Reproductive Toxicology 104 (2021) 114–124

Contents lists available at ScienceDirect

Reproductive Toxicology

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

A mixture of persistent organic pollutants detected in human follicular fluid increases progesterone secretion and mitochondrial activity in human granulosa HGrC1 cells

Kinga Krawczyk [a], Weronika Marynowicz [a], Justyna Gogola-Mruk [a], Klaudia Jakubowska [a], Wacław Tworzydło [b], Małgorzata Opydo-Chanek [c], Anna Ptak [a,*]

[a] Laboratory of Physiology and Toxicology of Reproduction, Jagiellonian University, Gronostajowa 9, 30-387 Krakow, Poland
[b] Department of Developmental Biology and Invertebrate Morphology, Jagiellonian University, Gronostajowa 9, 30-387 Krakow, Poland
[c] Laboratory of Experimental Hematology, Institute of Zoology and Biomedical Research, Faculty of Biology, Jagiellonian University, Gronostajowa 9, 30-387 Krakow, Poland

ARTICLE INFO

Handling Editor: Dr. Kempinas Wilma De Grava

Keywords:
Mixture of endocrine-disrupting chemicals
Granulosa cells
Steroidogenesis
Glucose uptake
Glycolysis
Mitochondrial activity

ABSTRACT

Disruption of granulosa cells (GCs), the main functional cells in the ovary, is associated with impaired female fertility. Epidemiological studies demonstrated that women have detectable levels of organic pollutants (e.g., perfluorooctanoate, perfluorooctane sulfonate, 2,2-dichlorodiphenyldichloroethylene, polychlorinated biphenyl 153, and hexachlorobenzene) in their follicular fluid (FF), and thus these compounds may directly affect the function of GCs in the ovary. Considering that humans are exposed to multiple pollutants simultaneously, we elucidated the effects of a mixture of endocrine-disrupting chemicals (EDCs) on human granulosa HGrC1 cells. The EDC mixture directly increased progesterone secretion by upregulating 3β-hydroxysteroid dehydrogenase (3βHSD) expression. Furthermore, the EDC mixture increased activity of mitochondria, which are the central sites for steroid hormone biosynthesis, and the ATP content. Unexpectedly, the EDC mixture reduced glucose transporter 4 (GLUT4) expression and perturbed glucose uptake; however, this did not affect the glycolytic rate. Moreover, inhibition of GLUT1 by STF-31 did not alter the effects of the EDC mixture on steroid secretion but decreased basal estradiol secretion. Taken together, our results demonstrate that the mixture of EDCs present in FF can alter the functions of human GCs by disrupting steroidogenesis and may thus adversely affect female reproductive health. This study highlights that the EDC mixture elicits its effects by targeting mitochondria and increases mitochondrial network formation, mitochondrial activity, and expression of 3βHSD, which is associated with the inner mitochondrial membrane.

1. Introduction

Normal ovarian steroid production and subsequent local steroid-mediated signaling are critical for maintenance of normal ovarian development and function. Disrupted steroidogenesis and/or steroid signaling disorders in the ovary can lead to profound ovarian pathologies and cause infertility in women of reproductive age. Endocrine-disrupting chemicals (EDCs) are natural or synthetic chemicals that can alter ovarian functions and thereby cause adverse health effects. Exposure to these chemicals occurs through ingestion, inhalation, and absorption. Several epidemiological studies indicated that EDCs accumulate in reproductive organs and ovarian follicular fluid (FF). The molecules identified as endocrine disruptors in human ovarian FF are highly heterogeneous and include synthetic chemicals used as industrial solvents/lubricants [polychlorinated biphenyls (PCBs)], pesticides [dichlorodiphenyldichloroethylene (p,p'-DDE) and hexachlorobenzene

Abbreviations: 2-DG, 2-deoxyglucose; 3βHSD, 3β-hydroxysteroid dehydrogenase; DMSO, dimethyl sulfoxide; ECAR, extracellular acidification rate; EDC, endocrine-disrupting chemical; FBS, fetal bovine serum; FF, follicular fluid; GC, granulosa cell; GLUT1, glucose transporter 1; GLUT4, glucose transporter 4; HCB, hexachlorobenzene; HK2, hexokinase 2; LDH, lactate dehydrogenase; OCR, oxygen consumption rate; OXPHOS, oxidative phosphorylation; p,p'-DDE, dichlorodiphenyldichloroethylene; PCB, polychlorinated biphenyl; PER, proton efflux rate; PFOA, perfluorooctanoate; PFOS, perfluorooctane sulfonate; StAR, steroidogenic acute regulatory protein.

* Corresponding author.
E-mail address: anna.ptak@uj.edu.pl (A. Ptak).

https://doi.org/10.1016/j.reprotox.2021.07.009
Received 10 May 2021; Received in revised form 28 June 2021; Accepted 21 July 2021
Available online 24 July 2021

(HCB)], and flame retardants [perfluorooctane sulfonate (PFOS) and perfluorooctanoate (PFOA)] [1,2]. The median concentrations of PFOS, PFOA, p,p'-DDE, PCB153, and HCB in FF of women undergoing assisted reproduction treatment were 7.5 ng/mL, 1.8 ng/mL, 392 pg/mL, 72 pg/mL, and 32 pg/mL, respectively [1,2]. These compounds have long half-lives, accumulate in fatty tissues, and are persistent organic pollutants. Moreover, high serum levels of PFOA, PFOS [3], PCB-153, and p,p'-DDE [4] are associated with reduced fecundability in women, whereas a high serum level of HCB is associated with infertility in women [5]. Importantly, humans are rarely exposed to a single chemical and are commonly exposed to a mixture of chemicals in the environment. Furthermore, the effects of different classes of EDCs may be additive or even synergistic.

