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Effects of endocrine disruptor mixtures on functions of cultured porcine ovarian follicular cells

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Objective. Involvement of various endocrine disruptors (EDs) in pathophysiology of reproductive system disorders has been suggested previously. The studies have shown adverse effects of the individual substances, however, in the real-life situation, numerous chemicals enter the organism on a daily basis. This points to the importance of examination of the combined effects of exogenous chemicals on biological systems, including reproductive system. The ovaries are a target of different EDs, which may impact the processes within the ovarian follicles. The aim of the present study was to examine the effects of binary or ternary mixtures combining selected non-persistent disruptors: bisphenol A (BPA), BPA-dimethacrylate (BPADM), benzyl butyl phthalate (BBP), 4-chloro-3-methylphenol (CMP), or alkylphenols (4-octylphenol, OP; 4-nonylphenol, NP; and tert-octylphenol, TOP) on ovarian follicular cell functions.

Methods. Porcine oocyte-cumulus complexes (OCCs) and granulosa cells (GCs) were treated with the tested ED mixtures (BPA+BBP, CMP+BBP, BPA+BPADM, BPA+BBP+CMP, and OP+NP+TOP) in a wide concentration range from 10^{-10} to 10^{-4} M. Follicle-stimulating hormone (FSH)-induced cumulus expansion was assessed after 24 h of culture according to a subjective scoring system. After 44-h treatment, oocyte nuclear maturation was evaluated. Basal and FSH-stimulated progesterone production by OCCs and GCs was measured by commercial radioimmunoassay after 44 h and 72 h of the culture, respectively. One-way ANOVA and Bonferroni post-test were used for statistical analysis of data.

Results. The results obtained showed that the lower concentrations of ED mixtures (10^{-10}–10^{-6} M) did not exert significant changes, while the highest concentration (10^{-4} M) significantly inhibited cumulus expansion, oocyte meiotic maturation, and progesterone production by OCCs and GCs. Moreover, the inhibitory effects of ED mixtures seem to be more profound than the effects caused by the individual substances.

Conclusion. The experimental approach of testing mixtures should provide a more comprehensive view on the effects of the ubiquitous EDs on various cell types of the reproductive organs.

Keywords: endocrine disruption, bisphenol, alkylphenols, phthalates, ovarian granulosa cells, oocyte, progesterone

The possible harmful effects of exposure to synthetic chemicals that can disrupt the endocrine system have become a matter of concern during recent years, and the female reproductive organs and functions have been identified as the targets of such chemicals. Because the reproductive system is complex, many sites, from the hypothalamic-pituitary-gonadal axis to germinal cells, may be vulnerable and susceptible to the action of endocrine disruptors (EDs) (Scsukova et al. 2016; Pan et al. 2024; Tzouma et al. 2025).

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The oocyte and companion granulosa cells (GCs) comprising the ovarian follicular unit maintain a close association throughout development from primordial to preovulatory stages. At the time of follicular antrum formation, the GC population becomes divided into mural layers, inner layers of GCs and cumulus cells surrounding the oocyte. Adequate production of steroids by ovarian somatic cells is an indispensable requisite for the female reproductive health since it plays a key role in the female phenotype maintenance, as well as is critical for regular ovarian processes, including follicle growth, oocyte maturation and ovulation (Richards and Pangas 2010). In response to the ovulatory surge of gonadotropins, the cumulus cell-oocyte complex (OCC) undergoes mucification and expands by depositing an extracellular matrix enriched in hyaluronan (HA) and HA binding matrix glycoproteins between the cumulus cells (Salustri et al. 1989). Simultaneously, the oocytes undergo meiotic resumption and cytoplasmic modification and attain the fertilizable metaphase II (MII) stage. These cellular events that immediately occur in OCCs in the ovulatory phase are strictly regulated by pituitary hormones, steroids, and growth factors (Kimura et al. 2007).

The fact that EDs have been detected in the biological samples of general population, and even directly in the follicular fluid of women, emphasizes the demands for testing the influence of EDs on the ovarian follicular cell functions (Li et al. 2024). There are several chemical groups of EDs, and phenolic compounds represent widespread substances with possible adverse health impact. These substances can be easily absorbed on frequent basis due to their widespread occurrence in consumer products (Cao 2010; Gerona et al. 2016).

