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Persistent organic pollutants affect steroidogenic and apoptotic activities in granulosa cells and reactive oxygen species concentrations in oocytes in the mouse

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ABSTRACT

Context. The destruction of granulosa cells (GCs), the main functional cell type in the ovary, prevents steroid hormone production, which in turn may damage oocytes, resulting in ovarian failure. The accumulation of a number of persistent organic pollutants (POPs) in the ovarian follicular fluid (FF) has been documented, which raises serious questions regarding their impact on female fertility. **Aims.** We aimed to determine whether a mixture of POPs reflecting the profile found in FF influences mouse GCs or oocyte function and viability. **Methods.** A mixture of POPs, comprising perfluorooctanoate, perfluorooctane sulfonate, 2,2-dichlorodiphenyldichloroethylene, polychlorinated biphenyl 153, and hexachlorobenzene, was used. In addition to using the exact concentration of POPs previously measured in human FF, we tested two other mixtures, one with10-fold lower and another with 10-fold higher concentrations of each POP. **Key results.** Steroidogenesis was disrupted in GCs by the POP mixture, as demonstrated by lower oestradiol and progesterone secretion and greater lipid droplet accumulation. Furthermore, the POP mixture reduced GC viability and increased apoptosis, assessed using caspase-3 activity. The POP mixture significantly increased the number of oocytes that successfully progressed to the second meiotic metaphase and the oocyte reactive oxygen species (ROS) concentration. **Conclusions.** Thus, a mixture of POPs that are typically present in human FF has detrimental effects on ovarian function: it reduces the viability of GCs, and increases the oocyte concentrations of ROS. **Implications.** These results indicate that chronic exposure to POPs adversely affects female reproductive health.

Keywords: apoptosis, granulosa cell, lipid accumulation, mitochondrial activity, mouse, oocyte, persistent organic pollutant, reactive oxygen species, steroidogenesis.

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Introduction

In recent years, environmental toxicants have become a serious health concern. Even though some of these chemicals have been regulated or banned in most countries for several decades, many remain major pollutants worldwide because of their persistence. A previous study demonstrated that many persistent organic pollutants (POPs) are present in the ovarian follicular fluid (FF) of humans (Petro *et al.* 2012, 2014) and farm animals (Kamarianos *et al.* 2003), as well as in other tissues and bodily fluids. The POPs present in the FF are highly heterogeneous and include synthetic chemicals that are used as industrial solvents or lubricants, such as polychlorinated biphenyls (PCBs); pesticides, including dichlorodiphenyldichloroethylene (p,p′-DDE) and hexachlorobenzene (HCB); and flame retardants, including perfluorooctane sulphonate (PFOS) and perfluorooctanoate (PFOA) (Petro *et al.* 2012, 2014). These chemicals have been detected in the FF of humans at mean concentrations of 72 pg/mL for PCB153, 392 pg/mL for p,p′-DDE, 32 pg/mL for HCB, 7.5 ng/mL for PFOS, and 1.8 ng/mL for PFOA (Petro *et al.* 2012, 2014). Their half-lives in the human body are as follows: 10–15 years for PCB (Ritter *et al.* 2011),

7–8.6 years for p,p′-DDE (Kirman *et al.* 2011; Saoudi *et al.* 2014), 6 years for HCB (Hardell *et al.* 2010; Mrema *et al.* 2013), 5 years for PFOS, and 4 years for PFOA (Olsen and Zobel 2007). The concentrations of pollutants in the FF of farm animals (Kamarianos *et al.* 2003) were higher than those reported for FF of humans (Petro *et al.* 2012, 2014). For example, HCB was detected at levels ranging from 780 pg/mL in pigs to 1.77 ng/mL in cattle; while pp-DDE was detected at levels ranging from 730 pg/mL in sheep to 1.50 ng/mL in cattle. Moreover, the median concentrations of total PCB were 450 pg/mL in pigs and 3.05 ng/mL in cattle (Kamarianos *et al.* 2003).

The presence of chemicals in the FF implies that the ovary has been exposed and that the ovarian cells and oocytes may experience effects. Within a follicle, oocyte development is dependent on the physiological interactions of the oocyte and granulosa cells (GCs); moreover, the surrounding FF forms a nutritive milieu around the oocyte and plays a critical role in determining oocyte quality, and the potential for the oocyte to be fertilised and develop into an embryo (Revelli *et al.* 2009). It is well known that the production of progesterone (P4) and 17β-oestradiol (E2) by GCs is essential for folliculogenesis and the development of the dominant antral follicle, as well as ovulation (Jamnongjit and Hammes 2006).

Previous studies have shown that HCB (Foster *et al.* 1992), PCB153 (Gregoraszczuk *et al.* 2003), p,p′-DDE (Nejaty *et al.* 2001), PFOA, and PFOS (Chaparro-Ortega *et al.* 2018), when administered individually, disrupt steroid hormone production within the ovary. However, humans and animals are exposed to mixtures of these compounds, and the biologic effect of such mixtures require elucidation.

Moreover, another previous study showed that chloro-organic compounds, such as PCBs and DDT, as well as their metabolites, alter the maturation and developmental competence of porcine cumulus-oocyte complexes (Campagna *et al.* 2001) and affect the ability of mouse oocytes to be fertilised (Kholkute *et al.* 1994). Other studies have shown that high total concentrations of POPs in the human follicular micro-environment are associated with a lower probability of an oocyte developing into a top-quality embryo, principally owing to a lower fertilisation rate (Petro *et al.* 2012, 2014).

Oocyte meiotic maturation and the quality of metaphase II (MII) oocytes are two key factors that affect the outcome of pregnancy. Even if the oocytes complete their meiotic maturation and reach MII, fertilisation may not occur because the quality of the MII oocytes is also affected by mitochondrial function (Babayev and Seli 2015). Mitochondria are especially sensitive to toxic insults because they are a major source of reactive oxygen species (ROS) and important sensors of overall cellular stress. In addition, the prevalence of defects in oocyte quality has been increasing in recent years, and it has been suggested that this adverse effect may be related to exposure to certain environmental pollutants (Kramer 2003; Silbergeld and Patrick 2005; Stillerman *et al.* 2008).

