



Perfluorooctanoic acid (PFOA) inhibits steroidogenesis and mitochondrial function in bovine granulosa cells *in vitro*[☆]

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ABSTRACT

Perfluorooctanoic acid (PFOA) is a persistent environmental contaminant. Due to the ubiquitous presence of PFOA in the environment, the impacts of PFOA exposure not only affect human reproductive health but may also affect livestock reproductive health. The focus of this study was to determine the effects of PFOA on the physiological functions of bovine granulosa cells *in vitro*. Primary bovine granulosa cells were exposed to 0, 4, and 40 μ M PFOA for 48 and 96 h followed by analysis of granulosa cell function including cell viability, steroidogenesis, and mitochondrial activity. Results revealed that PFOA inhibited steroid hormone secretion and altered the expression of key enzymes required for steroidogenesis. Gene expression analysis revealed decreases in mRNA transcripts for *CYP11A1*, *HSD3B*, and *CYP19A1* and an increase in *STAR* expression after PFOA exposure. Similarly, PFOA decreased levels of CYP11A1 and CYP19A1 protein. PFOA did not impact live cell number, alter the cell cycle, or induce apoptosis, although it reduced metabolic activity, indicative of mitochondrial dysfunction. We observed that PFOA treatment caused a loss of mitochondrial membrane potential and increases in PINK protein expression, suggestive of mitophagy and mitochondrial damage. Further analysis revealed that these changes were associated with increased levels of reactive oxygen species. Expression of autophagy related proteins phosphoULK1 and LAMP2 were increased after PFOA exposure, in addition to an increased abundance of lysosomes, characteristic of increased autophagy. Taken together, these findings suggest that PFOA can negatively impact granulosa cell steroidogenesis via mitochondrial dysfunction.

1. Introduction

Per- and polyfluoroalkyl substances (PFAS) are manmade chemicals that have been used in manufacturing consumer products such as textiles, paper, paint, footwear, carpeting, and industrial products for over 60 years (Buck et al., 2011). Common routes of exposure include contaminated food products and drinking water, inhalation of dust from treated textiles, dermal absorption from personal care products, or occupational exposures in industrial workers, firefighters, and military personnel (Sunderland et al., 2019). Because of their lipo- and

hydrophobic features with unique fluorocarbon bonds, PFAS are highly resistant to hydrolysis, photolysis, and thermal, chemical, or microbial degradation (Glüge et al., 2020). PFAS are rapidly absorbed and accumulate, with a half-life of approximately 3–5 years in humans (Olsen et al., 2007; Pérez et al., 2013). Therefore, PFAS are stable, persistent, and ubiquitous in the environment, which leads to their description as persistent organic pollutants.

Perfluorooctanoic acid (PFOA) is one of the most widely used PFAS compounds and has been detected in human serum at concentrations ranging from 3.4 ng/mL (0.01 μ M) to 22 μ g/mL (53 μ M) (Innes et al.,

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2011). The persistent nature of PFOA raises concerns about its potential impact on reproductive health. Observational studies in women suggest that PFOA/PFAS exposure delays menarche (Kristensen et al., 2013; Lopez-Espinosa et al., 2011), disrupts menstrual cyclicity (Lyngsø et al., 2014; Zhou et al., 2017; Fei et al., 2009), causes early menopause via premature ovarian failure (Knox et al., 2011; Zhang et al., 2018; Taylor et al., 2014), and alters steroid hormone levels (Knox et al., 2011; Zhang et al., 2018; Barrett et al., 2015). PFOA exposure is also associated with reduced fertility and increased miscarriage rates in women after maternal exposure (Fei et al., 2009; Liew et al., 2020; Whitworth et al., 2012).

Previous studies conducted with animal models indicate that PFOA exposure may lead to adverse effects on female reproduction in terms of ovarian function (Ding et al., 2020). Specific to the ovary, exposure to PFOA has been shown to impact the number of follicles in both juvenile and adult animal models (Chen et al., 2017; Du et al., 2019; Yang et al., 2022; Clark and Davis, 2022), alter the ovarian proteome (Estefanía González-Alvarez et al., 2022), and disrupt steroidogenesis in both *in vitro* and *in vivo* systems (Chen et al., 2017; Du et al., 2019; Yang et al., 2022; Chaparro-Ortega et al., 2018). Although human and experimental animal studies have demonstrated associations with PFOA exposure and ovotoxicity (Ding et al., 2020), there are only few studies reporting effects of PFAS on physiological functions of reproduction in livestock, which suggests the need for further comprehensive investigations (Basini et al., 2022; Chaparro-Ortega et al., 2018; Hallberg et al., 2021; Domínguez et al., 2016; Martínez-Quezada et al., 2021; Basini et al., 2023). Farm animals are also exposed to environmental toxic chemicals like PFOA, especially by ingestion, inhalation, or dermal contact, which can lead to serious and adverse effects on their health (Death et al., 2021; McGraw and Daigneault, 2022). However, exposure levels of many toxicants in livestock are not well defined, and hardly determined from human and/or rodent research due to different degradation and bioaccumulation patterns among species (Rhind, 2005). From this, there is a gap in our understanding of the adverse health effects among livestock despite studies that show PFAS are present and bioaccumulate in farm animals (Vestergren et al., 2013; Brake et al., 2023; Drew et al., 2021; Lupton et al., 2012; Lupton et al., 2022). Meat and dairy production depend greatly on the reproductive health of production animals, thus identifying potential exposures and their capacity to impact reproductive processes is imperative in mitigate these risks. Specific to cattle, PFAS exposure and accumulation have been reported via ingestion of contaminated feed (Guruge et al., 2008; Kowalczyk et al., 2013) and contaminated groundwater (Jha et al., 2021).

The granulosa cells of the ovarian follicle regulate and provide the physical support for the follicular microenvironment through the secretion of multiple growth factors and hormones (Hirshfield, 1991; Hsueh et al., 2015). Dysfunction of granulosa cells may lead to abnormal follicle development and ovulation, thus negatively impacting female fertility (Hirshfield, 1991; Hsueh et al., 2015). Mitochondria are a major cellular metabolic hub and are essential in steroid hormone biosynthesis (Miller, 2013; Sreerangaraja Urs et al., 2020). Further, mitochondria are an important source of reactive oxygen species (ROS) and regulators of autophagy/mitophagy after chemical exposures (Reddam et al., 2022). The distinct cellular effects and molecular mechanism of action of PFOA on ovarian cells, specifically granulosa cells, are mostly unknown. The purpose of this study is to investigate the effects of PFOA on primary bovine granulosa cells, including cell viability, steroidogenesis, and mitochondrial activity to identify potential mechanisms of action and further our understanding of the impact of PFOA exposure in reproductive cells.

2. Material and methods

2.1. Chemicals

PFOA (171468; purity 95%) was acquired from Sigma-Aldrich, USA.

PFOA was dissolved in 4.95 mL of Dulbecco's Modified Eagle Medium (DMEM)/F12 (Cytiva, Fisher Scientific, USA) and 50 μ L of dimethyl sulfoxide (DMSO; Fisher Scientific, USA), filter sterilized to make a 13.3 mM stock solution and diluted in culture media to obtain working solutions. The final concentration of DMSO in cell culture media was 0.01%.

2.2. Isolation of bovine granulosa cells

The use of domestic animals in biomedical research has proven to be beneficial to both agricultural and human health research (Abdal-Majed and Cupp, 2019). Bovine ovaries were collected from a local slaughterhouse and transported on ice to the laboratory. Follicles 2–5 mm in diameter were punctured and the linings gently scraped to collect granulosa cells into DMEM/F12 media containing 1% antibiotic/antimycotic solution (Gibco, ThermoFisher Scientific, USA). The isolated cells were washed twice with fresh DMEM/F12 media by centrifugation at 800 $\times$ g for 5 min, and then resuspended in DMEM/F12 culture media containing 10% fetal bovine serum (FBS; Gibco, ThermoFisher Scientific, USA) and 1% antibiotic/antimycotic solution. Total cell counts and cell viability were determined by trypan blue staining utilizing a Countess® Automated Cell Counter (Invitrogen, USA). Cell preparations with greater than 80% viability were used as described below.

2.3. Treatment of bovine granulosa cells with PFOA

Granulosa cells were seeded into 96-well culture plates at 1×10^4 cells/well for cell viability and steroid assays and 12-well culture plates at 2×10^5 cells/well for RNA and protein extraction. Granulosa cells were incubated in culture media containing 10% FBS as described above without any treatment for 24 h to allow for attachment. For treatment, cells were cultured in serum-free DMEM/F12 containing 1% insulin, transferrin, and selenium (ITS; Gibco, ThermoFisher Scientific, USA), 1% antibiotic/antimycotic, 0.1 μ M androstenedione as a precursor of estradiol, and PFOA at 0 – designated vehicle control, 4, and 40 μ M for 48 and 96 h. The media was replaced every 48 h. The bovine estrous cycle is ~21 days, in which 2 or 3 dominant follicles may emerge (Savio et al., 1988). Shortly after ovulation, a wave of follicles will be recruited and primed for maturation, a process that has been detected by ultrasound to take around 5 days (Roche and Boland, 1991). Thus, the time points of 48 and 96 h were selected to mimic exposure of granulosa cells during this period of follicle development. Further, the overall health and quality of our primary cells is maintained during 96 h incubations. The concentrations of PFOA used in this study were representative of the levels of PFOA reported to be present in biological fluids in humans (Innes et al., 2011; Gleason et al., 2015; Olsen et al., 2003; Olsen et al., 2007) and in cattle (Lupton et al., 2012).

2.4. Determination of live cell numbers

Experiments were conducted to measure the time- and concentration dependent effects of PFOA on the number of live cells. After 48 or 96 h of treatment, cells were trypsinized (TrypLE Express, Gibco, Denmark) after removing media and washing with phosphate buffered saline (PBS). Cells were collected and centrifuged at 800 $\times$ g for 5 min, then suspended in 1 mL PBS to determine cell numbers. An equal amount of cell suspension and trypan blue stain (10/10 μ L) were mixed in a micro tube and live cells were counted via automated cell counter (Invitrogen Countess®, ThermoFisher Scientific, USA).

