RESEARCH ARTICLE

Dibutyl Phthalate (DBP) Mediated Oxidative Stress, Cytotoxicity and Estradiol Synthesis in Cultured Ovine Granulosa Cell

Poonam K. Singh*, Sumanta Nandi, Paluru S.P. Gupta, Sukanta Mondal

Abstract

Plasticizer Dibutyl phthalate (DBP), a potent reproductive toxicant, can cause damage to the granulosa cells, the key cells for the production of steroidogenic hormones. It may also affect other antioxidant enzymes for follicular development, affecting oocyte quality. The present study examined the DBP-induced oxidative stress and hormone synthesis from granulosa cells. Biochemical assays such as MTT (3- (-4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) for the study of cell viability, proliferation, and cytotoxicity, cupric reducing antioxidant capacity (CUPRAC) assay for the total antioxidant capacity, malondialdehyde (MDA) assay for the lipid peroxidation, and Enzyme-linked immunosorbent assay (ELISA) for estradiol synthesis were employed to evaluate the effect of DBP at a concentration of 0 (control), 1, 10, 25, 50, 100 µM for 5 days in cultured bovine granulosa cells. Our results revealed that DBP decreased cell viability, cell proliferation, induced oxidative cellular stress, and affected estradiol secretion in a non-dose-dependent manner. The lowest concentration of DBP (1 µM) had a significant decrease in cell viability and increased lipid peroxidation compared to those observed in the control groups. The total antioxidant enzyme (CUPRAC) activity was non significantly induced in all concentrations of DBP in treated granulosa cells. The estradiol synthesis was increased significantly as the DBP concentrations increased. This study suggested that DBP at a very low concentration affected the steroidogenic capacity, cell viability, and proliferation by altering the antioxidant enzyme activity and causing oxidative stress to the cell.

Keywords: Biochemical assay, Dibutyl phthalate, Estradiol, Oxidative stress, Sheep.

Ind J Vet Sci and Biotech (2022): 10.21887/ijvsbt.18.2.7

Introduction

Di-butyl phthalate (DBP) is a widely used plasticizer recognized as an endocrine-disrupting chemical (EDC). Wild, farm, and domestic animals are exposed to this harmful chemical mainly through grazing, inhalation, or dermal contact, which may cause reproductive abnormalities (Rhind *et al*., 2002). DBP has endocrine-disrupting properties (Latini., 2005), and its elevated levels can cause reproductive damage in animals (Lovekamp-Swan and Davis., 2003). In mammalian cells, low molecular phthalates like DBP are degraded to monoesters, which are then transformed into oxidative metabolites by cytochrome p450 enzymes (phase 1 antioxidant enzymes system). Phase 2 antioxidant enzyme systems can also convert these metabolites to glucuronide conjugates, which are then excreted in the urine and feces (ATSDR, 2001, Silva *et al*., 2004). The ovarian follicles are made up of various somatic and germ cells (oocyte). These somatic cells secrete steroidogenic hormones, nutrients, and important growth factors to grow and ovulate, resulting in fertility and offspring production. As ovulation was reported to be inhibited and steroidogenic hormone synthesis was impaired due to toxic damage by plasticizer to the ovarian follicles (Jakub *et al*.; 2010, Guo *et al*.; 2010, Yaxian *et al*.; 2019). We hypothesized that DBP and its metabolites might reduce cell viability, steroidogenic and antioxidant potential, resulting in impaired reproduction and infertility. DBP's effect on ovarian granulosa cells in culture is little established. As a result, the current *in vitro* investigation was carried out to assess the effects of DBP on oxidative stress and hormone production in cultured bovine granulosa cells.

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

Corresponding Author: Poonam K. Singh, CSIR-UGC NET SRF, Jain University, Bengaluru, Karnataka, India, e-mail: poonam.s.shree@gmail.com

How to cite this article: Singh, P.K., Nandi, S., Gupta, P.S.P., & Mondal, S. (2022). Dibutyl Phthalate (DBP) Mediated Oxidative Stress, Cytotoxicity and Estradiol Synthesis in Cultured Ovine Granulosa Cell. Ind J Vet Sci and Biotech. 18(2), 36-39.

Source of support: Nil

Conflict of interest: None.