Therefore, in the present study, we aimed to determine whether a mixture of POPs affects the secretion of steroid hormones by mouse ovarian GCs and, if so, whether it influences the expression of steroidogenic enzymes and GC viability. Moreover, we aimed to evaluate the influence of POP mixtures on oocyte maturation *in vitro*, and on mitochondrial activity and ROS concentrations.

Materials and methods

Chemicals

All the materials were purchased from Sigma Chemical, Co. (St. Louis, MO, USA), unless stated otherwise. A stock solution of the test compounds PFOA, PFOS, HCB, p,p′-DDE, and PCB153 was dissolved in dimethyl sulfoxide (DMSO; 0.1 v/v), then aliquot and refrigerated. On the day of the experiment, the stock solution was serial diluted and added directly to the cells or oocytes.

Animals

Mature female outbred OF1 mice ($n = 30$) (3–6 months) were housed in an animal facility under controlled temperature (22°C ± 2°C) and light (12 h light/day) conditions, with free access to food and water. The experiments were performed in accordance with the State regulations on the protection of animals used for scientific and educational aims, and conformed with the European Union Council Directive 2010/63/EU of 22 September 2010. In each experiment, six ovaries from three animals were selected for GC cell and oocyte preparation.

Culture of ovarian granulosa cells

Mice were killed by cervical dislocation, and their ovaries were dissected and placed in complete medium containing phenol red-free Dulbecco's modified Eagle's/F12 medium (DMEM/F12), supplemented with 20% charcoal-stripped fetal bovine serum (FBS; Gibco, Waltham, MA, USA), 1% penicillin-streptomycin, and 0.1% bovine serum albumin (BSA; Gibco). The ovaries were punctured with insulin syringe needles to release the GCs into the medium. The medium was then centrifuged (500*g* for 5 min) and the cell pellet was resuspended in fresh complete cultivation medium. Cell number and viability were estimated after staining with trypan blue, and viable cells were seeded into 96-well tissues culture plates at 2.5×10^4 cells/well per 0.2 mL of complete medium for 12 h to allow adhesion. Thereafter, the medium was replaced with fresh complete medium and the cells were exposed to a mixture of the test compounds called Mix.

Assessment of oocyte meiotic maturation *in vitro*

Ovaries were placed in drops of M2 culture medium and punctured with the tips of sharp-pointed tweezers to release the oocytes from antral follicles into the medium. Cumulus cells, if present, were removed from the oocytes by pipetting. For further investigations, only oocytes that were at the germinal vesicle stage (GV) were selected. The GV oocytes (9–12 per group) were placed in drops of M2 medium containing 0.1% DMSO (control) or appropriate concentrations of POPs. Petri dishes containing these drops were covered with mineral oil and cultured at 37°C in 5% CO_2-containing air to allow meiotic maturation. The cultures were examined after 2 h to assess the dynamics of the resumption of meiosis, and after 18 h to evaluate the overall efficiency of the meiotic maturation. On this basis, the oocytes were placed into three categories: GV oocytes (oocytes that had not resumed meiosis), GVBD oocytes [oocytes that had resumed meiosis by progressing to the germinal vesicle breakdown stage (GVBD)], and MII oocytes (oocytes that had extruded a first polar body and had become arrested at the second meiotic metaphase, thereby completing meiotic maturation).

Treatments

We assessed the effect of a mixture of POPs using the following experimental groups: control; control in vehicle (0.1% DMSO); Mix 1 (2 ng/mL PFOA, 8 ng/mL PFOS, 50 pg/mL HCB, 1 ng/mL p,p′-DDE, and 100 pg/mL PCB153); Mix 0.1 (10-fold lower concentrations of the POPs than in Mix 1); and Mix 10 (10-fold higher concentrations of each of the same POPs). Mix 1 was formulated on the basis of the mean concentrations of individual POPs measured in samples of FF from women who were undergoing assisted reproduction (Petro *et al.* 2012, 2014). The cells were treated for 24 h for gene expression analysis, 48 h for protein expression analysis, and 72 h for steroid secretion analysis.

Evaluation of oocyte mitochondrial activity and reactive oxygen species concentrations

To assess mitochondrial activity and ROS concentrations in the oocytes after 18 h of culture, they were labelled with Mitotracker Orange CMTMRos (cat. no. 7510; Invitrogen, Waltham, MA, USA) and H_2DCFDA (Invitrogen, USA; cat no. D399), respectively. To permit simultaneous labelling with both reagents, the oocytes were incubated for 30 min at 37°C in M2 medium supplemented with Mitotracker Orange and H2DCFD at concentrations of 500 nM and 10 μM, respectively. Then, following two 5-min washes with M2, images of the oocytes were obtained using a TMS100f inverted microscope (Nikon, Tokyo, Japan) and the appropriate filters for each stain. The fluorescent images were analysed using ImageJ Software (National Institutes of Health, Bethesda, MD, USA). Single oocytes were manually outlined and the mean intensity of the signal was calculated. To determine the background signal intensity in each image, the intensities of signals in four areas adjacent to the oocytes were measured in four different sides and averaged to obtain the mean intensity. The mean intensity was considered the background intensity and was subtracted from the mean intensity signal of every oocyte analysed in the image. The data are reported relative to the mean fluorescence intensity of the control oocytes (set to 100) in each experiment.

Measurement of oestradiol and progesterone concentrations

To analyse 17β-oestradiol secretion, cells were grown in medium containing 10 μM androstenedione (a substrate for E2 synthesis). After 72 h of treatment with POP mixtures (0.1-, 1-, and 10-fold dilutions), the secretion of progesterone and 17β-oestradiol was assessed using enzyme-linked immunosorbent assays (DRG Instruments GmbH, Marburg, Germany, and My BioSource, San Diego, CA, USA), according to the manufacturers' instructions. All the samples were analysed in duplicate, and all the intra- and inter-assay coefficients of variation were <10%. Absorbances were measured using an ELx800 microplate reader (BioTek Instruments, Winooski, VT, USA) with a 450 nm filter. The data were recorded and analysed using KC Junior software (BioTek Instruments).