Ovarian steroidogenesis is a multistep process that biosynthesizes steroid hormones from cholesterol. In brief, androgens are synthesized from cholesterol in theca cells and then converted into estrogens in granulosa cells (GCs). Interestingly, GCs can synthesize pregnenolone and progesterone (P4) from cholesterol by expressing steroidogenic acute regulatory protein (StAR), side-chain cleavage enzyme (CYP11A1), and 3β-hydroxysteroid dehydrogenase (3βHSD). Importantly, mitochondria are the central sites for synthesis of lipids and steroid hormones in ovarian GCs. StAR is located on the outer mitochondrial membrane, and CYP11A1 and 3βHSD form a complex on the inner mitochondrial membrane [6]. Aromatization is the final step in ovarian steroidogenesis and is catalyzed by the aromatase CYP19A1 in GCs. These findings indicate that the state and function of mitochondria are related to the function of GCs. Therefore, there is a relationship between mitochondrial dysfunction and structural disorders and ovarian function [7]. Importantly, biological cues that provide information about energy status such as glucose levels can affect steroid production [8,9]. Moreover, some data suggest that there are relationships between steroid hormone synthesis and glycolysis [10].

Therefore, we investigated whether a mixture of EDCs similar to those found in human FF affects secretion of steroid hormones by a human ovarian GC line and, if so, whether it influences expression of steroidogenic enzymes and mitochondrial structure and activity. Moreover, we hypothesized that EDC mixtures disrupt steroidogenesis by modulating glucose availability and glycolysis.

2. Materials and methods

2.1. Cell culture and stimulation

The human non-luteinized GC line HGrC1 was a gift from Dr. Ikara Iwase (Nagoya University, Japan). HGrC1 cells were derived from GCs of antral follicles with a diameter of 3–5 mm. They were immortalized using the Tet-Off-inducible lentivirus system to introduce human telomerase reverse transcriptase, as well as mutant cyclin-dependent kinase 4, and cyclin D1, among others [11]. Cells were cultured in phenol red-free DMEM (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 2 mM L-glutamine and 10 % charcoal-stripped fetal bovine serum (FBS; Biowest, Nuaillé, France). Cells were kept at 37 °C in a humidified incubator containing 95 % air and 5% CO_2. The test compounds PFOA, PFOS, HCB, p,p'-DDE, and PCB153 were obtained from Sigma-Aldrich and dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO added to the cell growth medium was < 0.1 % (v/v). HGrC1 cells were seeded in 96-well plates at a density of 8×10^3 cells/well or 48-well plates at a density of 15×10^3 cells/well and exposed to a mixture of the test compounds called 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), which was formulated based on the mean concentrations of individual EDCs in FF samples [1,2]. In addition, cells were exposed to Mix 0.1 and Mix 10, in which the concentrations of the test compounds were 10-fold lower and higher than those in Mix 1, respectively. Cells were treated for 24 h to analyze gene expression, glucose uptake, and the glycolytic rate, and for 48 h to analyze protein expression, steroid secretion, cell viability, and mitochondrial activity, and to perform staining. To investigate the involvement of glycolysis in the effects of the test compounds, cells were pre-treated with the selective glucose transporter glucose transporter 1 (GLUT1) inhibitor STF-31 (Sigma-Aldrich) at concentrations of 0.01, 0.05, 0.1, and 0.5 μM for 1.5 h and then exposed to the test compounds for 24 or 48 h.

2.2. Measurement of estradiol and P4 secretion

Secretion of P4 and 17β-estradiol (E2) was measured using enzyme-linked immunosorbent assays (DRG Instruments GmbH, Marburg, Germany and My BioSource, San Diego, CA, USA), according to the manufacturers' instructions. Cells were grown in medium containing or lacking 10 μM androstenedione (as a substrate for E2 synthesis). All samples were run in duplicate in the same assay. The sensitivity limits of the P4 and E2 assays were 0.045 ng/mL and 9.714 pg/mL, respectively. Assessment of inter- and intra-run variability yielded coefficients of variation of 8.34 % and 6.99 % for P4, respectively, and 7.29 % and 5.71 % for E2, respectively. All other tested steroids for the P4 assay (17β-estradiol, estriol, testosterone, and pregnenolone) and E2 assay (estrone, estriol, testosterone, androstenedione, and progesterone) showed < 1% cross-reactivity. Absorbance was measured using an ELx800 microplate reader (BioTek Instruments, Winooski, VT, USA) with a 450 nm filter. Data were recorded and analyzed using KC Junior software (BioTek Instruments).

2.3. Quantification of cell viability and cell numbers

Cell viability was measured using an In Vitro Toxicology Assay Kit, Lactic Dehydrogenase based (Sigma-Aldrich). Supernatants were collected after treatment for 48 h and handled according to the manufacturer's instructions. Absorbance was measured at 490 nm using a micro-ELISA plate reader (BioTek Instruments). Cell numbers were determined using a Beckman Coulter Z2 Particle Counter (Beckman Coulter, Inc., USA). HGrC1 cells were collected after treatment for 48 h and a cell suspension (500 μl) was diluted with 4.5 ml of ISOTON II (filtered electrolyte solution based on 0.9 % saline, Beckman Coulter) and immediately analyzed using a Z2 Coulter counter equipped with a 100 μm diameter orifice (Beckman Coulter). The number and volume of GCs were determined at 550–8000 fL using Z2 AccuComp software (Beckman Coulter).

