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Are the new phthalates safe? Evaluation of Diisononilphtalate (DINP) effects in porcine ovarian cell cultures

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A R T I C L E I N F O

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A B S T R A C T

Phthalates are plasticizing chemicals, widely used in packaging materials and consumer products for several decades. These molecules have raised concerns because of their toxicity and their use have been restricted in several countries. Therefore, novel phthalates have been introduced. Among these, diisononilphtalate (DINP) is widely employed. However, its safety has not been properly addressed. Therefore, using a well validated granulosa cell model, collected from swine ovaries with a translational value, we studied potential DINP effects on important cellular functional parameters. In particular, we studied cell growth, steroidogenesis and redox status. Collected data showed that DINP stimulates ($p < 0.05$) cell growth, increases estrogen and inhibits progesterone production ($p < 0.05$), disrupts redox balance stimulating free radicals ($p < 0.05$) while reducing scavenger activities ($p < 0.05$). Taken together, DINP's impact on cultured swine granulosa cells provides cause for concern regarding its potential adverse effects on reproductive and endocrine functions.

1. Introduction

Phthalates are esters of phthalic acid and represent a family of chemical compounds used in the plastics industry as plasticizing agents, i.e. as substances added to the polymer to improve its flexibility and mouldability (Kumari and Pulimi, 2023). In terms of production volume, they are mainly used in PVC synthesis (Czogała et al., 2021). Phthalate allows the polymer molecules to flow over each other, making the material soft and malleable even at low temperatures (Harmon and Otter, 2022). Typical products are films, floor coverings, pipes, cables, paints, nail polishes and hair sprays. They are also used as auxiliary substances, e.g. in the gastro-resistant coating of active drugs (Arrigo et al., 2023). Phthalates represent important chemicals for industry and are therefore produced in large quantities (Baneshi et al., 2023). In the past, the most commonly used phthalate was diethylphthalate (DEHP) (Landrigan et al., 2023). Unfortunately, since plasticizers do not bind covalently to polymers they can be easily released from plastic products through volatilization, abrasion and subsequent leaching into the environmental (Kumari and Pulimi and, 2023). Living organisms are mainly exposed by oral and respiratory route as well as by dermal absorption (Wang et al., 2023a; b; Mérida et al., 2023). Phthalate presence has been documented in human blood, amniotic fluid and urine (Kumari and Pulimi, 2023). Since 2011, numerous studies have highlighted the risks deriving from exposure to these substances such as the development of cancer, asthma, diabetes, and metabolic disorders (Benjamin et al., 2017; Yu and Wang, 2022; Wang et al., 2023a,b; Wu et al., 2023; Mariana and Cairrao, 2023); in addition, immune (Zhang et al., 2022), nervous (Wylie and Short, 2023) and reproductive systems (Basso et al., 2022) appear to be affected. Above all, they have been indicated as endocrine-disrupting chemicals (EDCs) i.e. chemicals that mimic, block, or interfere with hormones (Ahn and Jeung, 2023). In particular, severe effects have been reported on male as well as female reproductive system (Adegoke et al., 2023; Levine and Hall, 2023; Interdonato et al., 2023) thus representing a real threath to fertility outcome (Panagopoulos et al., 2023). However, to date most of the research refers to DEHP, while more recent novel phtalate derivative effects have been poorly studied. Due to their environmental presence and critical effects, the use of several phthalates, mainly represented by ortho-phthalates, has been restricted in cosmetics, children's clothing and toys, and medical devices in several European countries, Japan and USA (Landrigan et al., 2023). Alternative plasticizers such as Diisononyl phthalate (DINP), a long-chain phthalate, have been incorporated into manufacturing processes largely in the absence of any conclusive information as to their safety or toxicity, prior to their launch onto the market. Therefore, the present investigation was undertaken to explore the effect of DINP using a well validated translational model of endocrine and reproductive cells represented by swine