Submitted: 20/10/2021 **Accepted:** 21/02/2022 **Published:** 10/04/2022

Materials and Methods

All kits were procured from Himedia chemicals, Mumbai, India. The cell culture chemicals were procured from Sigma Chemicals, MO, USA. Estradiol ELISA kits were procured from Calbiotech, India. Biochemical assays included in this study were MTT (3- (4,5-dimethylthiazol-2-yl-2,5-diphenyl tetrazolium bromide), MDA (Malondialdehyde), and CUPRAC (cupric reducing antioxidant capacity) assay.

The stock solution of Di-butyl phthalate (DBP 3.7 M) was prepared by diluting 1 µL of DBP liquid dissolved in 99 µL of acetone. The stock solution was immediately kept at –20°C until use. The control medium consisted of MEM-199 supplemented with bovine serum albumin (0.3%) + insulin transferrin selenium (1%) + gentamicin (50 μg/ml) + sodium bicarbonate (10 mM) + HEPES, 20 mM (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) + nonessential amino acids (1.1 mM) + androstenedione (10^{-6} M) + insulin growth factor (IGF-I) (2 ng/mL). The effect of DBP was evaluated at a concentration of 0 (control), 1, 10, 25, 50, 100 µM. The concentration of DBP was adjusted by 0.26 µL of DBP with 999.74 µL of control media, 2.6 µL of DBP with 997.4 µL of control media, 6.5 µL of DBP with 993.5 µL of control media, 13 µL of DBP with 987 µL of MEM media and 26 µL of DBP with 974 µL of control media respectively to make the final concentration of DBP as 1,10,25,50,100 µM respectively.

Granulosa Cell Collection and Culture

Ovine ovaries (n = 242) were collected from the civil slaughterhouse, Bengaluru, aseptically in sterile 0.9% saline supplemented with 100 X antibiotic- antimycotic solutions (10,000 U/mL Penicillin, 10 mg/mL, Streptomycin and 25 µg/mL Amphotericin B). Isolation and culture of granulosa cells were carried out as outlined by Nandi *et al.* (2016). The viability of the granulosa cells after culture was determined by the trypan blue exclusion test (Nandi *et al*., 2016). The cells ($1X10^5$ per ml) were cultured at 37°C in an atmosphere of 5% CO_2 with 95% humidity in a CO_2 incubator for 5 days. Every alternate day the growth was checked in a microscope, and media was renewed with fresh media along with the treatment on day 2. The cultured cells and media of day 5 were used for performing the biochemical assays and for the estimation of Estradiol-17β concentration, respectively.

Preparation of Granulosa Cell Lysis Supernatant

On day 5, the isolated granulosa cells of all treatments were homogenized separately using a sonicator (50 W for 1-min) in 1X (400 µL) of lysis buffer (pH 8) containing 10 mM Tris-HCl, 20 mM EDTA, and 0.25% V/V Triton X-100. After that, the lysate was centrifuged for 20 minutes at 4°C at 10,000 X *g*, and the supernatant was transferred for biochemical analysis.

Granulosa Cell Viability and Proliferation (MTT assay)

The MTT metabolic activity test was done by MTT kit following the manufacturer's instructions to assess the viability and proliferation of granulosa cells. The absorbance intensity was measured at 570 nm using an ELISA microplate reader. All studies were conducted in triplicate, and the relative cell viability (%) was calculated.

Total Antioxidant Activity (CUPRAC)

The freshly harvested cells (day-5) of the control and the treatment groups were used for the total antioxidant activity assessed by the CUPRAC kit. The absorbance was measured at 450 nm using an ELISA microplate reader. All studies were conducted in triplicate, and CUPRAC (total antioxidant activity) of each sample was calculated as Trolox Equivalents using the equation of the standard curve in µM.

MDA Assay (Lipid Peroxidation Test)

The cell lysate of freshly harvested cells (day-5) of control and treatment groups were used for lipid peroxidation and assessed by MDA kit. The absorbance was measured at 532 nm using an ELISA microplate reader. All studies were conducted in triplicate, and the calibration was performed using 1,1,3,3-tetra methoxy propane as a standard. The levels of lipid peroxides were presented as µM MDA.

Estradiol-17β Estimation by ELISA

The conditioned spent medium from all treatment groups of DBP (0,1,10,25,50, and 100 µM) of day 5 was collected after treatment. The estradiol 17β hormone concentrations were evaluated using the ELISA kit following the instructions as per the kit protocol. All samples were repeated in triplicate. The data were presented in pg/ml.