Bisphenol A (4,4'-isopropylidene-2-diphenol; BPA) has become one of the most studied EDs because of its extensive use in the manufacture of plastics. Previously, it has been found in the food storage containers, the inner lining of food cans, dental fillings, thermal paper receipts, etc. (Brotons et al. 1995; Gerona et al. 2016). BPA has been detected in 70–100% of population samples including ovarian follicular fluid (Ikezuki et al. 2002; Gerona et al. 2016). Disrupting potential of BPA on reproduction has been demonstrated by numerous studies, revealing that BPA is an ovarian toxicant affecting steroidogenesis (Peretz et al. 2014). Unfortunately, indications point that several disorders arising from the impaired ovarian steroid synthesis might be linked to the action of BPA, including disrupted estrous cyclicity and implantation (Ziv-Gal and Flaws 2016), higher risk of miscarriage (Lathi et al. 2014), or polycystic ovarian syndrome (Peretz et al. 2014). BPA-dimethacrylate (BPADM) is a key monomer used in material science, particularly in the formulation of polymers and composite materials, e.g. in dental applications (Mlynarcikova et al. 2005).

Benzyl butyl phthalate (BBP) is a plasticizer used in vinyl, adhesives, sealants, household products and cosmetics, although its potential toxicity has led to restrictions in some consumer products (Cao 2010; EC Regulation 2018/2005). According to several biomonitoring studies, BBP and its metabolites have been detected in various human biological samples including urine, semen, blood, umbilical cord blood, breast milk, and amniotic fluid (Wang et al. 2019; Zhang et al. 2021).

4-chlor-3-methylphenol (CMP, chlorocresol) is known as a potent disinfectant and antiseptic. It is also used as a preservative in cosmetics and medicinal products for both humans and animals. CMP was extensively detected indoor in the samples of house and care centers dusts (Mane et al. 2024).

Alkylphenols (including 4-octylphenol, OP; 4-nonylphenol, NP, tert-octylphenol, TOP) are organic compounds used in detergents, emulsifiers, paints, herbicides, or pesticides. As shown by several studies, alkylphenols appear to be prevalent in human samples (Acir and Guenther 2018). Chronic exposure to the mixture of environmentally relevant doses of NP and OP modified reproductive parameters in female mice (Patino-Garcia et al. 2018).

Numerous studies have shown that ovarian steroid hormone production is recognized as an important target for the action of EDs (Mlynarcikova et al. 2014). Furthermore, several studies have highlighted a correlation between ED exposure and increased incidence of meiotic aneuploidy, a phenomenon that underlies a large percentage of reproductive health issues (Fujimoto et al. 2011). However, while previous studies have focused on the action of the individual substances, organisms are exposed to ED cocktail in everyday life. Thus, there is an increasing tendency to examine the effects of chemical mixtures on various biological processes, including reproductive functions.

Several *in vivo* reproductive toxicity studies have investigated mixtures of chemicals, most of which have employed chemicals from the same families, such as phthalates (Gill et al. 2021; Gonsioroski et al. 2022), brominated flame retardants (Karpeta and Gregoraszczuk 2010; Lefevre et al. 2016), pesticides (Dopavogui et al. 2022) or fertilizers (Fowler et al.

2008; Lea et al. 2016). They confirm the threat of mixtures exposure for the ovary on different levels but additional studies need to be performed on mixtures combining compounds from different chemical families. Further research has shown that *in vitro* exposure to ED mixtures may adversely affect the ovarian functions by altering the expression of cell cycle regulators, apoptotic factors, steroidogenic enzymes, receptors, and antioxidant enzymes in cultured mouse antral follicles as well as may induce changes in sex steroid hormone concentrations in the serum of treated animals (El Fouikar et al. 2024; Henderson et al. 2024).

The aim of the present study was to explore how binary and ternary mixtures of selected EDs can affect production of steroid hormones by porcine ovarian GCs and maturation of porcine OCCs.