2.4. Quantification of mitochondrial activity

The Alamar blue assay (Invitrogen, Paisley, UK) was performed to quantify mitochondrial activity in HGrC1 cells [12]. Conversion of resazurin to resorufin by mitochondrial enzymes was measured with a spectrofluorometer (FLx800, BioTek Instruments) using an excitation wavelength of 560 nm and an emission wavelength of 590 nm. Data were analyzed using KC Junior software (BioTek Instruments). The Cell Titer-Glo Assay (Promega, Charbonnieresles-Bains, France) was performed to quantify the amount of ATP, which indicates the presence of metabolically active cells. Cells were lysed according to the manufacturer's instructions. Luminescence was measured using a SpectraMax L luminometer (Molecular Devices, San Jose CA, USA). Staining with MitoTracker Red was performed to investigate the number and structure of mitochondria in HGrC1 cells and to quantify their activity. MitoTracker Red passively diffuses across the plasma membrane and accumulates in active mitochondria. Cultured live HGrC1 cells were incubated with 500 nM MitoTracker Deep Red (Invitrogen) for 30 min at 37 °C in the dark in serum-free medium and then the medium was replaced by fresh medium. Fluorescence was detected in live cells using an Axiocam 503 bright field/fluorescence microscope (Zeiss, Jena, Germany) with excitation and emission wavelengths of 644 and 665 nm, respectively. Additionally, JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide) staining was performed as an

indicator of the electrochemical potential of the inner mitochondrial membrane. This fluorescent dye can distinguish active and inactive mitochondria in the same cell. After entering mitochondria, its fluorescence changes from green (JC1 monomers, indicative of inactive mitochondria) to red as the mitochondrial membrane becomes polarized and aggregates of JC-1 form (indicative of active mitochondria). Cultured HGrC1 cells were incubated with 10 μg/mL JC-1 (Sigma-Aldrich) at 37 °C in serum-free medium for 10 min. Thereafter, the medium was replaced by fresh medium and fluorescence was detected using an Axiocam 503 bright field/fluorescence microscope (Zeiss). Both monomeric (excitation wavelength, 490 nm; emission wavelength, 500–550 nm) and aggregated (excitation wavelength, 555 nm; emission wavelength, 575–620 nm) forms of JC-1 were detected. The obtained images were merged using ImageJ. Predominance of red fluorescence was indicative of active mitochondria, while predominance of green fluorescence was indicative of inactive mitochondria.

2.5. Assessment of glucose uptake

Cells were treated with Mix 0.1, Mix 1, and Mix 10 for 24 h. The culture medium was removed, and cells were washed with phosphate-buffered saline and incubated with 1 mM 2-deoxyglucose (2-DG) for 10 min at room temperature. Uptake was stopped by acid detergent solution, acid was neutralized by the Neutralization Buffer, 2DG6P Detection Reagent was added for 1 h and luciferase activity was measured using a Glucose Uptake-Glo Assay (Promega) according to the manufacturer's instructions and a SpectraMax L luminometer (Molecular Devices).

2.6. Seahorse analysis of the glycolytic rate

The extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) were determined using a XFp Extracellular Flux analyzer (Seahorse Bioscience, Agilent, USA) and a XFp Glycolytic Rate Assay Kit (Seahorse Bioscience) following the manufacturer's instructions. HGrC1 cells were seeded into a XFp cell culture microplate at a density of 8000 cells/well in phenol red-free DMEM (Sigma-Aldrich) supplemented with 2 mM L-glutamine and 10 % charcoal-stripped FBS (Biowest), and treated with Mix 1 for 24 h. Before the start of the assay, the medium was removed and cells were washed with freshly prepared non-buffered Seahorse XF DMEM at pH 7.4 supplemented with 1 mM pyruvate, 2 mM glutamine, and 10 mM glucose. About 180 μl of the same XF assay media was added to cells and samples were incubated in a CO_2-free incubator for a minimum of 1 h. ECAR and OCR measurements were recorded after each port injection starting with Mix 1, followed by rotenone and antimycin D (Rot/AA) and finally 2DG. The assay plates included control blank wells containing only media supplemented with the various reagents. The values obtained for blank wells were automatically subtracted from those obtained for experimental wells using the instrument's software. The measurements were normalized by cell number. XFp assays involved sequential mixing, pausing, and measurement cycles, and were performed three times in triplicate. The data were analyzed and exported into GraphPad Prism using Seahorse Wave software (Seahorse) to obtain graphs and bar charts and to perform statistical analyses.

2.7. Gene analysis by RT-qPCR

Expression levels of GLUT1 (*SLC2A1*; Hs00892681_m1), glucose transporter 4 (GLUT4; *SLC2A4*; Hs00168966_m1), CYP19A1 (Hs00240671_m1), 3βHSD (*HSD3B1*; Hs04194787_g1), hexokinase 2 (HK2; *HK2;* Hs00606086_m1), and LDHA (*LDHA;* Hs00855332_g1) after treatment with Mix 0.1, Mix 1, or Mix 10 for 24 h were measured by real-time PCR using the TaqMan Gene Expression Assay (Applied Biosystems/ThermoFisher Scientific, Waltham, MA, USA) as described in our previous study [13]. Expression levels were normalized to that of *GAPDH* (4310884E). Relative expression was quantified using the $2^{-\Delta\Delta Ct}$ method [14].

2.8. Protein analysis by western blotting

Antibodies against aromatase (CYP19A1) (ab18995; Abcam, Cambridge, UK), 3βHSD (ab55268; Abcam), GLUT1 (ab652; Abcam), and GLUT4 (#2213S; Cell Signaling Technology, Danvers, Massachusetts, USA) were used to analyze expression of these proteins after treatment with Mix 0.1, Mix 1, Mix 10, or STF-31 (0.1 μM) for 48 h. Anti-rabbit (#7074) and anti-mouse (#7076) (Cell Signaling Technology) secondary antibodies were used. An antibody against β-actin (A5316; Sigma-Aldrich) was used as a loading control. Western blot analysis was performed as described in our previous study [13].

2.9. Statistical analysis

Numerical data are presented as the mean ± SEM of three independent experiments performed in triplicate. Statistical analysis was performed using a one-way ANOVA followed by Tukey's test or the nonparametric Student's *t*-test (GraphPad Software, La Jolla, CA, USA). The level of significance was set at $P < 0.05$.