2.5. Cell cycle analysis

Flow cytometry analysis of vehicle control and PFOA treated cells was used to determine cell distributions in the cell cycle. Granulosa cells (4.0×10^5 /mL) cultured in 12-well culture plates were detached by

trypsinization (TrypLE Express, Gibco, Denmark), washed in PBS, fixed in 66% ice-cold ethanol, and stored at 4 °C until flow cytometry analysis. Before the analysis, cells were stained with Propidium Iodide in PBS according to manufacturer's instructions (Propidium Iodide Flow Cytometric Kit; ab139418, Abcam, USA). Cells were then analyzed for cell cycle distribution via flow cytometry using a BD LSRII flow cytometer (BD Biosciences, USA).

2.6. Measurement of estradiol and progesterone secretion

Media was collected at the end of each treatment period and stored at –80 °C until analysis. The progesterone and estradiol secreted by granulosa cells into the media were measured using commercial ELISA kits (Progesterone ELISA - EIA-1561, Estradiol ELISA - EIA-2693, DRG, USA) according to manufacturer's instructions.

2.7. Quantitative real time PCR (qRT-PCR) analysis

Total RNA from granulosa cells was isolated using a commercial RNA extraction kit (Zymo Direct-zol Microprep Kit, Zymo Research Corporation, USA). The quality and concentration of RNA was determined using a NanoDrop (ND1000, Nanodrop Technologies, USA). Total RNA (500 ng) was reverse transcribed into cDNA with iScript Reverse Transcription Supremix (Bio-Rad, USA). Quantitative RT-PCR was performed using Sso Advanced Universal SYBR Green Supremix (Bio-Rad, USA) with Bio-Rad CFX96 Real-Time System (Bio-Rad, USA) as previously described (Clark et al., 2022). Gene-specific primer pairs were designed using the NCBI primer designing tool (<http://www.ncbi.nlm.gov/tools/primer-blast/>) and synthesized by Eurofins Genomics. The primers used in this study are shown in Supplemental Table 1. A melting curve analysis was performed to ensure a single and expected product was amplified for each primer pair. Relative quantification of mRNA levels of target genes was calculated using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) and normalized to GAPDH as a housekeeping gene.

2.8. Western blot analysis

Western blot analysis was performed as previously described (Przygrodzka et al., 2021) with some modification. Briefly, cells were rinsed with ice cold phosphate buffered saline (PBS) and lysed with cold RIPA buffer containing protease and phosphatase inhibitor cocktails (ThermoFisher, USA). Following sonication for 3 s, the lysate was cleared by centrifugation at 14,000×g for 5 min at 4 °C. Protein content of the samples was determined using a commercial protein assay kit (Pierce™ BCA Assay Kit, ThermoFisher, USA), and 30 µg protein per lane was subjected to 10% SDS-PAGE, and then transferred to a nitrocellulose membrane. Nonspecific binding was blocked with 5% milk in tris-buffered saline with 0.1% Tween 20 (TBS-t) at room temperature for 30 min. Membranes were then incubated with primary antibodies (Supplemental Table 2) in 5% bovine serum albumin (BSA) overnight at 4 °C. Following 3 × 10-min washes with TBS-t, membranes were incubated with the secondary antibodies (Supplemental Table 2) in 5% BSA in TBS-t for 45 min followed by 3 × 10-min washes. Bound antibodies were detected using ECL Western Blotting Substrate (SuperSignal West Femto, ThermoFisher, USA) and signals were captured on an iBright Imaging System (ThermoFisher, USA). Densitometry of the appropriately sized bands was measured using ImageJ software (<https://imagej.nih.gov>) and normalized to ACTB prior to calculation of fold induction.

2.9. MTT assay

To evaluate mitochondrial/metabolic activity of cells, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution (5 mg/mL in PBS) was added to each well in a 96-well plate for 3 h prior to the end of incubation time. Media was then discarded, and formazan crystals dissolved in DMSO. Absorbance was measured at 590 nm and

650 nm via automated multi-well spectrophotometer (SpectraMax Plus 384, Molecular Devices, USA).

2.10. Confocal microscopy

Bovine granulosa cells were cultured in four-chamber glass bottom culture dishes at 1.0×10^5 cells/dish for 24 h prior to PFOA treatment as described above. Following attachment, conditioned media was replaced with serum-free media (DMEM/F12) containing 1% ITS, 1% antibiotic/antimycotic, androstenedione (0.1 µM), and PFOA (0, 4 and 40 µM) for 48 and 96 h. The media was replaced every 48 h. High-resolution live-cell images were collected using a Zeiss LSM800 confocal microscope with Airyscan equipped with a 20 × objective or a 63 × oil immersion objective (1.4 N. A) and acquisition image size of 512 × 512 pixel. The appropriate filters were used to excite each fluorophore and emission of light was collected between 450 and 1000 nm. Cells were randomly selected from each well and 30–45 z-stacked (0.30 µm) images were generated from bottom to top of each experiment. Images were converted to maximum intensity projections and processed utilizing ImageJ (<https://imagej.nih.gov>) analysis software.

2.10.1. Determination of cell number

Live-cell imaging was used to establish the effects of PFOA on cellular proliferation. Bovine granulosa cells were incubated in media containing PFOA as described above. Following incubation, cells were washed with fresh culture media and incubated with MitoTracker Deep Red and 4',6-diamidino-2-phenylindole (DAPI) for 45 min at 37 °C. Following incubation, cells were washed with fresh culture media and observed under confocal microscopy. To evaluate the effects of PFOA exposure on cell number, images were converted to maximum intensity projections and DAPI positive cells were quantified utilizing ImageJ (<https://imagej.nih.gov>) analysis software.

2.10.2. Determination of mitochondrial membrane potential

Using confocal microscopy, we set out to establish the effects of PFOA on mitochondrial membrane potential. JC-1 dye is a cationic dye that exhibits membrane potential-dependent accumulation in mitochondria, indicated by a fluorescence emission shift from red (JC-1 dimer; aggregate) to green (JC-1 monomer) as the damaged mitochondria lose their membrane. Cells were incubated in PFOA as described above. Following incubation, cells were washed with calcium-free Hank's balanced salt solution (HBSS) and incubated with JC-1 dye (10 µg/mL) for 10 min at 37 °C. Cells were washed with HBSS and observed under confocal microscopy. The ratios of red (JC-1 dimer) to green (JC-1 monomer) were generated from each individual cell image and images converted to maximum intensity projections and processed utilizing ImageJ (<https://imagej.nih.gov>) analysis software.

2.10.3. Determination of reactive oxygen species

To establish the effects of PFOA on reactive oxygen species (ROS), we utilized CellROX, a weakly fluorescent cell-permeant dye that exhibits bright green photostable-fluorescence following oxidation by ROS production. Cells were incubated in media containing increasing concentrations of PFOA as described above. Following incubation, cells were washed with calcium-free HBSS and incubated with fresh serum free culture medium supplemented with CellROX reagent (5 µM) for 30 min prior to live-cell imaging using confocal microscopy. Images were converted to maximum intensity projections and processed utilizing ImageJ (<https://imagej.nih.gov>) analysis software. Mean fluorescence intensity (MFI) was determined as previously described (Plewes et al., 2017).

2.10.4. Determination of autophagy and lysosomes

CytoID Green Detection Reagent and LysoTracker were used to evaluate the effects of PFOA exposure on autophagy and lysosomal number, respectively. Primary bovine granulosa cells were cultured as described above. Following 96 h incubation with increasing

concentrations of PFOA (0, 4, or 40 μM), adhered cells were equilibrated in fresh culture medium for 2 h. Thirty minutes prior to termination, CytoID and LysoTracker were added to each well according to manufacturer's protocol and maintained at 37 °C in an atmosphere of 95% humidified air and 5% CO_2 . Following incubation, media containing detection reagents were replaced with fresh culture medium and granulosa cells were immediately imaged using a confocal microscope. To determine the effects of PFOA exposure on mean fluorescence intensity of CytoID (autophagy) and LysoTracker (lysosomes), images were converted to maximum intensity projections, processed utilizing ImageJ (<https://imagej.nih.gov>) analysis software and MFI determined as previously described (Plewes et al., 2017).

2.11. Statistical analysis

All experiments were replicated independently at least three times with different granulosa cell populations. All statistical analyses were performed using GraphPad Prism 9.0 (GraphPad Software, USA). Data are reported as average percentage increases or fold change over controls and expressed as mean $\pm$ standard error of the mean (SEM). Changes in levels of measured parameters between the groups were compared using one-way ANOVA and post-hoc Dunnett test. The significance level was set at $P < 0.05$ and a trend for a statistical difference was considered at $P < 0.1$.

3. Results

3.1. Effects of PFOA on steroid hormone secretion

Treatment with 4 μM PFOA did not impact progesterone or estradiol production at 48 h, though treatment with 40 μM PFOA decreased ($P <$

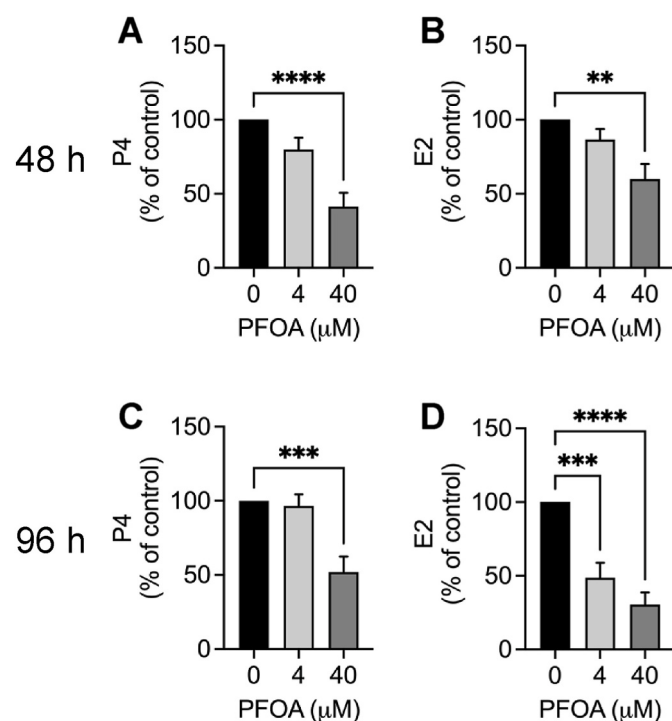


Fig. 1. PFOA exposure inhibits steroid secretion by bovine granulosa cells. Primary cultures of bovine granulosa cells were incubated for 48 h or 96 h with increasing concentrations of PFOA (0, 4 and 40 μM). Progesterone (P4) secretion (Panel A) in conditioned media and estradiol (E2) secretion (Panel B) in conditioned media following 48 h incubation. P4 secretion (Panel C) in conditioned media and E2 secretion (Panel D) in conditioned media following 96 h incubation. Results are percent of control means $\pm$ SEM, $n = 4$. ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$ compared to control.

