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Poonam K Singh
1. ICAR-National Institute of Animal Nutrition and Physiology, Adugodi, Bengaluru, Karnataka, India
2. Jain University, Bangalore, Karnataka, India

B Sampath Kumar
College of Veterinary Science, Mamnoor, Warangal, PVNRTVU, Telangana, India

Sumanta Nandi
ICAR-National Institute of Animal Nutrition and Physiology, Adugodi, Bengaluru, Karnataka, India

Paluru SP Gupta
ICAR-National Institute of Animal Nutrition and Physiology, Adugodi, Bengaluru, Karnataka, India

Sukanta Mondal
ICAR-National Institute of Animal Nutrition and Physiology, Adugodi, Bengaluru, Karnataka, India

Corresponding Author:
Sumanta Nandi
ICAR-National Institute of Animal Nutrition and Physiology, Adugodi, Bengaluru, Karnataka, India

Analysis of Dibutyl Phthalate (DBP) induced ovine granulosa cell viability by neutral red uptake, lactate dehydrogenase and sulforhodamine B assays

Poonam K Singh, B Sampath Kumar, Sumanta Nandi, Paluru SP Gupta and Sukanta Mondal

Abstract
Dibutyl Phthalate (DBP) is one of the commercially used plasticizer globally. The available research on cellular cytotoxicity of DBP in mammalian cells is not completely understood. The present experiments hence examined the consequences of cell viability and cytotoxicity in the DBP induced ovine granulosa cells at various dose levels. Cytotoxic assays: Neutral red uptake (NRU), Lactate dehydrogenase (LDH) and Sulforhodamine B (SRB) assays were employed to measure the cell viability and cytotoxicity. Cytotoxicity (%) measured by NRU in the GCs exposed to various concentrations of DBP was found to be significantly higher at 50µM dose onwards. Cytotoxicity (%) measured by LDH in the GCs exposed to various concentrations of DBP was found to be significantly higher only at 50µM dose specifically. Similarly, cytotoxicity (%) measured by SRB in the GCs exposed to various concentrations of DBP was found to be significantly higher from 10µM dose onwards linearly compared to the control groups respectively. Overall, DBP induce cellular toxicity by targeting not only plasma membrane but also subcelluar targets such as lysosomal membranes.

Keywords: Dibutyl Phthalate, NRU, LDH, SRB, assays, viability

Introduction
Colloquially referred to as "diester chemicals," phthalates are the diesters of orthophthalic acid (1, 2-benzene carboxylic acid: Perico *et al*., 2022) [1]. Among them Dibutyl phthalate (DBP) is a chemical that belongs to the phthalate ester class and is used in a wide range of industrial and consumer products (paints, printing ink, paper, carpet, concrete, nail polish, and cosmetics: HSDB, 2017) [2]. DBP is frequently employed as a plasticizer due to its low toxicity and broad liquid range (Rahman & Brazel, 2004) [3]. Herein, studies on animals show that exposure to phthalates can alter the levels of circulating hormones, which may have an adverse effect on reproductive physiology and the growth of target tissues that are estrogen-sensitive (Kay & Foster 2013) [4]. Indeed, DBP is one of the endocrine disruptors and reproductive toxins that also impair cell development and viability, according to *in vivo* toxicological investigations (Hu *et al*., 2020) [5]. Similarly, according to the *in vitro* toxicological studies, ovarian granulosa cells, which are essential for producing steroidogenic hormones, can be affected by DBP (Singh *et al*., 2022) [6]. Furthermore DBP was proven as a toxicant to the ovarian follicle and oocyte development by disrupting the antioxidant enzymes (Singh *et al*., 2022) [6]. Toxicological studies revealed DBP as an ovarian toxicant, but the dose remains obscure (Liu & Craig, 2019) [7]. Herein, cytotoxicity assays, are the precedence to investigate the viability of the ovarian cells in a conventional culture system with the inclusion of various doses of DBP (Al-Saleh *et al*., 2020) [8]. The MTT(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay is often used to determine cytotoxicity following exposure to toxic substances based on the viability and/or a number of mitochondria in a cell, as well as their integrity and activity (Vinken & Blaauboer., 2017) [9]. The NRU (Neutral red uptake) is also used to measure cell viability and is considered a better choice to measure the toxicity in the cells over those chemicals which cannot reduce the tetrazolium salts (Repetto *et al*., 2008) [10]. It is based on the ability of viable cells to incorporate and bind the supravital dye neutral red (Tolosa *et al*., 2015) [11]. The LDH (Lactate dehydrogenase) leakage assay is based on the measurement of lactate dehydrogenase activity in the extracellular membrane. Hence LDH assay demonstrates the cell plasma membrane damage (Maes *et al*., 2015) [12]. The SRB (Sulphorodhamine B) assay is used for cell density determination, based on the measurement of cellular protein content (Vichai & Kirtikar, 2006) [13].