Materials and Methods

Chemicals and reagents. Tested chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). BPA (≥99% purity), BPADM (>98% purity), BBP (98% purity), CMP (99% purity), OP (99% purity), NP (≥97% purity), and TOP (97% purity) were initially dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) to stock solutions (10^{-7}–10^{-1} M) and at the time of use, they were diluted to the required concentrations with the culture medium. The final DMSO concentration in the culture media was 0.1% (v/v). Medium M199 with Earle's salts, fetal bovine serum (FBS), and cell culture reagents (L-glutamine, HEPES buffer, penicillin, streptomycin, fungizone) were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Porcine follicle-stimulating hormone (pFSH) was generously supplied by Dr. A.F. Parlow (National Hormone & Pituitary Program; Harbor-UCLA Medical Center, Torrance, CA, USA).

Isolation and culture of granulosa cells. The primary culture of porcine granulosa cells (GCs) is a valuable model for studying the effects of different chemicals on the functional characteristics of steroidogenically active cells (Mlynarcikova et al. 2005). Ovaries of prepubertal gilts were obtained from a local abattoir. Immediately after slaughter, the ovaries were collected and placed in physiological saline plus antibiotics (penicillin 100 U/ml, streptomycin 100 µg/ml), transported to the laboratory, and then were washed several times in sterile saline. GCs were aspirated by sterile syringe and needle from antral follicles (3–6 mm in diameter) and isolated by centrifugation for 10 min at 1000 × g. Cells were washed, resuspended in the culture medium M199 (with Earle´s salts, 1 mM L-glutamine, and HEPES buffer), supplemented with 1% antibiotic-antimycotic solution and 10% FBS at final density 1×10^7 cells/ml medium. The density of cells was measured by a handheld automated cell counter Scepter (Millipore, Billerica, MA, USA). The cell cultures were maintained at 37 °C in a humidified incubator in an atmosphere of 5% CO_2–95% air.

Treatment of granulosa cells. GCs were seeded in 24-well plates (TPP AG, Trasadingen, Switzerland) at a density of 1×10^6 cells/well, and treated with different concentrations (10^{-10}–10^{-4} M) of selected ED mixtures. Control cells were treated with an equal volume of vehicle alone (0.1% DMSO). For stimulated progesterone production, the treatment pattern was set in the same way, along with supplementation of culture media of all groups with pFSH (1 µg/ml), and the positive control group was treated with pFSH (1 µg/ml) alone (and the equal volume of vehicle). After 72 h exposure to the tested agents, the culture media were collected and stored at –20 °C until used for radioimmunoassay.

Isolation and culture of porcine OCC. Immediately after slaughter, porcine ovaries were collected in physiological saline plus antibiotics and transported to the laboratory in a thermos. Follicular fluid was aspirated from medium size follicles (3–6 mm in diameter) and OCCs were collected and washed three times in Medium 199 with Earl's salts buffered with 20 mM $NaHCO_3$ and 6.25 mM Hepes, and supplemented with 0.91 mM sodium pyruvate, 1.62 mM calcium lactate and antibiotics. Incubation of OCCs was carried out in 24-well dishes (SARSTEDT, Numbrecht, Germany) at 38.5 °C in the atmosphere of 5% CO_2 and 95% air. Groups of approximately 30 OCCs were incubated in 300 µl media/well with or without different concentrations of the tested mixture (BPA+BBP). The culture medium was supplemented with recombinant FSH (100 ng/ml) (Organon, Oss, The Netherlands) and 10% fetal calf serum (FCS) (GIBCO BRL, Ivitrogen, Paisley, UK).

Cumulus expansion assessment. Cumulus expansion was evaluated under a stereomicroscope at 24 h of culture. The degree of expansion was assessed according to a subjective scoring system: degree 0 – no expansion, +1 – separation of only the outermost layer of cumulus cells, +2 – further expansion involving the outer half of the cumulus oophorus, +3 – further expansion up to, but not including, the corona radiata, +4 – complete expansion including the corona radiata cells (Nagyova et al. 2000).

Expanded OCC rated as +2 or higher were included in assessment.

