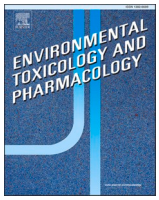
Environmental Toxicology and Pharmacology 101 (2023) 104169

Contents lists available at ScienceDirect

Environmental Toxicology and Pharmacology

journal homepage: www.elsevier.com/locate/etap



Perfluorooctanoic acid (PFOA) affects steroidogenesis and antioxidant defence in granulosa cells from swine ovary

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ARTICLE INFO

Edited by Dr. M.D. Coleman

Keywords:
PFAS
Ovary
Cell proliferation
Steroidogenesis
Antioxidant enzymes

ABSTRACT

PFOA is mainly employed in products with water and oil repellent properties. Due to its persistence, bioaccumulation and critical effects on health, its use has been restricted in several countries. This research was intended to explore PFOA action on the main functions of swine ovarian granulosa cells, a valuable model for translational medicine. Moreover, since we previously demonstrated a disruptive effect on free radical generation we sought to explore PFOA effects on the main antioxidant enzymes. PFOA inhibited cell proliferation ($p < 0.001$), assessed by BrdU uptake. Steroidogenesis was disrupted: PFOA also stimulated 17β-estradiol production ($p < 0.05$), increased progesterone production ($p < 0.05$) at the lowest dose while it displayed an inhibitory effect at higher concentrations ($p < 0.05$). SOD ($p < 0.001$), catalase ($p < 0.05$) and peroxidase ($p < 0.01$) activities were stimulated. Therefore, our study supports a disruptive effect of PFOA in cultured swine granulosa cells.

1. Introduction

Perfluorooctanoic acid (PFOA) is one of the main representatives of the per- and polyfluoroalkyl chemicals (PFAS). They are artificial chemicals present with increasing frequency in the food chain, due to environmental pollution mainly attributable to industrial activities. In particular, these substances are extensively employed in industrial applications and consumer goods, such as water-repellent and stain-resistant coatings for fabrics and carpets, oil-resistant coatings, paper products for food use, fire-fighting foams, floor paints and insecticides (Evich et al., 2022). They can accumulate in the body, entering via inhalation, food or with the intake of contaminated water. Due to their long persistence within the organism (DeLuca et al., 2022) they have been defined as part of a group of "forever chemicals" (Ko et al., 2021). The most well-known and studied PFAS is PFOA (ATSDR, 2018), which is consistently detectable even in wild animal serum (Giesy and Kannan, 2001). This substance has been shown to exert critical developmental effects at both high and low doses in rodents (Li et al., 2017). In man, after absorption, PFOA is not readily metabolized and has a half-life of 2.3–8.5 years (Li et al., 2017). Many studies on animal and human models show that PFOA accumulates mainly in the liver, kidneys and serum. (Vanden Heuvel et al., 1991), causing multiple deleterious effects, including hepatotoxicity, genotoxicity, immunotoxicity and neurotoxicity. Due to its long half-life in humans, the health risks have increasingly raised concerns worldwide.

The harmful effects on human health are exacerbated by the long half-life of this chemical as well as observations in animals exposed to PFOA, which have led to the creation of hypotheses based on potential impairment of endocrine signaling by this agent (Fenton et al., 2009): for example, Di Nisio et al. in 2019 (Di Nisio et al., 2019) showed that PFOA is able to play an antagonist role on the binding of testosterone to the androgen receptor. Chen et al. (Chen et al., 2017) documented that PFOA exposure in the mother impaired corpus luteum function, reduced serum progesterone concentrations and ovarian expression of Star, Cyp11a1 and Hsd3b1, enhanced ovarian tumor protein (p53) expression and Bax and re-duced Bcl-2 expression in the ovary. These effects resulted in embryo resorption, diminished foetal growth and newborn survival (Chen et al., 2017). The increasing concern regarding potential negative consequences on human health, wildlife, and the environment promoted the establishment of the PFOA Stewardship Program in 2006 by the U.S. Environmental Protection Agency (US EPA, 2022). By means of this program, the major PFAS manufacturing companies resolved to phase out PFOA, its precursors and related higher homologues from US production by 2015. Nevertheless, it is worth recording that international production of PFOA is still active. Recently, the U.S. Centers for Disease Control and Prevention (CDC) published a review outlining that