Statistical Analysis

All data were analyzed statistically using Graph Pad prism (8.0) software, and data were expressed as Mean ± SE from six separate experiments. The difference in means was determined using Tukey's multiple comparison test. A probability of $p < 0.05$ was considered statistically significant.

Table 1: Effect of different concentrations of DBP on granulosa cell viability, antioxidant enzyme activity, lipid peroxidation, and estradiol synthesis

Treatments DBP (µM)	*MTT(% viability)*	*MDA(µM)*	*CUPRAC activity (µM)*	*Estradiol(pg/mL)*
Control(0)	68.24±3.41[a]	13.76±0.48[a]	19.51±4.78[a]	20.99±0.58[a]
1	31.37±0.34[c]	18.92±0.54[b]	21.07±4.23[a]	32.92±1.35[a]
10	42.59±0.40[b]	22.54±1.66[c]	22.70±6.04[a]	57.04±7.41[b]
25	40.59±7.5[b]	19.49±0.51[b]	21.68±4.70[a]	99.67±6.06[c]
50	41.67±5.28[b]	21.49±1.21[c]	24.05±6.26[a]	154.8±15.81[d]
100	44.55±0.57[b]	19.14±0.16[b]	25.54±7.28[a]	268.1±74.28[e]

Values with different superscript letters differ significantly ($P < 0.05$) within the column.

Results and Discussion

Cell viability (MTT assay) and Lipid Peroxidation (MDA Assay)

Cell viability was significantly reduced at 1-µM concentration of DBP compared to that observed in the control group (Table 1). However, we observed significantly increased viability when the granulosa cell was exposed to 10 µM compared to that observed in the 1-µM group. As the DBP level was increased from 10 to 100 µM, no significant change in cell viability was observed between the treatment groups. A significant increase in lipid peroxidation was observed in the 1-µM level of DBP-treated granulosa cells compared to that observed in the control group (Table 1). The MDA concentrations observed in 10 and 50 µM of DPB (non-significant between them) treated granulosa cells were significantly higher than other DBP levels tested. The levels of MDA at 1,25 and 100 µM concentration of DBP were statistically similar, and at 10 and 50 µM DBP, the values were statistically higher compared to 0,1,25 and 100 µM DBP.

The expression of MDA was the biomarker of lipid peroxidation, which damages cell membrane (Ahmed *et al*., 2011) and reactive oxygen species (ROS), which reflected cellular stress in causing radical-mediated cellular stress. At the lowest concentration of 1 µM of DBP, cell viability and cell proliferation were decreased because this lower dose could activate cellular stress and result in activating other antioxidant systems for cell survivability. Studies on pharmacokinetics suggested that DBP was metabolized to form monobutyl phthalate (MBP) and phthalic acid in mammalian cells. MBP can then be excreted free or conjugated into a glucuronidated form or oxidized into mono-3-oxon-butyl phthalate, mono-3-hydroxy-n-butyl phthalate, and mono-3-carboxypropryl phthalate (Fennell *et al*.,2004) but these unreleased excessive metabolites also acted as free radicals in the cell, were highly reactive (unstable) and could cause lipid peroxidation (Ahmad *et al*.,2011). Lipid peroxidation directly harmed the cell membrane, which was composed of long-chain unsaturated fatty acids and membrane proteins and resulted in causing oxidative damage to long-chain unsaturated fatty acids and thus produced malondialdehyde (MDA), as a byproduct. This level of MDA was often used as an index of measurement of free radical activity (Lin *et al*., 2006).

Cupric Reducing Antioxidant Power (CUPRAC Assay)

There was no significant difference in the total antioxidant capacity as measured by CUPRAC activity; however, there was an increasing numerical trend from 1 to 100 µM DBP. (Table 1). Our results indicated that DBP exposure increased radicals in the granulosa cells that induced oxidative stress and caused cell membrane damage by inducing the lipid peroxidation in the cultured granulosa cells. Erkekoglu *et al*. (2011) reported that phthalate caused higher induction of ROS level, higher expression, and activity of antioxidant enzymes (e.g., superoxide dismutase and nitric oxide synthase). Elevated levels of ROS regulated the expression and activity of antioxidant enzymes by activating different signaling pathways in different biological systems, such as extracellular signal-regulated kinases (ERKs) and p38 mitogen-activated protein kinases (MAPKs), (Knock and Ward; 2011) and tyrosine kinase (El-Deeb *et al*.; 2010).