Cell viability assay

Cell viability was measured using Alamar Blue Cell Viability Reagent (Invitrogen, Paisley, UK), according to the manufacturer's instructions. The Alamar Blue stock solution was aseptically added to wells after 48 h of culture in amounts equal to 10% of the volume of the culture medium. After 4 h of incubation, the reduction of resazurin to resorufin was determined by measuring the fluorescence at an excitation wavelength of 560 nm and an emission wavelength of 590 nm using an FLx800 fluorescence microplate reader (BioTek Instruments, Winooski, VT, USA). Data were analysed using KC JUNIOR Software (BioTek Instruments).

Caspase-3 activity

After treatment of the GCs for 24 h with the POP mixtures (0.1-, 1-, and 10-fold dilutions), cells were lysed in Caspase Assay Buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 1 mM EDTA, 10% glycerol, and 10 mM dithiothreitol). Samples of the cytosolic fractions of the cells containing 100 μg protein were analysed. The assay was performed by adding 100 μM Ac-DEVDAMC (Sigma Chemical, Co., St. Louis, MO, USA) at 37°C. The quantity of fluorescent product was monitored continuously for

120 min using a spectrofluorometer (FLx800; BioTek Instruments), at an excitation wavelength of 355 nm and an emission wavelength of 460 nm. Data were analysed using KC Junior software (BioTek Instruments) and normalised to the fluorescence generated by vehicle-treated cells, and are expressed in fluorescence units.

Oil Red O staining

After 48 h of treatment with POP mixtures (0.1-, 1-, and 10-fold dilutions), the GCs were washed twice with PBS and fixed in 60% isopropanol for 20 min. The cells were then stained using Oil Red O solution (Sigma Aldrich, Darmstadt, Germany) for 5 min, and washed with 60% isopropanol and distilled water. Next, the nuclei were stained with haematoxylin for 30 s and then washed with distilled water. After drying, the cells were examined under a microscope (Axiocam 503; 40× objective; Zeiss, Oberkochen, Germany).

Fluorescent staining with Nile Red or Cell Tracker Green

After treatment of the GCs for 48 h with the POP mixtures (0.1-, 1-, and 10-fold dilutions), they were washed twice with PBS and incubated for 60 min in Nile Red dye (Invitrogen, USA) to stain intracellular lipids. A stock solution of dye was diluted 1:100 in medium not containing FBS. Then, after staining, the cells were washed with PBS and incubated for 30 min in a 2 μM solution of Cell Tracker Green CMFDA (Invitrogen, USA) in serum-free medium. The cells were then examined using an Axiocam503 (Zeiss) bright-field fluorescence microscope (excitation wavelengths: 590 nm for Nile Red and 495 nm for Cell Tracker Green). The fluorescent images were analysed using ImageJ Software. Single cells were manually outlined and the area, integrated density, and mean grey value were measured. The same outlining procedure was applied to each background zone.

RT-qPCR

The expression of the *Cyp19a1* (Mm00484049_m1) and *Hsd3b1* (Mm01261921_mH) genes after 24 h of treatment with the POP mixtures (0.1×, 1×, and 10× dilutions) was measured by real-time PCR, as described previously (Gogola *et al.* 2019). The expression of each was normalised to that of *18S* (4310893E), and their relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Primers for all these genes were used as ready-to-go format as TaqMan Gene Expression Assays from Applied Biosystems/ThermoFisher Scientific.

Western blot analysis

Aromatase (CYP19A1) (ab18995, Abcam, Cambridge, UK), 3β-hydroxysteroid dehydrogenase (3βHSD) (ab55268; Abcam), and Steroidogenic acute regulatory protein, (StAR) (ES10315, ELK Biotechnology, Hubei, China) protein expression in the GCs was analysed after treatment with the POP mixtures (0.1×, 1×, and 10× dilutions) for 48 h. Anti-rabbit (#7074) or anti-mouse (#7076) (Cell Signaling Technology, Danvers, MA, USA) secondary antibodies were used as appropriate, and anti-β-actin antibody (A5316; Sigma Aldrich) was used as a loading control. Western blot analysis was performed as described previously (Gogola *et al.* 2019).

Statistical analysis

All experiments were repeated at least three times in triplicate. Numerical data are presented as the mean ± s.e.m. Statistical analysis was performed using one-way ANOVA, followed by Tukey's (Figs 1, 2, 3, 4), or the non-parametric Student's

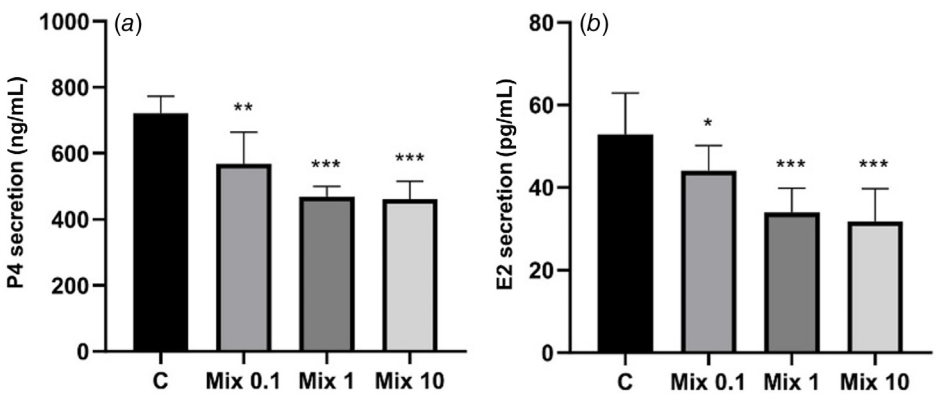

Fig. 1. The effects of 48 h incubations with POP mixtures (Mix 0.1, Mix 1, or Mix 10) on (*a*) progesterone (P4) and (*b*) 17β-oestradiol (E2) secretion by cultured granulosa cells obtained from mice. Data are mean ± s.e.m. of five independent experiments. C, control + 0.1% DMSO. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs C.