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https://doi.org/10.1016/j.etap.2023.104169
Received 27 February 2023; Received in revised form 30 May 2023; Accepted 4 June 2023
Available online 5 June 2023

PFAS exposure can be associated with several pathological states such as cancer, liver damage, and enhanced risk of asthma and thyroid disease (CDC, 2022). Severe concerns have been raised about negative reproductive effects, in particular decreased fertility (Chambers et al., 2021; Rickard et al., 2022; Calvert et al. 2022). Several studies explored PFOA effects on ovarian cell lines (Gogola et al., 2019; Clark et al., 2022), while the effect in primary granulosa cells have been investigated only by Zhou et al. (2020) and by Chaparro-Ortega et al. (2018). However, these studies were conducted culturing granulosa cells with serum, thus determining their luteinization. In a previous study performed using ovaries from the pig, an important animal model for translational medicine, we demonstrated that nitric oxide, superoxide anion and hydrogen peroxide were significantly inhibited by PFOA (Basini et al., 2022). These findings were important as free radicals are essential in folliculogenesis, which leads to successful ovulation. Therefore, to gain deeper insight into the PFOA effects on ovarian function, our current research was developed on primary cultures of granulosa cells isolated according to our well validated serum-free culture method which is useful to avoid granulosa cell luteinization (Basini et al., 2021a, 2021b). We tested the effect of PFOA on granulosa cell proliferation. Moreover, we measured for the first time to our knowledge the effects on steroidogenesis and on the activities of the main enzymatic radical scavengers, i.e. superoxide dismutase, catalase and peroxidase in a serum-free culture method thus avoiding their luteinization (Basini et al., 2021a, 2021b).

2. Materials and methods

Reagents were from Sigma (St. Louis, MO, USA) unless otherwise declared.

2.1. Collection of ovaries

The ovaries were taken from a local abattoir from 40 Large White hybrid gilts during each of the six collections performed. In all, 240 animals weighing approximately 180 kg each and aged between 8 and 9 months were used. A morphological study was then used to recognize the stage of the estrous cycle. (Akins and Morrissette, 1968; Babalola and Shapiro, 1988). Ovaries were put in PBS and treated as previously described (Bianco et al., 2005).

2.2. Granulosa cell collection

Ovarian follicles were categorized according to morphological criteria (Grasselli et al., 2003). In accordance with our and other previous research (Foxcroft and Hunter, 1985; Basini et al., 2014; Basini et al., 2017; Ciccimarra et al., 2018), granulosa cells were aspirated in sterility from healthy follicles at a later stage (> 5 mm diameter) in medium added with heparin (50 IU/mL). To pick the mural cells as well, the follicle wall was gently scraped with the needle. Purity was always higher than 90 % (Basini et al., 2021a, 2021b).

2.3. Granulosa cell culture and effects induced by PFOA

Previously validated culture medium DMEM/Ham's F12 modified for the growth of cells (Basini et al., 2022) indicated as culture medium (CM) has been used. After seeding, cells were subjected to a 48 h-incubation at 37 °C under humidified atmosphere (5 % CO_2) with different concentration of PFOA (2, 20 and 200 ng/mL) based on those tested proven to be not toxic in our (Basini et al., 2022) and tested also in previous works (Gogola et al., 2019; Gogola et al., 2020). The carrier solvent was represented by DMSO. Its final concentration was lower than 0.1 % v/v.