3. Results

3.1. The EDC mixture increases P4 secretion but does not affect E2 secretion

Steroidogenesis is one of the major physiological functions of GCs. Therefore, we first analyzed whether the EDC mixture altered steroid secretion by the human GC line HGrC1. In control cultures incubated in fresh medium containing 0.1 % DMSO, the level of P4 secreted into the medium after 48 h was 13.9 ± 1.3 ng/mL. Treatment with Mix 1 and Mix 10 significantly increased the level of P4 secreted into the medium (20.6 ± 3.7 and 22.4 ± 2.8 ng/mL, respectively) (Fig. 1A, $P < 0.05$ and $P < 0.01$). Moreover, treatment with the EDC mixture dose-dependently increased gene (1.7- and 2-fold upon treatment with Mix 1 and Mix 10, respectively) and protein (1.25- and 1.64-fold upon treatment with Mix 1 and Mix 10, respectively) expression of 3βHSD (Fig. 1B–C, $P < 0.05$, $P < 0.01$, and $P < 0.001$).

CYP17, which catalyzes conversion of P4 into androstenedione, is not expressed in HGrC1 cells [11]. Therefore, we supplemented the medium with androstenedione to measure E2 secretion. In androstenedione-supplemented HGrC1 cell cultures, treatment with Mix 0.1, Mix 1, and Mix 10 did not affect the level of E2 secreted into the medium after 48 h (24.71 ± 3.3, 26.0 ± 7.7, and 23.65 ± 4.5 pg/mL, respectively) compared with the control (28.12 ± 6.4 pg/mL) (Fig. 1D). Androgens (androstenedione and testosterone) are converted into estrogens by CYP19A1. Treatment with the EDC mixture did not significantly affect gene (Fig. 1E) or protein (Fig. 1F) expression of CYP19A in androstenedione-supplemented HGrC1 cell cultures.

3.2. The EDC mixture increases mitochondrial activity

Mitochondria play critical roles in steroidogenesis and are thus likely affected by the EDC mixture. Before investigating the effects of the EDC mixture on mitochondrial function in HGrC1 cells, we first explored its cytotoxicity. Mix 0.1, Mix 1, and Mix 10 did not elicit cytotoxic effects, as assessed by measurement of lactate dehydrogenase (LDH) release into the cell culture media (Fig. 2A).

Next, we used a Beckman Coulter Z2 Particle Counter to count the numbers of HGrC1 cells independent of the metabolic state upon treatment with Mix 0.1, Mix 1, and Mix 10. None of the mixtures affected the cell number (Fig. 2B).

The ATP content was measured by the Cell Titer-Glo Assay upon treatment with Mix 0.1, Mix 1, and Mix 10. The ATP content was