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granulosa cells (Tumbleson and Schook, 1996; Basini et al., 2013, 2021a, 2023; Berni et al., 2019). We tested the possible effects of DINP on the main functional features of ovarian granulosa cells i.e. cell growth and steroidogenesis; in particular, the synthesis of estradiol 17β (E2) and progesterone (P4) are crucial hallmarks of granulosa cell well being (Odermatt et al., 2016). Moreover, since redox balance is essential for granulosa cell function (Liu et al., 2023), we tested DINP effects on oxidative stress markers, considering superoxide anion (O_2^-) as reactive oxygen species and nitric oxide (NO) as reactive nitrogen species examples (Basini and Grasselli, 2015). Moreover, we also tested potential effects of DINP on antioxidant defence mechanisms, considering both enzymatic (superoxide dismutase activity, SOD) and non-enzymatic (Ferric Reducing Activity of Plasma, FRAP) ones (Basini et al., 2023).

2. Materials and methods

Reagents were purchased from Sigma (St. Louis, MO, USA) unless differently stated.

2.1. Granulosa cell culture

At a local abbatoir, six collections of ovaries were sourced and excised from 40 Large White hybrid gilts aged 8- 9 months. The oestrous cycle stage was determined on the base of a morphological study (Babalola and Shapiro, 1988). Ovaries were put in PBS and subjected to treatments as reported in previous studies (Bianco et al., 2006). Morphological criteria were used to classify ovarian follicles (Grasselli et al., 2003). Collection of granulosa cells was carried out in sterile conditions from healthy follicles >5 mm by employing medium containing 50 IU/mL heparin (Foxcroft and Hunter, 1985; Basini et al., 2014; Ciccimarra et al., 2018). Mural cells were obtained by gentle scraping of the follicle wall with the needle. Purity was always higher than 90 % (Basini et al., 2021a; Basini et al., 2021b). Cells were grown in a previously validated culture medium DMEM/Ham's F12 (Basini et al., 2022) (CM). Cells were treated for 48 h with different concentrations of DINP (1, 10 and 100 nM) at 37 °C under humidified atmosphere (5 % CO_2). The selected concentrations were lower than those tested to be effective in previous research (Mlynarcíková et al., 2007; Chen et al., 2022; Rajkumar et al., 2022). DMSO, at a final concentration lower than 0.1 % v/v, was the carrier solvent.

2.2. Granulosa cell proliferation and metabolic activity

ELISA BrdU (Roche Diagnostic, Indianapolis, In, USA) was employed. 10^4 cells/200 μL of CM were seeded in 96-well plates (Sarstedt, Nümbrecht, Germany) and treated with DINP. The Victor Nivo spectrophotometer (Perkin Elmer, Groningen, The Netherlands) was employed to assess the absorbance values at 450 nm. Viable cells were quantified comparing absorbance with a standard curve (Gigante et al., 2018) which was prepared by culturing granulosa cells at different plating densities (10^3 to 10^5 viable/200 μL) for 48 h. The curve was repeated four times. The relationship between cell number and absorbance was linear ($r = 0.92$). The resulting linear regression equation was employed to quantify the number of cells/well, which was used to correct for the experimental data. The detection limit of the assay was 10^3 cells/well and the coefficient of variation was less than 5 %.

In order to evaluate cell metabolic activity we quantified intracellular ATP levels by means of a bioluminescent assay (ATP-lites; Packard Bioscience, Groningen, Netherlands). ATP represents a well known marker of viability which rapidly decreases during cell necrosis or apoptosis (Basini et al., 2020). 2×10^5 viable cells/200 μL CM were grown in 96-well plates (Perkin Elmer, Buckinghamshire, UK) and subjected to a 48 h treatment with DINP as previously illustrated. The ATP lite-M assay system is based on the detection of light produced by the luciferase catalysed reaction of ATP with D-luciferin. The emitted light is proportional to the ATP concentration. Briefly, 100 μL of medium were removed from the well and 50 μL of mammalian cell lysis were added to the cell aggregates, attached to the culture plate by enlarged, flattened fibroblast-like cell, that formed the feet of the clump. This is the physiological morphology of granulosa cells that could be retained thanks to our previously validated serum free culture system (Basini and Tamanini, 2000). Finally, we supplemented the wells with 50 μL of substrate solution. Victor Nivo reader was employed to assess the luminescence.