2.3.1. Granulosa cell proliferation

ELISA BrdU (Roche Diagnostic, Indianapolis, In, USA) was used. After seeding the cells in 96-well plates (Sarstedt, Nümbrecht, Germany) (10^4 cells/200 μL of CM), treatment with PFOA follows. The absorbance values were evaluated at 450 nm using the Victor Nivo spectrophotometer (Perkin Elmer, Groningen, The Netherlands). To quantify the number of viable cells, the absorbance was compared with a standard curve as already described (Gigante et al., 2018); this 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 number of cells/well was estimated from the resulting linear regression equation and 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 %.

2.3.2. Granulosa cell steroidogenesis

Viable cells (10^4 /well) were seeded in 96-well plates (Sarstedt, Nümbrecht, Germany), with 200 μL CM with the addition of 28 ng/mL androstenedione (Basini et al., 2020; Basini and Tamanini 2000), and incubated with PFOA as above stated. At the end, media were collected, frozen and stored at − 20 °C until progesterone (P4) and estradiol 17β (E2) were determined. For the detection of hormones, the Estradiol ELISA and Progesterone ELISA kits (Dia.Metra s.r.l, Spello, PG, Italy), based on competitive colorimetric immunological methods, were used. For the first assay the sensitivity of the test is 8.6 pg/mL and the intra-assay CV is < 9 %; for the second one, the sensitivity is 0.05 ng/mL and the intra-assay CV is < 4 %. The absorbance is read at 450 nm against a reference wavelength of 620–630 nm using Victor Nivo reader (Perkin Elmer, Groningen, The Netherlands) (Pacentra et al., 2020).

2.3.3. Granulosa cell superoxide dismutase (SOD) activity

SOD Assay Kit (Dojindo Molecular Technologies, Japan) has been employed. 2 × 10^5 cells/200 μL CM were grown in 96-well plates (Sarstedt, Nümbrecht, Germany) and treated with PFOA as described above. Cell lysates were tested without dilution and a standard SOD curve was set between 0.156 and 20 U/mL. The assay was performed as previously described. The absorbance was read with Victor Nivo reader at 450 nm versus 620 nm (Perkin Elmer, Groningen, The Netherlands) (Basini et al., 2007).

2.3.4. Granulosa cell Peroxidase activity

Amplex Red Peroxidase Assay Kit (Molecular Probes, Poort Gebouw, The Netherlands) has been used. 2 × 10^5 cells/200 μL CM were seeded in 96-well plates (Sarstedt, Nümbrecht, Germany) and treated with PFOA as previously described. Peroxidase activity was tested on cell lysates and read against a standard curve of the peroxidase between 0.078 and 10 mU/mL. Absorbance was determined with Victor Nivo reader at 540 nm (Perkin Elmer, Groningen, The Netherlands) (Basini et al., 2008).

2.3.5. Granulosa cell catalase (CAT) activity

Catalase activity was measured using assay kit (Molecular Probes, PoortGebouw, The Netherlands). 2 × 10^5 cells/200 μL CM were grown in 96-well plates (Sarstedt, Nümbrecht, Germany) and treated with PFOA as previously described. Reagent working solution was added to cell lysates and read against a curve ranging from 62.5 to 1000 mU/mL. Absorbance was determined with Victor Nivo reader at 540 nm (Perkin Elmer, Groningen, The Netherlands) (Basini et al., 2008).

2.4. Statistical analysis

The experiments were repeated at least 6 times. Six replicates of each treatment with PFOA at different concentrations were performed in each experiment. The data achieved are given as mean ± SEM; statistical differences between treatments were computed by ANOVA using the Statgraphics package (STSC Inc., Rockville, MD, USA). In the presence of significant differences, the means were related with Scheffè's F test, a post-hoc test useful to set all possible contrasts among the factor level

means; p-values < 0.05 were considered statistically significant.

3. Results

3.1. Granulosa cell proliferation

The proliferation of granulosa cells, determined on the basis of BrdU incorporation into newly synthesized DNA, was significantly inhibited ($p < 0.001$) by all the examined PFOA treatments (Fig. 1).