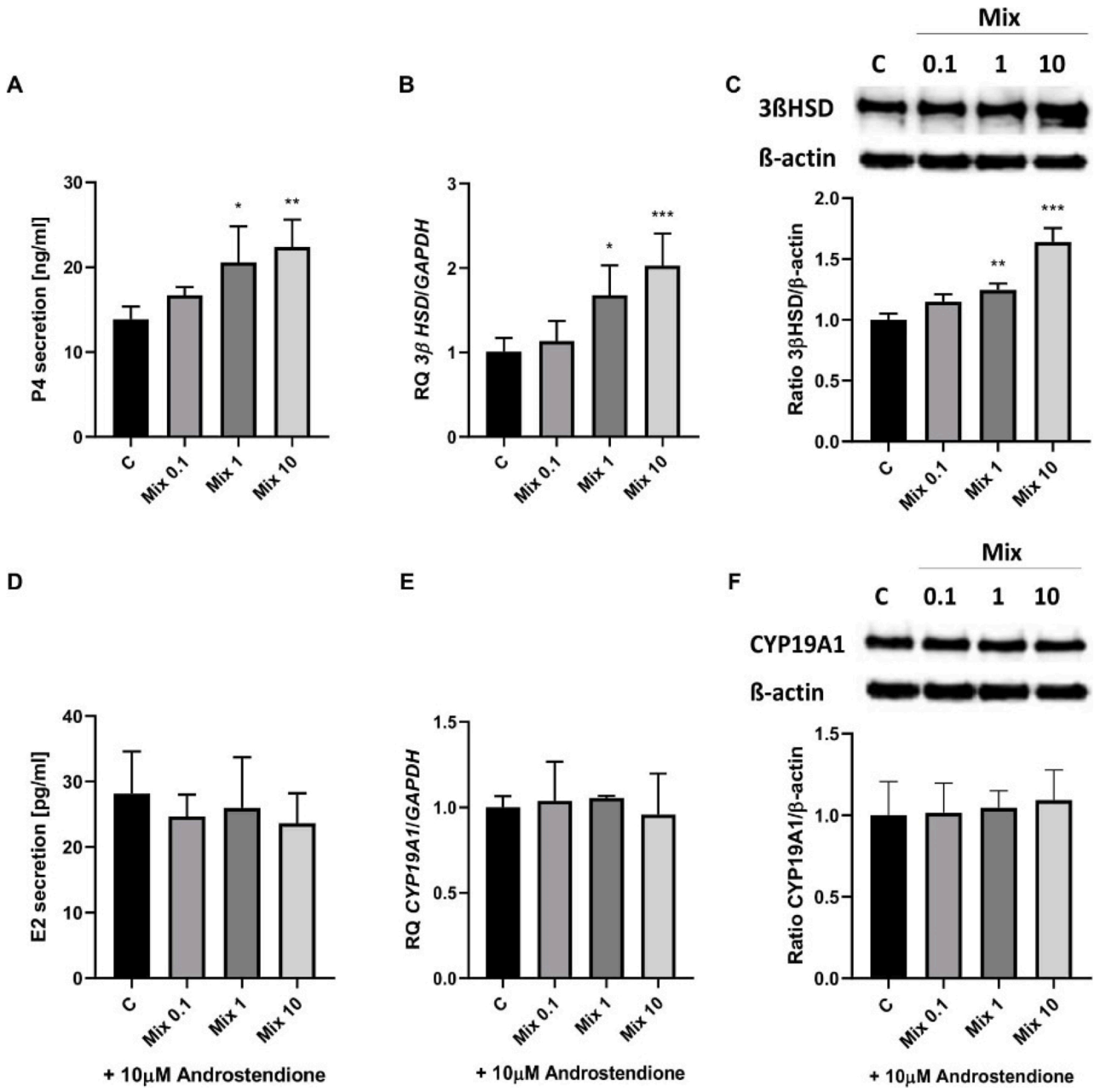

Fig. 1. Effects of the EDC mixture (Mix 0.1, Mix 1, and Mix 10) on (A) P4 secretion, (B) mRNA and (C) protein expression of 3βHSD, (D) androstenedione-stimulated E2 secretion, and (E) mRNA and (F) protein expression of CYP19A1 in HGrC1 cells after 24 h (gene expression) or 48 h (protein expression and steroid secretion). mRNA expression of *3βHSD* and *CYP19A1* in vehicle-treated cells was set to 1.0. RQ, relative quantity. Each bar represents the mean ± SEM of three independent experiments. C, control (0.01 % DMSO). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

significantly increased in HGrC1 cells treated with Mix 0.1, Mix 1, and Mix 10 (1.17-, 1.17-, and 1.28-fold, respectively) (Fig. 2C, $P < 0.05$ and $P < 0.001$).

The Alamar blue assay is commonly used to quantify cell viability, and is also a sensitive and simple indicator of mitochondrial function [12]. Treatment with Mix 0.1, Mix 1, and Mix 10 for 48 h increased mitochondrial activity (1.22-, 1.24-, and 1.35-fold, respectively) compared with control cells (Fig. 2D, $P < 0.001$).

We used the fluorescent dye MitoTracker Deep Red to visualize the effects of the EDC mixture on the number, structure, and function of mitochondria. Mix 0.1, Mix 1, and Mix 10 did not markedly affect the fluorescence intensity when samples were imaged at low magnification (Fig. 3A–D), indicating that they do not affect the number of active mitochondria. Unexpectedly, marked alterations in the mitochondrial structure were noticed when samples were imaged at higher magnification. In control cells, the vast majority of mitochondria were observed as individual, frequently elongated spots (Fig. 3A'). Treatment with the EDC mixture altered the structure of mitochondria, and they became connected and formed complicated and extensive networks (Fig. 3B'–D', arrows).

To detect changes in the electrochemical potential of the inner mitochondrial membrane, we stained control and EDC mixture-treated cells with JC-1. Inactive mitochondria with a low membrane potential emitted green fluorescence (Fig. 4A–D), while active mitochondria with a high electrochemical potential emitted red fluorescence (Fig. 4A'–D'; see Materials and Methods for further clarification). Treatment with the EDC mixture decreased green fluorescence representing inactive mitochondria with a low potential (Fig. 4A–D) and markedly increased red

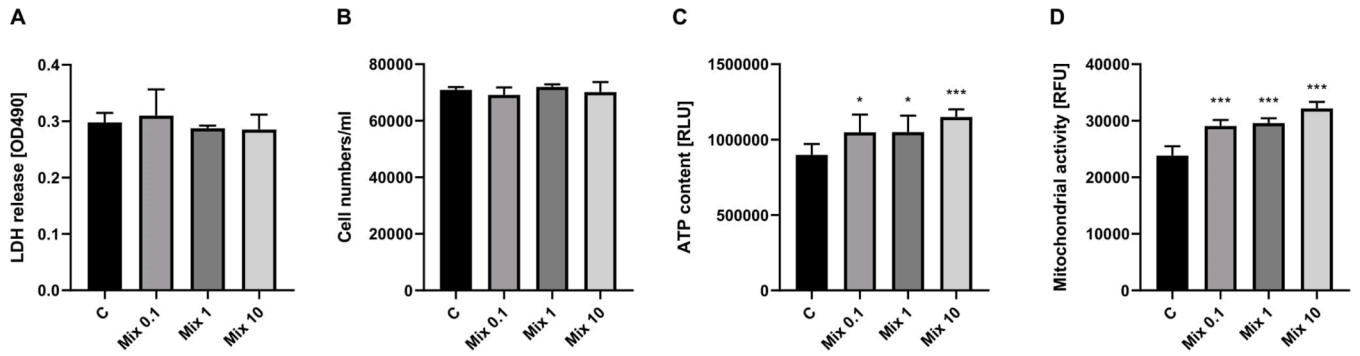

Fig. 2. Effects of the EDC mixture (Mix 0.1, Mix 1, and Mix 10) on (A) LDH release, (B) the cell number determined by a Coulter Z2 Particle Counter, (C) the ATP content determined by the Cell Titer-Glo Assay, and (D) mitochondrial activity determined by the Alamar blue assay in HGrC1 cells after 48 h. C, control (0.01 % DMSO). Data represent the mean ± SEM of three independent experiments. RFU, relative fluorescence units; RLU, relative luminescence units. *$P < 0.05$ and ***$P < 0.001$.

