

Research Article

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Abbreviations

AOacridine orange, ARTassisted reproduction technique, COCscumulus–oocyte complexes, CTCFcorrected total cell fluorescence, DEHPdi(2-ethylhexyl)phthalate, DCFH-DAdichlorofluorescein diacetate, EDCsendocrine-disrupting chemicals, JC-15,5,6,6-tetra-chloro-1,1,3,3-tetra-ethyl-benz-imidazo-carbocyanine iodide, MEHPmono(2-ethylhexyl)phthalate, OSoxidative stress, ROSreactive oxygen species, Plpropidium iodide, TCM-199tissue culture medium

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Encircling granulosa cells protects against di-(2-ethylhexyl)phthalate-induced apoptosis in rat oocytes cultured *in vitro*

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Summary

The present study investigated if the presence of encircling granulosa cells protected against di(2-ethylhexyl)phthalate (DEHP)-induced oxidative stress in rat oocytes cultured *in vitro*. Denuded oocytes and cumulus–oocyte complexes (COCs) were treated with or without various doses of DEHP (0.0, 25.0, 50.0, 100, 200, 400 and 800 μM) *in vitro*. Morphological apoptotic changes, levels of oxidative stress and reactive oxygen species (ROS), mitochondrial membrane potential, and expression levels of apoptotic markers (Bcl2, Bax, cytochrome *c*) were analyzed. Our results showed that DEHP induced morphological apoptotic changes in a dose-dependent manner in denuded oocytes cultured *in vitro*. The effective dose of DEHP (400 μg) significantly ($P > 0.05$) increased oxidative stress by elevating ROS levels and the mitochondrial membrane potential with higher mRNA expression and protein levels of apoptotic markers (Bax, cytochrome *c*). Encircling granulosa cells protected oocytes from DEHP-induced morphological changes, increased oxidative stress and ROS levels, as well as increased expression of apoptotic markers. Taken together our data suggested that encircling granulosa cells protected oocytes against DEHP-induced apoptosis and that the presence of granulosa cells could act positively towards the survival of oocytes under *in vitro* culture conditions and may be helpful during assisted reproductive technique programmes.

Introduction

In mammals, the ovary is a metabolically active organ that provides the necessary micro-environment for germ cell development via folliculogenesis. During this process, any functional disruption may lead to reproductive problems that ultimately affect the reproductive potential of an individual. Di-(2-ethylhexyl)phthalate (DEHP) is one of the most common endocrine-disrupting chemicals (EDCs) and is widely used to make vinyl plastics softer and more flexible (Bauer & Herrmann, 1997; Kambia *et al.*, 2004; Sampson & de Korte, 2011). A metabolite of DEHP, mono(2-ethylhexyl)phthalate (MEHP), has been found in human urine samples (Kato *et al.*, 2004; Heudorf *et al.*, 2007; Hogberg *et al.*, 2008), amniotic fluid (Huang *et al.*, 2009), breast milk (Hogberg *et al.*, 2008) and ovarian follicular fluid (Krotz *et al.*, 2012) indicating that even a limited exposure to DEHP in daily life from any source may become a potential risk factor to modulate ovarian physiology. As the major route for exposure to DEHP in the general population is food and water intake, this indicates its ubiquitous presence and therefore it seems impossible for many mammalian species including human to avoid daily exposure to DEHP.

It has been reported that exposure to DEHP leads to depletion of primordial follicle pools, affects oocyte maturation, decreases sex steroid hormone production and changes the DNA methylation status of imprinted genes (Ambruosi *et al.*, 2011; Li *et al.*, 2016). Studies in animals have suggested that DEHP exposure impairs mouse primordial follicle assembly (Zhang *et al.*, 2013, 2014) and the heritable modification of imprint gene DNA methylation in mouse oocytes (Li *et al.*, 2014; Liu *et al.*, 2017). DEHP has shown hepatotoxicity, reproductive toxicity, and developmental toxicity in experimental animals (Sircar *et al.*, 2008; Engel & Wolff, 2013; Abd-Ellah *et al.*, 2015; Hannon *et al.*, 2015). Recently, Sun *et al.* (2018) reported that DEHP has the ability to interfere with the hypothalamic–pituitary–thyroid axis, with downregulation of the homeostasis of thyroid-related hormones in adolescent rat. Although, the exact mechanism by which DEHP alters ovarian physiology remains unknown, a few studies have indicated that reactive oxygen species (ROS) plays an important role during DEHP-mediated toxicity (Ames, 1999; Erkekoglu *et al.*, 2010). ROS acts as a signalling molecule in various physiological processes and has a potential role in ovarian physiology

(Tripathi *et al.*, 2009). Oocytes, such as other aerobic cells, produce ATP and ROS by mitochondrial oxidative phosphorylation. Therefore, any perturbation in mitochondria or in the activity of scavenger systems can lead to oxidative stress (OS) induction and therefore ROS generation and mitochondrial cytochrome *c* release, which is an important sign of apoptosis. It seems that generation of ROS and/or depletion of the antioxidant system due to DEHP toxicity could result in OS and therefore apoptosis in follicular cells and/or oocytes. However, very little information is available on the precise mechanisms, timing and toxicity of DEHP that are responsible for several aberrations in reproductive functions in mammalian species.

Granulosa cells act as a potent antioxidant system and provide nutrient and growth factors to the developing oocyte via crosstalk between granulosa cells and oocyte (Huang & Wells, 2010; Shaeib *et al.*, 2013; Tiwari *et al.*, 2017). Further granulosa cells also protect the oocytes from harsh environments (Shaeib *et al.*, 2013; Tiwari *et al.*, 2017). Therefore, in this study, we hypothesized that encircling granulosa cells could protect against DEHP-induced oocyte toxicity.

Materials and methods

Chemicals

All chemicals used in this study were obtained from Sigma Chemical Co. (St. Louis, MO, USA) unless stated otherwise. DEHP (99% purity, analytical grade; cat no. 36735) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The DEHP stock solution (2.56 mM) was prepared by diluting 1 mg of DEHP powder in 1.0 ml of dimethyl sulfoxide (DMSO). The stock solution was immediately aliquoted and kept at -20°C until use.

Experimental animal

Sexually immature female rats (50 ± 5 g body weight), Charles-Foster strain, were purchased from the Central Animal House, Institute of Medical Science, Banaras Hindu University, Varanasi, India and housed in air-conditioned, light-controlled rooms, with food and water *ad libitum*.

Preparation of culture medium and DEHP working concentrations

Culture medium (TCM-199) was prepared according to a published protocol (Tripathi & Chaube, 2015). TCM-199 was supplemented with sodium bicarbonate (0.035% w/v), penicillin (100 IU/ml) and streptomycin (100 mg/ml) and the pH was adjusted to 7.2 ± 0.10 . To find out the optimum effective dose of DEHP, different working concentrations (0.0, 25.0, 50.0, 100, 200, 400 and 800 μM) of DEHP were prepared by diluting the stock solution (2.56 mM) with freshly prepared working culture medium. The final concentrations of DEHP did not alter the osmolarity (290 ± 5 mOsmol) and pH 7.2 ± 0.10 of the culture medium. As DMSO was used as a solvent for the DEHP stock solution, an equivalent dilution of the highest concentration (0.02% DMSO) was used in the control group.