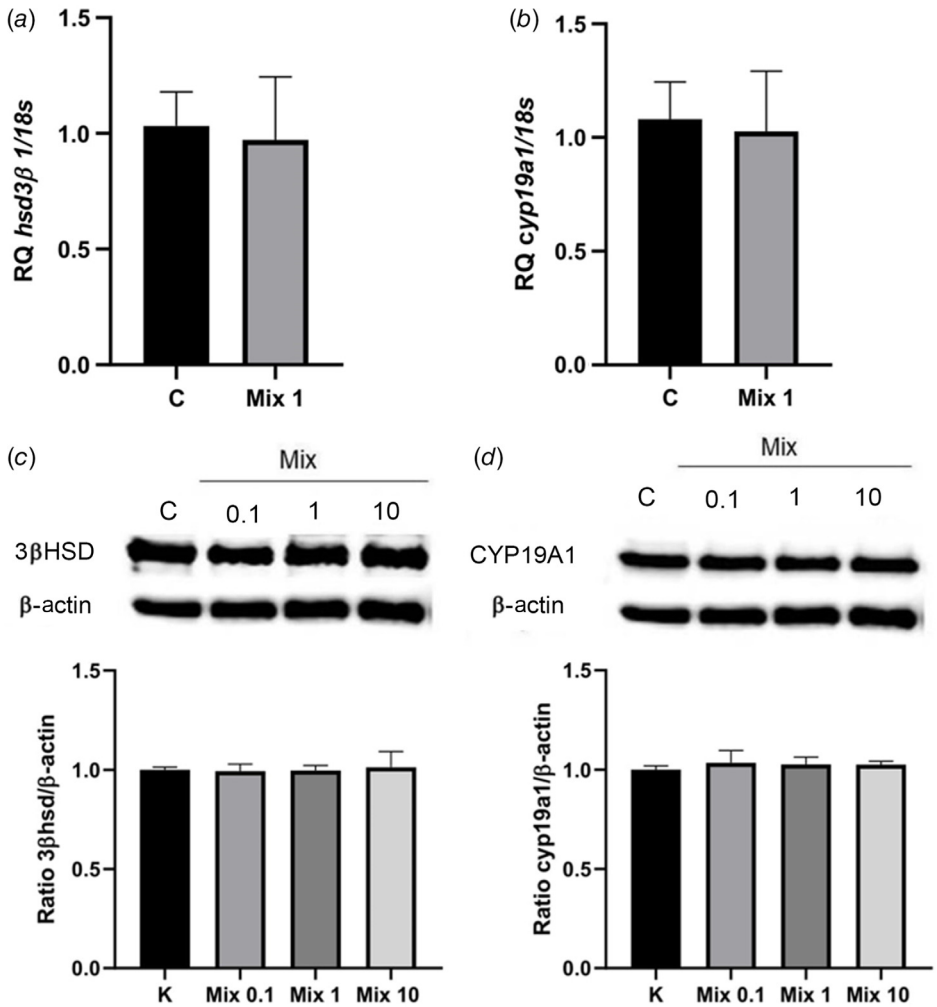

Fig. 2. Effects of POP mixtures (Mix 0.1, Mix 1, or Mix 10) on the mRNA and protein expression of 3βHSD1 and CYP19A1 in mice granulosa cells (*a*, *b*) after 24 h (gene expression) and (*c*, *d*) 48 h (protein expression). The mRNA expression of vehicle-treated cells was set to 1.0; RQ, relative quantity. The data in the graphs are representative of at least three independent experiments and are expressed as the mean ± s.e.m. C, control + 0.1% DMSO.

t-test (Figs 6, 7) (GraphPad Software, La Jolla, CA, USA), and the level of significance was set at $P < 0.05$.

Results

A mixture of POPs reduces the secretion of progesterone and oestradiol by granulosa cells

To determine whether the POP mixtures affect steroidogenesis, we obtained ovarian GCs from the mice and measured the concentrations of the two principal female sex steroid hormones, 17β-oestradiol and progesterone, in the media after 48 h of treatment. As shown in Fig. 1*a*, the P4 concentration was dose-dependently reduced by Mix 0.1, Mix 1, or Mix 10 exposure (568 ± 95, 488 ± 31, and 461 ± 53 ng/mL, respectively) vs the control group (721 ± 51 ng/mL) (Fig. 1*a*; $P < 0.001$). Similarly, in androstenedione supplemented cultures, all three POP mixtures (Mix 0.1–10) reduced E2 secretion (44.1 ± 6.1, 34.0 ± 5.9, and 31.8 ± 7.9 pg/mL, respectively) vs the control culture (52.9 ± 10.1 pg/mL; Fig. 1*b*; $P < 0.05$, $P < 0.001$).

POP mixtures do not affect the expression of enzymes responsible for P4 and E2 synthesis

Next, we assessed the effects of Mix 1 on *Hsd3b1* and *Cyp19a1* mRNA expression and found that it had no effect on the expression of either (Fig. 2*a*, *b*). These findings were corroborated by western blotting analysis, which showed that none of the tested mixtures affected the protein expression of 3βHSD1 or CYP19A1 (Fig. 2*b*, *c*).