Meiotic maturation assessment. After 44 h of incubation, oocytes were denuded of their surrounding cumulus cells by repeated pipetting. Oocytes were then fixed in acetic acid/ethanol (1:3, v/v), stained with 1% orcein, and examined under a phase-contrast microscope for the stage of maturation. Oocytes with no visible chromatin or with unrecognizable chromatin configuration were considered degenerated and were discarded. Oocytes, in which diffuse or slightly condensed chromatin could be identified, were classified as being in the germinal vesicle (GV) stage. Oocytes that possessed clumped or strongly condensed chromatin which formed an irregular network of individual bivalents (prometaphase) or a metaphase plate but no polar body, were classified as being in the metaphase I (M I) stage. Oocytes with either a polar body or two bright chromatin spots were classified as being in the metaphase II (MII) stage (Algriany et al. 2004). Overall, oocytes with reinitiated meiosis, reaching MI or MII, irrespectively, were classified as underwent GV breakdown (GVBD).

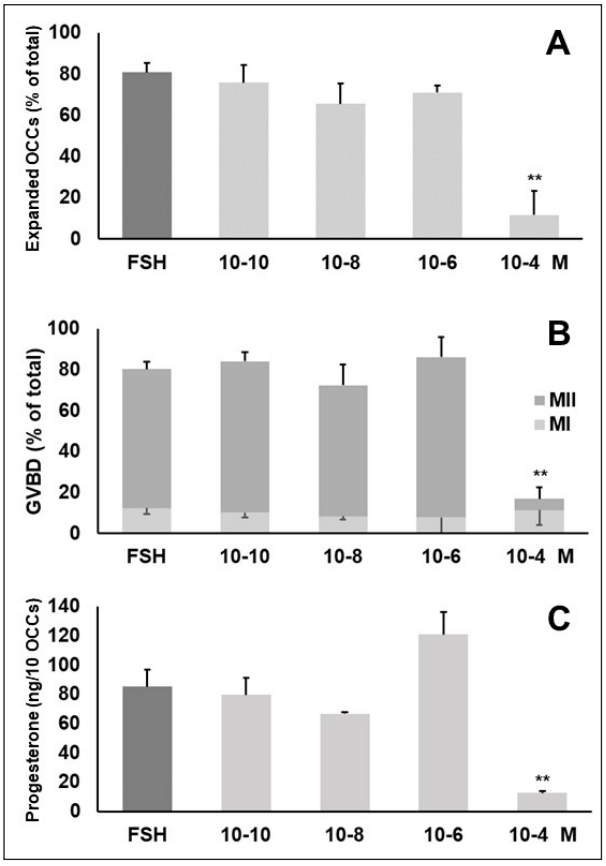

Figure 1. Effects of the mixture of bisphenol A (BPA) with benzyl butyl phthalate (BBP) on (**A**) follicle-stimulating hormone (FSH)-induced (100 ng/ml) expansion of porcine oocyte-cumulus complexes (OCCs) after 24-h culture; (**B**) nuclear maturation of cumulus-enclosed porcine oocytes after 44-h incubation; (**C**) progesterone production by FSH-stimulated porcine OCCs after 44-h culture. FSH represents a control group, i.e. OCCs treated with FSH alone. The results represent mean±S.E.M. of three independent experiments (**p<0.01 vs. FSH). Abbreviations: GVBD – germinal vesicle breakdown; MI – metaphase I; MII – metaphase II.

Radioimmunoassay. Progesterone levels in the culture media (collected after 44 h and 72 h of OCCs and GCs culture, respectively) were measured with a commercial [125I]-progesterone radioimmunoassay without extraction (IZOTOP, Budapest, Hungary) according to the manufacturer´s instructions, using WIZARD2 Gamma Counter (PerkinElmer, Waltham, MA, USA). The assay sensitivity was 0.04 ng/ml, the intra-assay and inter-assay coefficients of variation were <6.7% and <17.3%, respectively.

Statistical analysis. The data are presented as mean ± standard error of the mean (SEM) of 3 independent experiments. Data were analyzed by one-way ANOVA, and Bonferroni post-test was performed using GraphPad InStat version 3.05 (GraphPad Software, Inc.; San Diego, CA, USA) to identify statistical differences between relevant groups. Differences were considered to be significant when $p<0.05$.

