated at room temperature for 10 min, and spectrophotometer readings were taken at 410 nm (JASCO, v-530, Tokyo, Japan). The result was expressed as micromoles of GSH per milligram of tissue protein[20].

Catalase activity levels were determined in the ovarian post-mitochondrial fraction (PMF), obtained as the supernatant from ovarian homogenate centrifuged at 10000 *g* for 30 min at 4 °C. The PMF was mixed with 0.05 M phosphate buffer at pH 7.0. The decrease in optical density per minute for three readings was

measured using a spectrophotometer at 240 nm (JASCO, v-530, Tokyo, Japan), with the initial optical density set at 0.5. The results were expressed as international units (IU) per milligram of protein[21].

SOD activity was assessed in the ovarian PMF. The PMF was mixed with a solution containing 0.1 mM EDTA, 50 mM sodium carbonate (pH 10), 1.5 mM nitroblue tetrazolium (NBT), 0.6% Triton X-100, and hydroxylamine hydrochloride. The kinetic readings were recorded for 3 min at an optical density of 560 nm using a spectrophotometer (JASCO, v-530, Tokyo, Japan). The activity of SOD was expressed as IU per milligram of protein[22].

LPO was evaluated in ovarian tissue homogenate. The homogenate was subjected to incubation in Tris HCl buffer (pH 7.4) containing 0.3 moles of NADPH in a metabolite shaker at 37 °C for 60 min. Subsequently, the reaction was halted using an ice-cold mixture of TCA-HCl. The supernatant was obtained by centrifuging the mixture for 10 min at 3000 *g*. This supernatant was then combined with 1% thiobarbituric acid (TBA) and incubated in a boiling water bath for 15 min. The concentration of MDA-TBA was determined using a plate reader at 535 nm, utilizing an extinction coefficient of $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$. The outcomes were expressed as nanomoles of MDA-TBA chromophores formed per minute per milligram of protein. The levels of the MDA-TBA complex formed served as an indicator for estimating the levels of LPO[23].

2.6. Histopathological examination

The ovarian tissue, initially fixed in 10% neutral buffered formalin, underwent a series of steps including processing and embedding in paraffin wax. Subsequently, 5-micron thick paraffin sections were prepared, followed by deparaffinization and staining with hematoxylin and eosin. These stained sections were then examined using a Nikon microscope Eclipse 80i (Nikon Instruments Inc., Japan).

For each experimental group, four distinct ovarian tissue samples were analyzed, with two sections (middle covering all follicles) examined per ovary. Follicles at various stages of development, including growing follicles, were manually counted in two separate sections of each ovary under magnifications of 40×, 100×, and 400×. Additionally, atretic follicles and corpus luteum follicles were manually quantified from two sections of each ovary and presented[24].

2.7. In situ apoptosis staining

Paraffin-embedded tissue sections were subjected to deparaffinization and treated with 20 µg/mL proteinase K on slides, followed by a 15-minute incubation at room temperature. Afterwards, the slides were washed with PBS. A labelling reaction mixture, consisting of TdT enzyme and labelling safe buffer, was

applied to the slide, and the slides were then incubated in a 37 °C humidified chamber. The reaction was halted by washing the slides three times for 5 min each with PBS. Subsequently, an Olympus BX 41 microscope (Olympus Corporation, Tokyo, Japan) equipped with cell sense software was utilized to capture images of the FITC-labelled slides. The percentage of TUNEL-positive (+) cells relative to a total of 10 000 granulosa cell counts from three distinct biological ovarian sections in each group was recorded as the apoptotic index[25].

2.8. Statistical analysis

Statistical analyses were made using GraphPad Prism 11.0 software (GraphPad Software, San Diego, CA, USA). Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by *post hoc* comparison using Dunnett's test for multiple comparisons. All data were presented as the mean ± standard difference (mean ± SD). Statistical significance was considered at $P < 0.05$.

2.9. Ethics statement

This study was approved by the Institutional Animal Ethics Committee (IAEC/KMC/66/2020; ethics date: 20.05.2020). Animals were housed in accordance with guidelines set forth by the Committee for Control and Supervision on Experiments on Animals.

3. Results

3.1. Effect of ascorbic acid on DEHP-induced serum hormonal alterations

The serum FSH level showed a significant decrease in the high-dose DEHP-treated group (group 3) compared to the control group (group 1) ($P = 0.01$). A significant increase in FSH levels was observed in the high-dose DEHP with ascorbic acid group (group 5) compared to the high dose DEHP group ($P = 0.01$). Though there was a decline in FSH levels in the low-dose DEHP-treated group compared to the control group and the mitigating effect was observed in the low-dose DEHP-treated group with ascorbic acid (group 4), compared to low dose DEHP group, the levels were not significant (Figure 1A).