Collection of COCs and denuded oocytes

The ovary along with the oviduct was collected from experimental animals that had been subjected to a superovulation induction protocol (Tripathi *et al.*, 2013). Ovulated cumulus-enclosed

oocyte (COCs) were isolated in pre-warmed culture medium under a dissecting microscope (Wild (Heerbrugg), Switzerland) by puncturing the oviduct using a 26-gauge needle attached to a 1 ml syringe. Ovulated cumulus-enclosed oocytes were picked up using microtubing (inner diameter 2 mm) attached to a glass micropipette (inner diameter, 100 μm ; Clay Adams, NJ, USA) and transferred to culture medium with or without 0.01% hyaluronidase at room temperature. After 2 min of treatment, denuded and cumulus containing oocyte were removed, washed three times with fresh culture medium and used for *in vitro* studies.

In vitro exposure of DEHP to COCs and denuded oocytes

Groups of COCs and denuded oocytes (4–6 per group) were placed in culture medium containing different concentrations of DEHP (0.0, 25.0, 50.0, 100, 200, 400 and 800 μM) for 3 h in a CO_2 in air incubator (Model; Galaxy 170 R, New Brunswick, Eppendorf AG, Hamburg, Germany). For the dose- and time-dependent study, oocytes from different treatments groups were evaluated every 1 h for morphological changes using a Phase-Contrast Microscope (EVOS FL, Life Technologies) at $\times 400$ magnification. Based on the dose- and time-dependent study, the dose of 400 μM DEHP was selected to analyze the levels of intracellular ROS, OS, mitochondrial membrane potential (JC1 staining), and analyse apoptotic marker gene expression in both denuded and COCs. All experiments were performed in triplicate.

Oxidative stress detection

The redox status of control/stressed cells was assessed using an OS (ENZ-51042; Enzo Life Sciences, NY, USA) assay kit according to the manufacturer's instructions. In brief, after 3 h DEHP (400 μM) treatment, denuded oocytes and COCs were washed with phosphate-buffered saline (PBS) and incubated with an OS detection mix for 30 min at 37°C in a CO_2 in air incubator, respectively. After incubation, cells were washed with PBS and observed under a fluorescence microscope (EVOS FL, Life Technologies). As per the manufacturer's instructions, OS were detected using a fluorescein isothiocyanate (FITC) filter at 490/525 nm. The ROS inducer (Pyocyanin) was used as the positive control and a sample without the detection mix was used as the negative control for the assay. The experiment was repeated three times to confirm the results.

Intracellular ROS detection

For analysis of intracellular ROS levels in DEHP-treated denuded oocytes and COCs, 10 μM dichlorofluorescein diacetate (DCFH-DA, Sigma Chemical) were used. In detail, after 3 h of DEHP (400 μM) exposure, denuded oocyte and COCs were washed in PBS and incubated with 10 μM DCFH-DA prepared in PBS for 15 min at 37°C respectively. After incubation, oocytes were observed under a fluorescence microscope (EVOS FL, Life Technologies) for fluorescence intensity measurement. The negative control was prepared without adding DCFH-DA in incubation solution.

Assessment of mitochondrial membrane potential

DEHP-treated denuded oocytes and COCs were stained with inner mitochondrial membrane potential reporter dye JC-1 (5,5,6,6-tetrachloro-1,1,3,3-tetra-ethyl-benz-imidazo-carbocyanine iodide) (BD Bioscience) as per manufacturer's instruction. In brief, JC-1 was

prepared to a final concentration of 1 μM in diluting buffer and denuded oocytes and COCs were incubated for 20 min. The stained oocytes were examined under an epifluorescence microscopy in the FITC and rhodamine-B isothiocyanate (RITC) channels using an inverted fluorescence microscope (EVOS FL, Life Technologies). The specific characteristics of JC-1 dye are as follows. When the mitochondrial membrane potential is a low, JC-1 exists as a monomer, and green fluorescence can be detected in the FITC channel. By contrast, when the potential increases, JC-1 monomers assemble into arrays termed J-aggregates that exhibit red fluorescence. Therefore, at high mitochondrial membrane potential ($\Delta\Psi\text{m}$), red fluorescence is detected in the RITC channel. Fluorescence analysis was carried out by measuring the total fluorescence of the entire oocyte. The mean value for each fluorescence was normalized to area of measurement using the corrected total cell fluorescence (CTCF) method (Parry & Hemstreet, 1988). Values were expressed as total red CTCF for individual oocytes from three different treatments.

Acridine orange/propidium iodide nuclear staining

Acridine orange (AO) and propidium iodide (PI) staining were performed to confirm the apoptotic profile as a result of morphological changes in the DEHP-treated denuded oocytes and COCs. In brief, DEHP-treated oocytes and COCs were rinsed with PBS and fixed with 3.7% formaldehyde in PBS (pH 7.4) at room temperature for 20 min. Then, cells were rinsed in PBS twice and stained with AO/PI mix (10 $\mu\text{g}/\text{ml}$ in PBS) for 10 min. Photographs were taken at $\times 200$ and $\times 400$ magnification under an inverted fluorescence microscope (EVOS FL, Life Technologies) from three separate experiments.

Annexin-V/PI staining

Apoptosis was detected in all treated groups using an annexin V-FITC/PI apoptosis detection kit. In detail, the denuded oocytes and COCs were harvested after DEHP (400 μM) treatment and incubated with 200 μl of binding buffer (HEPES-buffered PBS supplemented with 2.5 mM CaCl_2), 2 μl of annexin V-FITC and 5 μl of PI at room temperature for 15 min in the dark, following the manufacturer's instructions. After a final wash in PBS, denuded oocytes and COCs were immediately analyzed under a fluorescence microscope. Cells were excited by light at a wavelength of 488 nm with barrier filters of 525 nm and 575 nm for FITC fluorescence and PI detection, respectively. Data were analyzed and plotted for annexin V-FITC using appropriate software.

Detection of Bax, Bcl-2 and cytochrome c expression

DEHP-treated denuded oocytes and COCs were immediately fixed with 3.7% formaldehyde for 10 min at room temperature. The fixed cells were exposed to sodium tricitrate (0.01 M; pH 6.0) for antigen retrieval. After washing with Triton X-100 (0.01% in PBS) supplemented PBS, cells were then incubated with blocking buffer (5% PBS-BSA solution) at 37°C for 30 min. Thereafter, cells were exposed to 50 μl of their respective primary antibodies (Bax, Bcl₂, Cyt-c, 1:500 dilution in blocking buffer; Santa Cruz Biotechnology) at 37°C for 2 h. After five washes with pre-warmed PBS, slides were exposed to 500 μl fluorescein isothiocyanate (FITC)-labelled secondary antibody (1:1000 dilution in blocking buffer; Santa Cruz Biotechnology) for 1 h at 37°C in a humidified chamber. After 1 h of incubation, cells were washed three times with pre-warmed PBS and then observed under an inverted fluorescence microscope (EVOS FL, Life Technologies).