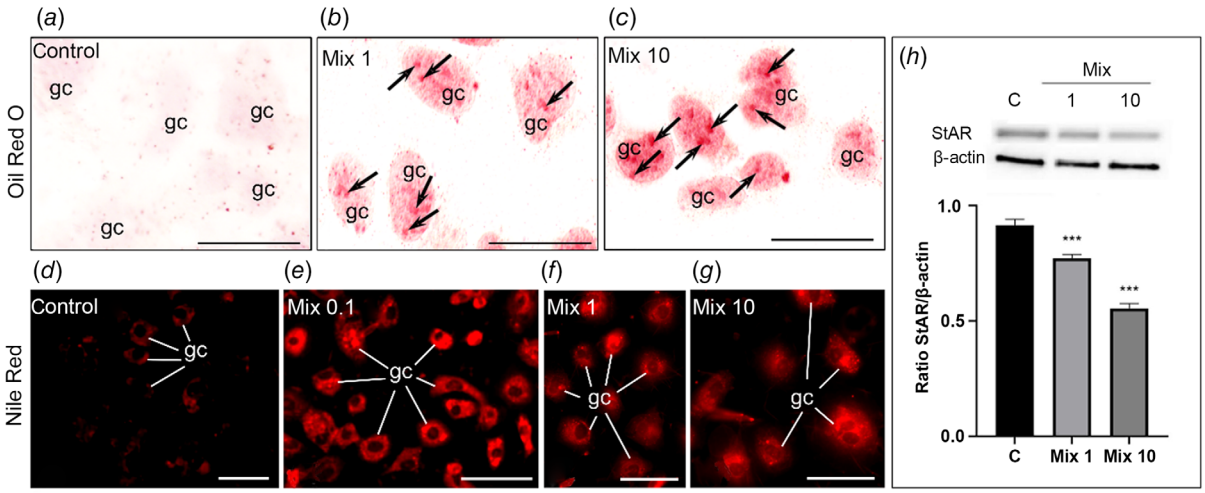

Fig. 3. The effects of POP mixtures (Mix 0.1, Mix 1, and Mix 10) on the lipid droplet content and the StAR protein expression of cultured mouse granulosa cells. (*a–c*) Oil Red O staining, (*d–g*) Nile Red staining, (*h*) protein expression. gc, granulosa cell; arrows indicate lipid droplets. The data in the graphs are representative of at least three independent experiments and are expressed as the mean ± s.e.m. C, control + 0.1% DMSO. ***$P < 0.001$ vs C. Scale bars = 100 μm.

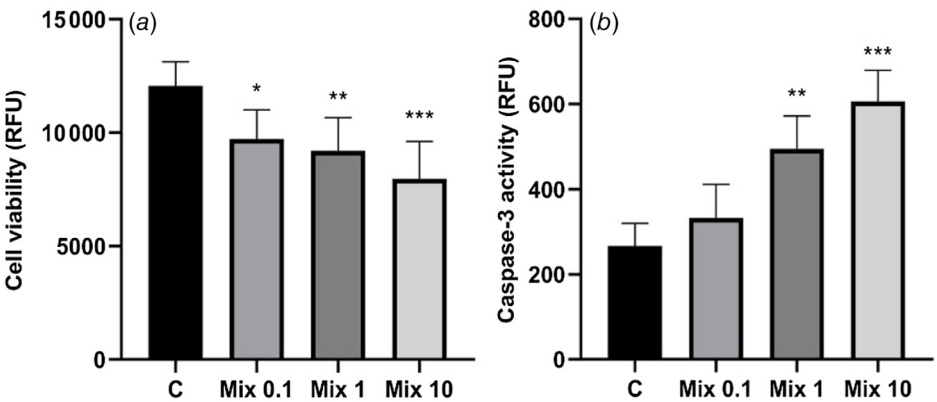

Fig. 4. Effects of POP mixtures (Mix 0.1, Mix 1, or Mix 10) on mouse granulosa cell (*a*) viability after 48 h and (*b*) caspase-3 activity after 24 h. C, control + 0.1% DMSO. RFU, relative fluorescence units. Data are mean ± s.e.m. of three independent experiments. C, control + 0.1% DMSO. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs C.

POP mixtures increase lipid droplet accumulation and decrease expression of steroidogenic acute regulatory protein

We next sought to determine whether the POP mixture affected lipid droplet content as well as StAR protein expression. Careful analyses of microphotographs of control and treated GCs revealed that the Mix-treated cells were more intensely stained (Fig. 3*a–c*), which implies the presence of numerous lipid droplets within their cytoplasm. Spherical dots of various sizes can be seen within the cells in Fig. 3*b, c* (arrows) but not in control cells (Fig. 3*a*). To further confirm this effect of Mix treatment on GCs, we also stained the cells with Nile Red, a fluorescent lipophilic stain. Again, the control cells did not show any fluorescence, or the signal was very weak (Fig. 3*d*), whereas there was a strong cytoplasmic signal in Mix-treated cells (Fig. 3*g*). No dose-dependent relationship between the POP concentrations and the staining of lipid droplets using either stain was identified, but with respect to Oil Red O staining, treatment with Mix 10 seemed to result in slightly more intense staining than Mix 1 treatment.

Next, we analysed the effect of the POP mixture (Mix 1 and Mix 10) on protein expression of StAR, which transports cholesterol to the inner mitochondrial membrane for steroidogenesis. We found that exposure to the POP mixtures (Mix 1 and Mix 10) decreased the expression of StAR protein (0.8- and 0.6-fold, respectively) in GCs (Fig. 3*h*; $P < 0.001$).

POP mixtures reduce the viability of mouse granulosa cells by stimulating caspase-3 activity

We next analysed the effects of the POP mixtures on GC viability by assessing mitochondrial activity and apoptosis (caspase-3 activity). The Alamar Blue assay is commonly used to quantify cell viability, and is also a sensitive and simple indicator of mitochondrial function (Springer *et al.* 1998). The POP mixtures dose-dependently (Mix 0.1–10) reduced cell viability (9724 ± 1352, 9204 ± 1544, and 7962 ± 1747 RFU, respectively) vs controls (12059 ± 1144 RFU; Fig. 4*a*; $P < 0.01$, $P < 0.001$). Caspase-3-mediated proteolysis is a key mediator of GC apoptosis, and we found that all the test mixtures stimulated caspase-3 activity in a dose-dependent manner (494 ± 77 and 606 ± 73 RFU after treatment with Mix 1 and Mix 10, respectively) vs controls (266 ± 53 RFU; Fig. 4*b*; $P < 0.01$ and $P < 0.001$).

POP mixtures reduce the adhesion and disrupt the morphology of mouse granulosa cells

Because the biochemical tests clearly showed that Mix treatment altered the viability of the cells, we decided to use a fluorescent dye, Cell Tracker Green, to stain GCs, in order to characterise the shapes of the cells and their ability to flatten, attach to a surface, and generate projections. Analysis of the microphotographs revealed that control cells demonstrated flattening and had all the features characteristic of normal, healthy cells (Fig. 5*a*). They covered the surface quite evenly, and normal cellular projections were observed (Fig. 5*a*). However, some of the cells treated with POP mixtures demonstrated alterations in morphology, poor attachment to the surface, and a lack of characteristic cellular projections (Fig. 5*b–d*, white arrows). The affected cells appeared microscopically as very bright, spherical dots.