Serum LH levels exhibited a significant decrease in both the low-dose (group 2) ($P < 0.001$) and high-dose (group 3) ($P < 0.001$) DEHP groups compared to the control group. A significant increase in LH levels was observed in the high-dose DEHP+ascorbic acid treated group (group 5) compared to the high-dose DEHP exposure group ($P = 0.007$). Though there was an alleviating effect observed in the low-dose DEHP-treated group with ascorbic acid (group 4), compared to low dose DEHP group, the levels were not significant (Figure 1B).

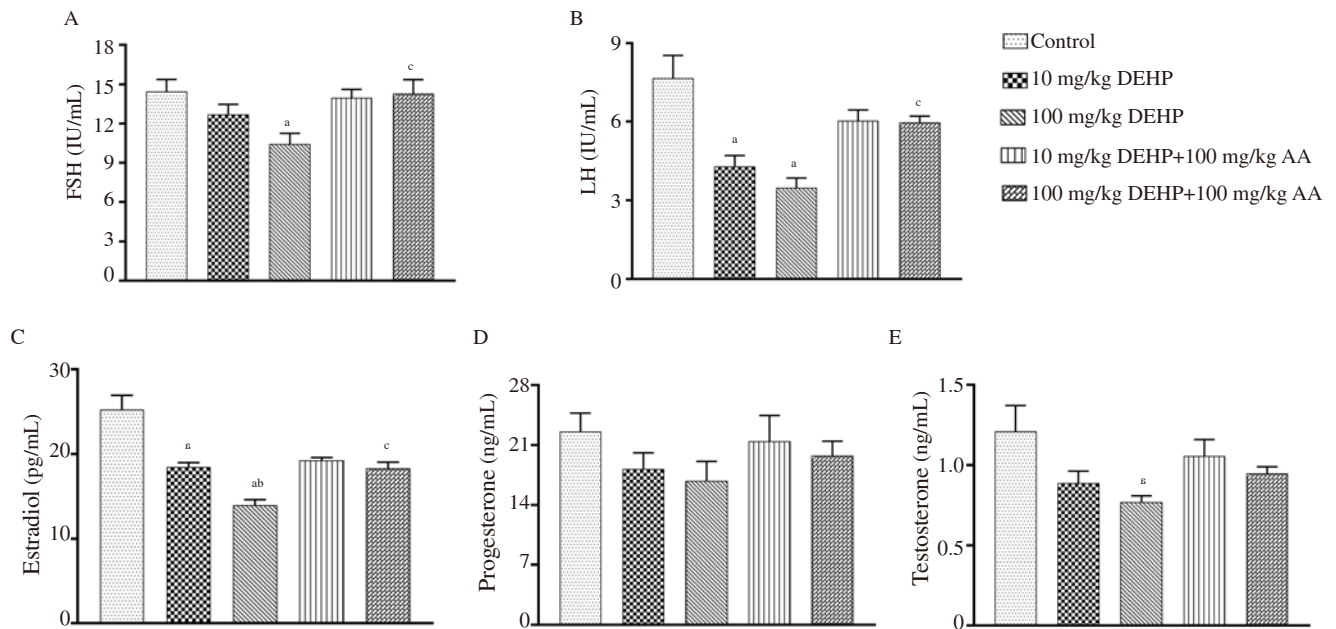


Figure 1. Effect of ascorbic acid (AA) on di-2-ethylhexyl phthalate (DEHP)-induced serum ovarian hormone alterations. A) Follicle-stimulating hormone (FSH); B) Luteinizing hormone (LH); C) Estradiol; D) Progesterone; and E) Testosterone. ^a $P<0.05$: compared to the control group; ^b $P<0.05$: compared to the 10 mg/kg DEHP group; ^c $P<0.05$: compared to the 100 mg/kg DEHP group.