As a result, the current study used cytotoxic assays such as NRU (lysosomal disintegration), LDH (cell membrane damage), and SRB (total protein assay) to assess the viability and cytotoxic effect of DBP at various doses on ovarian granulosa cells biochemically.

Materials and Methods

Chemicals

A stock solution of Di-butyl phthalate (DBP 3.7 M) was prepared in acetone by diluting one µL of DBP (3.7M) liquid dissolved in 99 µL of acetone. The stock solution (1mM) was maintained at −20 °C until use. The working solutions of DBP ranging from 1-100 µM (0.26-26.0 µg/ml) were prepared in MEM-199 supplemented with bovine serum albumin (0.3%) + insulin transferrin selenium (1%) + gentamicin (50µg/ml) before granulosa cell culture. DMSO concentrations in the medium did not exceed 0.5% including for the control and treatment groups. DBP was examined at the levels of 0, 1, 10, 25, 50 AND 100µM concentration. All kits (NRU, LDH and SRB) kits for Biochemical assays were procured from Himedia chemicals, Mumbai, India. The cell culture chemicals were procured from Sigma Chemicals, MO, USA. The purchase of plasticware was made in Tarsons, Kolkata, India.

Granulosa cell collection and culture

Ovine ovaries (n=265) were collected from the slaughterhouse in Bangalore, India and immediately collected in sterile 0.9% saline, supplemented with gentamicin 50µg/ml. Ovaries were washed three times in normal saline and then cleaned with 75% ethanol for 3-5 seconds. Using a syringe with an 18 G sterile needle, the ovaries were punctured, and the follicular fluid was aspirated from medium-sized follicles 300-500 µm in aspiration media containing MEM 199, gentamicin (50µg/mL), and heparin (10 µg/µL) further the mixture of GCs and oocytes in the follicular fluid was separated using 40µm cell strainers.

GCs were centrifuged at 400 g for 5 min at 4 ºC, suspended in 1% PBS and again the cells were then washed three times in MEM 199 supplemented with gentamicin (50µg/mL) and BSA 0.3% and centrifuged at 200 g for 10 min and then finally resuspended in 1ml of MEM199 + 0.3% BSA. Granulosa cell viability and the culture of granulosa cells were carried out as outlined by Nandi *et al*. (2016) [14]. Cells were then cultured for 72 hours at 37 °C in an atmosphere of 5% CO^2 with 95% humidity in a CO^2 incubator. Every other day the growth was examined under a microscope, and fresh media was added.

Assessment of cellular toxicity evaluation

Three spectrophotometric methods were applied to determine the cell viability and cellular toxicity of DBP. The absorbance values at appropriate wavelengths were recorded using a Spectrophotometer microplate reader**.**

NRU Assay

Neutral red uptake into the lysosomes of viable cells was evaluated by a kit method. Briefly, after 72h, the control and DBP-treated cultured granulosa cells were harvested and then 0.33% Neutral Red Solution (150µl) was added to each well with an equal volume of culture medium and incubated for 4 hours at 37 ºC and then the medium was removed and the cells were washed with 1 X PBS and finally 150 µl of 1 X Neutral Red Assay Solubilization Solution was added to each well. The culture plates of treated cells are allowed to stand for 10 minutes at room temperature and absorbance was measured at a wavelength of 540 nm. Each experiment was repeated 6 times in 2 replicates. The cytotoxicity is measured by following the formula % Cytotoxicity = O.D. DMSO - O.D. sample O.D. DMSO * 100%.

LDH Assay

Percentage cytotoxicity was estimated by measurement of lactate Dehydrogenase enzymes (LDH) efflux from damaged cells into the culture medium. The granulosa cells at 1 ×105 cells/mL were treated with different concentrations of DBP (0, 1, 10, 25, 50 and 100µM) for 72 hours, at 37 ºC in 5% CO2. The LDH efflux released from granulosa cells was determined using an LDH kit, according to the manufacturer's instructions. Each experiment was repeated 6 times in 2 replicates. Cytotoxicity was calculated using the following formula: Percentage Cytotoxicity = (E – B)/(M L – B) X 100 where E is experimental LDH release from DBP treated cells, BC is background control, ML is maximal LDH release caused by triton X-100.