CTCF measurement for oocytes and COCs was carried out as per the published protocol (Tiwari *et al.*, 2016). All parameters were kept constant for each oocyte, fluorescence intensity was analyzed using ImageJ software (version 1.44 from the National Institutes of Health, Bethesda, USA). For this purpose, a minimum of three different areas for each oocyte cytoplasm, as well as its corresponding background, was selected. Total fluorescence per oocyte was calculated on a Microsoft Excel[®] sheet, by applying the measurements obtained from the analyzed cell using the formula: $\text{CTCF} = \text{integrated density} - (\text{area of selected cell} \times \text{mean fluorescence of background readings})$.

Gene expression analysis by PCR

mRNA was isolated from control as well as DEHP-treated denuded oocytes and COCs using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and then reversed transcribed using a cDNA synthesis kit (Thermo Scientific Inc). RTPCR for different genes: Bax, Bcl₂, cytochrome c and GAPDH was performed using the Maxima hot start green master mix (Thermo Scientific, Rockford, IL, USA) in a thermal cycler (Applied Biosystem). Semi-quantitative RT-PCR was chosen to estimate the transcript level of analyzed genes according to Dubey *et al.* (2012). To control variation in the efficiencies of the RT step among different experimental samples, mRNA concentrations of GAPDH, a housekeeping gene presumed to be expressed at constant amounts in all samples, were also calculated, along with the mRNA concentrations of targeted genes, by densitometry analysis using ImageJ 1.43U software (NIH, Bethesda, Maryland, USA). Relative expression was determined as arbitrary units, defined as the ratio of mRNA level to the corresponding GAPDH mRNA level after subtraction of background intensity:

$$\text{value} = \frac{(\text{intensity; gene of interest} - \text{intensity; background})}{(\text{intensity; GAPDH} - \text{intensity; background})}$$

Mean values of three measurements for each gene in each sample band were taken for analysis.

Statistical analysis

Data were expressed as the mean \pm standard error of the mean (SEM) of three independent experiments. All percentage data were subjected to arcsine square-root transformation before statistical analysis. Data were analyzed by one-way analysis of variance (ANOVA) using SPSS software, version 17.0 (SPSS, Inc., Chicago, IL, USA) followed by Bonferroni post hoc analysis. A probability of $P < 0.05$ was considered to be statistically significant.

Results

DEHP-induced morphological apoptotic changes in denuded oocyte (DOs) and COCs

DEHP (100, 200 and 400 μM) significantly ($P < 0.05$) induced morphological apoptotic changes (shrinkage, cytoplasmic granulation and fragmentation) in denuded oocytes (DOs) as well as in COCs in a dose-dependent manner (one-way ANOVA, $F = 114.28$, $P < 0.05$), while higher doses of DEHP (800 μM) induced degeneration in few denuded oocytes (Fig. 1 and Tables 1 and 2). We did not observe any apoptotic features in denuded oocytes or in COCs at the lower dose of DEHP (25 μM). However, we observed less morphological apoptotic changes in DEHP-treated COCs (Fig. 2) compared with denuded oocytes. Based on dose- and time-

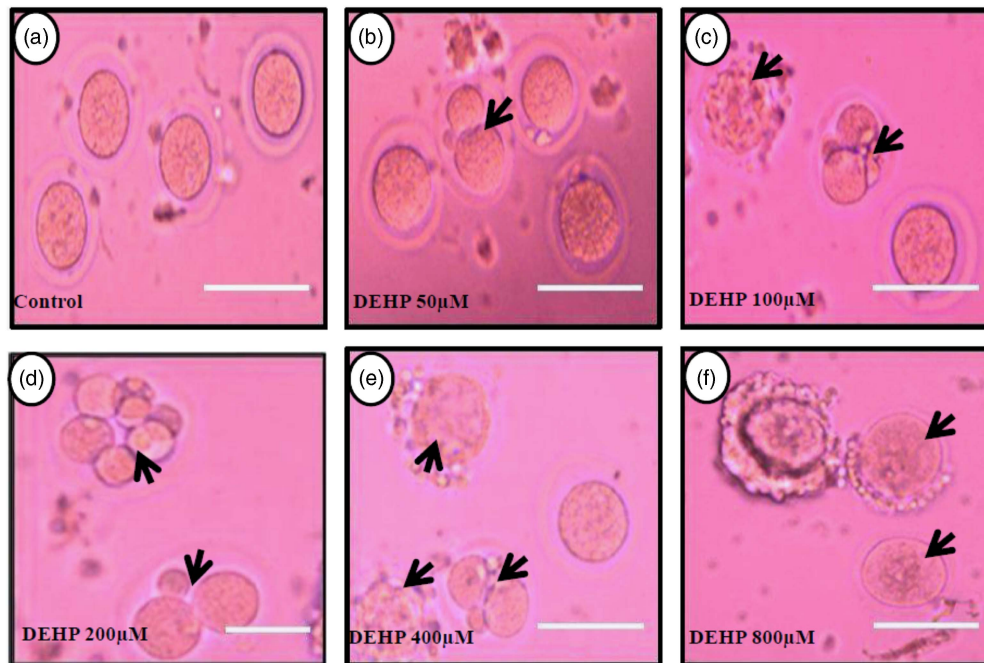


Figure 1. Representative photographs showing DEHP-induced morphological apoptotic changes in a dose-dependent manner in denuded oocytes cultured *in vitro*. Higher doses of DEHP (100, 200, 400 and 800 μM) induced shrinkage, cytoplasmic fragmentation, granulation and degeneration (c-f; black arrows) compared with control and lower dose of DEHP (50 μM) treated oocyte showing normal morphology (a, b). Three independent experiments were conducted to confirm the results. Bar represent 200 μm .

Table 1. Effect of DEHP exposure on morphological apoptotic changes in rat DOs and COCs cultured *in vitro*

Treatment/apoptotic features	Total no. of oocytes		Morphological apoptotic changes						Total no. of apoptotic oocytes		Apoptosis (%)	
			Shrinkage		Cytoplasmic fragmentation		Cytoplasmic granulation					
	DOs	COCs	DOs	COCs	DOs	COCs	DOs	COCs	DOs	COCs	DOs	COCs
Control	27	24	0	0	0	0	0	0	0	0	0.0	0.0
DEHP 25 μg	25	26	0	0	0	0	0	0	0	0	0.0	0.0
DEHP 50 μg	24	22	1	1	1	0	1	0	3	1	12.5 ^a	4.54 ^b
DEHP 100 μg	26	27	5	2	2	0	1	1	8	3	30.76 ^c	11.11 ^d
DEHP 200 μg	25	25	4	3	3	0	3	2	10	5	40.0 ^e	20.0 ^f
DEHP 400 μg	24	23	5	2	4	3	4	1	13	6	54.16 ^g	26.08 ^h
DEHP 800 μg	26	25	1	3	1	2	9	3	11	8	42.30 ^{e,i}	32.00 ^j

^{a-j}Different superscript letters represent significant difference (DOs vs. COCs) to each other ($P < 0.05$).

dependent studies (Tables 1 and 2), we selected the concentration of 400 μM DEHP for analysis of cell survival, intracellular ROS level, OS, mitochondrial membrane potential (JC1 staining), apoptosis and gene expression in denuded oocytes, as well as in COCs.