2.3. Granulosa cell steroidogenesis

10^4 viable cells were cultured in 96-well plates (Sarstedt, Nümbrecht, Germany) with 200 μL CM supplemented with 28 ng/mL androstenedione (Basini and Tamanini, 2000) in the presence of DINP as previously described. Media were then collected, frozen and stored at –20 °C for progesterone (P4) and estradiol 17β (E2) quantification by means of Estradiol and Progesterone ELISA kits (Dia.Metra s.r.l, Spello, PG, Italy). As for E2 assay, sensitivity is 8.6 pg/mL and intra-assay CV is <9 %; P4 assay sensitivity is 0.05 ng/mL and the intra-assay CV is < 4 %. Victor Nivo reader (Perkin Elmer, Groningen, The Netherlands) (Pacentra et al., 2020) was used to read absorbance at 450 nm against a reference wavelength of 620–630 nm.

2.4. Granulosa cell redox status

WST-1 (4-[3-(4-iodophenyl)–2-(4-nitrophenyl)–2 H-5-tetrazolium]–1,3-benzene disulfonate) test (Roche, Mannheim, Germany) was employed to assess O_2^- production (Basini et al., 2009). After seeding 10^4 cells/200 μL CM in 96-well plates, treatment with DINP was carried out as previously illustrated. After the addition of 20 μL WST-1 to each well during the last 4 h of incubation, the Victor Nivo was employed to assess absorbance of the color developed at a wavelength of 450 nm against 620 nm. The coefficients of variation were less than 3 %.

Nitrite in culture media were quantified to assess NO levels using a microplate method based on the formation of a chromophore after reaction with Griess reagent. After seeding 2×10^5 viable cells/200 μL CM in 96-well plates, DINP tretment was carried out as above detailed. Plates were then centrifuged for 10 min at 400g and we collected supernatants. At the end of Griess reagent incubation, the Victor Nivo was employed to assess absorbance by means of a 540 nm against 620 nm filter. By diluting sodium nitrite in CM (Dong and Yallampalli, 1996) we prepaired a calibration curve ranging from 25 to 0.39 μM.

Non-enzymatic scavenging activity was tested by the Ferric Reducing Activity of Plasma (FRAP) assay, a colorimetric method based on the reduction of ferric-tripiridyltriazine (Fe^{3+} TPTZ) to a ferrous form (Fe^{2+} TPTZ) by antioxidant molecules present in a biological matrix. The assessment of Fe^{2+} colored complex with 2,4,6-Tris(2-pyridyl)-s-triazine (Fe^{2+} TPTZ) by a spectrophotometer allows Fe^{2+} quantification. TPTZ reagent was obtained by mixing 25 mL of acetate buffer, 2.5 mL of 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) 10 mM in HCl 40 mM and $FeCl_3 – 6H_2O$ solution. After seeding 2×10^5 cells/200 μL CM in 96-well plates, DINP tratment was carried out. Plates were then centrifuged for 10 min at 400g, supernatants were discarded and cell lysis was obtained by the addition, in ice bath for 30 min, of cold Triton 0.5 % + PMSF in PBS (200 μL/well). 40 μL of cell lysates added to Fe^{3+} TPTZ reagent were used. Victor Nivo was used to assess absorbance of Fe^{2+} TPTZ at 595 nm after a 30 min incubation at 37 °C. The ferric reducing ability of cell lysates was quantified by plotting a standard curve of absorbance against $FeSO_4 – 7H_2O$ standard solution (Gigante et al., 2018).