3.2. Granulosa cell steroidogenesis

The production of 17β-estradiol (E2) (basal 715 ± 46 pg/mL, mean ± SEM) was significantly stimulated ($p < 0.05$) by PFOA at all concentrations without significant differences attributable to dosages (Fig. 2A). Progesterone (P4) production (basal 11 ± 1 ng/mL, mean ± SEM was stimulated by the lowest treatment, while it resulted significantly inhibited by the highest treatments ($p < 0.05$) (Fig. 2B).

3.3. Granulosa cell scavenger enzyme activities

Superoxide dismutase (SOD) enzyme activity was significantly stimulated ($p < 0.001$) in granulosa cells by all concentrations of PFOA, particularly by the highest concentration (200 ng/mL) (Fig. 3A). The activity of catalase (CAT) and peroxidase was significantly stimulated by all concentrations of PFOA ($p < 0.05$ and $p < 0.01$ respectively) without differences (Fig. 3B and C).

4. Discussion

The impact of exposure to PFAS on reproductive health has been extensively studied: exposure to PFOS (perfluorooctane sulfonic acid) reduced newborn survival in rats and also resulted in retarded growth and postnatal development, and caused teratogenic effects in the offspring (Lau et al., 2003). In women, high serum PFOA levels are linked to menstrual cycle impairment and a slightly prolonged period of pregnancy (Fei et al., 2008); furthermore, an in vitro study by Zhao et al. (2014) showed that rat Leydig cells exposed to PFOA displayed a lower activity of 3-β and 17-β-hydroxysteroid dehydrogenase. According to these observations, it is possible to hypothesize that exposure to PFOA may impair the hypothalamic-pituitary-gonadal axis. Several studies show that PFOA alters the hormone-dependent processes and the function of this endocrine gland: among these, a cross-sectional study on 212 males exposed in the Veneto region demonstrated that the increase in PFOA levels in serum and sperm is correlated with the increase of circulating testosterone and luteinizing hormone (LH), resulting in poor sperm quality, reduction of testicular volume, penile length and anogenital distance (Di Nisio et al., 2019). Another crosssectional study performed in Nanjing, China on 664 adult men documented that seminal levels of PFOA were really related with a decreased sperm and an increased DNA fragmentation (Pan et al., 2019). On these bases, the chemicals have been included in the group of endocrine disruptor molecules. In the ovary, theca cells, granulosa cells and the oocyte interact in coordination thus ensuring follicular development, oocyte maturation and ovulation. Therefore, the first aim of this work was to get a better insight on some issues which was previously studied (Basini et al., 2022). We tested the effect of 2, 20 and 200 ng/mL. These concentrations mimic a real exposure since in the human serum levels of 3.4–34 ng/mL have been documented (Zhao et al., 2010), while serum levels of up to 691 ng/mL were detected in exposed workers (Olsen et al., 2007). We studied the effects of PFOA in sow granulosa cell cultures, as a model of endocrine reproductive cells. We analyzed the basic parameters that allow to study the complex phenomenon of cell growth, the ability to produce hormones and to scavenge free radicals (Basini et al., 2021a, 2020). Within the ovary, germ cells or oocytes are produced and released, as well as the secretion of sex steroid hormones takes place. Primordial follicles in mammalian females, which are present in a finite number, contain an immature oocyte surrounded by a single layer of somatic cells known as granulosa cells (Hirshfield, 1991). During folliculogenesis, primordial follicles must evolve to the antral stage in order to release an egg for fertilization. Furthermore, antral follicles produce large amounts of sex steroid hormones, particularly estrogen. The data collected in the present study indicate that granulosa cell proliferation is inhibited by PFOA exposure. In a study by Yang et al. (2022), it was documented that the higher concentration of PFOA significantly inhibits the proliferation of antral follicles in female mice. On the contrary, Gogola et al. (2019) and Clark et al. (2022) showed stimulatory effect caused by PFOA exposure. In our opinion, this discrepancy can be related to cell specific differences, since these studies were conducted using cell lines, while our research was undertaken in primary normal granulosa cells.