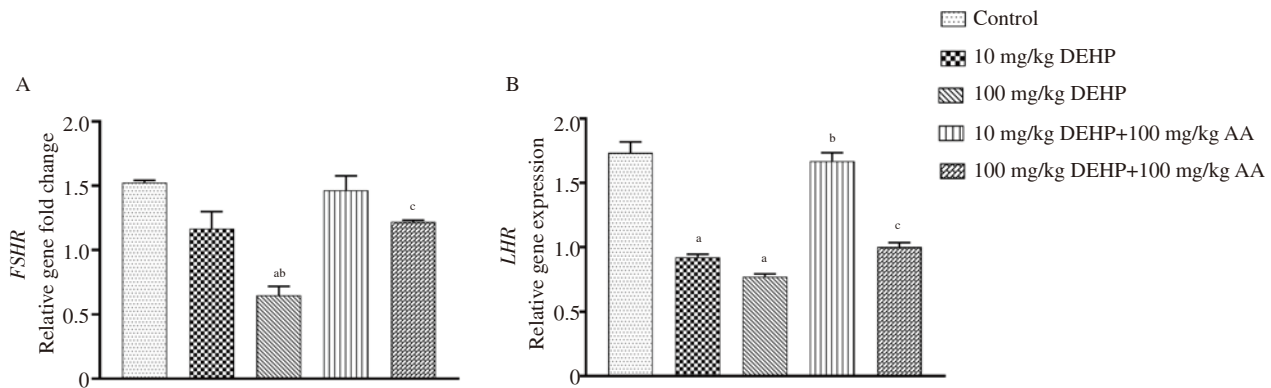


Figure 2. Effect of ascorbic acid (AA) on di-2-ethylhexyl phthalate (DEHP)-induced changes in FSH and LH receptor gene expression. A) *FSHR* relative expression levels; B) *LHR* relative expression levels. ^a $P<0.05$: compared to the control group; ^b $P<0.05$: compared to the 10 mg/kg DEHP group; ^c $P<0.05$: compared to the 100 mg/kg DEHP group. FSHR: follicle stimulating hormone receptor; LHR: luteinizing hormone receptor.

Serum estradiol hormone levels displayed a significant decrease in both the low-dose (group 2) ($P<0.001$) and high-dose DEHP-treated groups (group 3) ($P<0.001$) compared to the control group (group 1). The estradiol levels were significantly increased in the high-dose DEHP+ascorbic acid group (group 5) ($P=0.01$) compared to the high-dose DEHP group (group 3). Though the incline of estradiol levels was observed in the low-dose DEHP+ascorbic acid group (group 4) compared with the low-dose DEHP group (group 2), the levels were not significant. The high dose DEHP (group 3) showed significant decline level of estradiol compared to low dose DEHP (group 2) which is a dose dependent effect (Figure 1C).

Serum progesterone levels did not demonstrate any significance in multiple group comparisons, although there was a decreased level of progesterone in the high-dose DEHP group (group 3) compared to the control group (Figure 1D). Serum testosterone levels exhibited a

significant decrease in the high-dose DEHP-treated group (group 3) compared to the control group ($P=0.029$) (Figure 1E).

3.2. Effect of ascorbic acid on DEHP-induced FSH and LH receptor gene expression alterations

Ovarian *FSHR* relative gene expression showed a significant decrease in high-dose DEHP group (group 3) ($P<0.001$) compared to the control group (group 1). The group treated with high-dose DEHP+ascorbic acid (group 5) showed increased *FSHR* expression compared to the high dose DEHP treated group (group 3) ($P=0.003$). Low-dose DEHP (group 2) did not show significant decrease compared to the control group. High-dose DEHP (group 3) showed significant increase in *FSHR* expression levels when compared with low-dose DEHP group (group 2) (Figure 2A).