Results

Cumulus expansion in the presence of binary mixture (BPA+BBP). It has been well established that supplementation of the culture media with FSH stimulates full expansion of intact porcine OCCs incubated *in vitro* (Nagyova et al. 1999). In the present study, porcine OCCs treated with FSH were considered expanded when their cumulus layers spread to the stage of expansion +2 and higher. After 24 h of FSH (100 ng/ml) stimulation, more than 80% of the OCCs were expanded to such stages (Figure 1A). No changes in cumulus expansion were observed in the presence of low concentrations (10^{-10}, 10^{-8}, and 10^{-6} M) of the binary mixture of BPA with BBP. In contrast, the highest tested concentration of the mixture significantly impaired cumulus expansion of FSH-stimulated OCCs. By the action of 10^{-4} M of BPA+BBP mixture, only 12% of the OCCs were observed as expanded ($p<0.01$) (Figure 1A).

Oocyte meiotic maturation in the presence of binary mixture (BPA+BBP). At the time of isolation (0 h), most of the oocytes were at the GV nuclear stage (data not shown). After 44 h of *in vitro* culture,

less than 20% of the oocytes from the OCCs cultured in the medium supplemented with FSH alone (control group) remained at the GV stage, while 80% of oocytes underwent GVBD and 68% reached MII stage (Figure 1B).

At low concentrations (10^{-10}, 10^{-8}, and 10^{-6} M), the binary mixture of BPA with BBP did not disrupt nuclear maturation of *in vitro* cultured porcine oocytes. However, at the highest concentration, the mixture significantly affected oocyte nuclear maturation. After exposure to 10^{-4} M, number of oocytes that remained at the GV stage was markedly higher when compared to the control group (83% vs. 20%). Thus, the number of oocytes that underwent GVBD as well as the percentage of oocytes that reached MII stage were significantly decreased (6% vs. 68% in control; $p<0.01$; Figure 1B).

Production of progesterone by complexes in the presence of binary mixture (BPA+BBP). Stimulatory effect of FSH on the oocyte-cumulus complex is accompanied by the increased cumulus cell progesterone production (Jezova et al. 2001). Treatment of OCCs with the BPA+BBP mixture at the lower exposures did not affect the amount of progesterone released to the culture media (Figure 1C). The highest concentration of BPA+BBP mixture (10^{-4} M) significantly ($p<0.01$) reduced progesterone production by FSH-stimulated complexes after 44-h incubation (Figure 1C).

Production of progesterone by granulosa cells in the presence of ED mixtures. In these experiments, levels of progesterone were determined in the culture media after 72-h incubation of GCs with different concentrations (10^{-9} M to 10^{-4} M) of the selected