POP mixtures increase the number of oocytes maturing to metaphase II and increases the production of reactive oxygen species

Finally, we determined whether the POP mixture affects oocytes. In all the treatment groups, the majority of oocytes resumed meiosis and progressed to the GVBD stage during the first 2 h of culture *in vitro* (Fig. 6*a*). The proportion of oocytes that resumed meiosis increased slightly during a further 16 h of culture. There were no significant differences in the proportions of oocytes that resumed meiosis among the treatment groups after either 2 or 18 h of culture. Of the oocytes that resumed meiosis, most underwent full meiotic maturation to the MII stage in all the treatment groups (Fig. 6*b*). However, when compared with the control cells, the percentage of MII oocytes at the end of the culture period was high in the Mix 10 group (Fig. 6*b*; 76% vs 88%; $P < 0.02$, *t*-test).

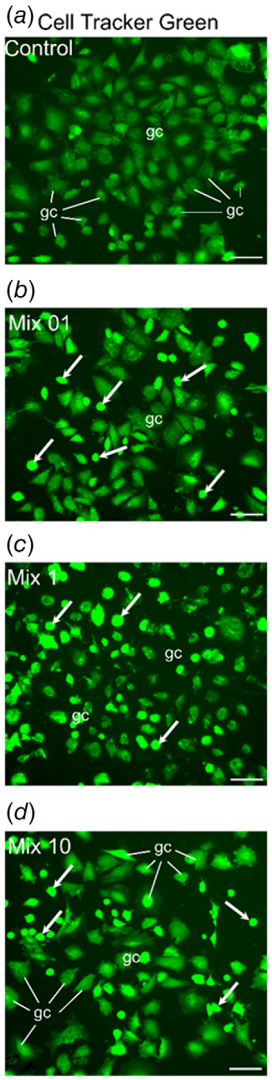

Fig. 5. Mouse granulosa cells stained with Cell Tracker Green to assess their shape and adhesion. (*a*) Control and (*b–d*) POP mixture (Mix 0.1, Mix 1, or Mix 10)-treated (48 h) cells. gc, granulosa cell; arrows indicate non-adhering cells. The data in the photos are representative of at least three independent experiments. Scale bars = 100 μm.

After 18 h of culture, the oocytes were labelled with Mitotracker Orange and H2DCFDA to assess the activity of their mitochondria and the intracellular concentrations of ROS, respectively (Fig. 7). There were no significant differences in the mean Mitotracker Orange fluorescence intensity between control oocytes and oocytes exposed to any of the POP mixtures (Fig. 7*d–f*, *j*). The intensity of H2DCFDA labelling (Fig. 7*g–i*) depended on the stage of the oocyte, with the fluorescence intensity of the GV oocytes being the lowest (Fig. 7*g*; oocytes marked with arrows), that of the GVB oocytes being intermediate, and that of the MII oocytes being highest. Notably, the oocytes that underwent full maturation to the MII stage demonstrated significantly higher H2DCFDA fluorescence intensity in

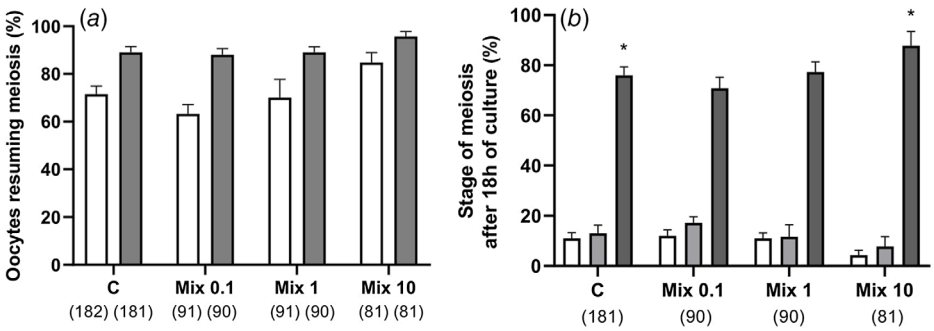

Fig. 6. Meiotic maturation of oocytes cultured in the presence or absence of POP mixtures (Mix 0.1, Mix 1, or Mix 10). (*a*) Proportion of oocytes that had resumed meiosis after 2 h (empty bars) and 18 h (black bars) of culture. (*b*) Stage of meiosis after 18 h of culture. White bars, GV oocytes; grey bars, GVBD oocytes; black bars, MII oocytes (fully mature). Data are mean ± s.e.m. of six or 11 independent experiments. The numbers of oocytes analysed are shown in parentheses. *$P < 0.02$, *t*-test.