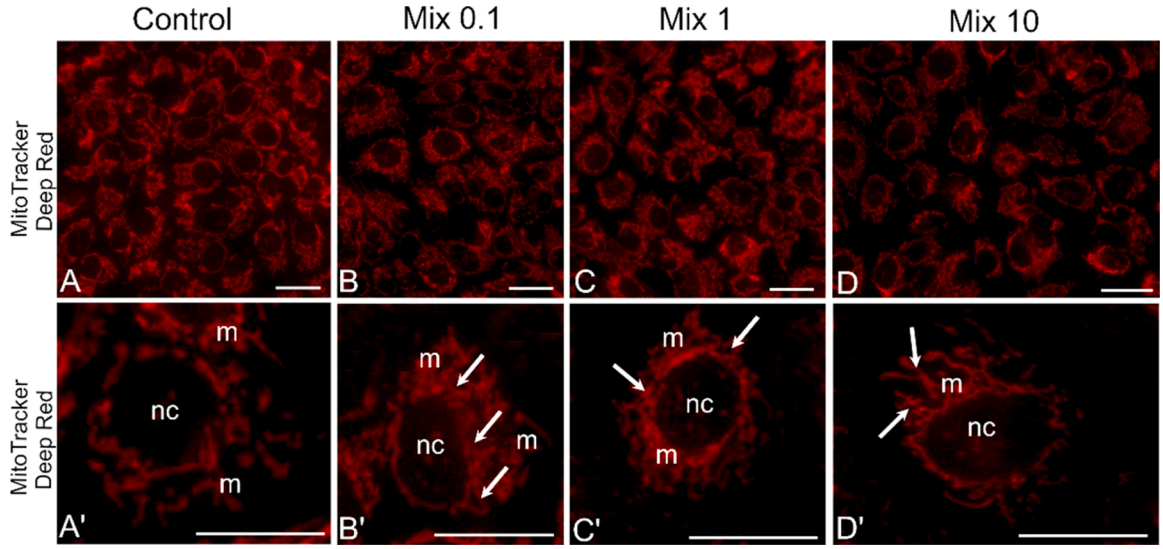

Fig. 3. Visualization of the mitochondrial structure in (A and A') control and (B–D') EDC mixture-treated HGrC1 cells. Mix 0.1, Mix 1, and Mix 10 did not affect the relative number of mitochondria, but profoundly altered the mitochondrial structure. These mixtures elongated mitochondria and induced formation of complicated mitochondrial networks (arrows) in the vicinity of the nucleus, while mitochondria were detected as individual, slightly elongated spots in control cells (A'). Nc, nucleus; m, mitochondria. Scale bar: 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

fluorescence representing active mitochondria with a higher membrane potential (Fig. 4A'–D'). In merged images (Fig. 4A"–D"), green (Fig. 4A") or red (Fig. 4D") fluorescence was predominant. These results indicate that the EDC mixture increases mitochondrial activity in a dose-dependent manner.

3.3. The EDC mixture decreases GLUT4 expression and glucose uptake

Steroid production by GCs is primarily stimulated by gonadotropins but can also be affected by biological cues that provide information about energy status. We tested whether the EDC mixture directly altered glucose availability in HGrC1 cells.

We first examined the effect of the EDC mixture on expression of glucose transporters in HGrC1 cells. None of the mixtures affected expression of GLUT1 (Fig. 5A–B), but they decreased mRNA (Fig. 5C, $P < 0.01$) and protein (Fig. 5D, $P < 0.05$ and $P<0.01$) expression of GLUT4.

Moreover, we analyzed glucose uptake upon treatment with the EDC mixture. Treatment with Mix 1 and Mix 10 significantly decreased glucose uptake by HGrC1 cells (0.96- and 0.93-fold, respectively) (Fig. 5E, $P < 0.01$ and $P < 0.001$). However, treatment with Mix 1 did not affect expression of HK2, which catalyzes the first step of glycolysis, or LDHA, which converts the glycolytic product pyruvate into lactate (Fig. 5F–G).

Next, we measured the glycolytic rate and maximum glycolytic capacity in control and Mix 1-treated cells by extracellular flux analysis. This assay involved calculation of the proton efflux rate (PER) in the presence of glucose followed by inhibition of the electron transport chain using Rot and AA, which inhibit NADH ubiquinone reductase and cytochrome c reductase, respectively. This was followed by inhibition of all glycolysis using the glucose analogue 2-DG. The PER at baseline and in response to injection of Rot/AA and 2-DG did not differ between control and Mix 1-treated HGrC1 cells (Fig. 5H), similar to basal glycolysis (Fig. 5I). However, the mitoOCR/glycoPER ratio was increased in Mix 1-treated cells, indicative of a shift from aerobic glycolysis to mitochondrial oxidative phosphorylation (OXPHOS) (Fig. 5J, $P < 0.05$).