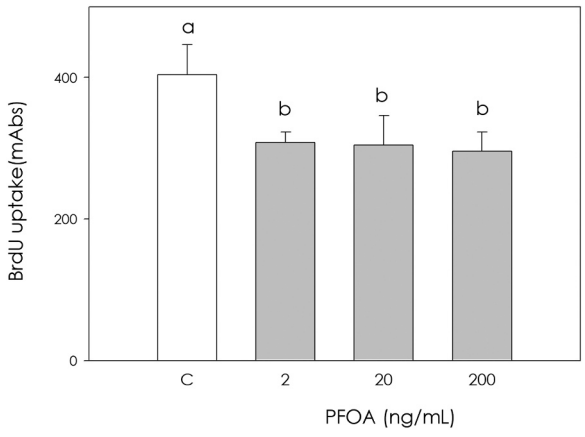

Fig. 1. Result of 5-bromo-2′deoxyuridine (BrdU) tests carried out on porcine granulosa cells following treatment with PFOA (2, 20 and 200 ng/mL) for 48 h. The test evaluates proliferation of treated cells. Data are expressed as milli-Abs units and represent the mean ± SEM of six replicates/treatment repeated in six experiments. Different letters placed on the bars indicate that the data, after having been subjected to statistical ANOVA and Scheffè F test, show significant differences ($p < 0.001$).

A primary function of the ovary is represented by the production and secretion of sex steroid hormones. Steroidogenesis requires the close coordination of ovarian cells with the hypothalamus-pituitary complex. Regarding steroidogenesis, our data show an enhancement of estrogen production in granulosa cells exposed to PFOA, an increase in P4 only at the lowest concentration, while its inhibition at higher concentrations. A previous study by Chaparro-Ortega et al. (2018), on granulosa cells and porcine theca cells documented that in cells not stimulated by gonadotropins, the effects of PFAS depend on the cell type. In fact, PFOA inhibited E2 secretion in granulosa but did not display any effect on theca cells. In line with our findings, various studies (Biegel et al., 1995; Lau et al., 2007; Cook et al., 1992) reported that PFOA significantly increased estradiol level in rodents, but studies on other animal models, including humans, are still very limited. Regarding the biphasic effect shown on P4 synthesis, a 2018 study by Tian et al. (2019), on mouse Leydig tumor cells (MLTC-1), showed a similar result: the content of 17 -OH progesterone after exposure to PFOA lowest concentrations (50 and 100 μM) was significantly increased compared to the control, while steroid levels were significantly reduced by 200 μM PFOA. One explanation may be that PFOA, as well as other endocrine-disrupting chemicals, challenge traditional concepts in toxicology, namely the dogma of "the dose makes the poison". At low concentrations, EDCs can display effects that are not predicted by their action at higher doses (Vandenberg et al., 2012). Non-monotonicity represents a challenge to fundamental concepts in toxicology and risk assessment. Indeed, environmental risk assessment approaches used by regulatory agencies around the world were developed on the basis of a methodology