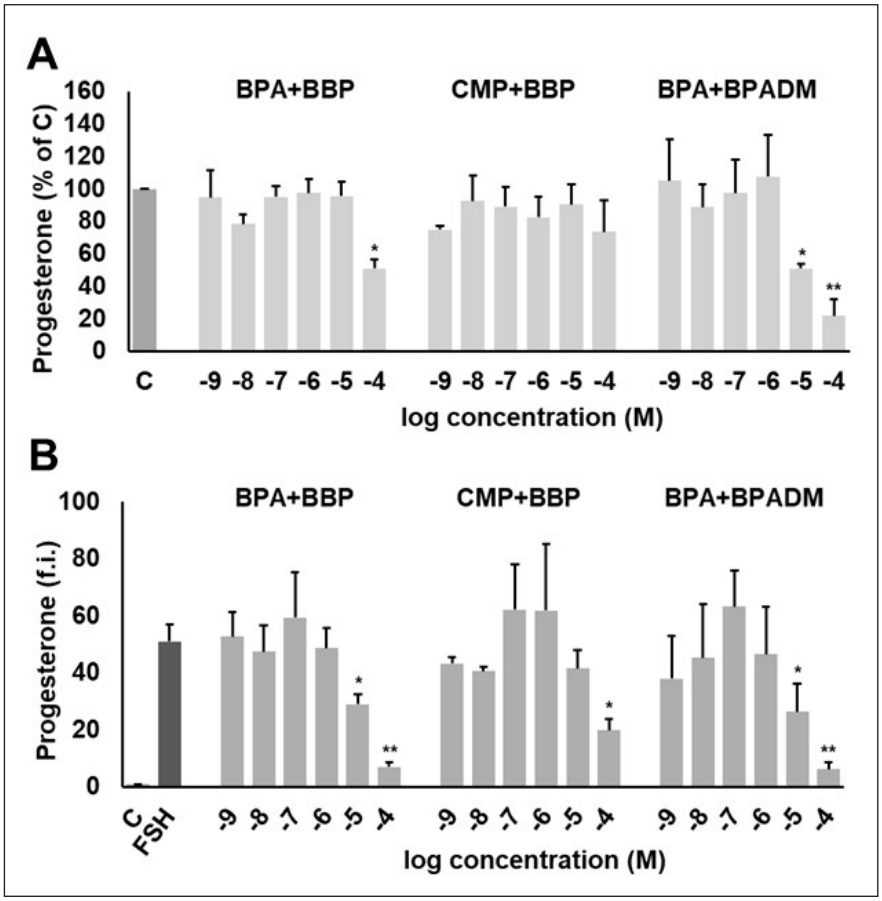

Figure 2. Effect of binary mixtures on basal (**A**) and follicle-stimulating hormone (FSH)-stimulated (1 μg/ml) (**B**) progesterone production by porcine ovarian granulosa cells after 72-h culture. Values represent the mean±S.E.M. of three independent experiments ($^{*}p<0.05$, $^{**}p<0.01$ vs. control (A) or FSH (B), respectively. Abbreviation: f.i. – fold induction; BPA – bisphenol A; BPADM – BPA-dimethacrylate; BBP – benzyl butyl phthalate; CMP – 4-chloro-3-methylphenol.

mixtures. We tested three binary mixtures of phenolic EDs (BPA+BBP, CMP+BBP, BPA+BPADM) and two ternary mixtures (BPA+BBP+CMP, OP+NP+TOP).

As shown in Figure 2A, basal progesterone synthesis by GCs did not change in the presence of lower concentrations of the mixtures, while the highest concentration (10^{-4} M) of both mixtures containing BPA significantly inhibited progesterone production. Moreover, the mixture of BPA with BPADM suppressed progesterone synthesis even at 10^{-5} M (Figure 2A). It is well known that gonadotropin stimulation of GCs results in a marked enhancement of steroidogenesis (Kolena and Channing 1972). In the present experiments, FSH supplementation (1 μg/ml) of the culture media resulted in 51-fold induction of progesterone production by the cells (Figures 2B, 3B). In the conditions of FSH-stimulation, we observed significant inhibition of progesterone production in the presence of all tested binary mixtures at 10^{-4} M concentrations (Figure 2B). In addition, the mixtures containing BPA (BPA+BBP and BPA+BPADM) exerted inhibitory effects at 10^{-5} M (Figure 2B).

Interestingly, tested ternary mixtures did not cause profound inhibition of either basal or FSH-induced progesterone production. Progesterone level was significantly lower after the treatment of FSH-stimulated GCs with the highest concentration of the BPA+BBP+CMP mixture (Figure 3B). The mixture of three alkylphenols (OP+NP+TOP) did not significantly change amounts of progesterone released by the cultured cells (Figure 3).