0.05) both progesterone and estradiol production at 48 h (Fig. 1A and B). At 96 h, treatment with the higher concentration of PFOA (40 μM) decreased ($P < 0.05$) progesterone and estradiol secretion, and significantly decreased ($P < 0.05$) estrogen production at the 4 μM PFOA concentration (Fig. 1C and D).

3.2. Impact of PFOA on steroidogenic gene and protein expression

To further assess the effects of PFOA on steroidogenesis, the abundance of mRNA and proteins in the steroidogenic pathway was evaluated [steroidogenic acute regulatory protein (STAR), cytochrome P450 11A1 (CYP11A1), 3-beta-hydroxysteroid dehydrogenase 1 (HSD3B1), and aromatase (CYP19A1)]. Treatment with PFOA (40 μM) at 48 h significantly increased ($P < 0.05$) STAR mRNA, with no effects seen at the 4 μM concentration relative to control (Fig. 2A). Treatment with 4 μM or 40 μM PFOA for 48 h decreased ($P < 0.05$) expression of CYP11A1 mRNA transcripts (Fig. 2B). The abundance of HSD3B1 and CYP19A1 mRNA was unaltered at 48 h of incubation with either concentration of PFOA (Fig. 2C and D). In contrast to our observations at 48 h, no changes in STAR or CYP11A1 mRNA transcripts were observed at 96 h with 4 μM or 40 μM PFOA (Fig. 2E and F). A decrease ($P < 0.05$) in HSD3B1 transcripts was observed with 4 and 40 μM at 96 h, and transcripts for CYP19A1 mRNA were decreased ($P < 0.05$) with 40 μM PFOA (Fig. 2G and H).

Western blot analysis (Fig. 3A) showed that at 48 h of exposure, STAR was not affected by either concentration of PFOA (Fig. 3B). CYP11A1 was significantly decreased ($P < 0.05$) by 4 μM and 40 μM PFOA (Fig. 3C). Levels of HSD3B protein were not altered by either PFOA concentrations (Fig. 3D). After 48 h of PFOA exposure, CYP19A1 was decreased ($P < 0.05$) in response to 40 μM PFOA, but not 4 μM PFOA (Fig. 3E). At 96 h, STAR protein expression was not affected by PFOA exposure (Fig. 3F). However, CYP11A1 was decreased ($P < 0.05$) by treatment with 4 and 40 μM PFOA (Fig. 3G). HSD3B or CYP19A1 protein expression did not vary between the controls and the PFOA treatments at 96 h (Fig. 3H and I).

3.3. Effects of PFOA on live cell numbers and the cell cycle

The impact of PFOA exposure on cell viability was evaluated by determining the number of live cells present after treatment with PFOA for 48 or 96 h. Under the experimental conditions, PFOA did not lead to changes in live cell numbers at either timepoint (Fig. 4A and B). Microscopic analysis revealed similar numbers of intact nuclei following treatment with PFOA (Fig. 4C–E). To determine the impacts of PFOA on cell cycle regulation and cell growth, flow cytometry was performed. The mean percentage of cells for each cell cycle fraction (G_0/G_1 , S, G_2/M) is shown in Table 1. The exposure to PFOA did not change the percentage of cells in each phase of the cell cycle at either 48 or 96 h (Table 1).

3.4. Effects of PFOA on metabolic activity

To determine metabolic/mitochondrial activity of cells after PFOA exposure, we employed the MTT assay. Metabolic activity was reduced ($P < 0.05$) at both 4 and 40 μM of PFOA at 48 and 96 h of incubation, respectively (Fig. 5A and B).

3.5. The effects of PFOA on mitochondrial membrane potential

To determine the impact of PFOA exposure on mitochondrial membrane potential, bovine granulosa cells were incubated in media containing increasing concentrations of PFOA (0, 4, or 40 μM) for 48 or 96 h and immunostained for JC-1 dimer (aggregate; red) and JC-1 monomer (green) (Fig. 6A). Treatment with both 4 and 40 μM PFOA for 48 h resulted in a decreased ratio ($P < 0.05$) of fluorescence emission from red to green when compared to control cells (Fig. 6B), indicating a

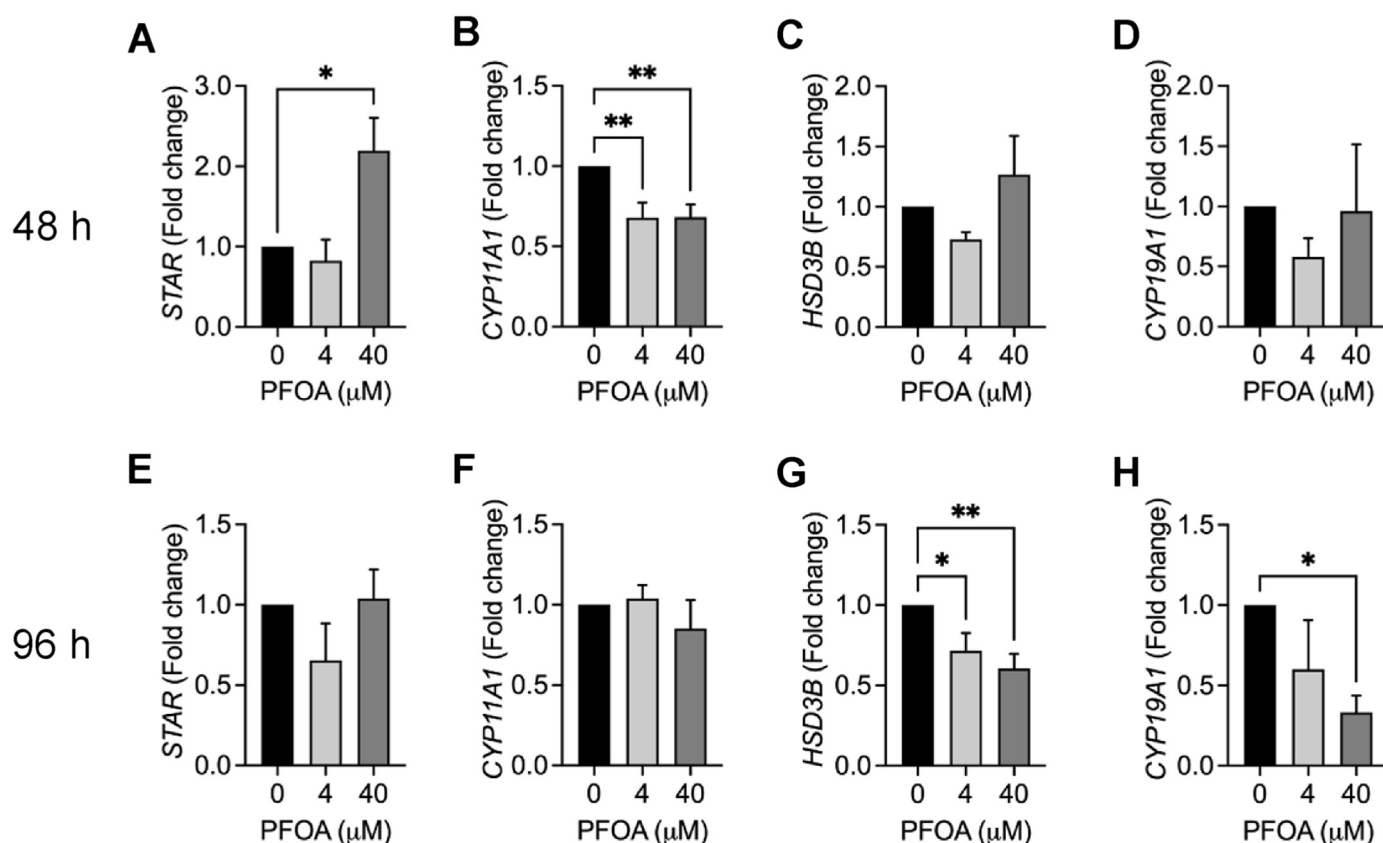


Fig. 2. PFOA inhibits the expression of genes required for steroid production by granulosa cells. Primary cultures of bovine granulosa cells were incubated for 48 h or 96 h with increasing concentration of PFOA (0, 4 and 40 μM). mRNA was collected and prepared for RT-PCR. Quantification of (Panel A) *STAR*, (Panel B) *CYP11A1*, (Panel C) *HSD3B*, and (Panel D) *CYP19A1* mRNA expression following 48 h exposure to PFOA. Quantification of (Panel E) *STAR*, (Panel F) *CYP11A1*, (Panel G) *HSD3B*, and (Panel H) *CYP19A1* mRNA expression following 96 h exposure to PFOA. The results are average relative fold-changes compared to controls and presented means $\pm$ SEM, $n = 4$. * $P < 0.05$; ** $P < 0.01$.

decrease in mitochondrial membrane potential. Treatment with 4 or 40 μM PFOA for 96 h resulted in a 75% decreased ($P < 0.05$) in the ratio of fluorescence emission from red to green when compared to the vehicle control cells (Fig. 6C).

3.6. Effects of PFOA on mitophagy

Because we observed a decrease in mitochondrial membrane potential following treatment with PFOA, we evaluated potential mitochondria damage using the expression of PTEN Induced Kinase 1 (PINK1) expression, an indicator of mitophagy (Quarato et al., 2023). There were no differences in the expression of PINK1 following 48 h treatment with either 4 or 40 μM PFOA when compared to vehicle control cells (Fig. 7A and B). However, an increase ($P < 0.05$) in the expression of PINK1 was observed 96 h after treatment with 40 μM PFOA (Fig. 7C and D).