Acridine orange/propidium iodide nuclear staining

We performed AO/PI staining to confirm apoptosis as a result of morphological changes in DEHP-treated (400 μM) denuded oocytes and COCs. The results showed that DEHP treatment induced apoptosis in denuded oocytes (Fig. 3g) as evidenced by a more red fluorescence intensity for PI compared with control denuded oocytes (Fig. 3c). However, as shown in Fig. 3j, oocytes within the COCs showed AO green fluorescence compared with PI red fluorescence in the same encircling granulosa cells, indicating the protective role of

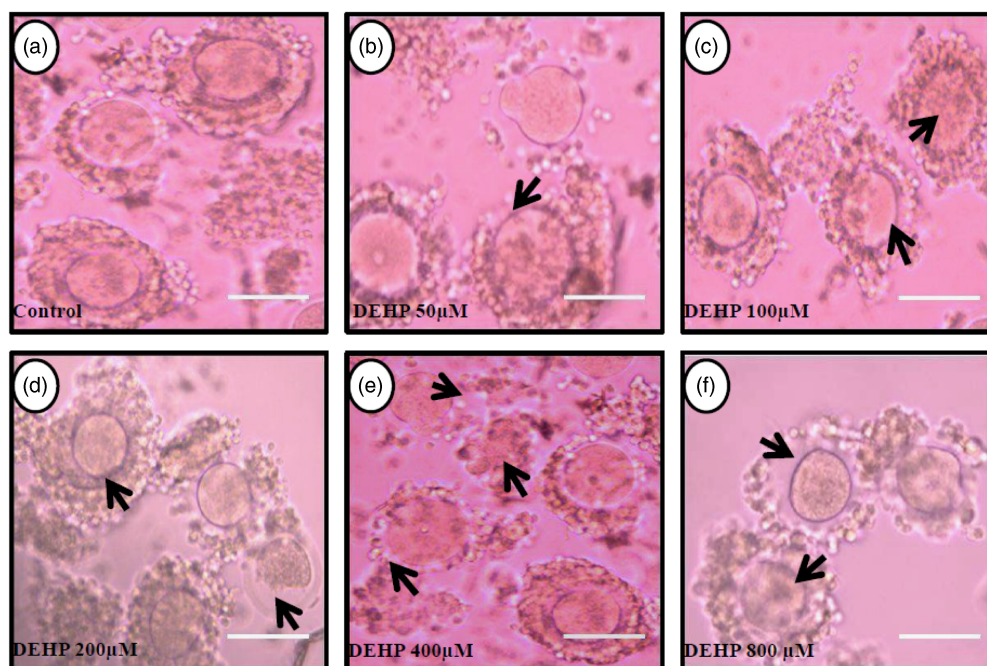
encircling granulosa cells towards oocyte. Interestingly, as shown in Fig. 3l, granulosa cells in COCs showed more intense PI red fluorescence, while oocytes did not take up the PI stain.

Encircling granulosa cells protect against DEHP-induced oxidative stress

In order to explore if encircling granulosa cells protected against DEHP-induced OS, COCs and denuded oocytes were exposed to DEHP (400 μM) and the OS level analyzed. As shown in Fig. 4e, treatment with DEHP (400 μM) was significantly ($P < 0.05$) associated with increased immunofluorescence intensity in the denuded oocyte compared with the control denuded oocyte (Fig. 4b). Furthermore, as shown in Fig. 4h, encircling granulosa cells protected oocytes from DEHP induced OS, as evidenced by

Table 2. Time and dose-dependent effect of DEHP exposure on morphological apoptotic changes in rat denuded oocytes (DOs) cultured *in vitro*

Treatment/apoptotic features	Total no. of oocytes	1 h	2 h	3 h	Total no. of apoptotic oocytes
Control	27	0	0	0	0
DEHP 25 μg	25	0	0	1	1
DEHP 50 μg	24	0	1	2	3
DEHP 100 μg	26	1	2	5	8
DEHP 200 μg	25	2	3	5	10
DEHP 400 μg	24	4	5	4	13
DEHP 800 μg	26	4	5	2	11

**Figure 2.** Representative photographs showing the protective effects of encircling granulosa cells on DEHP-induced morphological apoptotic changes in COCs cultured *in vitro*. The presence of encircling granulosa cells protected the oocyte against higher doses (100, 200, 400 and 800 μM) of DEHP-induced morphological apoptotic changes (c–f; black arrows) compared with control and lower dose of DHEP (50 μM) treated COCs showing normal morphology (a, b). Encircling granulosa cells protected DEHP-induced morphological apoptotic features in DEHP-treated oocytes. Three independent experiments were conducted to confirm the results. Bar represents 200 μm .

low fluorescence intensity compared with DEHP-treated denuded oocytes. CTCF analysis for measurement of OS level was carried out using 6–8 denuded oocytes/COCs from three independent experiments.

Encircling granulosa cells protect against DEHP-induced generation of ROS

As we measured OS levels in DEHP-treated in denuded oocytes and COCs, we were interested to find out whether OS could be associated with ROS generation. Therefore, we analyzed ROS levels in DEHP-treated (400 μM) denuded oocytes, as well as in COCs using the ROS-specific fluorescence probe DCFH-DA. As shown in Fig. 5d, DEHP treatment significantly ($P < 0.05$) increased the ROS level in denuded oocytes compared with control denuded oocytes (Fig. 5b). However, encircling granulosa cells protected the oocyte from DEHP-induced increased ROS levels (Fig. 5f), as evidenced by low fluorescence intensity compared with DEHP-treated denuded

oocytes. CTCF analysis for measurement of oxidative ROS levels was carried using 6–8 denuded oocytes/COCs from three independent experiments.

DEHP exposure induced mitochondrial activity in denuded oocytes

Active mitochondria produce a more red fluorescence light by readily accumulating JC1 dye in mitochondrial compared with less active mitochondria that produce a green fluorescence light; this ratio of red/green fluorescence is indicative of mitochondrial activity in a given cell (Reers *et al.*, 1995). As shown in Fig. 6h, DEHP (400 μM) treatment significantly ($P < 0.05$) increased mitochondrial activity in denuded oocytes compared with control denuded oocyte (Fig. 6d), as evidenced by the high ratio of red/green fluorescence. However, DEHP-treated COCs significantly reduced mitochondria activity, as evidenced by a low red/green fluorescence ratio (Fig. 6l) compared with DEHP-treated denuded oocytes, indicating a protective role for the encircling granulosa