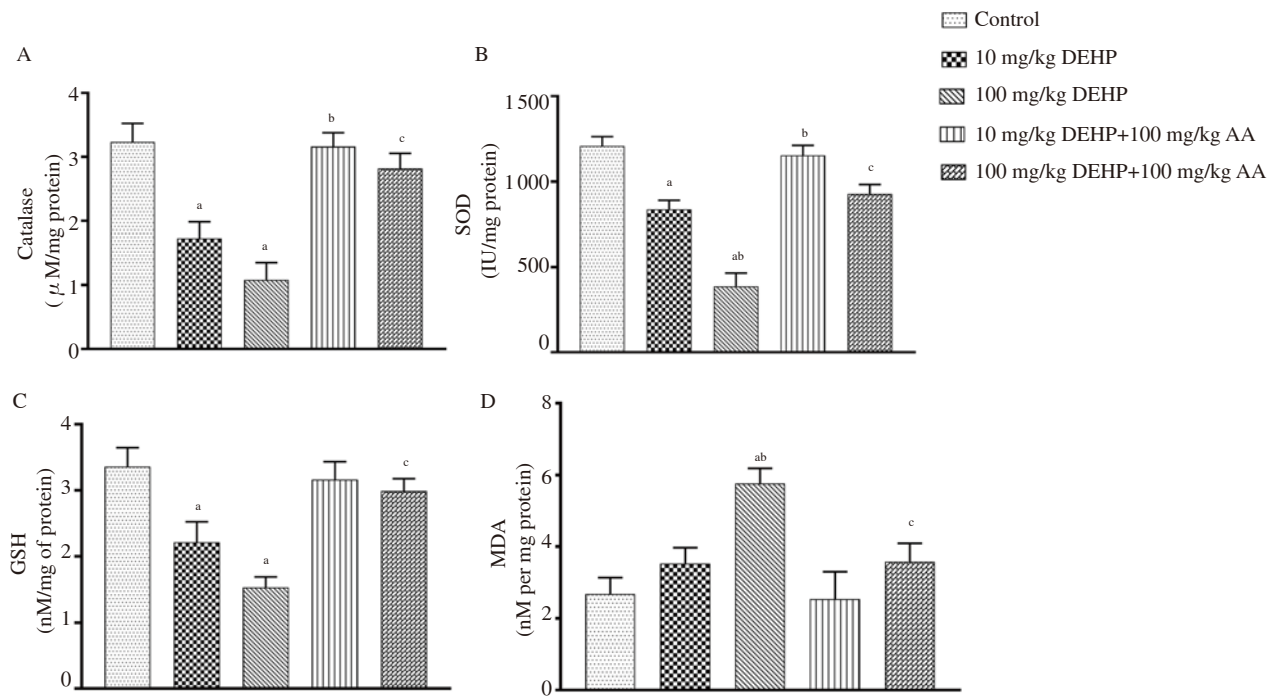


Figure 3. Effect of ascorbic acid (AA) on di-2-ethylhexyl phthalate (DEHP)-induced ovarian oxidative stress. A) Catalase; B) Superoxide dismutase (SOD); C) Glutathione (GSH); and D) Malondialdehyde (MDA). ^a $P < 0.05$: compared to the control group; ^b $P < 0.05$: compared to the 10 mg/kg DEHP group; ^c $P < 0.05$: compared to the 100 mg/kg DEHP group.

Ovarian *LHR* relative gene expression decreased in both the low-dose (group 2) ($P < 0.001$) and high-dose DEHP group (group 3) ($P < 0.001$) compared to the control group (group 1). The inclined levels of *LHR* expression levels were observed in the low-dose DEHP+ascorbic acid (group 4) ($P < 0.001$) compared with low-dose DEHP group (group 2). Also increased level of *LHR* in the high-dose DEHP+ascorbic acid group (group 5) ($P = 0.009$) compared with high-dose DEHP group (group 3) was noted (Figure 2B).

3.3. Effect of ascorbic acid on DEHP-induced oxidative marker alterations

Catalase levels showed a significant decrease in both the low-dose ($P = 0.002$) and high-dose ($P < 0.001$) DEHP-treated groups compared to the control group. In contrast, a significant increase was observed in both the low-dose ($P < 0.001$) and high-dose ($P = 0.004$) DEHP cotreated with ascorbic acid groups compared with the low- and high-dose DEHP-treated groups (Figure 3A).

SOD levels exhibited a significant decrease in both the low-dose ($P = 0.002$) and high-dose ($P < 0.001$) DEHP-treated groups compared to the control group. Furthermore, the high-dose DEHP-treated group demonstrated a significant decrease compared to the low-dose DEHP-treated group ($P < 0.001$). Conversely, a significant increase was observed in both the low-dose ($P = 0.007$) and high-dose ($P < 0.001$) DEHP cotreated with ascorbic acid groups compared with low- and high-dose DEHP groups (Figure 3B).

GSH levels showed a significant decrease in both the low-dose

($P = 0.022$) and high-dose ($P < 0.001$) DEHP-treated groups compared to the control group. Additionally, a significant increase was observed in the group cotreated with high-dose DEHP and ascorbic acid ($P = 0.003$) compared with the group treated with high-dose DEHP group (Figure 3C).

LPO, the levels of MDA a marker of lipid peroxidation, exhibited a significant increase in the high-dose DEHP-treated group ($P = 0.003$) compared with the control group. Moreover, the high-dose DEHP-treated group also demonstrated a significant increase compared with the low-dose DEHP-treated group ($P = 0.038$). Conversely, a significant decrease was observed in the high-dose DEHP cotreated with ascorbic acid group ($P = 0.044$) compared with the high-dose DEHP group (Figure 3D).

3.4. Effect of ascorbic acid on DEHP-induced ovarian histopathological alterations

The animals treated with low-dose DEHP showed an ovarian stromal cortex containing follicles at various stages of maturation, occasionally displaying Graafian follicle formation, in comparison to the control-treated tissues. A slight reduction in follicles with atrophic changes and disrupted layers was observed in the low-dose DEHP-treated group (Figure 4).

Significant changes were observed in the tissues treated with high-dose DEHP, characterized by a decrease in the number of follicles and accompanying stroma showed atrophic changes, and increased corpus luteum (Figure 4). However, the diameter of mature follicles