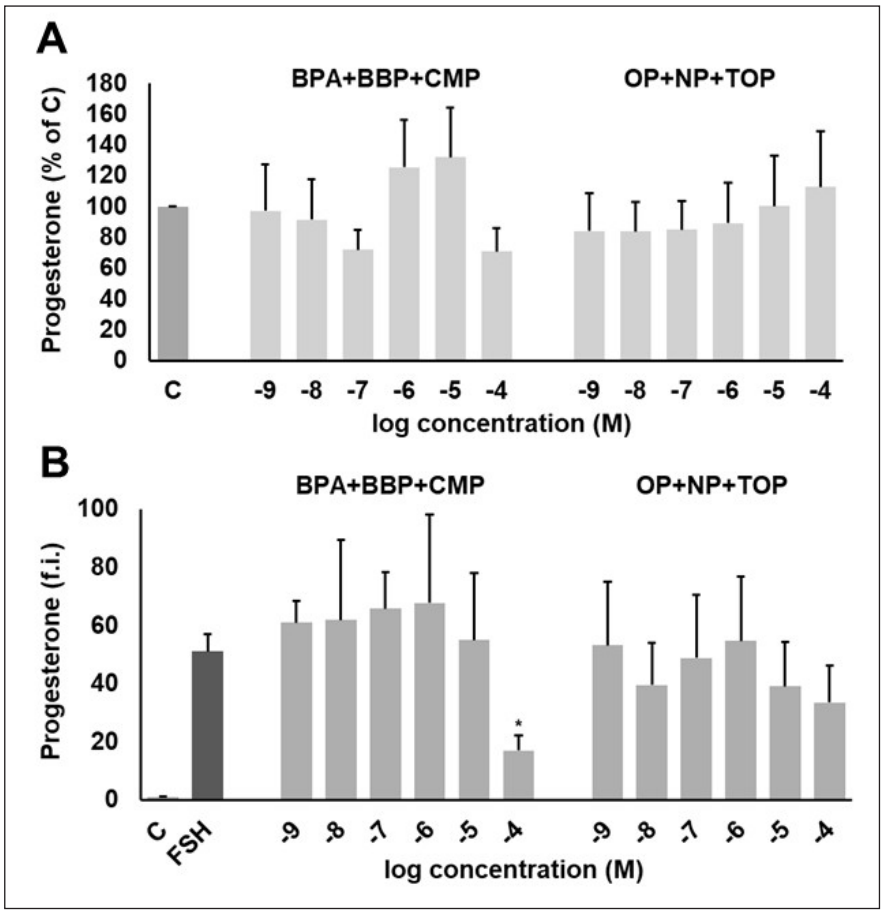

Figure 3. Effect of ternary mixtures on basal (**A**) and follicle-stimulating hormone (FSH)-stimulated (1 μg/ml) (**B**) progesterone production by porcine ovarian granulosa cells after 72-h culture. Values represent the mean ± S.E.M. of three independent experiments (*$p<0.05$ vs. FSH). Abbreviation: f.i. – fold induction; BPA – bisphenol A; BBP – benzyl butyl phthalate; CMP – 4-chloro-3-methylphenol; OP – 4-octylphenol; NP – 4-nonylphenol; TOP – tert-octylphenol.

Discussion

The possible role of various EDs in pathophysiology of reproductive system disorders has been suggested previously. Traditionally, chemical toxicity assessment is based on single substance studies. However, approaches that include consideration of mixture effects are needed to adapt to real life situations (Tralau et al. 2021). Nevertheless, since there is a myriad of exogenous chemicals in the environment, such simulations pose a difficult challenge. The present study aimed at examination of the effects of the mixtures combining selected EDs: BPA, BPADM, BBP, CMP, or alkylphenols (OP, NP, TOP), using the model system of *in vitro* cultured porcine OCCs and GCs, respectively. These cultures represent valuable models for studying the effects of different chemicals on the functional characteristics of ovarian cells (Mlynarcikova et al. 2007). We tested the broad range of concentrations, from environmentally relevant nanomolar doses to high micromolar doses.

Proper maturation of the OCC is an indispensable requisite for the release of the fertilizable oocyte by the ovary (Richards and Pangas 2010). Previously, we have employed the porcine cultures to examine the effects of the individual substances. We have observed that both BPA and BBP at the concentration of 10^{-4} M impaired cumulus expansion of FSH-stimulated complexes. In addition, after 10^{-4} M BPA exposure, number of oocytes from these complexes that underwent GVBD as well as the percentage of oocytes that reached MII stage were significantly decreased, and the amount of progesterone produced by cumulus cells was diminished when compared to the FSH control group (Mlynarcikova et al. 2009). Similar impairment of oocyte maturation in a concentration-dependent mode has been reported even in the cultured human oocytes (Machtinger et al. 2013). In accordance, the present results show that the mixture of BPA+BBP (10^{-4} M) exerted significant inhibitory action on porcine cumulus expansion, nuclear oocyte maturation, and cumulus progesterone production. Moreover, this inhibition seems to be more profound than the effects caused by the BPA or BBP alone. Thus, we might assume that these substances may have additive effects on the cultured cells. Nevertheless, since such concentration represents high supraphysiological doses, the impairment observed might partially result from the cytotoxic effects of these chemicals, which have been detected previously in several cell types (Bujnakova Mlynarcikova and Scsukova 2018; Kim et al. 2019; Kim et al. 2024).