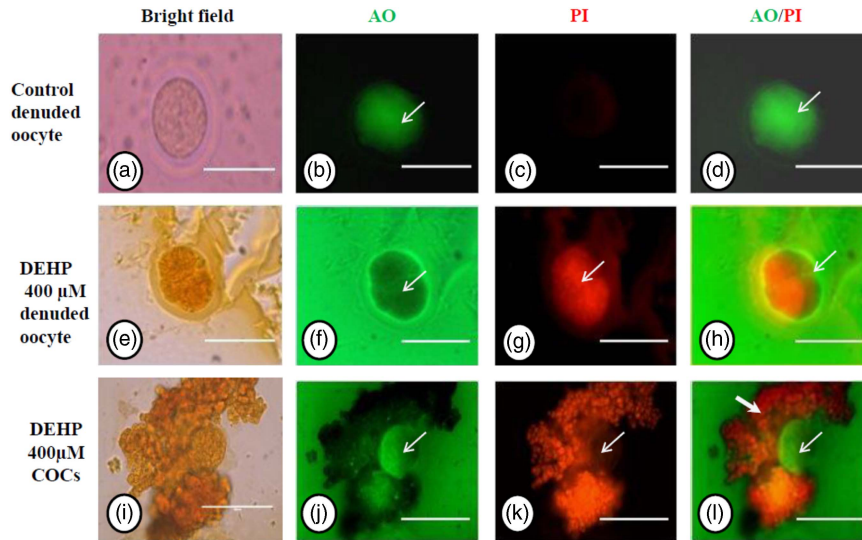


Figure 3. Representative photographs showing acridine orange/PI staining for analysis of cell viability after DEHP treatment in denuded oocytes and in COCs. DEHP treatment (400 μM) induced apoptotic morphological changes in denuded oocyte as evidenced by increased PI staining and reduced AO staining (e–h) compared with control denuded oocyte (a–d). The presence of encircling granulosa cells protected oocytes (green fluorescence in oocyte) from DEHP-induced apoptotic changes (j, white arrows). However, encircling granulosa themselves showed deterioration in their morphology because of DEHP toxicity as evidenced by higher PI staining (i, k, l). Three independent experiments were conducted to confirm the results. Bar represents 200 μm.

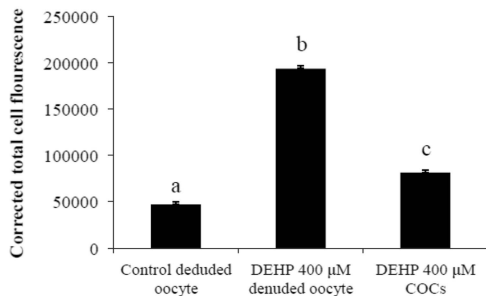
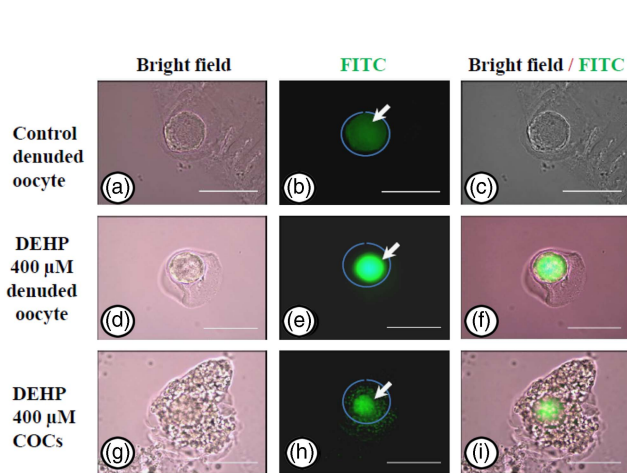


Figure 4. Representative photographs showing total oxidative stress level in COCs as well as in denuded oocyte cultured *in vitro*. (Upper) DEHP (400 μM) significantly increased oxidative stress levels in denuded oocyte (d–f) as evidenced by an increased CTCF value compared with the control denuded oocyte (a–c). The presence of encircling granulosa cells protected the oocytes against DEHP-induced increased oxidative stress level in treated COCs (g–i). (Lower) The level of oxidative stress is measured using oxidative stress detection kit. Data are presented as mean ± standard error of the mean (SEM) of three independent experiments. Bar represents 200 μm. ^{a–c}Different letters of superscript showing significant difference ($P < 0.05$) (denuded oocyte vs. COCs).

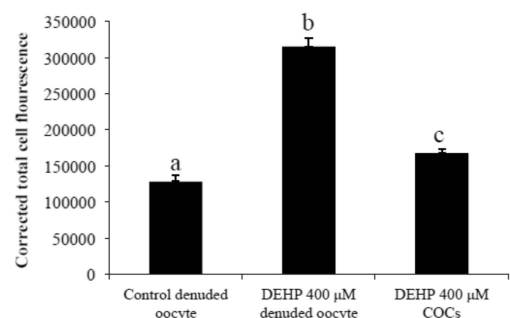
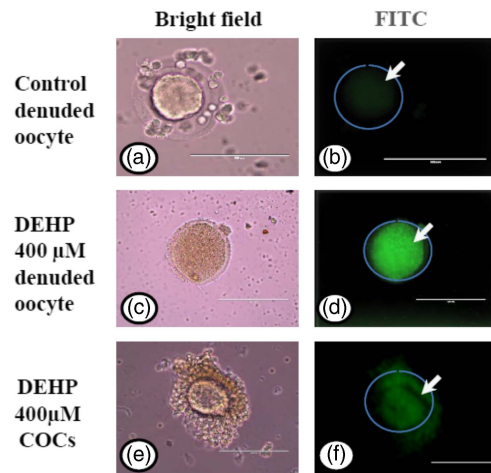


Figure 5. Representative photographs showing total intracellular ROS levels in COCs, as well as in denuded oocytes cultured *in vitro*. (Upper) DEHP (400 μM) significantly increased ROS levels in denuded oocyte (c, d) as evidenced by increased CTCF values compared with control denuded oocytes (a, b). The presence of encircling granulosa cells protected oocyte against DEHP-induced increased ROS level in treated COCs (e, f). (Lower) The level of ROS was measured using a DCFH-DA dye. Data are presented as mean ± standard error of the mean (SEM) of three independent experiments. Bar represents 100 μm. ^{a–c}Different superscript letters show significant differences ($P < 0.05$) (denuded oocyte vs. COCs).