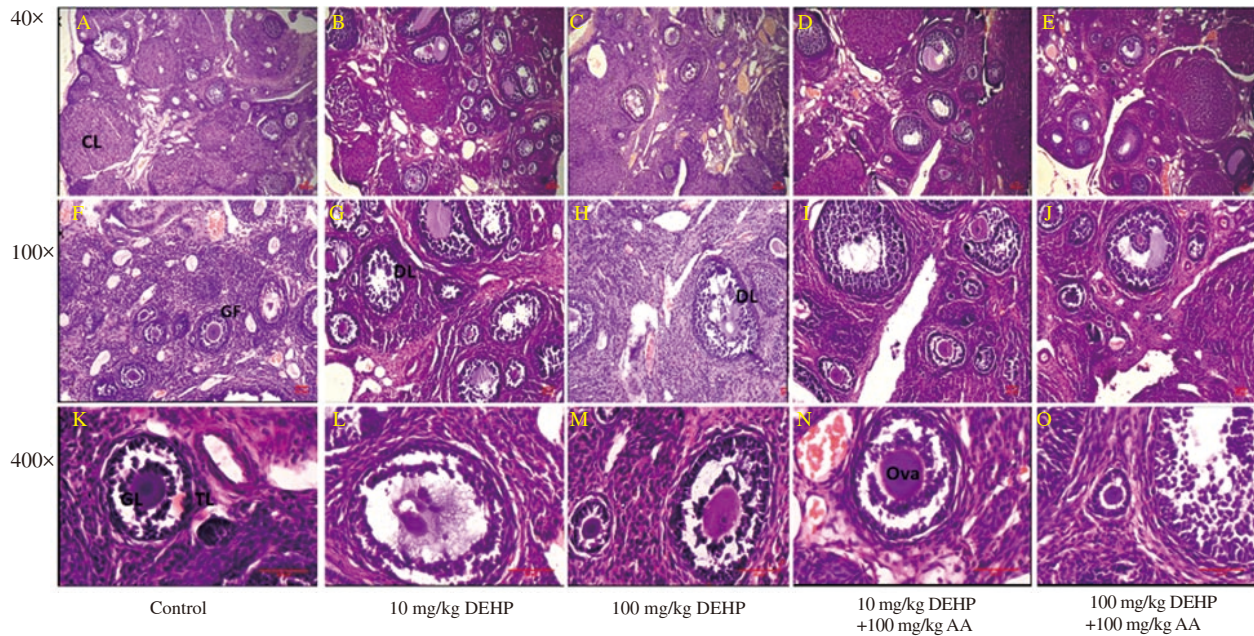


Figure 4. Effect of ascorbic acid (AA) on di-2-ethylhexyl phthalate (DEHP)-induced ovarian morphological alterations under magnification 40×, 100× and 400× (hematoxylin and eosin staining). Low 10 mg/kg DEHP & high dose 100 mg/kg DEHP showed decreased growing follicles (GF), increased granulosa (GL) & theca layer (TL) disruption, disrupted layer (DL), corpus luteum (CL) and atresia (A) which is comparable with the control group. Low-dose DEHP treated with AA and high-dose DEHP treated with AA have mitigated this morphological alteration, and the follicles are in comparison to the control group. A, F, K: the control group at magnification 40×, 100× and 400×, respectively; B, G, L: 10 mg/kg DEHP at magnification 40×, 100× and 400×, respectively; C, H, M: 100 mg/kg DEHP at magnification 40×, 100× and 400×, respectively; D, I, N: 10 mg/kg DEHP+100 mg/kg AA at magnification 40×, 100× and 400×, respectively; E, J, O: 100 mg/kg DEHP+100 mg/kg AA at magnification 40×, 100× and 400×, respectively.

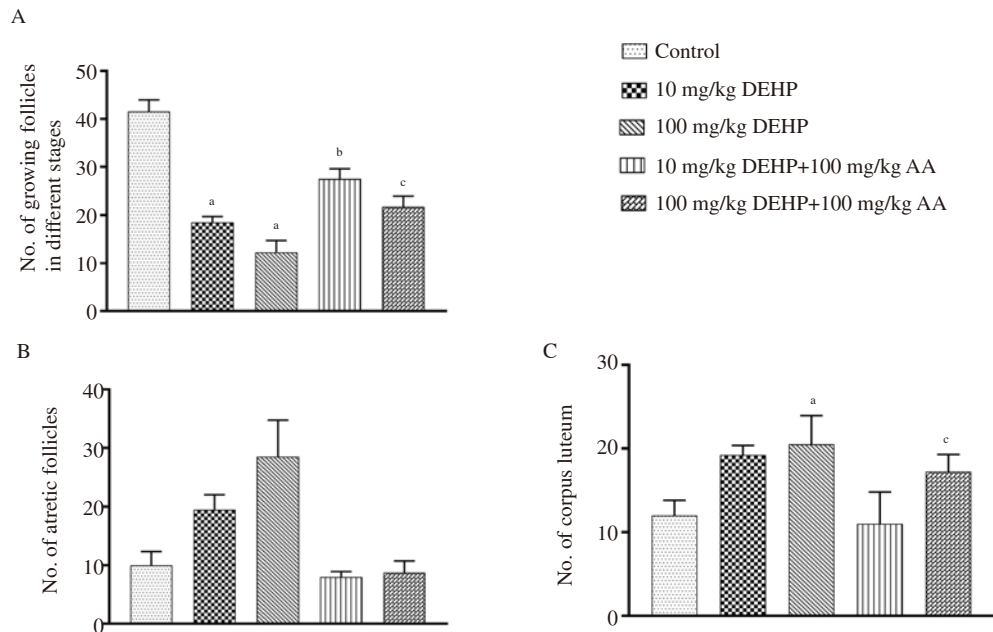


Figure 5. Effect of ascorbic acid (AA) on di-2-ethylhexyl phthalate (DEHP)-induced histopathological changes. A) Number (No.) of growing follicles in different stages; B) Number of atretic follicles; and C) number of corpus luteum. ^a*P*<0.05: compared to the control group; ^b*P*<0.05: compared to the 10 mg/kg DEHP group; ^c*P*<0.05: compared to the 100 mg/kg DEHP group.

remained comparable to that of the control group.