3.4. The GLUT1 inhibitor STF-31 decreases GLUT4 expression in HGrC1 cells

To investigate whether HGrC1 cells respond to low glucose, we

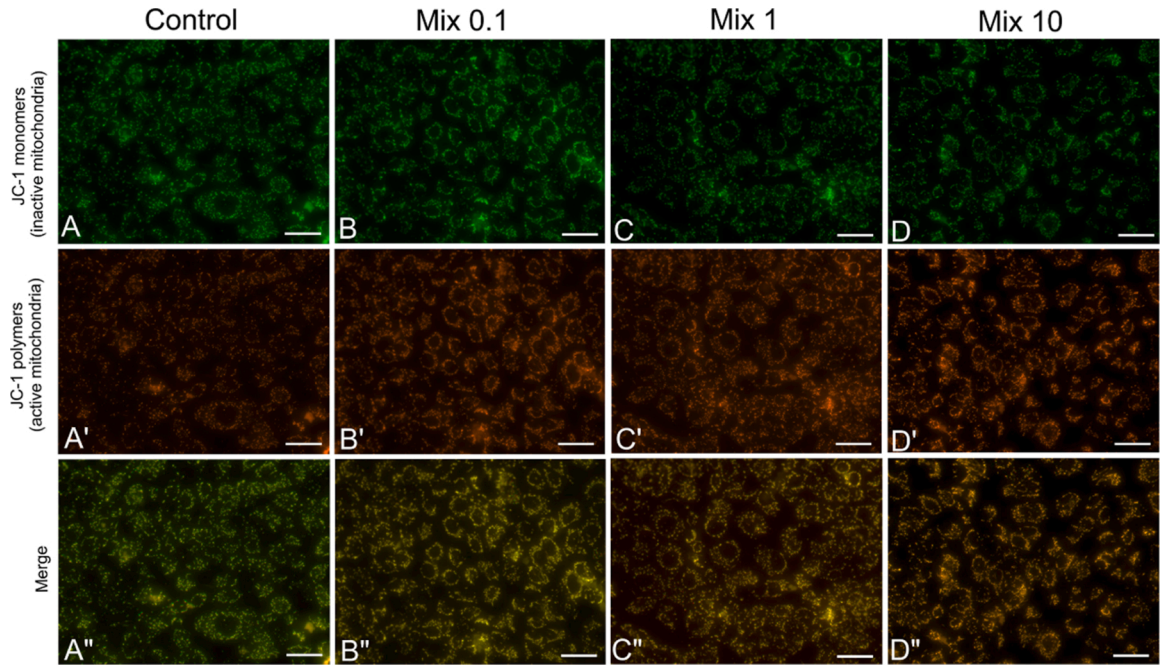

Fig. 4. Mitochondrial activity in (A, A', and A") control and (B–D") EDC mixture-treated HGrC1 cells. Treatment with Mix 0.1, Mix 1, and Mix 10 decreased green fluorescence representing inactive mitochondria with a low potential (B–D) and increased red fluorescence representing active mitochondria with a higher potential (B'–D'). In merged images (A"–D"), green (A") or red (D") fluorescence was predominant, suggesting that the EDC mixture increases mitochondrial activity in a dose-dependent manner. Scale bar: 100 μm (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

utilized STF-31, which binds directly to the glucose transporter GLUT1 and blocks glucose uptake.

To select the appropriate dose of STF-31, cells were incubated with increasing concentrations (0.01, 0.05, 0.1, and 0.5 μM) of STF-31 for 48 h. Basal mitochondrial activity of HGrC1 cells was unaffected by treatment with 0.01, 0.05, and 0.1 μM STF-31, and significantly decreased by treatment with 0.5 μM STF-31 (75 % below the control level) (Fig. 6A, $P < 0.05$). Thus, a concentration of 0.1 μM was chosen for further experiments.

Treatment with 0.1 μM STF-31 did not alter mRNA (Fig. 6B) or protein (Fig. 6C) expression of GLUT1, but significantly decreased GLUT4 expression (2-fold vs. control) (Fig. 6D, $P < 0.001$). These results were confirmed by western blot analysis (Fig. 6E, $P < 0.001$).

3.5. Inhibition of glucose uptake by STF-31 does not change basal or Mix 1-induced P4 secretion, but reduces basal E2 secretion

Next, we tested whether glucose availability directly influenced basal steroid secretion or abrogated the effects of Mix 1 on steroid secretion. We pre-treated HGrC1 cells with STF-31 (0.1 μM) for 1.5 h and then exposed them to Mix 1 for 48 h. STF-31 did not change basal or Mix-1-induced P4 secretion (Fig. 7A) or mRNA and protein expression of 3βHSD (Fig. 7C–D) in HGrC1 cells. However, STF-31 decreased basal E2 secretion (Fig. 7B, $P < 0.001$) and mRNA and protein expression of CYP19A1 (Fig. 7E–F, $P < 0.01$ and $P < 0.001$) in androstenedione-supplemented HGrC1 cell cultures.

4. Discussion

GCs, the main functional cells in the ovary, are involved in steroid secretion, are essential for follicular growth, and play a role in determining the survival of follicles. However, GCs may be affected by exogenous chemicals found in FF, including p,p'-DDE, HCB, PCB153, PFOA, and PFOS [1,2]. GCs are exposed to these compounds simultaneously; therefore, the biological effects of mixtures of these chemicals on GC steroidogenesis may be an important public health issue. Our study is the first to evaluate the effect of a mixture of EDCs that reflect the chemicals found in FF on a human GC line. The concentrations of components in Mix 1 reflect the levels of chemicals detected in human FF [1,2].

Production of P4 and E2 by GCs is essential for folliculogenesis, development of the dominant antral follicle, and ovulation [15]. Mitochondria are the central sites for steroid hormone biosynthesis. The first and rate-limiting step in biosynthesis of steroid hormones occurs in mitochondria of GCs. The 3βHSD enzyme binds to the cholesterol side-chain cleavage enzyme and thereby forms a complex that is inserted into the inner mitochondrial membrane to synthesize P4 [6]. Interestingly, our results demonstrated that the EDC mixture increased P4 secretion but did not affect E2 secretion. To gain insight into the mechanism underlying the effects of the EDC mixture on P4 secretion by GCs, we investigated steroidogenic enzyme expression. HGrC1 cells produce P4 by expressing 3βHSD [16]. The EDC mixture increased P4 secretion by upregulating 3βHSD expression in HGrC1 cells. HGrC1 cells do not express CYP17 enzymes to convert P4 into androstenedione [11]; therefore, androstenedione supplementation is required to induce estrogen production. However, the EDC mixture did not affect E2 secretion or CYP19 expression upon androstenedione supplementation. The present study is the first to report that chemicals found in human FF act as paracrine factors and disrupt steroidogenesis in human GCs. The EDC mixture altered hormones involved in the initial stages of the steroidogenic pathway and thus disrupted the balance between P4 and E2 secretion, leading to an increase in P4. Disruption of ovarian steroid secretion and thus local steroid signaling can lead to significant ovarian pathologies. The concentration of P4 in ovarian cells is increased in women with ovarian hyperstimulation syndrome [17]. Furthermore, a high local P4 level is associated with formation of ovarian follicular cysts [18] and an irregular menstrual cycle [19]. Moreover, a high level of P4 promotes development and growth of breast cancer and uterine fibroids [20,21]. Thus, our results support the hypothesis that EDCs that accumulate in human FF negatively affect female reproductive health and may lead to ovarian pathologies.