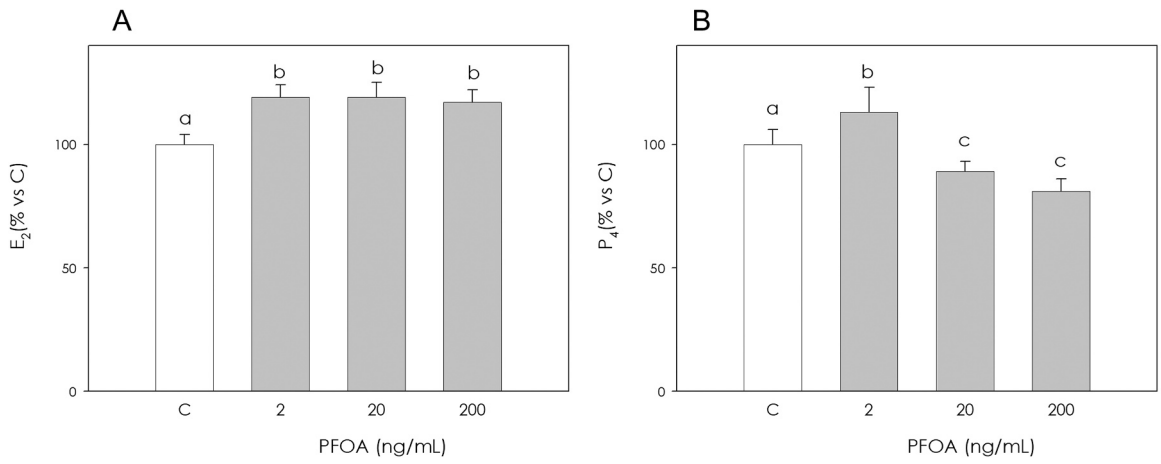

Fig. 2. Results of the production of 17β estradiol (E2) (A) and progesterone (B) by porcine granulosa cells treated 48 h with PFOA (2, 20 and 200 ng/mL) detected by ELISA assay. Data are expressed as % vs control and represent the mean ± SEM of six replicates/treatment repeated in six different experiments. Different letters placed on the bars indicate that the data, after having been subjected to statistical ANOVA and Scheffè F test, show significant differences ($p < 0.05$).

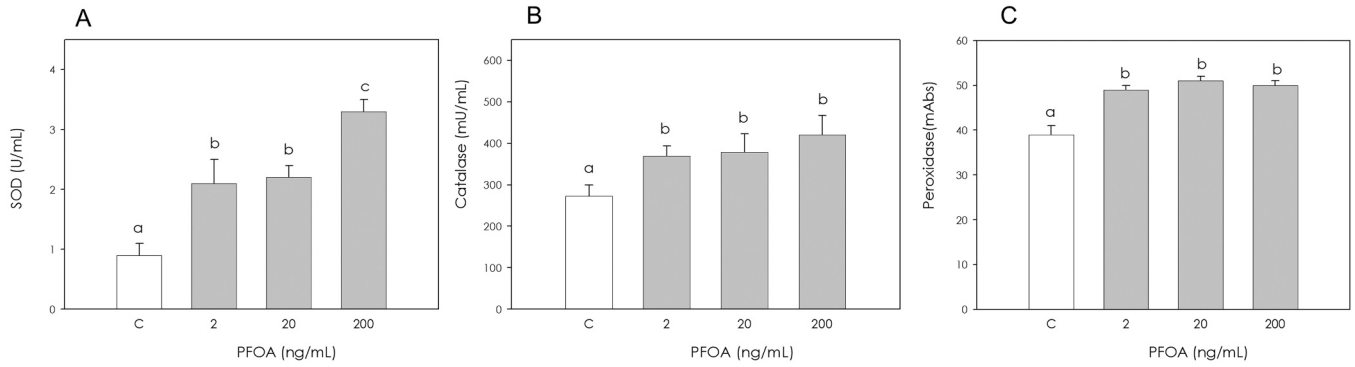

Fig. 3. Results of enzymatic scavenging activity by porcine granulosa cells treated for 48 h with PFOA ((2, 20 and 200 ng/mL): superoxide dismutase activity (A; U/mL), catalase activity (B; mU/mL), peroxidase activity (C; milliAbs). Data represents the mean ± SEM of six replicates/treatment repeated in six different experiments. Different letters placed on the bars indicate that the data, after having been subjected to statistical ANOVA and Scheffè F test, show significant differences ($p < 0.05$).

published by the National Academy of Sciences. For the hazard characterization step, it is generally accepted that, if detectable, a response of an organism to a toxicant increases proportionally to the level of exposure until reaching an upper-limit or maximal-effect level (Emax) beyond which higher toxicant dose will not increase the response (known as a monotonic dose-response). Experimental studies investigating the effects of endocrine disruptors frequently identify potential unconventional dose-response relationships called non-monotonic dose-response (NMDR) relationships. Standardized approaches for investigating NMDR relationships in a risk assessment context are missing. Several times, "hormetic responses" to endocrine disruptors have been demonstrated (Vandenberg et al., 2012), namely a dose response phenomenon to xenobiotics or other stressors characterized by a low-dose stimulation, with zero dose and high-dose inhibition, thus resulting in a J-shaped or an inverted U-shaped dose response. This phenomenon has been already described for PFBA, (Omagamre et al., 2022) as well as for PFOA (Manera et al., 2019). Further research is necessary to unravel this aspect.