A SOD Assay Kit (Dojindo Molecular Technologies, Japan) was used to assess superoxide dismutase activity. After seeding 2×10^5 cells/200 μL CM in 96-well plates, the DINP treatment was carried out for 48 h. After centrifugation for 10 min at 400g, the surnatants were discarded and cell lysis was carried out by adding cold Triton 1 % in TRIS HCl (100 μL/10^5 cells) and incubating on ice for 30 min. The test was carried out on cell lysates without dilution, employing a standard curve of SOD

ranging from 0.156 to 20 U/mL. Formazan production resulting from the reaction beetwen a tetrazolium salt and a superoxide anion (O_2^-), generated by the reaction of an exogenous xantine oxidase, was assessed by a colorimetric assay. The remaining O_2^- is an indirect hint of the endogenous SOD activity. Victor Reader reading at 450 nm against 620 nm was employed to assess absorbance (Basini et al., 2007).

2.5. Statistical analysis

Six experiments were carried out, with a total of 240 gilts from which ovaries were collected. Treatments with DINP at different concentrations were performed with six replicate wells. Data are presented as mean ± SEM. Statistical differences were calculated by ANOVA using Statgraphics software (STSC Inc., Rockville, MD, USA). When significant differences were found, means were compared by Scheffè 's F test; p values < 0.05 were considered to be statistically significant.

3. Results

3.1. Granulosa cell proliferation and metabolic activity

DINP significantly increased (Fig. 1 A) ($p < 0.01$) cell proliferation, monitored by BrdU uptake, at all evaluated concentrations. No differences were shown among the different concentrations tested.

In addition, DINP treatment significantly enhanced (Fig. 1B) ($p < 0.05$) cell metabolic activity, evaluated as ATP production, at the concentration of 1 and 10 nM.

3.2. Granulosa cell steroidogenesis

DINP altered granulosa cell steroidogenesis. Specifically, E2 production was significantly stimulated ($p < 0.05$) without distinction between the different DINP doses (Fig. 2A) ($p < 0.05$).

On the contrary, P4 production was inhibited ($p < 0.001$) at all tested concentrations, without differences between the induced effects (Fig. 2B) ($p < 0.001$).

3.3. Granulosa cell redox status

As for the redox status parameters, NO generation was significantly increased ($p < 0.05$) by the highest concentration tested (Fig. 3A) while O_2^- was significantly enhanced ($p < 0.01$) (Fig. 3B) by DINP but only by 10 nM.

Scavenger activity was significantly reduced by DINP. Enzymatic activity as well as non-enzymatic antioxidant power, (Fig. 4) were significantly reduced ($p < 0.05$) at all the concentrations tested, without significant differences among them ($p < 0.05$).

4. Discussion

Ovarian follicles are specialized compartments where growth and development of oocytes take place. Germ cells are surrounded by multiple layers of somatic granulosa cells which closely regulate proliferation and metabolic activity are responsible for the fate of oocyte development until ovulation. Therefore, an ordered and complex series of signaling events take place during follicular growth. Endocrine disruptors have been pointed out as responsible of severe disturbancies in the normal control of cell growth that cause alteration in brain development (Demeneix, 2019), mammary gland (Kass et al., 2020; duPlessis et al., 2023) and male reproductive tract function (Adegoke et al., 2020; Corti et al., 2022). In details, disruption in the fine tuning of cell growth regulation has been associated to most of the toxic reproductive effects attributed to phthalates (Zhang et al., 2023). In contrast with our present findings, Inada et al. (2012) and Li et al. (2018) demonstrated that mono-(2-ethylhexyl) phthalate (MEHP), the active metabolite of DEHP, markedly attenuated proliferation of rat ovarian granulosa cells. These contrasting results could be attributable to specific actions displayed by different phtalates. Further studies are necessary to unravel specific molecular events involved in signalling mechanisms of various phthalates. To our knowledge, present study investigated for the first time the effect of DINP on granulosa cell growth. Interestingly, Chen et al. (2022) studied DINP effect on granulosa cell apoptosis and autophagy in mouse granulosa cells, reporting a dramatic increase which appears to be attributable to the concomitant oxidative stress. Data collected in our research confirm the imbalance in redox status, since reactive species increased while antioxidant defence was reduced by DINP. During aerobic metabolism prooxidant molecules called free radicals can be generated. The complex interaction between prooxidants and antioxidants is necessary for the maintenance of cellular homeostasis. The imbalance between prooxidants and antioxidants results in oxidative stress (Agarwal et al., 2012) which is detrimental for fertility outcome. Granulosa cell oxidative stress can be associated with reproductive disorders such as polycystic ovary syndrome and premature ovarian failure (Liu et al., 2023) and aging (Wang et al., 2023a,b). Oocytes have been shown to be particularly susceptible to free radical damage (Shi et al., 2023), mainly due to the molecules present in follicular fluid, which contains secretory products from granulosa cells. The oocyte and the surrounding granulosa cells are tightly coupled by means of the microenvironment represented by follicular fluid, which allows nutrient exchange and biological signal transmission.