Tissues treated with both low and high doses of DEHP cotreated with ascorbic acid displayed maintained cortical stroma, exhibiting a cellular, spindly appearance with mitotic figures, suggestive of regeneration compared to the low- and high-dose DEHP groups. No

features of atrophy or inflammation were noted (Figure 4). Follicles at various stages of maturation, including occasional Graafian follicle formation, were also observed, comparable to the control group (Figure 5).

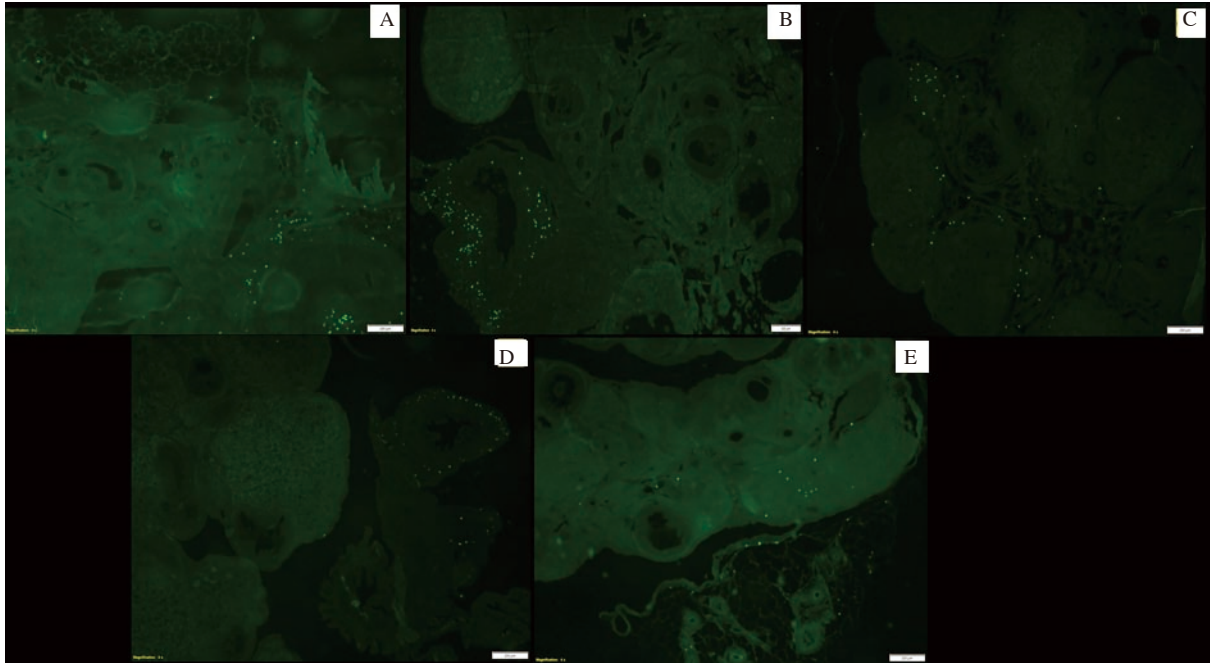


Figure 6. Effect of ascorbic acid (AA) on di-2-ethylhexyl phthalate (DEHP)-induced ovarian apoptosis assay (TUNEL staining; magnification 40×). An increase in apoptotic cells in low-dose and high-dose DEHP-treated ovarian section are observed compared to the control group section which is mitigated by DEHP+AA treatment in both low- and high-dose DEHP groups. A: the control group; B: 10 mg/kg DEHP; C: 100 mg/kg DEHP; D: 10 mg/kg DEHP+100 mg/kg AA; E: 100 mg/kg DEHP+100 mg/kg AA.

3.5. Effect of ascorbic acid on DEHP-induced apoptotic changes

The control group exhibited minimal fluorescence within the ovarian stroma. In contrast, both the low and high-dose DEHP-treated cases showed a notable increase in fluorescence, indicating an increase in apoptotic products. Conversely, the low- and high-dose DEHP groups treated with ascorbic acid displayed a reduction in fluorescence comparable to that of the DEHP-treated groups, suggesting a decrease in apoptosis (Figure 6). The apoptotic index of ovarian sections from each group was plotted as the percentage of TUNEL-positive cells (Figure 7).