An adequate ovarian steroidogenesis and resultant steroid-mediated local signaling are important for a normal ovarian development and function. The impairment of steroid production and / or inadequate steroid signaling in the ovary can result in significant ovarian disease leading to infertility in women of reproductive age. For example, P4 levels secreted by ovarian cells is increased in women with ovarian hyperstimulation syndrome; high P4 local levels are associated with the formation of ovarian follicular cysts and irregular menstrual cycles. Furthermore, high P4 concentrations promote the development and growth of breast cancer and uterine fibroids (Poole et al., 2006; Donnez, 2020).

There are few studies conducted on the mechanisms involved in PFOA effects on oxidative stress in the ovary (Chen et al., 2017; López-Arellano et al., 2019; Teteltitla et al., 2022). In our previous study (Basini et al., 2022), we demonstrated an increase in granulosa cell free radical generation induced by PFOA. Therefore, present research was under-taken to explore the effect of PFOA on antioxidant defences which were found to be increased. This cellular response could be a specific challenge in order to counteract the augmented ROS generation demonstrated in our previous research. In general, most of the studies performed on other cell types, such as liver or thyroid cells, have documented that PFOA affects the free radicals-antioxidant balance; Liu et al. (2007), showed that cultured primary hepatocytes of freshwater tilapia (Oreochromis niloticus) treated with PFOA displayed dose-dependent generation of ROS. As for enzymatic antioxidant activity, SOD activity was significantly increased, while CAT activity was significantly in-creased only at the highest exposure concentration (Liu et al., 2007). A study on the HepG2 cell line, showed that PFOA induced a significant increase of intracellular generation of ROS at all tested concentrations (Yao and Zhong, 2005). Lin et al. (2020) found that exposure to PFOA significantly increased ROS levels on mouse GC-1 spermatogonial cells. Furthermore, the cellular antioxidant defense

offered by SOD, an important ROS scavenging enzyme, has been undermined by PFOA treatment. At present, data on the interaction of PFOA with porcine ovary cells are limited. Lopez-Arellano et al. (2019) documented that ROS levels increased significantly and in concentration-dependent manner in mouse oocytes exposed to PFOA. These results support those obtained in an in vivo study by Chen et al. (2017), in which the administration of PFOA decreased superoxide dismutase and catalase activities and enhanced the levels of hydrogen peroxide and malondialdehyde in pregnant mice ovaries. The partial divergence of these results with those obtained by us may be due to the variability of cell type, as well as to the different times of exposure and to the various concentrations of PFOA. Further studies are necessary and are in progress in our laboratory, in order to better investigate the molecular pathways involved in the disruption of redox status balance induced by PFOA. Our future studies will be focused on potential effects of PFOA on gene expression since absence of consensus exists at present (Wielsøe et al., 2015; Suh et al., 2017; Piva et al., 2022).

In conclusion, our data show that PFOA inhibits granulosa cell proliferation, disrupts steroidogenesis and redox status. These findings create concern about the critical effects that this substance can cause on reproductive function.

CRediT authorship contribution statement

G. Basini: Conceptualization, Methodology, Writing – original draft, Writing – review & editing, Supervision. **S. Bussolati:** Data collection. **V. Torcianti:** Data collection. **F. Grasselli:** Writing – original draft, Writing – review & editing. All authors have read and agreed to the published version of the manuscript.

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.

Acknowledgements

This research was supported by the Program “FIL” of University of Parma.

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