3.7. Effects of PFOA on reactive oxygen species (ROS)

To determine the impact of PFOA exposure on mitochondrial ROS production, bovine granulosa cells were incubated in media containing increasing concentrations of PFOA (0, 4, or 40 μM) for 48 or 96 h. Intracellular ROS was visualized by confocal microscopy using CellROX Green Reagent (Fig. 8A). Upon oxidation by ROS, CellROX subsequently binds to DNA, promoting aggregated bright green photostable fluorescence. Reactive oxygen species present in cytoplasmic compartments accumulate on nuclear DNA, while mitochondrial ROS convene on mitochondrial DNA. Treatment with 4 and 40 μM PFOA for 48 and 96 h increased ($P < 0.05$) the mean fluorescent intensity of CellROX, a

marker of intracellular ROS, when compared to vehicle control (Fig. 8A–C). Furthermore, the increased ROS production observed appears to be cytosolic rather than mitochondrial as seen by localization with nuclei (Fig. 8A).

3.8. Effects of PFOA on activation of autophagy and lysosome number

To determine the effects of PFOA on activation of autophagy and lysosome number, bovine granulosa cells were treated in media containing increasing concentrations of PFOA (0, 4, or 40 μM) for 48 or 96 h. Activation of autophagy was visualized via CytoID Autophagy Detection reagent and lysosomes were observed by Lysotracker (Fig. 9A and D). We observed an increase ($P < 0.05$) in the mean fluorescent activity of CytoID in cells treated with either 4 or 40 μM concentrations of PFOA at 48 h (Fig. 9A and B) and 96 h (Fig. 9D and E) when compared to cells treated with vehicle control. Additionally, 48 h treatment with 40 μM PFOA increased the mean fluorescent intensity of Lysotracker when compared to control cells (Fig. 9A and C). Following 96 h of treatment with either 4 or 40 μM PFOA, we observed an increase ($P < 0.05$) in the mean fluorescent intensity of Lysotracker when compared to control cells (Fig. 9D and F). To confirm activation of autophagy, protein expression was evaluated via Western blot for the autophagy initiation protein Unc-51-like autophagy activating kinase 1 (ULK1), lysosomal associated membrane protein 2 (LAMP2), and microtubule associated protein light chain 3 beta (LC3B). Treatment for 48 h with 4 and 40 μM PFOA resulted in significantly increased ($P < 0.05$) the phosphorylated (active) ULKser555/total ULK1 ratio and LAMP2 protein expression at both concentrations relative to controls (Fig. 10A–C). After 96 h of PFOA treatment, LAMP2 was increased ($P < 0.05$) at 4 μM PFOA, and the

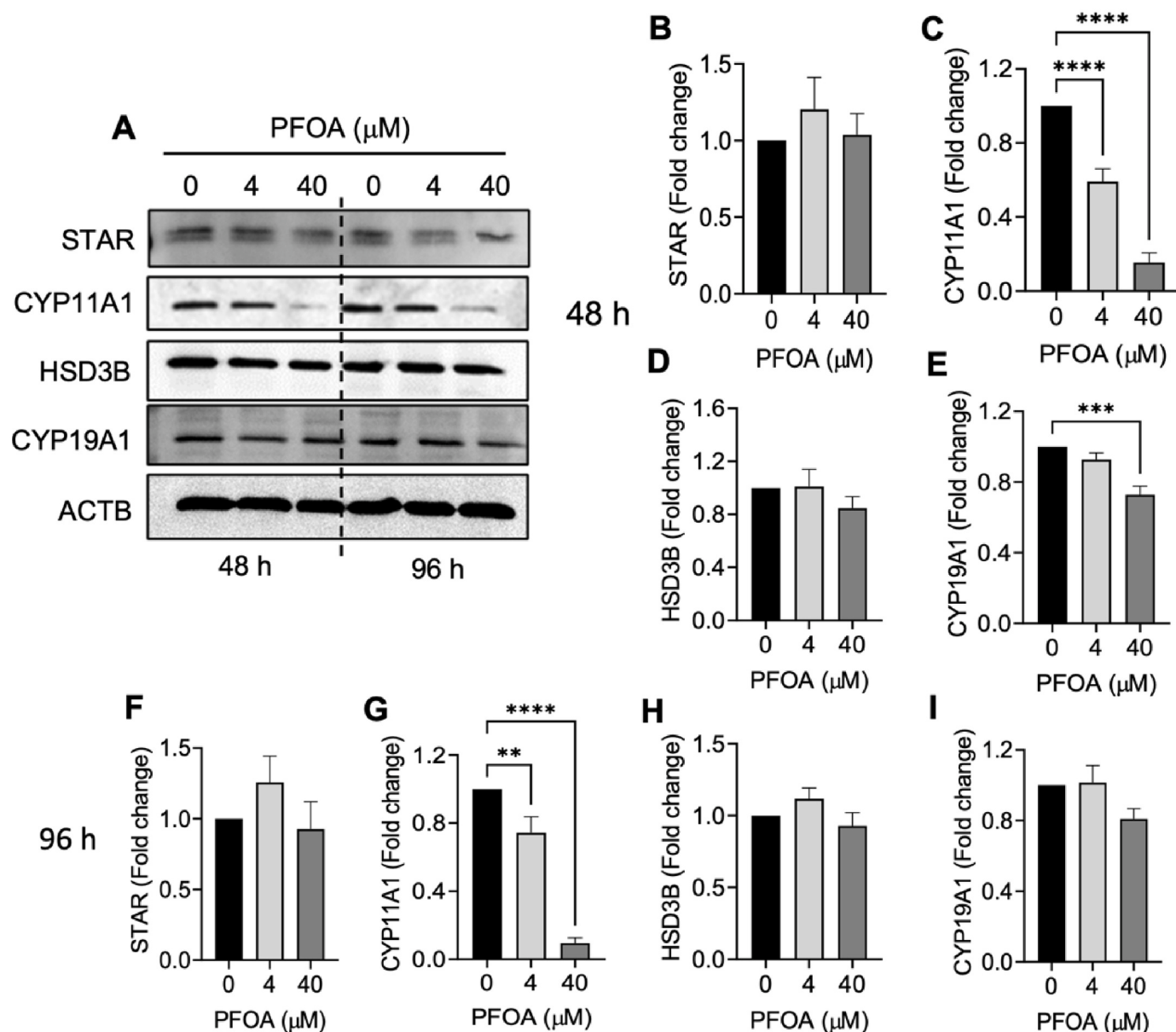


Fig. 3. PFOA inhibits the expression of proteins required for steroid production by granulosa cells. Primary cultures of bovine granulosa cells were incubated for 48 h or 96 h with increasing concentration of PFOA (0, 4 and 40 μ M). Protein lysates were collected and subject to Western blotting. (**Panel A**) Representative Western blot of enzymes associated with steroid synthesis following 48 h exposure to PFOA. Quantification of STAR (**Panel B**), CYP11A1 (**Panel C**), HSD3B (**Panel D**), and CYP19A1 (**Panel E**) protein expression following 48 h exposure to PFOA. Quantification of STAR (**Panel F**), CYP11A1 (**Panel G**), HSD3B (**Panel H**), and CYP19A1 (**Panel I**) protein expression following 96 h exposure to PFOA. Results are averages of the fold-changes compared to control in each experiment. Data are shown as means $\pm$ SEM, $n = 4$. ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

pULK/ULK1 ratio was also increased at 4 μ M PFOA (Fig. 10D–F). In contrast, no changes in LAMP2 or pULKser555 were observed between controls and 40 μ M PFOA after 96 h (Fig. 10D–F). The ratio of LC3B II/I and the total expression of LC3B was statistically unchanged at both timepoints and both PFOA concentrations, though slight elevations were observed (Supplemental Figs. 1A–F).

4. Discussion

In recent years PFOA has attracted the attention of researchers as it may cause adverse effects on human and animal health due to its long-term persistence in the environment and bioaccumulation in the body. To our knowledge, this is the first study to examine the effects of PFOA exposure on bovine ovarian cells. This study was designed to investigate the possible adverse effects of PFOA on viability and cytotoxicity,

proliferation, steroidogenesis, mitochondrial function, and autophagy of primary cultures of bovine granulosa cells. The results of the present study indicate that PFOA exposure alters mitochondrial function and inhibits the steroidogenic capacity of bovine granulosa cells, which has the potential to disrupt follicular function and fertility in both humans and livestock.

Although PFAS were previously considered metabolically inert and/or nontoxic substances in their early years, they are presently viewed as endocrine disrupting compounds (White et al., 2011). Several studies have shown that these compounds can alter the physiological functions of the endocrine system by mimicking hormones and/or interfering with their mechanisms (Chaparro-Ortega et al., 2018; Henry and Fair, 2013; Huang et al., 2022; Krawczyk et al., 2021; Yang et al., 2022). Steroid hormones are essential for many physiological processes including development, reproduction, and metabolism. Endocrine disruptor