Steroid hormones progesterone and estradiol, produced by ovarian GCs, play important roles in coordination and proper function of the reproductive system. Alteration in the production of sex steroids can therefore have deleterious effects on the reproductive processes (Richards and Pangas 2010). In our previous studies, we have revealed possible deleterious effects of the individual phenolic EDs. From the tested substances, BPA appeared to exert the most notable effects on steroidogenesis (Mlynarcikova et al. 2005; Mlynarcikova et al. 2009). Consistently, here we have observed that all tested mixtures containing this compound (BPA+BBP, BPA+BPADM, BPA+BBP+CMP) significantly inhibited progesterone production by porcine GCs, although the effect occurred at higher doses (10^{-5} and 10^{-4} M). The mechanism of steroidogenesis disruption in the presence of BPA have been shown to include alterations in the expression of several steroidogenesis-related genes (Bloom and Mok-Lin 2016; Mukhopadhyay et al. 2022). Overall, we can see more profound effects on FSH-stimulated progesterone production what indicates that these chemicals might interfere with gonadotropin signaling in GCs. Except the adverse effects on reproductive functions, BPA has been linked to several other disorders, and finally, European Union has adopted a ban on the use of BPA in food contact materials and thermal paper to further lower human exposure (EC Regulation 2016/2235; 2024/3190).

Only a few studies have investigated the direct effects of alkylphenols on progesterone production in GC cultures. Our present results with the tested ternary mixture of OP+NP+TOP are in accordance with our previous experiments, where the individual substances did not cause any substantial alterations in progesterone production (Mlynarcikova et al. 2007). However, models using different experimental design, culture conditions and species, have observed possible changes in steroidogenesis after exposure to alkylphenols. In rat granulosa-luteal cells, OP increased both basal and FSH-stimulated progesterone production (Nejaty et al. 2001). On the other hand, OP inhibited human chorionic gonadotropin (hCG)-stimulated progesterone production by cultured mouse Leydig tumor cells (Nikula et al. 1999).

Assessment of mixture actions on GC functions has gained increased efforts during recent years also with regard to other groups of EDs. Study of Hannon et al. (2023) characterized the effects of a phthalate mixture on ovulatory progesterone/progesterone receptor (PGR) action using primary human GCs. The findings suggest that exposure

to the phthalate mixture decreased production of progesterone and *PGR* levels, and dysregulated PGR action by decreasing *ADAMTS1*, *CXCR4*, *PTX3*, and *RGS2* levels, all of which are essential for successful ovulation and fertility (Robker et al. 2000; Duffy et al. 2019). Findings of the study of Clark et al. (2024) testing a mixture of per- and polyfluoroalkyl substances (PFAS) revealed increased proliferation of human GCs and also altered steroid hormone synthesis, with increased both FSH-stimulated and basal progesterone secretion and concomitantly upregulated STAR protein. RNA sequencing revealed inherent differences in transcriptomic profiles in hGCs after PFAS exposure. These findings suggest that PFAS compounds can disrupt normal GC function with possible long-term consequences on overall reproductive health (Clark et al. 2024).

Selection of the substances to be tested in the mixture represent a complex challenge, since the intake and ratios of ED chemicals can vary across different populations. Thus, experimental studies may not completely simulate real-life scenarios for humans, characterized by daily exposures. The present study involved just a very limited ED combinations, and the future studies should broaden the range of the chemicals tested. Still, the use of epidemiologically defined mixtures of EDs is considered a beneficial strategy to estimate exposure risks when compared with traditional toxicological approaches based on the assessment of single chemicals, and may reveal cumulative effects of EDs (Curi et al. 2023).

Conclusions. The data obtained in the present as well as in many other studies clearly show that both somatic and germinal ovarian cells are targets of different EDs. The experimental approach of testing mixtures should provide a more comprehensive view on the effects of the ubiquitous EDs on female reproductive functions.

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Conflict of interest: *The authors declare no conflict of interest.*

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