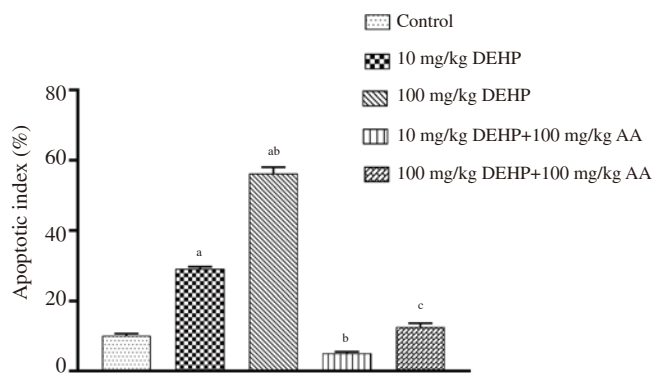


Figure 7. Effect of ascorbic acid (AA) on di-2-ethylhexyl phthalate (DEHP)-induced percentage change in apoptotic index. ^a $P < 0.05$: compared to the control group; ^b $P < 0.05$: compared to the 10 mg/kg DEHP group; ^c $P < 0.05$: compared to the 100 mg/kg DEHP group.

4. Discussion

Widespread exposure to DEHP, a well-known plasticizer component, results in endocrine disruption at different toxicity levels in both human and animal populations[26]. Exposure to DEHP increases the risk of ovarian dysfunction in women, which is a serious threat to the public's health. A significant correlation was observed in a large cross-sectional study among *in vitro* fertilization patients between phthalate levels (ranging from 0.73 ng/mL to 2.20 ng/mL) that include DEHP in human follicular fluid and ovarian hormonal disturbances[27].

Reports indicate that ascorbic acid may potentially mitigate the harmful effects of DEHP in male laboratory rats[16]. In neonatal mouse ovaries exposed to MEHP, a significant increase in primary follicles and the activation of the PI3K signaling pathway were observed. Additionally, there was an inhibition of ovarian steroidogenesis, indicated by a reduction in the number of mature follicles[28]. Li *et al* showed that DEHP could prolong the estrous cycle with increased follicular atresia by promoting granulosa cell apoptosis, inhibiting sex hormone secretion, and reducing steroidogenic enzyme expression[29]. The current study investigated the effects of ascorbic acid against DEHP-induced female reproductive toxicity. The high-dose DEHP group exhibited decreased hormonal levels of FSH, LH, estradiol, progesterone and testosterone, confirming the DEHP effect on steroidogenesis, thereby leading to toxicity, which was in line with previous studies[28,29]. Exposure to microplastics and DEHP disrupted granulosa cells, leading to follicular fragmentation and atresia in mice. This disruption was accompanied by increased levels of ROS[30]. Elevated

ROS induce OS, leading to the activation of nuclear factor kappa B, hypoxia-inducible factor-1, and activator protein-1, which further leads to hyperandrogenism and inflammation in the ovaries, potentially resulting in conditions such as polycystic ovarian syndrome and infertility[31]. In our study, the low or high dose DEHP-treated group showed significant disruption to follicles with an increased count of atretic follicles and corpus luteum in the animal ovaries. Furthermore, antioxidant levels such as catalase, SOD, and GSH in ovarian tissues were decreased in both the low and high-dose DEHP-treated groups, while the MDA was notably increased in both groups which indicates the effect on folliculogenesis and OS. Examination of ovarian sections stained with TUNEL exhibited an increased percentage of apoptotic cells in the DEHP-treated groups, indicating its toxicity, as consistently reported in the previous animal studies[28,30].

The activation and binding of FSH to its receptor, as part of the hypothalamic-pituitary-gonadal (HPG) axis, stimulates the production of the aromatase enzyme. This enzyme is crucial in steroidogenesis, follicle maturation, and the initiation of ovulation[31]. Mutations in the *FSHR* and *LHR* can disrupt folliculogenesis and hormonal regulation, potentially leading to infertility and polycystic ovarian syndrome[32]. In this regard, while we investigated the expression levels of *FSHR* and *LHR* levels in ovarian tissues, we could see the levels reduced in both low and high-dose DEHP groups, confirming the disruption in folliculogenesis including the HPG axis.

Antioxidants are pivotal in maintaining female reproductive function as they aid in the metabolism of oocytes and support the vasculature of the uterus. Moreover, antioxidants play a vital role in shielding reproductive tissues from oxidative damage, thus enhancing overall reproductive health and function[33]. Ascorbic acid serves as a crucial antioxidant with diverse roles in reproductive processes. It contributes significantly to follicle development, hormone synthesis, tissue remodelling, follicle repair, and protection from oxidative damage. These multifaceted functions highlight the essential role of ascorbic acid in sustaining reproductive health and function[34]. Ascorbic acid reserves in the granulosa cells and theca interna of growing and luteinizing follicles are vital for maintaining the redox balance within these follicles. Ascorbic acid acts as a scavenger for ROS, thereby protecting the oocyte from oxidative damage. This helps in preserving the integrity of the oocyte and ensures proper reproductive function[35]. The role of ascorbic acid beyond its antioxidant function also has a role in collagen biosynthesis. Ascorbic acid is essential for the hydroxylation of proline and lysine residues in the synthesis of collagen, a key structural protein found in various tissues including the ovarian stroma and follicles[36]. Studies have shown that ascorbic acid can decrease the expression levels of apoptotic markers such as caspase-3 and caspase-8 in both ovarian and uterine tissues. Further, ascorbic acid has been observed to decrease anti-Mullerian hormone levels, which is indicative of its role in modulating reproductive processes and preserving ovarian

and uterine health[37].