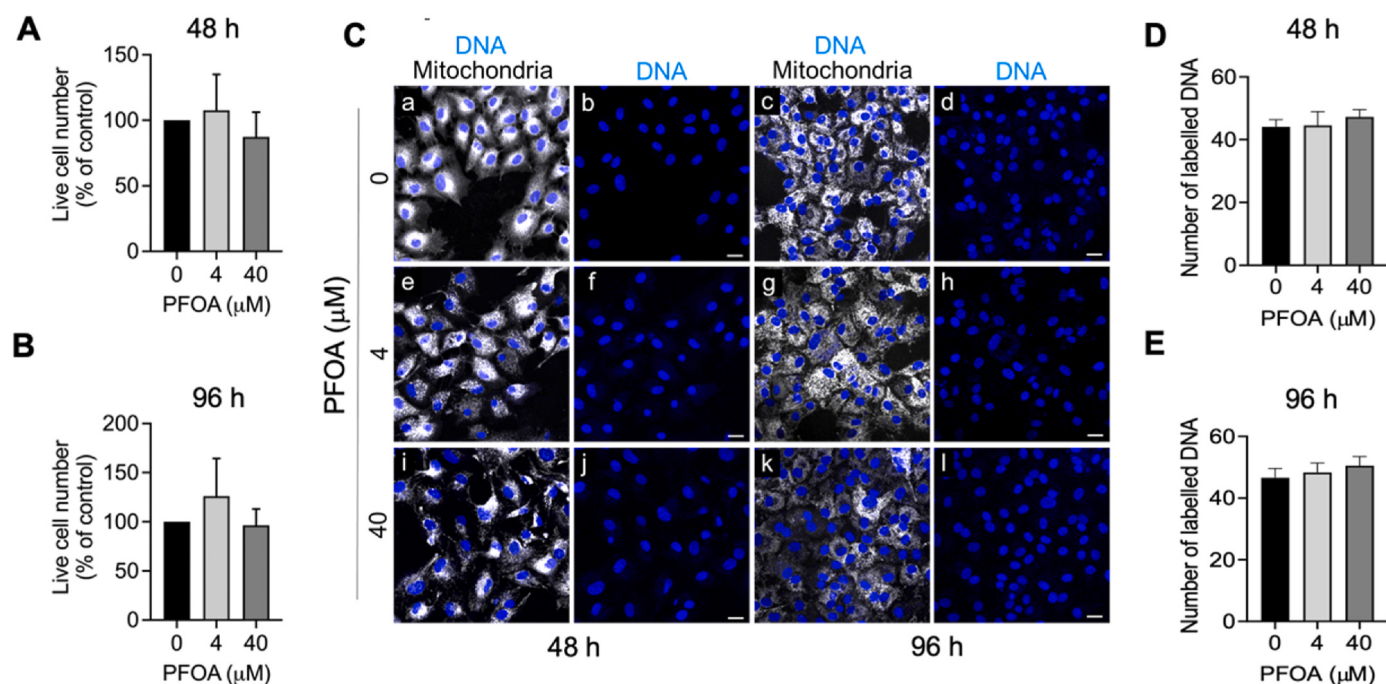


Fig. 4. PFOA does not stimulate proliferation of bovine granulosa cells. Primary cultures of bovine granulosa cells were incubated for 48 h or 96 h with increasing concentrations of PFOA (0, 4 and 40 μM). Cell counts were performed by Trypan blue staining to determine effects of PFOA on proliferation. Cell counts following 48 h of PFOA exposure (**Panel A**) and following 96 h of PFOA exposure (**Panel B**). (**Panel C**) Representative micrographs showing nuclear DNA staining using DAPI (blue) and mitochondria staining (white) as described in the Methods. Concentrations of PFOA (top to bottom); incubation time (left to right). Quantification of labelled DNA following 48 h PFOA exposure (**Panel D**) and following 96 h PFOA exposure (**Panel E**). Micron bar represents 20 μm . Results are expressed as means $\pm$ SEM, $n = 3$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Effects of PFOA on the cell cycle of bovine granulosa cells.

	48 h			96 h		
	G ₀ /G ₁	S	G ₂ /M	G ₀ /G ₁	S	G ₂ /M
Control (0)	74.2 $\pm$ 2.1	10.8 $\pm$ 0.9	15.0 $\pm$ 1.2	79.0 $\pm$ 2.5	7.9 $\pm$ 0.5	13.9 $\pm$ 2.0
4 μM PFOA	71.2 $\pm$ 0.2	12.5 $\pm$ 0.4	16.4 $\pm$ 0.3	73.5 $\pm$ 0.1	9.1 $\pm$ 0.3	17.4 $\pm$ 0.1
40 μM PFOA	73.3 $\pm$ 2.8	11.6 $\pm$ 0.9	15.1 $\pm$ 2.2	81.3 $\pm$ 2.2	7.6 $\pm$ 0.8	11.1 $\pm$ 1.7

Percentage of cells in G₀/G₁, S, or G₂/M phase of the cell cycle. Data are means $\pm$ SEM.F.

effects of toxic chemicals may occur at the cellular and/or molecular level via interference with steroid hormone biosynthesis in the ovary. Exposure of the human granulosa cell line HGrC1 to a mix of endocrine disruptors including PFOA (2 ng/mL; 4 nM) increased progesterone production (Krawczyk et al., 2021). Furthermore, *in vitro* culture of murine antral follicles with PFOA (100 $\mu\text{g}/\text{mL}$; 2.4 μM) reduced follicle growth, estradiol and estrone levels (Yang et al., 2022). In this same study, *in vivo* administration of 1 mg/kg PFOA to mice caused an elevation in serum testosterone while 5 mg/kg resulted in reduced progesterone and pregnenolone levels (Yang et al., 2022). In the present study, we found that PFOA decreased the ability of bovine granulosa cells to secrete progesterone and estradiol. Similar findings were reported in porcine granulosa cells in which basal or gonadotropin stimulated progesterone and estradiol production was inhibited with exposure to 1.2 μM PFOA and 0.012 μM PFOA, respectively (Chaparro-Ortega et al., 2018). Interestingly, another study utilizing porcine granulosa cells showed increased levels of estradiol and progesterone production in response to PFOA (2, 20, 200 ng/mL; 4 nM, 48 nM, 0.4 μM), albeit progesterone did decrease with increasing concentrations of PFOA (20, 200 ng/mL; 48 nM, 0.4 μM) (Basini et al., 2023).

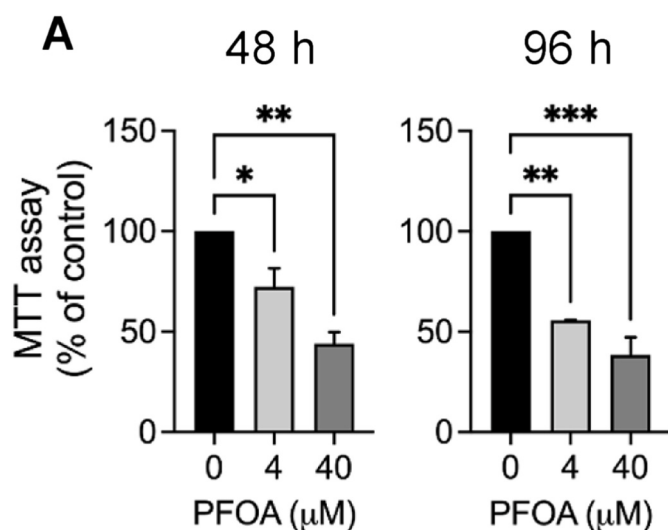


Fig. 5. PFOA exposure inhibits metabolic activity of bovine granulosa cells. Primary cultures of bovine granulosa cells were incubated for 48 h or 96 h with increasing concentration of PFOA (0, 4 and 40 μM). MTT assays were performed to determine cellular metabolic activity. Results are expressed as average percent of control in each experiment. Data are represented as means $\pm$ SEM, $n = 3$. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

To further investigate the impact of PFOA on steroidogenesis, we measured mRNA and protein expression of enzymes related to steroid production. We found that the PFOA-induced reductions in CYP11A1 protein were more closely associated with PFOS-induced reductions in progesterone synthesis, than alterations in STAR or HSD3B. Surprisingly, levels of CYP19A1 protein expression following treatment with PFOA, were not associated with reductions in estradiol secretion,