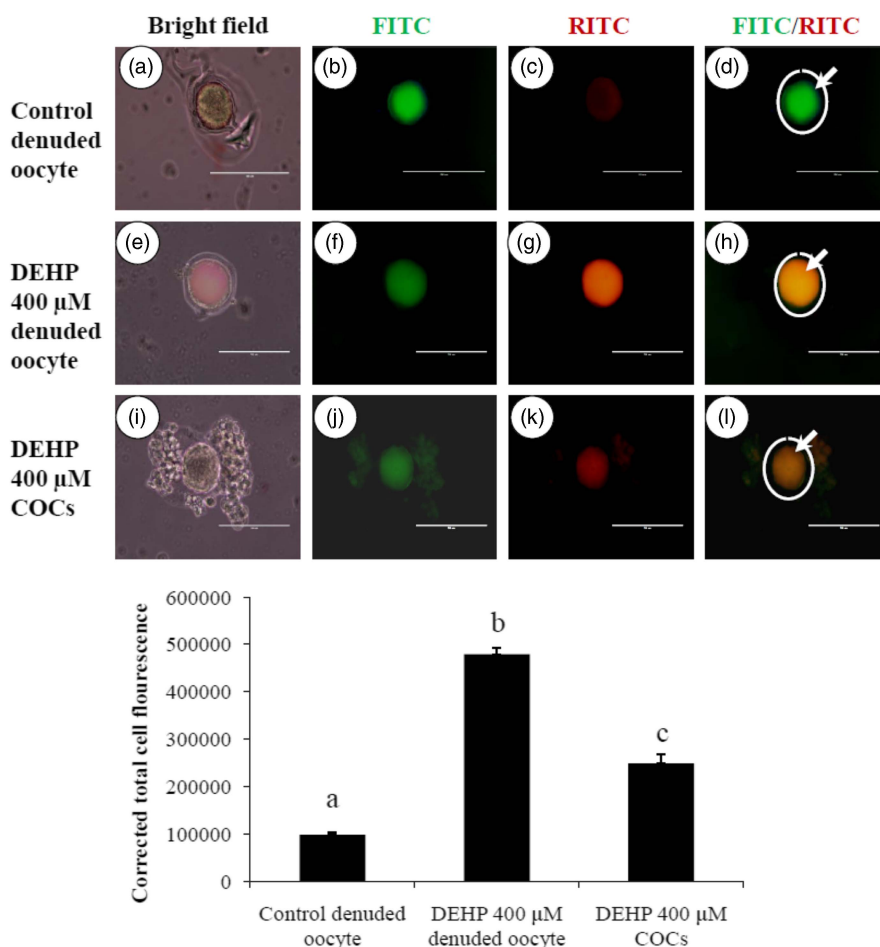


Figure 6. Representative photographs showing mitochondrial membrane potential in COCs as well as in denuded oocytes cultured *in vitro*. (Upper) DEHP (400 μ M) significantly increased mitochondrial activity in denuded oocyte (e–h) as evidenced by increased CTCF values compared with control denuded oocytes (a–d). The presence of encircling granulosa cells protected oocytes against DEHP-induced increased activity of mitochondria in treated COCs (i–l). (Lower) Mitochondrial activity is measured using the mitochondrion-specific dye JC1. Data are presented as mean \pm standard error of the mean (SEM) of three independent experiments. Bar represents 100 μ m. ^{a–c}Different letters of superscript showing significant differences ($P < 0.05$) (denuded oocyte vs. COCs).

cells. CTCF analysis for measurement of mitochondrial activity levels was carried out using 6–8 denuded oocyte/COCs from three independent experiments.

Encircling granulosa cells protect against DEHP-induced apoptosis

We used an annexin V-FITC/PI apoptosis detection kit to further confirm our data on apoptosis obtained by AO/PI staining. As shown in Fig. 7g, DEHP treatment (400 μ M) induced apoptosis in denuded oocytes, as evidenced by a more red PI fluorescence compared with control denuded oocytes, which produced very faint red fluorescence (Fig. 7c). However, encircling granulosa cells protected oocytes against DEHP-induced apoptosis, as evidenced by annexin-V-FITC green fluorescence, suggesting the protective role of granulosa cells that underwent apoptosis, evidenced by red fluorescence in encircling granulosa cells (Fig. 7k, l).

Encircling granulosa cells protect against DEHP-induced expression of apoptotic proteins

We were interested to find out whether DEHP-induced morphological apoptotic changes were associated with the altered expression of apoptotic proteins. Our data showed that DEHP

treatment (400 μ M) significantly increased Bax (Fig. 8b) and cytochrome *c* (Fig. 8f) expression levels in denuded oocyte, as evidenced by high fluorescence intensities compared with their respective COCs that had low green fluorescence (Fig. 8d, h). However, expression of the anti-apoptotic protein Bcl2 was reduced in DEHP-treated (400 μ M) denuded oocyte (Fig. 8j), evidenced by low intensity green fluorescence and compared with a slightly increased intensity of green fluorescence in COCs (Fig. 8l). CTCF analysis for measurement of mitochondrial activity level was performed using 6–8 denuded oocyte/COCs from three independent experiments.

Analysis of mRNA expression of apoptotic genes

We performed semi-quantitative RT-PCR to evaluate the expression of apoptosis genes in DEHP-treated denuded oocytes and COCs. The results showed that mRNA expression levels of Bax and cytochrome *c* were higher in DEHP-treated (400 μ M) denuded oocyte compared with control denuded oocytes (Fig. 9c, d). However, mRNA expression of Bax and cytochrome *c* was slightly reduced in DEHP-treated COCs (Fig. 9C, D), indicating the protective role of encircling granulosa cells. In addition, mRNA expression levels of anti-apoptotic gene *bcl2* were reduced in DEHP-treated denuded oocyte compared with control

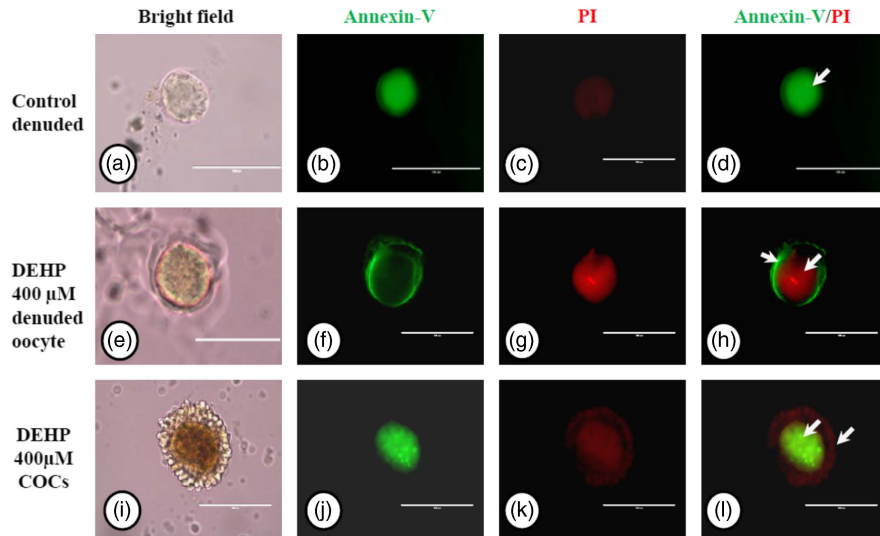


Figure 7. Representative photographs showing annexin-V/PI staining for analysis of apoptosis in DEHP-treated denuded oocytes and in COCs. DEHP treatment (400 μM) induced DNA fragmentation in denuded oocytes as evidenced by increased PI staining and reduced annexin-V staining (e–h) compared with control denuded oocytes (a–d). The presence of encircling granulosa cells protected the oocyte from DEHP-induced apoptotic changes (i–l). However, encircling granulosa cells showed apoptosis as evidenced by higher staining of PI (k, l). Three independent experiments were conducted to confirm the results. Bar represents 200 μm .