SRB Assay

The Sulforhodamine B Assay measures total biomass by staining cellular proteins with the Sulforhodamine B stain. The control and DBP treated cultured granulosa cells were harvested and 25µl of cold fixative was added to 50 µl of the culture medium volume to each well and incubated for 1 hour at 2-8 ºC and then fixative was discarded and gently 100 µl of washing solution was added 2-3 times and wells were then air dried and 50 µl of sulphorhodamine stain was added to each well and incubate for 20-30 min at room temperature and then stain was discard and 25 µl of washing solution to each well and 1X of Solubilization solution was added (100 µl) and allow the plate to stand at room temperature for 10 min and absorbance was read at 565 nm. Each experiment was repeated 6 times in 2 replicates. The cytotoxicity is measured by following the formula

% Cytotoxicity = O.D. DMSO - O.D. sample O.D. DMSO * 100%.

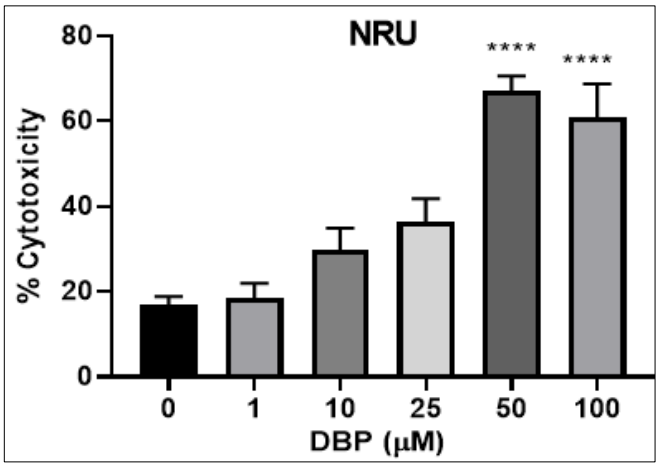
Statistical Analysis

Multiple comparisons using Tukey statistical hypothesis were used to analyze the data. The p-value < 0.05 as compared to the control was considered significant.

Results

NRU Assay

Cytotoxicity (%) measured by NRU in the GCs exposed to various concentrations of DBP was found to be significantly higher at 50µM dose compared to control and lower doses. Further exposure of DBP to the GCs in a culture medium i.e, 100µM dose also produced a significant difference in the percent of toxicity. The concentrations below 50µM dose of DBP did not produce any significant difference in the percent of toxicity when exposed to DBP in a culture medium with various concentrations (Fig.1).

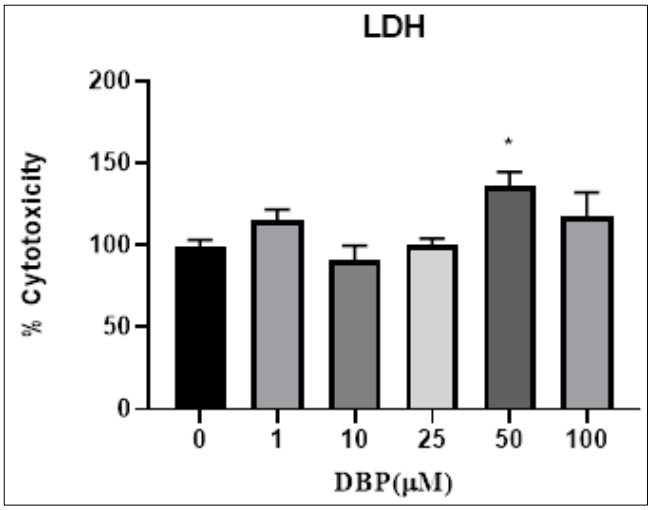


Absorbance unit (540 nm/10^5cells) values are represented as mean ±SE.
Values with different asterisks (*) differ significantly.

Fig 1: Measuring the cytotoxicity (%) of DBP induced ovarian GC on NRU assay

LDH assay

Cytotoxicity (%) measured by LDH in the GCs exposed to various concentrations of DBP was found to be significantly higher only at 50µM dose among all the concentrations. The concentrations above and below 50µM dose of DBP did not produce any significant difference in the percent of toxicity when exposed to DBP in a culture medium with various concentrations (Fig.2).

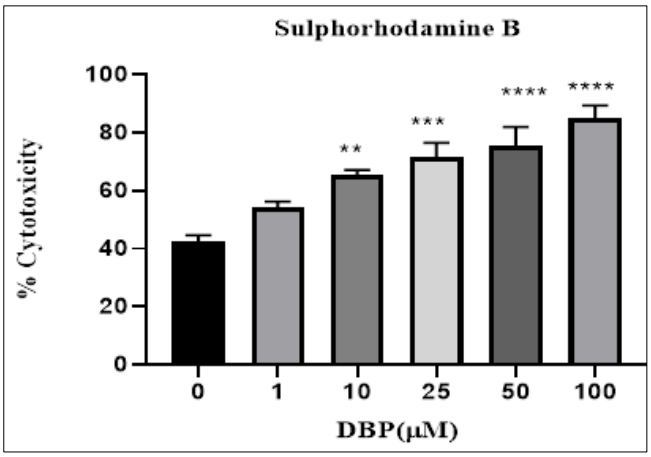


Absorbance unit (580 nm/10^5cells) values are represented as mean ±SE.
Values with different asterisk (*) differ significantly.