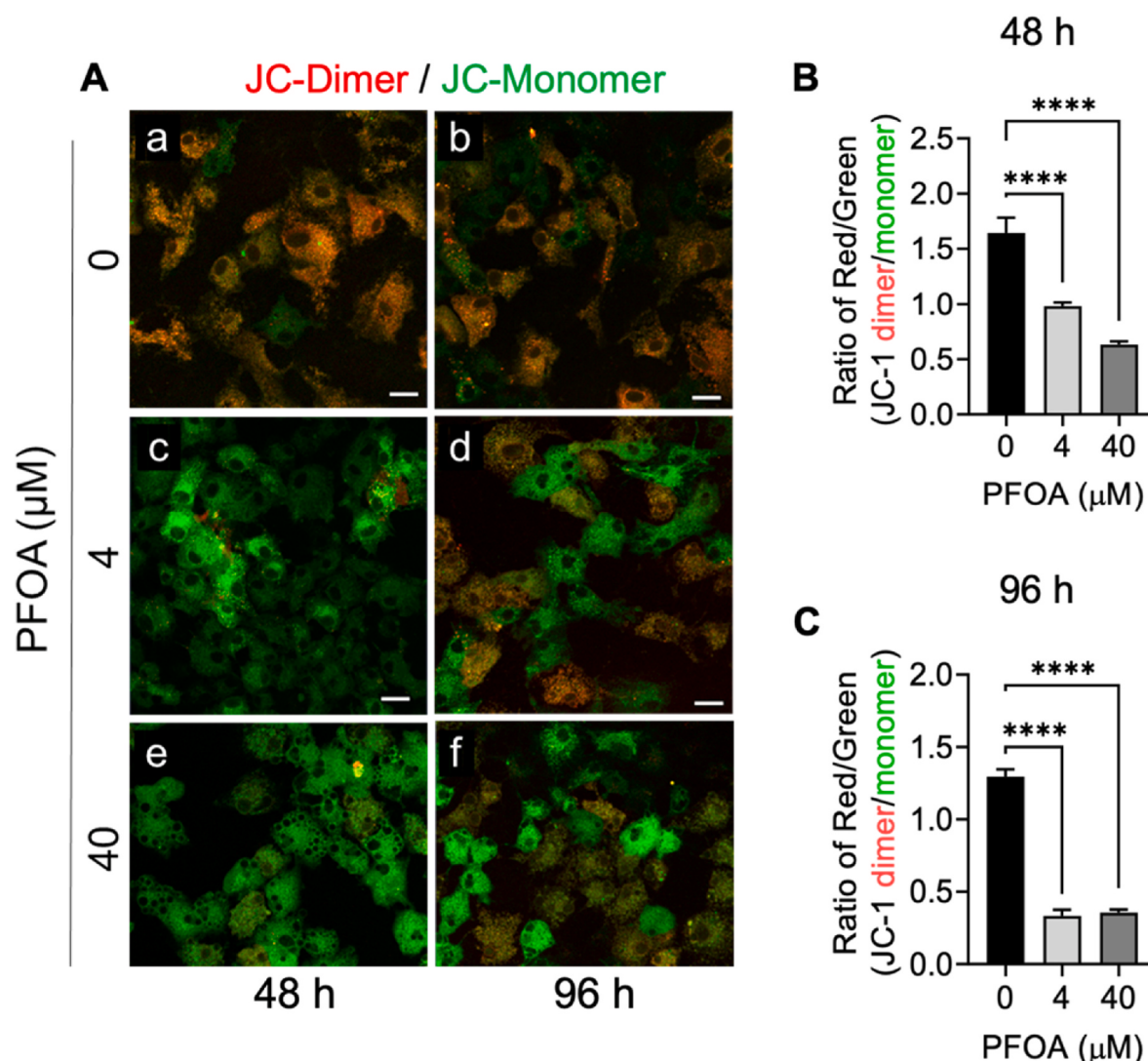


Fig. 6. Treatment with PFOA decreases mitochondrial membrane potential in bovine granulosa cells. Bovine granulosa cells were treated with increasing concentrations of PFOA (0, 4, or 40 μM) for 48 h and 96 h and subject to confocal microscopy. (**Panel A**) Representative micrographs showing the effects of PFOA on mitochondrial membrane potential as measured by JC-1. From left to right; 48 h exposure to PFOA (merge of red and green; panels a, c, and e) and 96 h exposure to PFOA (merge of red and green; panels b, d, and f). Quantitative analyses of the mean fluorescence intensity of JC-1 red/green following 48 h exposure to PFOA (**Panel B**) and following 96 h exposure to PFOA (**Panel C**). Micron bar represents 20 μm. Results are means ± SEM, n = 3. ****P < 0.0001. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

particularly after 96 h of treatment, suggesting that estradiol may be degrading at a faster rate than usual. Our findings of disrupted hormone levels and steroidogenic gene and protein expression are consistent with previous studies on perfluoroalkyl compounds in rodent gonadal tissues and cell culture models. Yang et al. (2022) reported that *in vitro* exposure of 100 μg/mL (2.4 μM) PFOA significantly inhibited estradiol and progesterone production by antral follicles. These inhibitory responses were associated with decreased expression of transcripts for *Star*, *Cyp11a1*, *Hsd3b1* as well as *Cyp19a1* in mouse antral follicles. Conversely, these authors showed that *in vivo* treatment of female mice with 1 mg/kg PFOA for 10 days reduced serum progesterone without alterations in expression of ovarian steroidogenic enzymes. Paradoxically, this study also reported that levels of estradiol were unchanged despite an increase in ovarian *Cyp19a1* mRNA levels. In another study, exposure of the human non-luteinized granulosa cell line, HGrC1, to a mix of endocrine disruptors including PFOA (2 ng/mL; 4 nM) resulted in upregulation of *HSD3B* mRNA expression and progesterone secretion, while no effect on CYP19A1 expression and estradiol production (Krawczyk et al., 2021). Similar confounding findings have also been reported in studies

investigating PFOA exposure and steroidogenesis in the male (Han et al., 2022; Huang et al., 2022; Tian et al., 2019). Upregulation of *Star* mRNA and coinciding decrease in other steroidogenic genes has also been observed after PFOA exposure in male rat testes, while increasing overall serum hormone levels (Han et al., 2022). Similarly, another study found that PFOA increased expression of steroidogenic enzymes and steroid hormone levels in both rat and mouse Leydig cells *in vitro* and rat testes *in vivo* (Huang et al., 2022; Tian et al., 2019). In contrast, PFOA has been shown to decrease steroid hormone levels and corresponding protein/gene expression in rat and mouse testes (Lu et al., 2019; Zhang et al., 2014; Eggert et al., 2019). The differences in our results of gene and protein expression changes could be attributed to translation rates, translation rate modifications, modulation of protein half-life, protein synthesis delay, and protein transport, as multiple complex temporal processes can influence the relationships between transcript abundance and protein expression (Liu et al., 2016). Differences in culture conditions and/or cell line specific physiologic characteristics may also account for differences in sensitivity to chemical exposures, so these parameters should be taken into consideration when

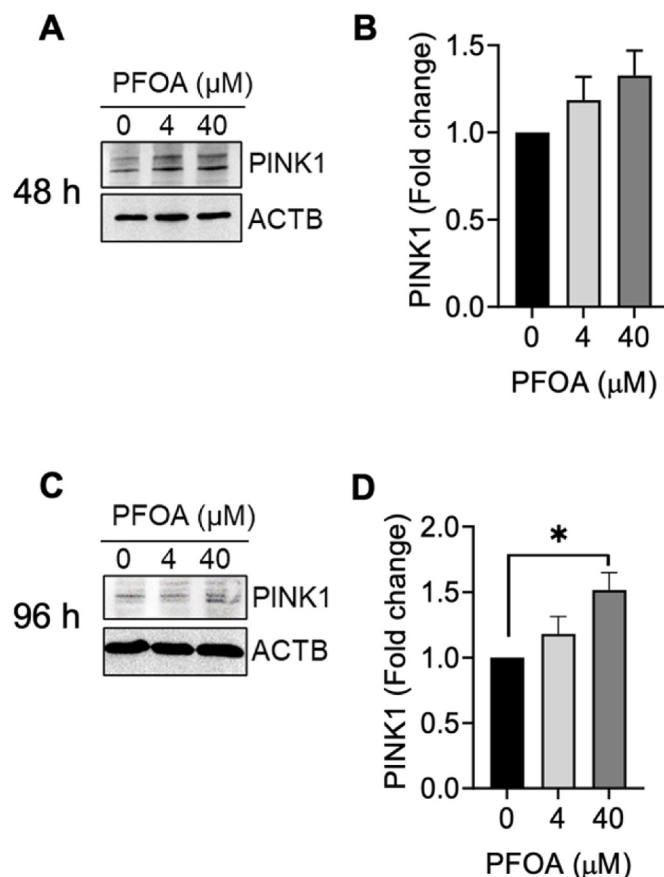


Fig. 7. PFOA exposure stimulates the expression of mitophagy associated protein PINK1 granulosa cells. Primary cultures of bovine granulosa cells were incubated for 48 h or 96 h with increasing concentration of PFOA (0, 4 and 40 μM). Protein lysates were collected and subject to Western blotting. **(Panel A)** Representative Western blot of PINK1 expression following 48 h exposure to PFOA. **(Panel B)** Quantification of PINK1 protein expression following 48 h treatment with PFOA. **(Panel C)** Representative Western blot of PINK1 expression following 96 h exposure to PFOA. **(Panel D)** Quantification of PINK1 protein expression following 96 h treatment with PFOA. Results are average relative fold-change means $\pm$ SEM, $n = 4$. * $P < 0.05$.

designing future experiments and evaluating the toxicity of PFAS. Differences observed *in vivo* and *in vitro* may also reflect systemic responses due to alterations in the hypothalamic/pituitary axis rather than direct effects on the gonad. Taken together, these results suggest that PFOA may have negative impacts on reproductive health by interfering with the production and/or balance of steroid hormones in gonadal tissues.

A number of studies have reported that PFOA has proliferative effects on granulosa cells (Basini et al., 2022; Clark et al., 2022; Krawczyk et al., 2021). Therefore, we evaluated the effects of PFOA on the number of live cells to determine whether reduction in steroid secretion is a result of cell death. We observed that treatment with up to 40 μM PFOA for 48 and 96 h did not significantly change the number of live cells. We confirmed this result by cell cycle analysis via flow cytometry, which revealed no significant changes in cell cycle phases between the vehicle control and PFOA treated groups. Moreover, our results of live cell nuclei staining via confocal microscopy were consistent with the results of cell counts and cell cycle analysis. While we did not find any evidence of cell death in our study using primary cultures of granulosa cells, other studies utilizing granulosa cell lines have determined that 30 and 300 μM PFOA treatment for 24 h reduced cell viability and induced apoptosis in KGN cells (Zhou et al., 2020) and that exposure to 100 μM PFOA was cytotoxic to HGrC1 cells (Clark et al., 2022). We did not see any significant differences between the cellular responses at 48 and 96 h. We

believe that the toxicity of PFOA has potentially reached a steady state, though this remains to be explored. Time-dependent toxicokinetic studies in future experiments will be critical in understanding the toxicity of PFOA.

Despite the observation that the live cell number did not change, we observed a significant decrease in cellular metabolic activity after PFOA exposure. Similar results have been found in our previous study conducted with testicular cells and tebuconazole pesticide (Kabakci et al., 2021). Although the MTT assay is commonly used to measure cytotoxicity through measures of proliferation, viability, and mitochondrial/metabolic activity of cells (Stockert et al., 2018), the basis for the MTT assay is mitochondrial enzyme activity that converts MTT salts to formazan crystals. Because we did not observe a change in granulosa cell number following treatment with PFOA, the observed reduction in formazan crystal formation suggests that PFOA inhibits the mitochondrial/metabolic activity of bovine granulosa cells. Because reduced mitochondrial membrane potential is often a key indicator of aberrant mitochondrial function, we investigated the effects of PFOA exposure on mitochondrial membrane potential and ROS production. In the present study, we report that PFOA exposure results in a significant reduction of mitochondrial membrane potential. Generation of ROS is also a critical function during ovulation and steroidogenesis (Agarwal et al., 2012; Shkolnik et al., 2011; Goutami et al., 2022). Further, impairment of ROS generation can impact ovulation (Shkolnik et al., 2011). Thus, we evaluated the effects of PFOA incubation of ROS production using live-cell imaging. We report an increase in ROS production following exposure to PFOA, confirming the occurrence of aberrant mitochondrial function and oxidative stress. The alteration in mitochondrial function may explain, at least in part, the reduction observed in the MTT assay. Further, the increase in ROS may lead to increased oxidative stress which would influence progesterone and/or estradiol (Behrman et al., 2001; Luderer, 2014), thus impacting enzymatic activity of HSD3B or CYP19A1, respectively. For example, the activity of CYP19A1 depends on the availability of certain cofactors such as NADPH (Flück and Pandey, 2017), increased ROS and oxidative stress can disrupt cellular redox balance and affect the availability of these cofactors, potentially impacting the enzymatic activity of CYP19A1. These results are in agreement with others who reported that PFOA exposure induces oxidative stress and increased ROS production in mouse oocytes (Zhou et al., 2022; Jiao et al., 2021; Guo et al., 2021; López-Arellano et al., 2019; Zhang et al., 2022), porcine cumulus granulosa cells (Basini et al., 2022; Basini et al., 2023), mouse primary hepatocytes (Xu et al., 2019), and pancreatic β-cells (Suh et al., 2017).