Estradiol-17β production

There was no significant difference in estradiol production between control and 1-µM DBP-treated groups (Table 1). However, further increment of DBP to 10 to 100 µM level caused a significant increase in estradiol production from the granulosa cells compared to those observed in a lower concentration of DBP-treated groups. DBP is an environmental endocrine disruptor with comparatively strong estrogenic and mitogenic properties (Hu *et al*., 2013). Roszak *et al*. (2017) found that DBP induced the proliferation of estrogen receptor-positive human breast cancer MCF-7/BUS cells. DBP has structural and estrogenic similarities with estrogen (Parveen *et al*., 2008). Our study indicated that DBP had strong estrogenic properties. It was also reported that DBP might bind to steroid receptors (ERβ1) and give a receptor-mediated additive effect (Catherine *et al*.,1997).

Natural hormones responded to their targets at low concentrations and might exert their effect at the nano and picomolar range. So it was often predicted that endocrine-disrupting chemicals such as DBP often exerted its effect at a deficient concentration, resulting in a non-monotonic dose-response and failed to provide similar results predicted for higher doses (Welshons *et al*., 2003 and Vandenberg *et al*., 2012). Non-monotonic dose-response curves are usually U-shaped, indicating that maximum responses of the measured endpoint were observed at low and high doses or an inverted U-shaped graph, which indicated maximal responses are observed at intermediate doses (Vandenberg *et al*., 2012). Welshons *et al.* (2003) found in their study that a 10-fold increase in hormone concentration and a 9-fold increase in receptor occupancy at the lower dose of EDC exposure, whereas a 10-fold rise in hormone concentration and more petite than 1.1-fold increase in receptor occupancy at high doses of EDC exposure. This study gave an idea to understand that even moderate changes in EDC concentration in the low-dose range produced substantial changes in receptor occupancy and therefore generated significant changes in biological effects.

DBP might induce its toxicity in two ways: disturbing the normal oxidative systems via lipid peroxidation of the cell membrane or, therefore, leading to granulosa cell apoptosis or disturbing normal synthesis of steroid hormone-like estradiol. Finally, the short-term exposure of

DBP may interact with transcription factor pathways such as Peroxisome proliferator-activated receptors (PPARs) and Aryl hydrocarbon receptor (AHR) to cause these gene expression changes, which need to be studied further in detail at the cellular and gene level.

Conclusion

This study found that the exposure of DBP induced oxidative stress and caused cellular stress by decreasing cell viability and thereby modulating the steroidogenesis hormone production in granulosa cells. This study demonstrated ovarian toxicity in the very low dose exposures to DBP in ovine granulosa cells.

Acknowledgment

We are grateful to the UGC and Director, ICAR-NIANP, Bangalore, for providing the necessary facility to carry out the research work.

References

Ahmed, T., Pathak, R., Mustafa, M.D., Kar, R., Tripathi, A.K., Ahmed, R.S., & Banerjee, B.D. (2011). Ameliorating effect of N-acetylcysteine and curcumin on pesticide-induced oxidative DNA damage in human peripheral blood mononuclear cells. - *Environmental Monitoring and Assessment*. *179,*293–309.

ATSDR. (2001). Toxicological Profile for di-n-butyl phthalate (DBP). Atlanta, GA. Available online at: http://www.atsdr.cdc.gov/toxprofiles.

Catherine, A., Pirkko, H., Malcolm, G. P., & Sumpter, J. P. (1997). The Estrogenic Activity of Phthalate Esters *in Vitro*. *Annals of Clinical & Laboratory Science,105,*802-811.

El-Deeb , I.M., Yoo, K., & Lee, H. (2010). ROS receptor tyrosine kinase: a new potential target for anticancer drugs. *Medical Care Research and Review*. *31,*794–818.

Erkekoglu, P., Rachidi, W., Yuzugullu, O.G., Giray, B., Ozturk, M., Favier, A., & Hincal, F. (2011). Induction of ROS, p53, p21 in DEHP- and MEHP-exposed LNCaP cells-protection by selenium compounds. *Food and Chemical Toxicology*. *49,*1565–1571. Fennell, T., Krol, W., Sumner, S., & Snyder, R. (2004).Pharmacokinetics of dibutyl phthalate in pregnant rats. *Toxicological Sciences*. *82,* 407–418.