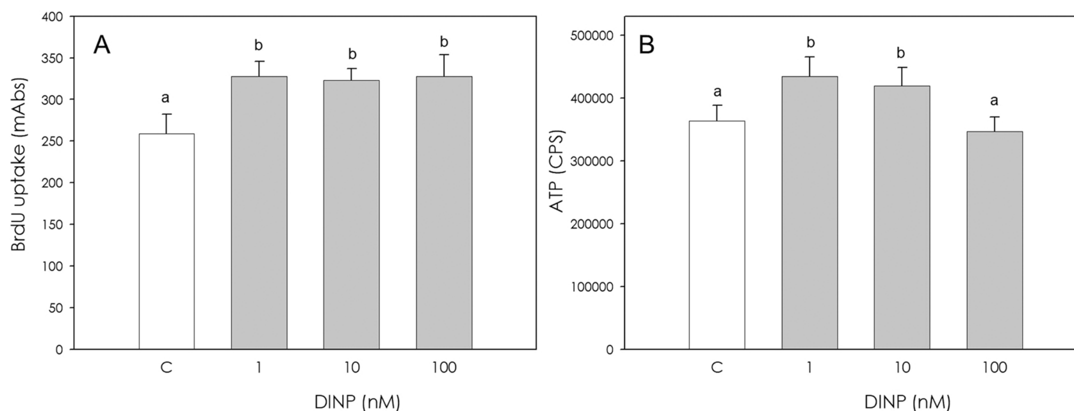

Fig. 1. Effect of the treatment with Diisononilphtalate (DINP 1, 10 and 100 nM) for 48 h on swine granulosa cell proliferation using 5-bromo-2'-deoxyuridine (BrdU) incorporation assay test (panel A) and on swine granulosa cell metabolic activity using ATP content assay test (panel B). Data, expressed as milliAbs units (panel A) or as counts per second (panel B), represent the mean ± SEM of six replicates/treatment repeated in six different experiments. Different letters on the bars indicate a significant difference ($p < 0.05$) among treatments as calculated by ANOVA and Scheffè' F test.