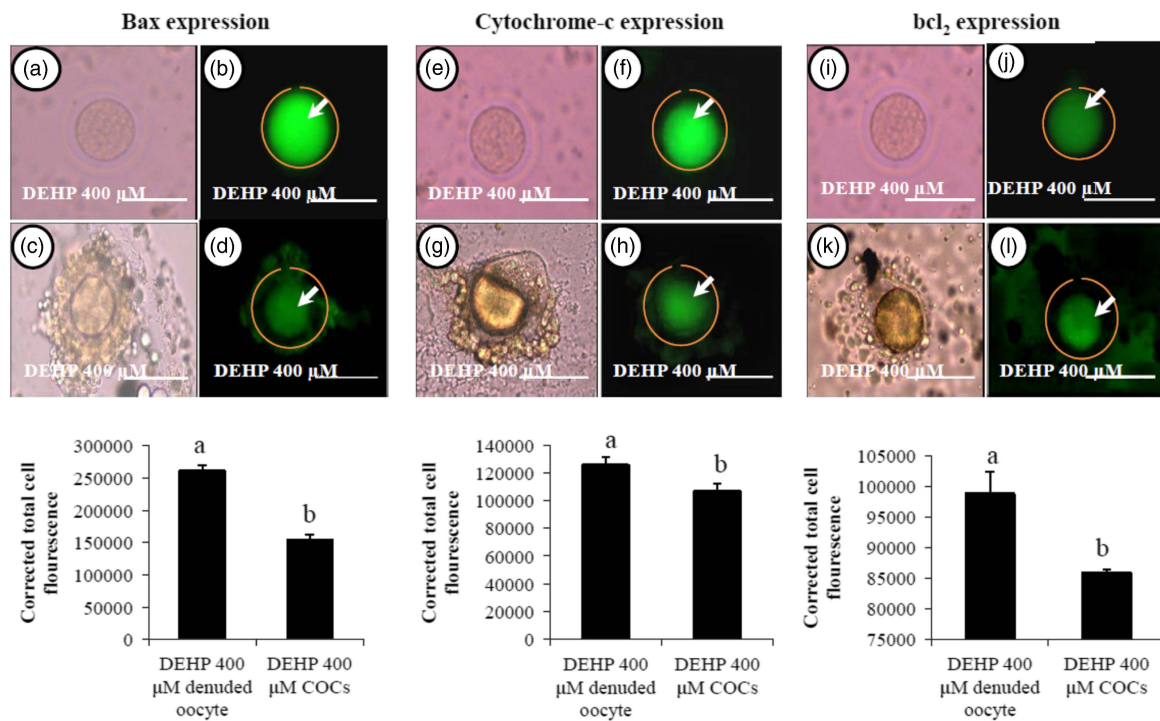


Figure 8. Representative photographs showing the expression level of apoptotic (Bax and cytochrome *c*) and anti-apoptotic (Bcl2) marker protein in DEHP-treated denuded oocyte and in COCs cultured *in vitro*. (Upper) DEHP significantly increased the expression of pro-apoptotic protein Bax (a, b) and cytochrome *c* (e, f) protein expression levels in treated denuded oocyte. Encircling granulosa cells protected the oocyte against DEHP-induced increase of Bax and cytochrome *c* expression in oocytes of COCs as evidenced by low corrected fluorescence intensity value (c, d) and (g, h) respectively. Conversely, DEHP treatment significantly reduced anti-apoptotic Bcl-2 expression level in COCs (i, j) compared with denuded oocytes (k, l). Encircling granulosa cells protected DEHP-induced increase of Bax and cytochrome *c* expression levels in COCs, as evidenced by low CTF values, compared with denuded oocytes. (Lower) Three independent experiments were conducted to confirm the results. Bar represents 200 μm . ^{a,b}Different superscript letters showing significant difference ($P < 0.05$) (denuded oocyte vs. COCs).

denuded oocytes (Fig. 9b). However, the respective COCs showed slightly higher *bcl2* mRNA expression levels, again suggesting the protective role of encircling granulosa cells (Fig. 9b). However, the expression level of GAPDH remained unchanged in all samples and did not differ significantly between samples (Fig. 9a).

Discussion

In this study, we used an *in vitro* culture system to investigate whether DEHP induces OS and, if yes, whether the presence of encircling granulosa cells protects against DEHP-induced OS and apoptosis. DEHP is a ubiquitous endocrine-disrupting chemical

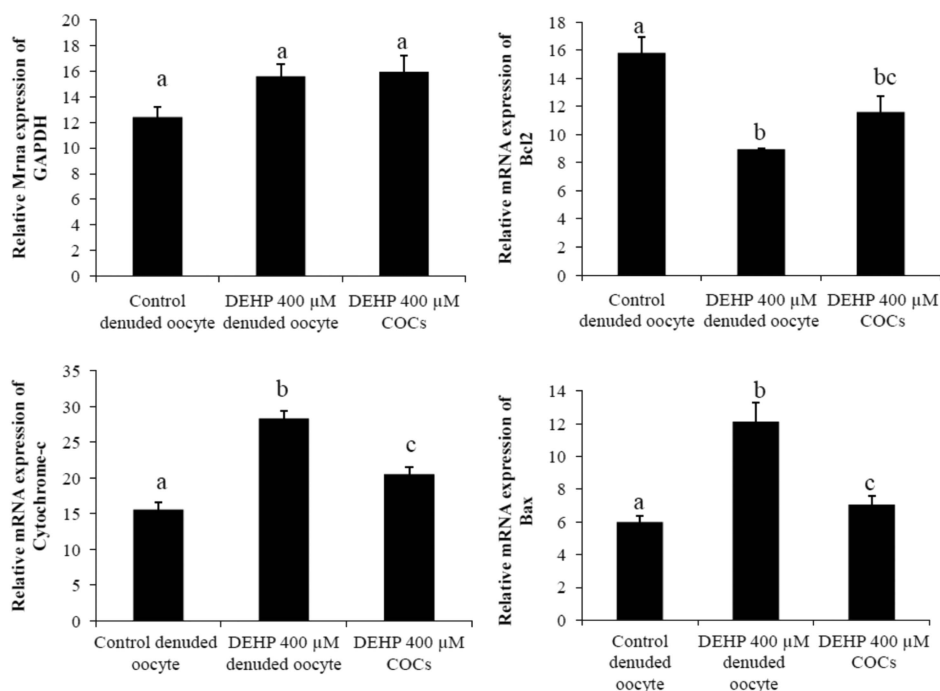


Figure 9. Representative photographs showing mRNA expression levels of apoptotic (Bax and cytochrome *c*) and anti-apoptotic (Bcl2) marker genes in DEHP-treated denuded oocytes and in COCs cultured *in vitro*. mRNA transcripts from control (denuded oocyte) and DEHP-treated groups (denuded oocyte and COCs) were converted into cDNA and amplified using pro-apoptotic (Bax and cytochrome *c*) and anti-apoptotic (Bcl2) primers. Treatment with DEHP significantly increased mRNA expression levels of Bax and cytochrome *c* in denuded oocytes. Encircling granulosa cells protected oocytes against any DEHP-induced increase in Bax and cytochrome *c* expression in oocytes and COCs. Furthermore, encircling granulosa cells prevented any decrease in Bcl2 expression levels in oocytes and COCs. Three independent experiments were conducted to confirm the results. Data are presented as mean \pm standard error of the mean (SEM) of three independent experiments. Bar represents 200 μ m. ^{a-c}Different superscript letters show significant difference ($P < 0.05$) (denuded oocyte vs. COCs).