Loss of mitochondrial membrane potential and increases in intracellular oxidative stress have been reported to trigger mitophagy and autophagy, two essential cellular processes responsible for breaking down cellular contents and safeguarding against accumulation of oxidative damage (Zhang, 2013). When mitochondrial membrane potential is dissipated, PINK1 accumulates on the outer mitochondrial membrane where it can recruit PARKIN to impaired mitochondria to initiate mitophagy (Durcan and Fon, 2015). Here, we report that loss of mitochondrial membrane potential was accompanied by an increase in PINK1 expression. We further investigated the effects of PFOA exposure on autophagy in primary bovine granulosa cells using live cell imaging. We report that PFOA exposure increases indices of autophagy as measured by an increase in autophagic vacuoles and lysosomal abundance. Furthermore, the observed increase in autophagy was accompanied by an increase in lysosome numbers and lysosomal associated membrane protein 2 (LAMP2), which plays an important role in the fusion of autophagosomes with lysosomes during autophagy. Additionally, we observed increases in phospho-ULK1, further confirming the initiation of autophagy after PFOA exposure. These observations are in agreement with others that report that PFOA exposure stimulates activation of autophagy in multiple tissues (Zeng et al., 2021; Tang et al., 2018; Weng et al., 2020).

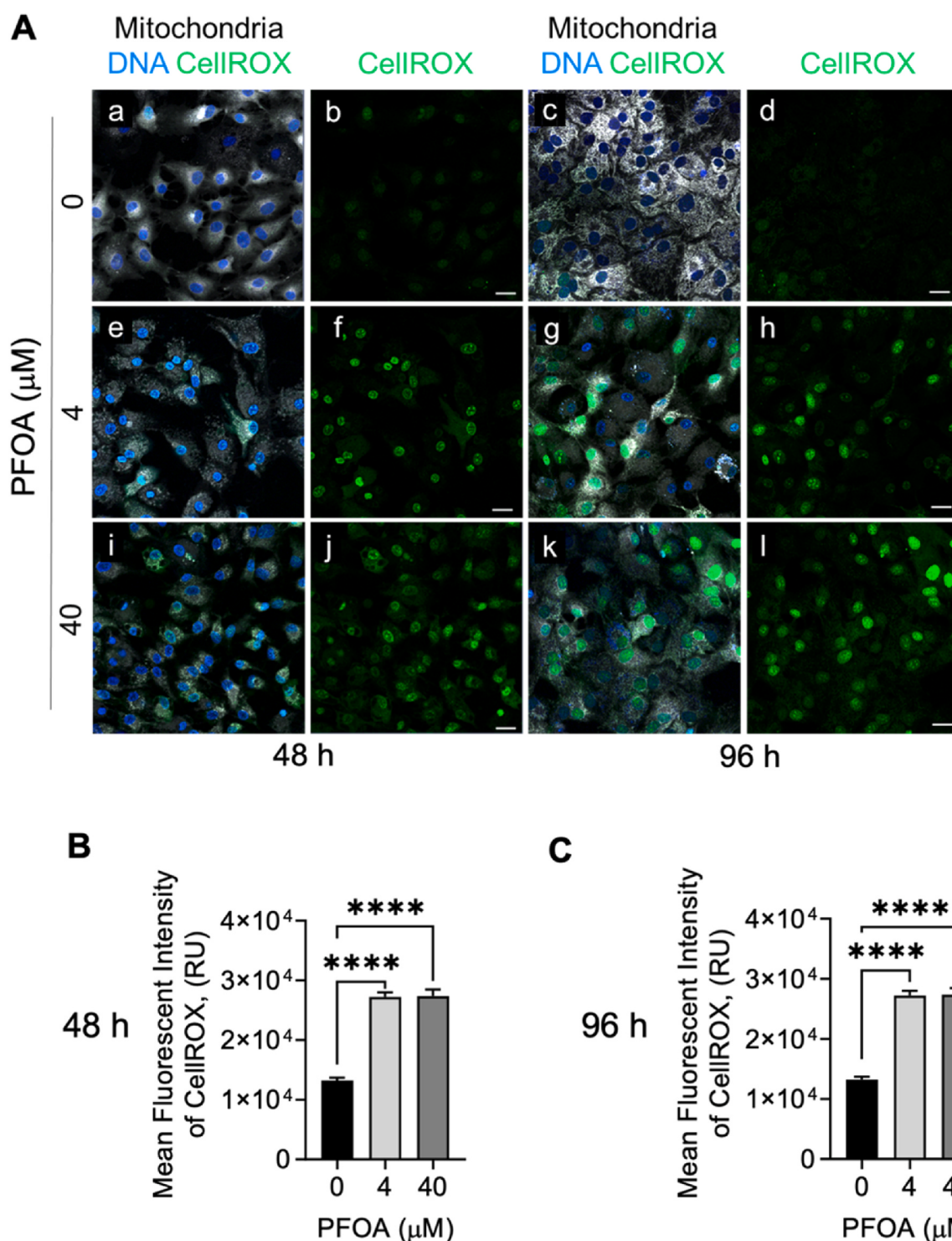


Fig. 8. Treatment with PFOA stimulates the production of reactive oxygen species (ROS) in bovine granulosa cells. Bovine granulosa cells were treated with increasing concentrations of PFOA (0, 4, or 40 μM) for 48 h and 96 h and subject to confocal microscopy. (**Panel A**) Representative micrographs showing the effects of PFOA on ROS production as measured by CellIROX. From left to right; Merge of Mitochondria, DNA, and CellIROX (48 h; panels a, e, and i), CellIROX (48 h; panels b, f, and j), Merge of Mitochondria, DNA, and CellIROX (96 h; panels c, g, and k), and CellIROX (96 h; panels d, h, and l). (**Panels B, C**) Quantitative analyses of the mean fluorescence intensity of CellIROX following 48 h exposure to PFOA (**Panel B**) and following 96 h exposure to PFOA (**Panel C**). Micron bar represents 20 μm. Results are means ± SEM, n = 3. ****P < 0.0001.

5. Conclusion

Due to its persistence in the environment, PFOA presents an increasing concern in terms of endocrine disruptive effects in humans and animals. In the present study, we aimed to elucidate the impact of

PFOA on bovine granulosa cell function. We demonstrated that PFOA significantly inhibited estradiol and progesterone secretion of bovine granulosa cells, as well as altered expression of key steroidogenic enzymes. According to our findings on cell viability and cycle analysis, these inhibitions were not related to reduced proliferation or increased