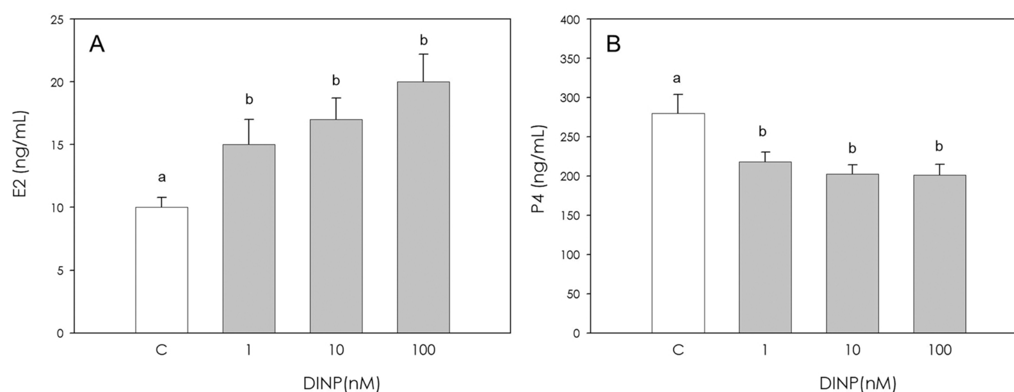

Fig. 2. Effect of the treatment with Diisononilphtalate (DINP 1, 10 and 100 nM) for 48 h on swine granulosa cell estradiol 17β (E2) production (panel A) or progesterone production (panel B) using ELISA assay. Data, expressed as ng/mL, represent the mean ± SEM of six replicates/treatment repeated in six different experiments. Different letters on the bars indicate a significant difference ($p < 0.05$) among treatments as calculated by ANOVA and Scheffè ' F test.

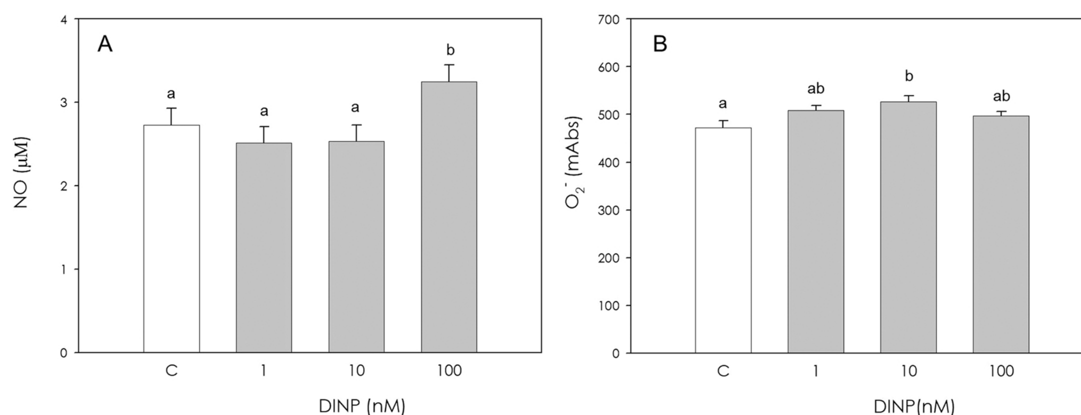

Fig. 3. Effect of the treatment with Diisononilphtalate (DINP 1, 10 and 100 nM) for 48 h on swine granulosa cell nitric oxide (panel A) and superoxide anion ($O_2^{\cdot -}$) generation (panel B) using colorimetric assays. Data, expressed as µM (panel A) or milliAbs units (panel B), represent the mean ± SEM of six replicates/treatment repeated in six different experiments. Different letters on the bars indicate a significant difference ($p < 0.05$) among treatments as calculated by ANOVA and Scheffè ' F test.