DEHP negatively impacts female reproductive health by disrupting ovarian function and inducing OS in the ovaries. Few studies have proposed that ascorbic acid could be utilized as a potential mitigating compound for the adverse reproductive effects associated with DEHP exposure in males. Choi *et al* demonstrated that vitamins C and E mitigated DEHP-induced male reproductive toxicity by restoring hormonal balance, enhancing antioxidant enzyme activity such as glutathione and SOD, and improving MDA concentrations[13]. Shen *et al* concluded that vitamin C can be beneficial for addressing male infertility induced by DEHP exposure[14].

All these reports are in male animal physiology; hence, the effect of ascorbic acid on DEHP-induced female animal reproductive physiology was elucidated in our findings. Our findings showed ascorbic acid mitigated the toxicity induced by DEHP in female animal reproduction. Both low- and high-dose DEHP plus ascorbic acid treated groups showed protection in serum reproduction hormones, gonadotropin receptor expression levels, MDA levels, and increased antioxidant enzyme activity, leading to the improved ovarian morphology and reduced apoptotic alterations, when compared with low- and high-dose DEHP groups.

In a previous study, low- and high-dose DEHP showed opposite effects on the onset of puberty and reproductive hormones through the hypothalamic kiss-1 peptide[38]. Our findings were in line with this study that the low dose had a less toxic effect on serum hormonal levels, gonadotropin receptor gene expression, and OS markers with decreased apoptosis products compared to high-dose DEHP treatment; thus, a significant protective effect of ascorbic acid in the high-dose DEHP group was observed. Ascorbic acid, as an antioxidant, alleviated the toxic effect of DEHP in female reproduction through the reversal of OS and restoring its folliculogenesis and steroidogenesis which was evident through the ovarian morphology, hormonal levels and receptor expression levels. Emojevwe *et al* investigated the protective effects of *N*-acetylcysteine and zinc sulphate against reproductive dysfunction induced by DEHP in rats, demonstrating their potential to enhance testicular antioxidant status and hormone levels while mitigating OS and receptor signaling disruptions[39]. There are few similar studies investigating on compounds protecting the dysfunction induced by DEHP in both male and female reproductive system.

The limitation of the current study lies in understanding the pathway through which DEHP exposure exerts its toxic effects on reproductive health and how ascorbic acid modulates these effects. This gap in knowledge presents challenges when translating the research findings into therapeutic applications for human reproductive disorders associated with DEHP exposure.

In conclusion, DEHP exhibits toxic effects on female reproduction. Our study also highlights the low and high dose toxicity which is relevant to daily and occupational exposure levels to DEHP, particularly concerning in female reproduction. The high doses pose a greater risk compared to low doses which necessitate therapeutic

interventions. While low-dose DEHP exhibits toxicity, concerning ovarian morphological and physiological alterations, the findings were not significant concerning hormones and receptor levels although OS and apoptosis were present significantly. The study confirms dysregulated steroidogenesis and folliculogenesis in high dose DEHP-treated group through findings such as decreased levels of serum reproductive hormones, FSH and LH receptor gene expression, increased ovarian OS, and apoptosis in ovaries accompanied by morphological changes. Our findings highlight the protective effect of ascorbic acid treatment with both low and high-dose DEHP compared to low and high-dose DEHP-induced female reproductive toxicity. Future investigations should focus on elucidating the underlying mechanisms of ascorbic acid's antagonistic action against DEHP toxicity, dose-response curve, and effect of ascorbic acid on mixed environmental pollutant exposure, offering valuable insights for clinical interventions.

Conflict of interest statement

The authors declare no conflict of interest.

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Authors' contributions

Kalaivani Manokaran, Ravi Sankar Bhaskaran, and Karkala Sreedhara Ranganath Pai contributed to conceptualization. Methodology were performed by Kalaivani Manokaran and Sachin Shetty. Investigations were carried out by Kalaivani Manokaran. The first draft of manuscript was written by Kalaivani Manokaran and all the authors commented on previous versions of the manuscript. Jayesh Mudgal and Vennila Jaganathan were involved in validation, data analysis, manuscript review and editing. Prabu Paramasivam and Dharani Abirama Sundari Shanmugam were involved in manuscript review and editing. Deepak Nayak and Sunitha Carnelio were involved in interpreting the pathological results and manuscript editing. All the authors approved the final manuscript.

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