Fig 2: Measuring the cytotoxicity (%) of DBP induced ovarian GC on LDH assay

SRB assay

Cytotoxicity (%) measured by SRB in the GCs exposed to various concentrations of DBP was found to be significantly higher at 10µM dose. Further exposure of DBP to the GCs in a culture medium i.e, starting from 25, 50 and 100µM doses a significant difference in the percent of toxicity was observed. The concentrations below 10µM dose of DBP did not produce any significant difference in the percent of toxicity when exposed to DBP in a culture medium with various concentrations (Fig.3).



Absorbance unit (565nm/10^5cells) values are represented as mean ±SE.
Values with different asterisks (*) differ significantly.

Fig 3: Measuring the cytotoxicity (%) of DBP induced ovarian GC on SRB assay

Discussion

In addition to being a useful tool for early toxicity assessments, cultured cells are also useful for the mechanistic analysis of toxic compounds (Zucco *et al*., 2004) [15]. To investigate the cytotoxicity, studies have been performed on ovarian GCs by exposing them to different concentrations of DBP.

DPB-induced toxicity to the GCs in an *in vitro* culture system certainly gives us information about the extent of damage to the plasma membrane, lysosome and other organelle inside the cell (Singh *et al*., 2022) [6]. Damage to the plasma membrane results from feeble cell plasma integrity, which in turn causes cytosolic compounds to leak into the medium of subsequent cultures of cells exposed to cytotoxicity chemicals (Sumantran, 2011) [16]. Likewise, LDH, also leaks into the culture medium from cells once the plasma membrane is damaged by the toxic compounds (Maes *et al*., 2015) [12]. Hence the percent of cytotoxicity directly correlates with the leakage of LDH into the medium. In the present experiment, the cytotoxicity (%) in the GCs exposed to DPB was observed only at 50µM concentration upon measuring with LDH assay. This demonstrates a dose-specific effect of DPB on the cell membrane. According to the finding of Hlisníková *et al*., 2020 [17] DBP impairs the Nuclear receptors, and peptide receptors through the cell membrane once exposed. DBP also alter cell proliferation and induces apoptosis via cross-talk between MAPK, NF-kB, Pi3K/Akt and NR (Hlisníková *et al*., 2020) [17].

In addition to the cell membrane, DBP also damages the lysosomes of the cells if exposed to a specific dose. This is evidenced in the present research by NRU assay by measuring the cytotoxicity (%) in the GCs exposed to various concentrations of DBP. Neutral Red is a weakly cationic dye that can penetrate cell membranes and accumulate in the lysosomes of viable cells where it binds by hydrophobic bonds with anionic sites. Viable cells can take up the dye, whereas non-viable cells cannot (Repetto *et al*., 2008) [18]. The obtained results in the present study revealed that cytotoxicity (%) was observed in the GCs when exposed to DBP at 50µM concentration. Further, an increase (>50 µM) in the exposure of DBP to the GCs also showed higher cytotoxicity (%). This demonstrates lysosomal damage in the GCs in a dose-

dependent manner from 50 µM onwards after exposure to DBP.

The total protein content in the cell works as a toxicity indicator for cell proliferation and/or detachment (Peropadre *et al*., 2013) [19]. Sulforhodamine B (SRB) is one such assay which measures the cell density based on the cellular protein content (Vichai & Kirtikar, 2006) [13]. The method is based on the ability of the SRB to bind cellular protein components stoichiometrically (Kasinski *et al*., 2015) [20]. However, this method does not distinguish the difference between viable and dead cells so also the protein content measured is the cumulative result of both viable and dead cells (Kuete *et al*., 2017) [21]. In the present experiment, cytotoxicity (%) in the GCs was linear as the dose of exposure increased from 10µM onwards. This represents a cytotoxic effect of DPB on the GCs in a dose-dependent manner.

Conclusion

Cytotoxicity (%) measured by LDH or NRU assays in the GCs exposed to various concentrations of DBP was found to be significantly higher only at 50µM dose suggesting a dose-specific effect. In contrast, cytotoxicity (%) measured by SRB in the GCs exposed to various concentrations of DBP was found to be significantly higher from the 10µM dose onwards. Results revealed that DBP induced cellular toxicity in the ovarian GCs by targeting not only plasma membrane but also subcellular organelle such as lysosomal membranes in a specific dose. Hence DPB can be considered as one of the potential ovarian toxicants albeit at a specific dose.

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