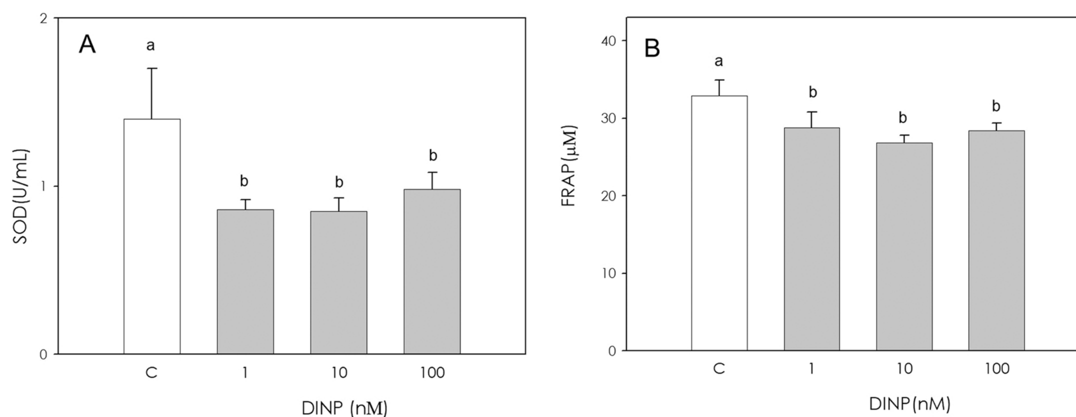

Fig. 4. Effect of the treatment with Diisononilphtalate (DINP 1, 10 and 100 nM) for 48 h on swine granulosa cell superoxide dismutase activity (SOD, panel A) or non-enzymatic scavenging activity (FRAP, panel B) by swine granulosa cells using colorimetric assays. Data, expressed as U/mL (panel A) or as µM (panel B), represent the mean ± SEM of six replicates/treatment repeated in six different experiments. Different letters on the bars indicate a significant difference ($p < 0.05$) among treatments as calculated by ANOVA and Scheffè ' F test.

Throughout the reproductive lifespan, the ovaries undergo cyclical structural and functional changes that are crucial for sex steroid hormone production. Granulosa layers primarily secrete estradiol and, to a lesser extent, progesterone (Casarini et al., 2022). The imbalance of sex steroid hormones is associated with adverse effects on female fertility and health such as polycystic ovary sindrome (Monget et al., 2021), primary ovarian insufficiency (Clark et al., 2022) and ovarian cell malignant neoplasia (Celar Sturm and Virant-Klun, 2023). Unfortunately, the peculiar biochemical processes occurring in granulosa cells provide many possible targets for endocrine disruptors, which have been

ascribed as the main responsibles of infertility which, according to the World Health Organization (WHO) affects 8–12 % of human couples in reproductive age worldwide (Silva et al., 2023). Even though pthalates have been severely categorized among endocrine disruptors (Zhang et al., 2023) the effect of DINP have been mostly ignored. Our data document an alteration of steroidogenesis in swine granulosa cells, a well-demonstrated translational model (Tumbleson and Schook, 1996; Basini et al., 2021a, 2023). Mlynarcíková et al. (2007) reported contrasting results which were obtained by employing different culture conditions. In particular, the effect of DINP on steroidogenesis was studied without the addition of an androgen precursor; moreover, the effect was examined in FSH-stimulated cells, not in basal condition as in our experimental setting. Finally, Mlynarcíková et al. cultured granulosa cells in the presence of FCS (10 %) which likely induces granulosa cell differentiation in luteal cells. On the contrary, we employed a serum free culture system, previously validated to avoid luteinization (Basini and Tamanini, 2000) and to maintain a physiological differentiation pattern.

The effects of phtalates on granulosa cell steroidogenesis have mostly been investigated in rat models, considering DEHP or its active metabolite, mono-(2-ethylhexyl) phthalate (MEHP). In general, estrogens appear to be inhibited (Davis et al., 1994; Lovekamp and Davis, 2001; Inada et al., 2012; Tripathi et al., 2019). The same effect was demonstrated in human granulosa cells (Reinsberg et al., 2009), while Li et al. (2018) reported a stimulatory effect in the rat. As for progesterone, Davis et al. (1994) and Tripathi et al. (2019) documented an inhibitory action, Inada et al. (2012), and Li et al. (2018) demostrated a stimulation, while no effect was reported by Reinsberg et al. (2009). Steroidogenesis appears to be suppressed by DBP both in rat (Wang et al., 2016) and human (2017). It should be noted that previous studies explored the effects of classical phtalates used at higher concentrations, while in the present work we focused on DINP which was employed at lower concentrations.

Together, our data indicate that the novel phthalate DINP can affect granulosa cell growth, redox balance and steroidogenesis, thus forecasting negative drawback on fertility and endocrine function. It should be noted that DINP concentrations do not exert a "dose-response" effect for most of the examined parameters but the substance appears to arise an "all or none" effect. Therefore, we argue that will be crucial to verify the effect of lower DINP concentrations to define more deeply its negative potential.

In conclusion, further studies are required to better clarify the effects of this pthalate on the ovary.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

Acknowledgement

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