Guo, Z.Y., Gai, P.P, Duan, J., Zhai, J.X., Zhao, S.S., Wang, S., & Wei, D.Y. (2010). Simultaneous determination of phthalates and adipates in human serum using gas chromatography-mass spectrometry with solid-phase extraction. Biomedical Chromatography. 24,1094-1099.

Hu, J., Du, G., Zhang, W., Huang, H., Chen, D., Wu, D., Wang, X. (2013). Short-term neonatal/prepubertal exposure of dibutyl phthalate (DBP) advanced pubertal timing and affected hypothalamic kisspeptin/GPR54 expression differently in female rats. *Toxicology*. 314, 65-75.

Jakub, K., Yoshihiro, N., Toshihiko, Y., & Giudice, L. (2010). Peroxisome Proliferator-Activated Receptor-Mediates Bisphenol A Inhibition of FSH-Stimulated IGF-1, Aromatase, and Estradiol in Human Granulosa Cells. *Environmental Health Perspectives*.118, 400-406.

Knock, GA, & Ward, J.P. (2011). Redox regulation of protein kinases as a modulator of vascular function. Antioxidant Redox Signal. 15,1531-1547.

Latini, G. (2005). Monitoring phthalate exposure in humans. *Clinica Chimica Acta*. *361*, 20–29.

Lin, K.H.R., Tsou, C.C., Hwang, S.Y., Chen, L.F.O., & Lo, H.F. (2006). Paclobutrazol pretreatment enhanced flooding tolerance of sweet potato. *Journal of Plant Physiology*. *163*,750–760.

Lovekamp-Swan, T., & Davis, B. J. (2003). Mechanisms of phthalate ester toxicity in the female reproductive system. *Environmental Health Perspectives*. *111,* 139–145.

Nandi, S., Gupta, P.S.P., & Mondal, S. (2016). Ammonia concentrations in different size classes of ovarian follicles of sheep (Ovis aries): Possible mechanisms of accumulation and its effect on oocyte and granulosa cell growth *in vitro*. *Theriogenology*. *85,* 678–687.

Parveen, M., Inoue, A., Ise, , R., Tanji, M. & Kiyama, R. (2008). Evaluation of estrogenic activity of phthalate esters by gene expression profiling using a focused microarray. *Environmental Toxicology & Chemistry*. *27,* 1416-1425.

Rhind S.M. (2002). Endocrine-disrupting compounds and farm animals: their properties, actions, and routes of exposure. *Domestic Animal Endocrinology*. *23,* 179–187.

Roszak, J., Smokpieniazek, A., Domeradzkagajda, K., Grobelny, J., Tomaszewska , E., Ranosze-ksoliwoda., Celichowski, K., & Stepnik, M. G. (2017). Inhibitory effect of silver nanoparticles on proliferation of estrogen-dependent MCF-7/BUS human breast cancer cells induced by butylparaben or di-n-butyl phthalate. *Toxicology and Applied Pharmacology*. 337.

Silva, M. J., Reidy, J. A., Herbert, A. R., Preau, J. L., Needham, L. L., & Calafat, A.M. (2004). Detection of phthalate metabolites in human amniotic fluid. *Bulletin of Environmental Contamination* and *Toxicology*. 72, 1226–1231.

Vandenberg, L., Colborn, N.T., Hayes, T.B., Heindel, J.J., Lee, J.D., Shioda, T. A., Soto, M., Vomsaal, F.S. & Welshons, W.V. (2012): Hormones and endocrine-disrupting chemicals: low-dose effects and non-monotonic dose responses. *Endocrine Reviews*.*33,* 378-455.

Welshons, W., Thayer, V.K.A., Judy, B. M., Taylor, J.A., Curran, E.M. & VomSaal, F.S. (2003). Large effects from small exposures. Mechanisms for endocrine-disrupting chemicals with estrogenic activity. *Environmental Health Perspectives*. *111*, 994-1006.

Yaxian, M., Jun, Z., Rujun, Z., Xiaoyong, Q., Ran, C., Ying, N., Yunyao, L., Sicong, Li., Jing, Z.,Wenming, X., Liangzhi, X. & Ying, H. (2019). Effects of the Dibutyl Phthalate (DBP) on the Expression and Activity of Aromatase in Human Granulosa Cell Line KGN. *Annals of Clinical & Laboratory Science, 49*.