that is known to induce cytotoxicity in many mammalian species including human (Heudorf *et al.*, 2007; Hogberg *et al.*, 2008; Krotz *et al.*, 2012; Li *et al.*, 2016; Liu *et al.*, 2017). Previous studies have indicated that MEHP (a metabolite of DEHP) inhibits maturation and the growth of antral follicles and induces atresia in mouse *in vitro* (Anas *et al.*, 2003; Huang *et al.*, 2009; Li *et al.*, 2016). Furthermore, this result was confirmed in a subsequent study in which Eimani *et al.* (2005) reported the inhibition of meiotic maturation in the mouse after *in vivo* oral DEHP administration. Our data also indicated that treatment with DEHP induced morphological apoptotic changes in a dose-dependent manner. Lower doses of DEHP (50 and 100 μ M) induced the initiation of morphological apoptotic changes, while higher doses of DEHP (400 and 800 μ M) induced apoptosis and degeneration. However, encircling granulosa cells protected the oocyte from DEHP-induced morphological apoptotic changes. Data in the present study are further strengthened by previous observations that the presence of encircling granulosa cells prevented oocytes from ROS-mediated OS damage for mouse (Li *et al.*, 2011; Zhou *et al.*, 2012; Jiao *et al.*, 2013), rat (Jiao *et al.*, 2016), bovine (Morado *et al.*, 2009) and human (Goud *et al.*, 1998) oocytes cultured *in vitro*.

DEHP induced OS via ROS generation and affected oocyte physiology (Tatemoto *et al.*, 2000; Suna *et al.*, 2007; Tripathi *et al.*, 2013). Our findings also suggested that DEHP treatment (400 μ M) induced OS in denuded oocytes. Data from our findings are also supported by previous studies that suggested that DEHP induced OS (Tang *et al.*, 2016; Franken *et al.*, 2017). However, encircling granulosa cells protected oocytes from DEHP-induced OS. Our study corroborated findings that

granulosa cells protected the oocyte from oxidative damage (Tiwari *et al.*, 2017).

ROS are known to play important roles in many physiological processes (Ames, 1999; Erkekoglu *et al.*, 2010). In this study, we assumed that DEHP exerted their toxic effect and potentially affected the physiology of oocytes by increasing ROS levels. Therefore, we assessed the ROS levels in DEHP-treated groups using a ROS-specific probe DCFH-DA. Our results showed that ROS levels were significantly higher in DEHP-treated denuded oocytes. DEHP might have induced ROS levels by increasing OS and, therefore, by induction of morphological apoptotic changes in treated denuded oocytes. Interestingly, we found that encircling granulosa cells protected the oocyte from DEHP-induced ROS generation. Granulosa cells encircling the oocyte somehow reduced the DEHP-induced OS and by lowering the level of intracellular ROS production. Taken together these data suggest the protective role of encircling granulosa cells towards DEHP-induced ROS-mediated apoptosis under *in vitro* culture conditions. These findings are further supported by previous studies in which encircling granulosa cells prevented an increase in ROS levels in oocytes under *in vitro* culture conditions (Huang & Wells, 2010; Shaib *et al.*, 2013).

Mitochondria respiration is the primary source of ATP generation. The balance between ROS generation and ATP synthesis in a healthy mitochondria is tightly regulated and any disturbance in this process can lead to mitochondrial membrane hyperpolarization in human and mouse embryos (Acton *et al.*, 2004). Studies have used the fluorescent $\Delta\Psi_m$ reporter dye JC-1 to determine metabolic activity and membrane potential (Ahn *et al.*, 2002; Van Blerkom *et al.*, 2003). In our study, we were interested

to find out the mitochondrial membrane potential (hyperpolarize status of mitochondria) in DEHP-treated oocytes and COCs using the mitochondria-specific dye JC1. Our results showed that, in DEHP-treated oocytes, mitochondria were hyperpolarized and fluoresced red due to the formation of J-aggregates. However, for COCs, mitochondria were less hyperpolarized, as indicated by a lesser intensity of red fluorescence. Our result indicated that the encircling granulosa cells protected the oocyte from the adverse effects of DEHP. Our results were further strengthened by this previous observation (Goud *et al.*, 2008; Tripathi *et al.*, 2013; Tiwari *et al.*, 2017).

It is well established that any change in mitochondrial membrane potential results in altered Bax/Bcl₂ ratios and therefore cytochrome *c* is released into the cell, this leads to apoptosis (Tripathi & Chaube, 2012). We analyzed the mRNA and protein expression levels of Bax, Bcl₂ and cytochrome *c* in DEHP-treated denuded oocytes, as well as in COCs. Our result showed that DEHP treatment increased mRNA and protein expression levels for Bax and cytochrome *c* (pro-apoptotic markers) and reduced the expression of anti-apoptotic Bcl2 protein. However, interestingly, we observed that encircling granulosa cells prevented an increase in mRNA and protein expression levels for these apoptotic markers. The expression levels of anti-apoptotic protein Bcl2 were only reduced slightly in the DEHP-treated denuded oocytes. Our findings are also supported by previous studies in that granulosa cells play a role in the prevention of ROS-induced apoptotic pathways in rat COCs cultured *in vitro* (Tripathi & Chaube, 2012; Tiwari *et al.*, 2015, 2017).

To further confirm our results that encircling granulosa cells protected the oocyte from DEHP-induced apoptosis, we further analyzed cell survival and apoptosis using AO/PI and annexin-V/PI staining. Our result showed that DEHP induced oocyte death, as evidenced by PI-positive staining. However, the oocytes surrounded by granulosa cells did not take up the PI stain, although granulosa cells were found to be positive for PI staining. This finding indicates that granulosa cells protect the oocyte from apoptosis induced by DEHP and are the cells that first enter apoptosis, rendering the oocyte protected from damage (Tripathi & Chaube, 2012; Tripathi *et al.*, 2013).

Conclusions

Our data suggest that the presence of encircling granulosa cells acts as a potential protection barrier and effectively masks the oocyte against the adverse effects of DEHP, which exerts a dose-dependent deleterious effect on denuded oocytes. This effect was mediated by oxidative stress-induced ROS generation, which altered mitochondrial membrane potential and resulted in disturbance in the Bax/Bcl-2 ratio that ultimately led to oocyte apoptosis. Considering the extensive use of DEHP-containing plastic products, especially in rural areas because of the lack of adequate primary healthcare facilities, unhygienic conditions and direct exposure to various kinds of endocrine disruptors, the presence of encircling granulosa cells might be beneficial for protecting the oocyte during *in vitro* manipulation against DEHP-induced reproductive toxicity.

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Conflict of interest. The authors have declared that no conflict of interest exists.

Ethical standards. Animal experimental work was carried out as according to regulations provided by the Animal Ethical Committee of the Banaras Hindu University, Varanasi, India. This study was approved by the Institutional Animal Ethical Committee of the University (see letter no. Dean/2015/CAEC/1517, dated 21 December 2015).

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