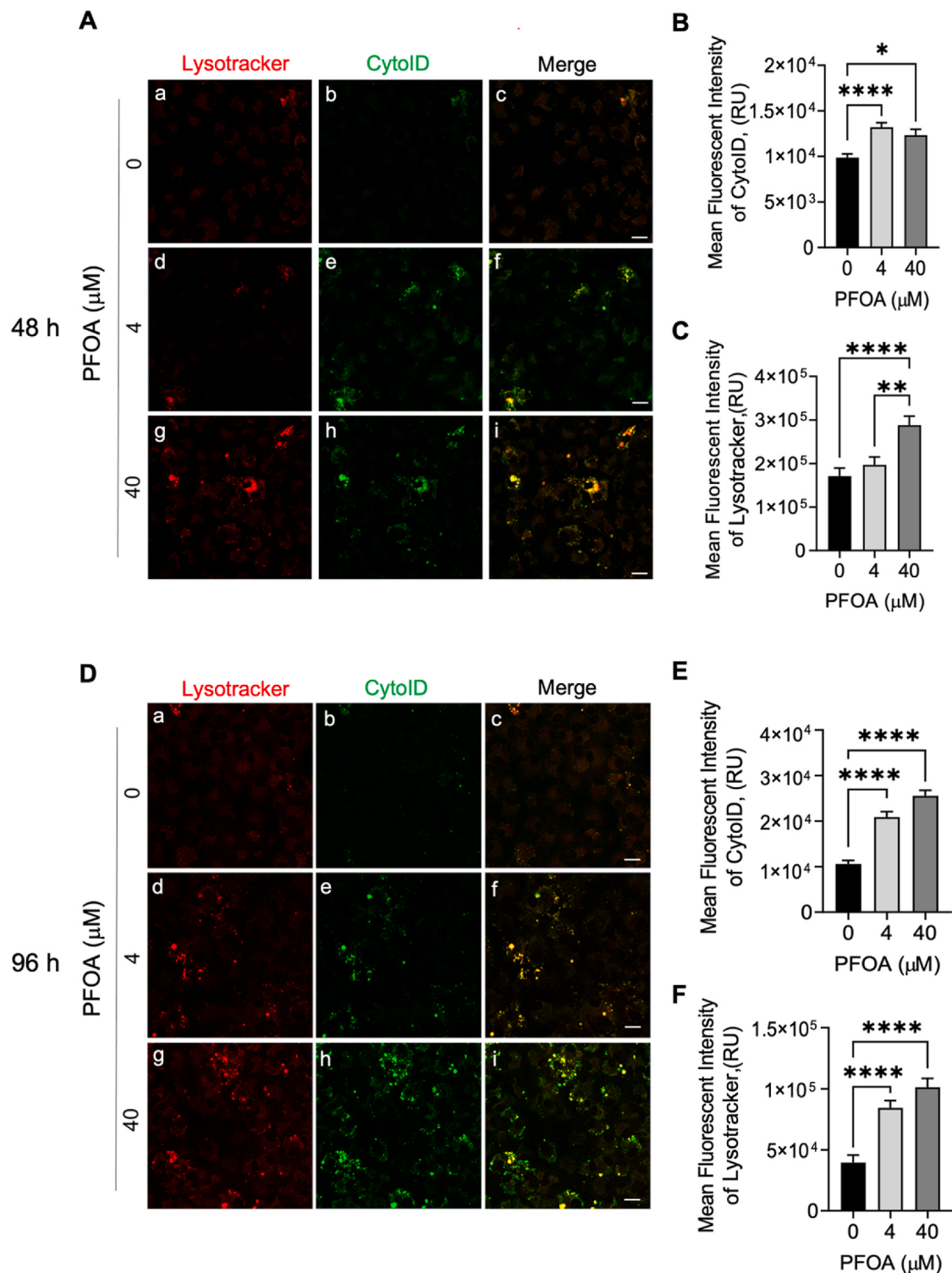


Fig. 9. Treatment with PFOA stimulates the activation of autophagy and accumulation of lysosomes in bovine granulosa cells. Bovine granulosa cells were treated with increasing concentrations of PFOA (0, 4, or 40 μ M) for 48 h and 96 h and subject to live-cell imaging using a confocal microscope. **(Panel A)** Representative micrographs showing the effects of PFOA on activation of autophagy (CytoID, green) and lysosomal accumulation (Lysotracker, red) following 48 h exposure. From left to right; Lysotracker (panels a, d, and g), CytoID (panels b, e, and h), Merge of Lysotracker with CytoID (panels c, f, and i). **(Panel B)** Quantitative analyses of the mean fluorescence intensity of CytoID following 48 h exposure to PFOA. **(Panel C)** Quantitative analyses of the mean fluorescence intensity of Lysotracker following 48 h exposure to PFOA. **(Panel D)** Representative micrographs showing the effects of PFOA on activation of autophagy (CytoID green reagent) and lysosomal (Lysotracker) accumulation following 96 h exposure. From left to right; Lysotracker (panels a, d, and g), CytoID (panels b, e, and h), Merge of Lysotracker with CytoID (panels c, f, and i). **(Panel E)** Quantitative analyses of the mean fluorescence intensity of CytoID following 96 h exposure to PFOA. **(Panel F)** Quantitative analyses of the mean fluorescence intensity of Lysotracker following 96 h exposure to PFOA. Micron bar represents 20 μ m. Results are means $\pm$ SEM, $n = 3$. * $P < 0.05$, **** $P < 0.0001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

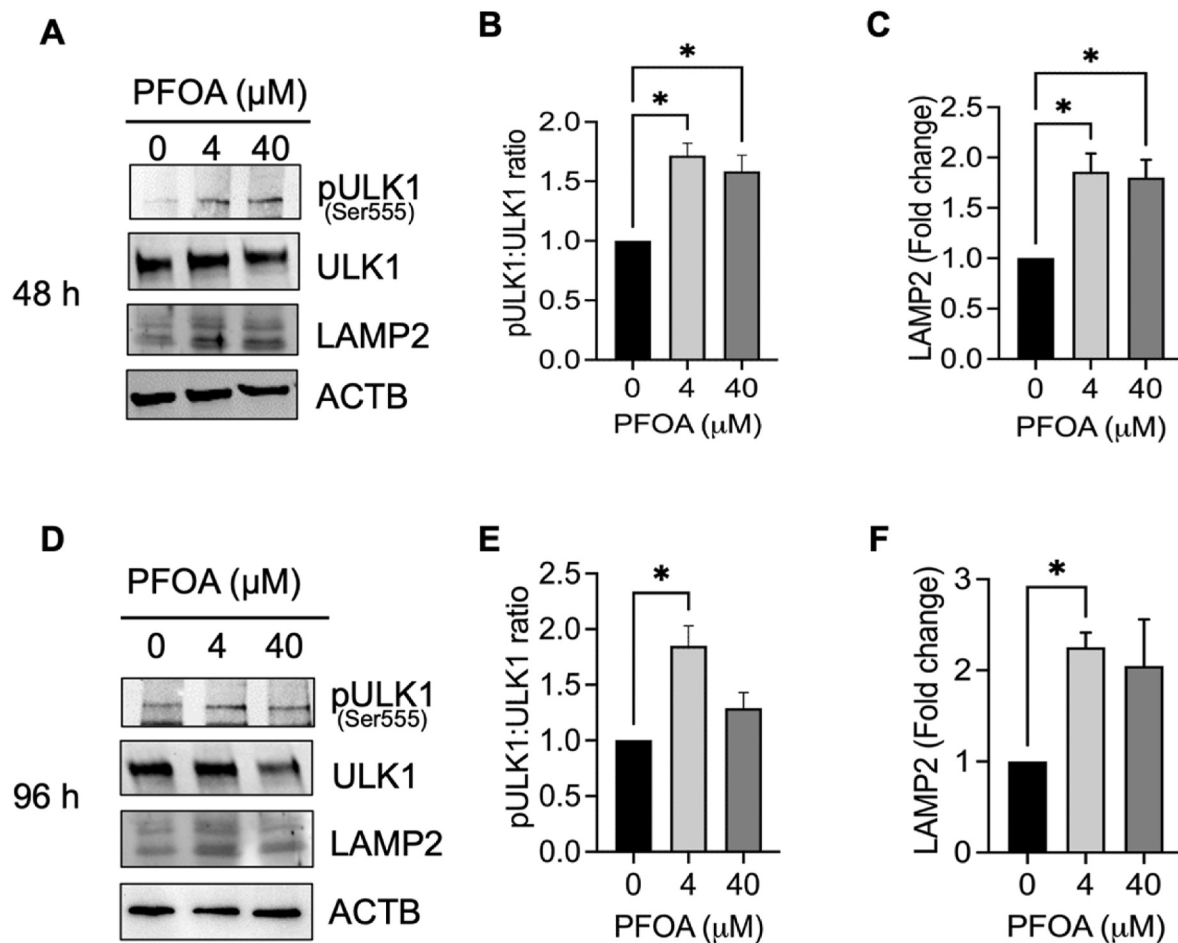


Fig. 10. PFOA triggers the expression of proteins involved in autophagy initiation. Primary cultures of bovine granulosa cells were incubated for 48 h or 96 h with increasing concentration of PFOA (0, 4 and 40 μ M). Protein lysates were collected and subject to Western blotting. (**Panel A**) Representative Western blot of proteins associated with autophagy initiation following 48 h exposure to PFOA. Quantification of phospho-ULK1 (Ser555) and total ULK1 ratio (**Panel B**) and LAMP2 (**Panel C**) protein expression. (**Panel D**) Representative Western blot of proteins associated with autophagy initiation following 96 h exposure to PFOA. Quantification of phospho-ULK1 (Ser555) and total ULK1 ratio (**Panel E**) and LAMP2 (**Panel F**) protein expression. Results are average relative fold-change means $\pm$ SEM, $n = 3$. # $P < 0.1$; * $P < 0.05$; ** $P < 0.01$.

cell death but may be a result from reduced mitochondrial activity. Lastly, our study provides evidence that PFOA induces mitochondrial dysfunction, autophagy and plays a potential role in the inhibition of granulosa cell steroidogenesis (Fig. 11). The study identifies increased ROS levels in response to PFOA exposure as a potential contributor to the adverse actions of PFOA on ovarian function in an important domestic farm animal. However, the detailed mechanistic pathways linking PFOA exposure, mitochondrial damage, autophagy, and steroidogenesis alterations are not fully elucidated. Further investigations are needed to establish causal relationships and identify intermediate steps in these processes.

While our findings shed light on the effects of PFOA on granulosa cells, applying these findings to the broader landscape of reproductive health requires careful consideration. Our investigation centered on PFOA's influence on granulosa cells within an *in vitro* context, which may not fully recapitulate the complex *in vivo* environment. Variations in species-specific physiology and metabolism, along with potential differences in PFOA metabolism and responsiveness, may limit the direct translation of these results. Moreover, the chosen PFOA concentrations (0, 4, and 40 μ M) and exposure durations (48 and 96 h) were based on experimental constraints, including cell viability within the *in vitro* culture system, but may not precisely reflect the actual exposure levels and durations that livestock encounter in their natural environment. Collectively, these findings add to a growing body of evidence that

PFOA may negatively impact human and animal reproductive health.

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CRediT authorship contribution statement

Michele R. Plewes: Conceptualization, Methodology, Investigation,

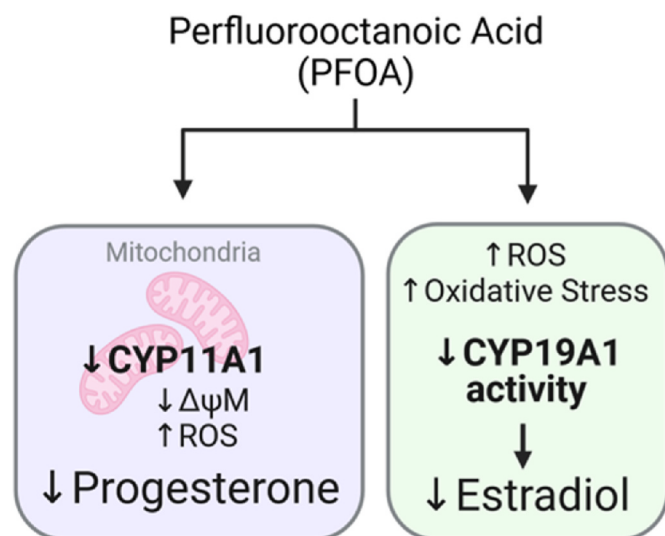


Fig. 11. Schematic model of how PFOA may inhibit steroid hormone synthesis in bovine granulosa cells. PFOA increases cellular levels of reactive oxygen species (ROS) and decreases mitochondrial membrane potential ($\Delta\psi M$), potentially impacting the enzymatic activity of the cholesterol side chain cleavage enzyme CYP11A1 and the aromatase enzyme CYP19A1. These changes result in reductions in progesterone and estradiol synthesis, respectively.

Writing – original draft, Visualization, Funding acquisition. **Corrine F. Monaco:** Methodology. **John S. Davis:** Conceptualization, Supervision, Funding acquisition, Writing – review & editing. **Ruhi Kabacki:** Conceptualization, Methodology, Investigation, Writing – original draft, Visualization. **Kendra L. Clark:** Conceptualization, Methodology, Investigation, Writing – original draft, Visualization.

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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envpol.2023.122698>.

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