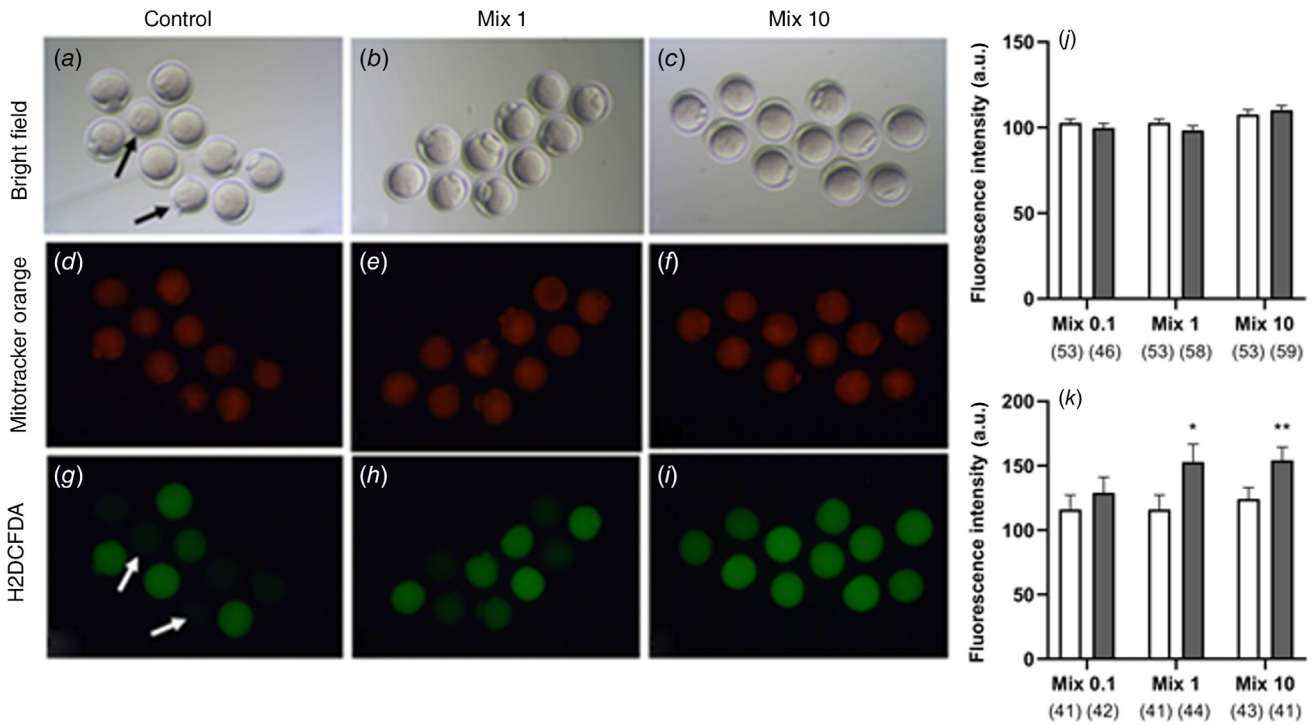

Fig. 7. Mitochondrial activity and ROS concentration in oocytes that matured *in vitro* in the presence of POP mixtures. (*a–i*) Representative examples of Mitotracker Orange labelling, indicating mitochondrial activity, and H2DCFDA labelling, indicating ROS concentration. Arrows indicate oocytes with low-intensity H2DCFDA labelling, implying a lack of resumption of meiosis (as indicated by the presence of the prophase nucleus) and continued presence in the GV stage. (*j*) The POPs had no effect on the mitochondrial activity of the MII oocytes but (*k*) significantly increased H2DCFDA staining intensity, reflecting high concentrations of ROS. White bars, control; grey bars, mixture. Data are mean ± s.e.m. of four or five independent experiments. The numbers of oocytes analysed are shown in parentheses. *$P < 0.05$ and **$P < 0.01$; *t*-test.

both the Mix 1 and Mix 10 groups than in the controls (Fig. 7*k*; $P < 0.05$, *t*-test), which is indicative of higher concentrations of ROS.

Also, we examined the individual effects of PFOA, PFOS, p, p′-DDE, HCB and PCB153 on oocyte maturation at their corresponding concentrations in the Mix 1. We observed that treatment with compound individually affected oocyte maturation (Supplementary Fig. S1), mitochondrial activity (Fig. S2), and ROS concentration (Fig. S3); however, these effects were different from the predicted additive effects

calculated for the POP mixture (calculated on the basis of the sum of the independent effects of the individual compounds).

Discussion

The ovary has two important roles: the delivery of the female gametes and the production of ovarian hormones. There is a growing body of evidence that in addition to the endocrine, paracrine and autocrine regulation of ovarian function, exogenous chemicals also regulate ovarian function. The present findings constitute evidence that a mixture of POPs that reflects the types of chemical that have been identified in human ovarian FF has substantial detrimental effects on mouse GCs and oocytes. These data are highly relevant to the question of why a high total POP concentration in the human follicular micro-environment is associated with a lower probability of an oocyte developing into a top-quality embryo (Petro *et al.* 2012, 2014).

Granulosa cells are exposed to a number of different exogenous compounds simultaneously, and therefore the effects of mixtures of chemicals on GC and oocyte function may represent an important public health issue. The present study is the first to evaluate the low-dose effects of a mixture of POPs that reflect the mixtures found in human FF on mouse GCs and oocytes. We found that the POP mixtures reduce the secretion of both P4 and E2 and the observed effects were a nonlinear monotonic dose-response. These low-dose effects have been described by many studies of endocrine disrupting chemicals (Vandenberg *et al.* 2012). We suggested that the mechanism of the nonlinear response induced by the mixture is due to the plurality of molecular targets as well as receptors saturation; however, the mechanism requires further elucidation. There is growing evidence that exogenous chemicals disrupt steroid hormone production in the ovary when administered individually. For example, exposure to p,p′-DDE stimulates P4 secretion in rat granulosa-luteal cells (Nejaty *et al.* 2001) and aromatase activity in human GCs (Younglai *et al.* 2004). Furthermore, HCB increases the serum concentration of P4, but has no effect on that of oestradiol, in superovulatory rats (Foster *et al.* 1992). PCB153 has been shown to reduce the secretion of P4 and testosterone by porcine luteal cells (Młynarczuk and Kotwica 2006) and to increase E2 secretion by porcine follicles (Gregoraszczuk *et al.* 2003). Moreover, E2 secretion by porcine ovaries is reduced by PFOA but unaffected by PFOS (Chaparro-Ortega *et al.* 2018). However, the individual effects of POPs cannot be used to accurately predict the effects of a mixture (Gogola *et al.* 2020), and this is important because humans are exposed to complex mixtures of POPs in their environments.

To gain insights into the mechanisms underlying the effects of the POP mixtures on steroid secretion by GCs, we measured the expression of steroidogenic enzymes. Ovarian steroidogenesis is a multi-step process whereby steroid hormones are synthesised from cholesterol. Steroidogenic cells must transport cholesterol from the outer mitochondrial membrane to the site of the first enzyme in the steroidogenesis pathway, which is a step that is largely mediated by the steroidogenic acute regulatory protein (StAR or STARD1) (Manna *et al.* 2016). In the present study, we demonstrated that the POP mixture decreases expression of StAR protein. Moreover, we found that the POP mixtures increased the accumulation of lipids in mouse GCs. However, the POP mixtures did not affect 3βHSD or CYP19A1 gene or protein expression. To the best of our knowledge, no *in vivo* or *in vitro* data have been published regarding the direct effects of POP mixtures on the accumulation of lipid droplets and StAR protein expression in GCs. However, one previous study showed that administered HCB causes mitochondrial condensation in the developing follicles of monkeys, with marked abnormal accumulation of cytoplasmic lipid droplets. Moreover, HCB has been shown to inhibit the activities of membrane enzymes and transport proteins that are responsible for the transfer of cholesterol from the outer to the inner mitochondrial membrane (Bourque *et al.* 1995). In addition, in mouse testes, StAR expression and the testosterone level decrease after PFOS (Qiu *et al.* 2021) or PFOS (Eggert *et al.* 2019) treatment. Our results indicate that the POP mixture inhibits StAR expression, and that this is paralleled by an increase in lipid droplet accumulation.

Excess lipid droplet accumulation may lead to cellular lipotoxicity, and the effects of lipotoxicity on GCs have been reported previously (Raviv *et al.* 2020). Fluorescence microscopy analysis showed that a POP mixture with a profile similar to that of human FF altered the morphology of GCs. Furthermore, the POP mixtures reduced GC viability and in parallel, induced the apoptosis of mouse GCs, which is consistent with the hypothesis that POPs have detrimental effects on reproductive health by causing the accumulation of lipid droplets. In addition, previous studies have shown that the intracellular accumulation of lipids results in high concentrations of free fatty acids, which cause oxidative damage and give rise to highly active oxidative metabolites that cause further irreversible damage to cells. Prolonged damage and oxidative processes impair cellular homeostasis and ultimately lead to apoptosis (Schaffer 2003). The impact of lipotoxicity on GCs has been shown in obese mice, in which excessive local lipid overload causes advanced follicular atresia, with apoptosis and defective steroidogenesis (Garris and Garris 2004; Serke *et al.* 2012). Therefore, the present finding, together with previously published findings, may indicate that POP mixtures cause excess lipid droplet accumulation, resulting in damage to GCs and impairment of GC steroidogenesis, which is required for correct gonadal function.

It is well known that the follicular environment influences oocyte growth and quality, and therefore their ability to develop. Thus, the destruction of GCs ultimately reduces

oocyte viability. However, little is known regarding the direct effects of POPs on oocytes. Interestingly, in the present study, H2DCFDA labelling revealed that the POP mixtures added to the culture medium at a range of concentrations, including those found in human FF and higher (Petro *et al.* 2012, 2014) was sufficient to increase significantly the level of ROS in oocytes which matured to the MII stage, when compared to the control oocytes not exposed to POP mixtures during maturation *in vitro*. To our knowledge, the effects of POP mixtures on the concentrations of ROS have not been previously reported, but previous studies have shown that ROS concentrations are affected by these chemicals when they are administered individually. Therefore, the present findings are in part consistent with the previous observation that treatment with PFOA (28.2 μM), increases ROS accumulation, as assessed using DCFH-DA, in fetal mouse ovaries *ex vivo* (López-Arellano *et al.* 2019), whereas PFOS (100 μM) exposure impairs mitochondrial dynamics and function, resulting in higher concentrations of ROS and the induction of apoptosis in porcine oocytes (Chen *et al.* 2021). However, in the present study, treatment with a POP mixture did not cause a loss of oocyte viability. The discrepancy in these results might be due to the different concentrations used, which were 10 000 times higher than those used by us. Furthermore, one limitation of the experimental model used is that environmentally relevant concentrations of POPs are difficult to reproduce in an *in vitro* oocyte culture model; women are subjected to chronic exposure, rather than a single 18 h exposure. The effects of chronic exposure of POPs are more difficult to determine. Therefore, the duration of treatment may have been too short to induce apoptotic effects in oocytes.

Recent studies have shown that meiotic maturation is associated with a moderate physiological increase in ROS concentration in oocytes (Kala *et al.* 2017). Therefore, we hypothesise that the rise in ROS concentration in oocytes treated with POP mixtures might induce the elevation of the fraction of the oocytes which attained the MII stage. It should be stressed, however, that whereas significant ROS elevation occurred at the POP dose corresponding to that one which was measured previously in human FF (Petro *et al.* 2012, 2014) the increase in the number of oocytes maturing to MII stage required 10 times higher concentration of POP. Moreover, the improvement in the rate of nuclear maturation *in vitro* induced by POP treatment, despite no detectable effect on viability, does not confirm that these cells are of high quality sufficient to support fertilisation and undisturbed development since the excessive release of ROS is one of the main obstacles in the production of high-quality embryos. It is well documented that detrimental effects of high ROS concentrations can manifest subsequently. An excess of ROS can have deleterious effects on many cellular structures and molecules, and in particular it may damage both the nuclear and mitochondrial genomes (Luderer 2014). ROS are one of the main causes of DNA single-strand breaks, which can be converted into very dangerous double strand breaks (DSBs) (Lindahl 1993). Such defects may lead to structural chromosomal anomalies in the progeny, a risk that is especially important in light of the results of a recent study, which showed that human oocytes do not possess an active checkpoint mechanism to eliminate oocytes with damaged DNA (Rémillard-Labrosse *et al.* 2020). Moreover, the efficiency of DNA repair in oocytes decreases with age (Titus *et al.* 2013; Govindaraj *et al.* 2015). This may increase the risk to the integrity of the oocyte genome through an accumulation of the effects of prolonged exposure to POPs and an impaired ability of older women to repair oocyte DNA. Through the introduction of defects in the mitochondrial genome, ROS may also affect the activity of mitochondria, which could in turn impair both fertilisation and early embryonic development (Stojkovic *et al.* 2001; Santos *et al.* 2006). Finally, the potential mutagenic effect of high ROS concentrations threatens the integrity of the oocyte mitochondrial genome, which is passed on to the progeny in its entirety, and may result in a spectrum of heritable metabolic disorders. For these reasons, the mechanisms whereby POPs cause an accumulation of ROS and the long-term effects of this require further elucidation.

In summary, we provide evidence that a mixture of POPs, reflecting the profile of POPs that has been identified in human FF, has direct detrimental effects on mouse GCs. Specifically, the POP mixture reduces steroid secretion, which manifests as the accumulation of lipid droplets and induces apoptosis. Furthermore, the oocytes of the mice demonstrate an accumulation of ROS. Because oocytes at all stages of their development are surrounded by follicular cells, such a POP mixture might also have indirect effects involving damage to the follicular wall, which would compromise its ability to maintain oocyte viability. These important effects of POPs on GCs and oocytes may result in reproductive disorders, female infertility, and anomalies affecting the progeny. Because under normal conditions women are exposed to POPs for the whole of their reproductive life, further studies should seek to identify the long-term effects of POPs on the female reproductive system.

Supplementary material

Supplementary material is available online.

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Data availability. The data that support this study are available in the article and accompanying online supplementary material.

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