There is growing evidence that EDCs may disrupt steroid hormone

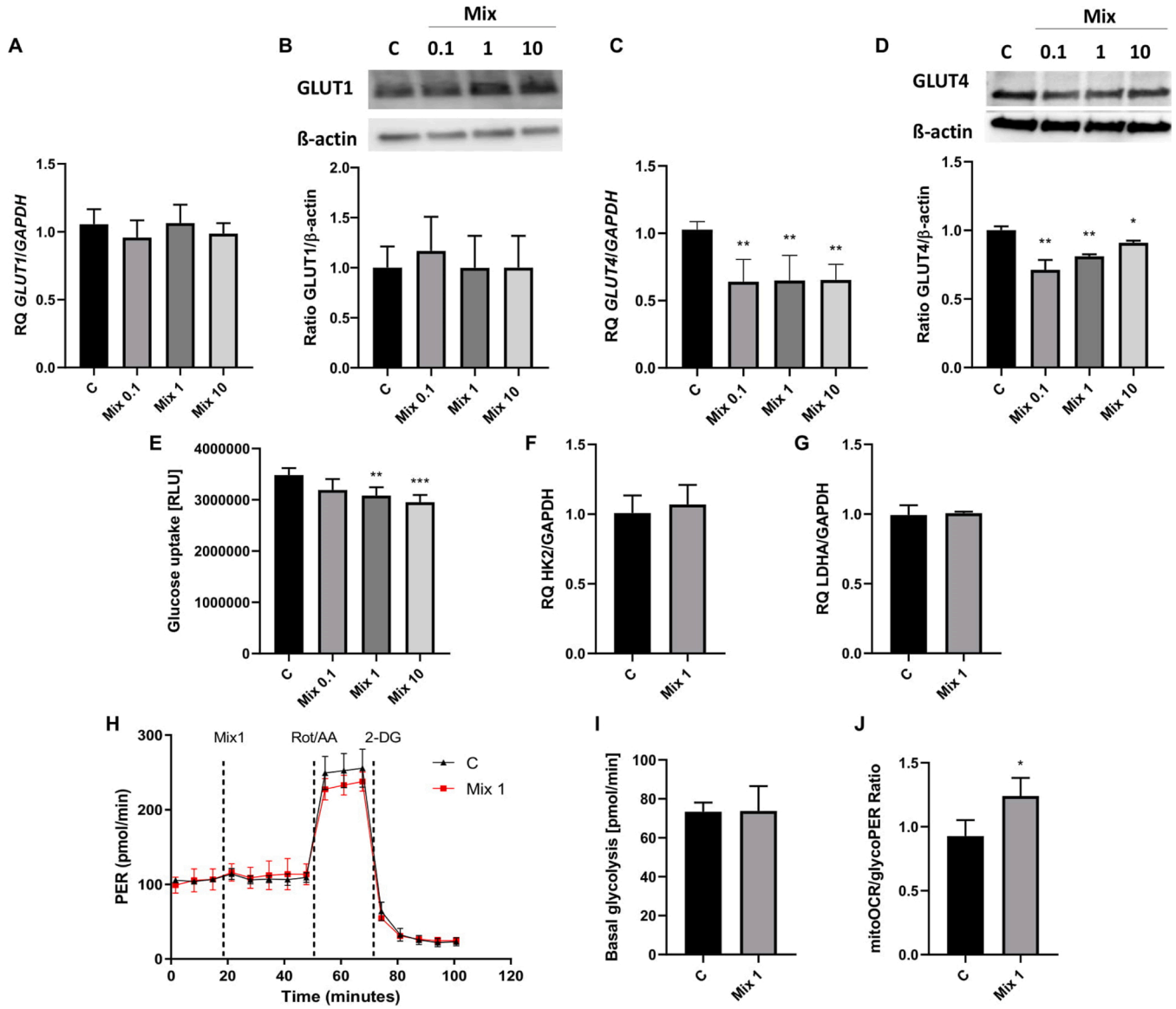

Fig. 5. Effects of the EDC mixture (Mix 0.1, Mix 1, and Mix 10) on (A) mRNA and (B) protein expression of GLUT1, (C) mRNA, and (D) protein expression of GLUT4, (E) glucose uptake, (F) mRNA expression of HK2, (G) mRNA expression of LDHA, (H) the PER, (I) basal glycolysis, and (J) the mitoOCR/glycoPER ratio in HGrC1 cells after 24 h (gene expression and the glycolytic rate) or 48 h (protein expression). mRNA expression of GLUT1 or GLUT4 in vehicle-treated cells was set to 1.0. RQ, relative quantity. Each bar represents the mean ± SEM of three independent experiments. C, control (0.01 % DMSO). RLU, relative luminescence units. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

production in the ovary when administered individually. For example, HCB reduces E2 secretion and CYP19 expression but does not affect P4 secretion [22], while PCB153 increases E2 secretion in porcine antral follicles [23]. p,p'-DDE disrupts ovarian steroidogenesis by enhancing basal and testosterone-stimulated E2 release in porcine follicles [24] and basal and follicle-stimulating hormone-stimulated aromatizing enzyme activity in human GCs [25]. Moreover, exposure to p,p-DDE stimulates P4 secretion in pig GCs [24,26], the stable pig GC line JC-410 [26], and rat granulosa-luteal cells [27]. In addition, E2 secretion by porcine ovaries is reduced by PFOA but unaffected by PFOS [28]. However, PFAS suppresses the CYP19 level as well as E2 and P4 secretion in human placental syncytiotrophoblasts [29]. Thus, in vitro studies provide important insight into the effects of individual EDCs on ovarian function; however, humans are often exposed to complex mixtures of EDCs in the environment. More studies are needed to determine the effects of EDC mixtures on ovarian function, which is central to female reproductive function and viability.

Respiratory competent mitochondria are required to facilitate steroidogenesis in GCs [30,31]. Therefore, we next analyzed the effect of the EDC mixture on mitochondrial activity. The EDC mixture increased mitochondrial activity and the ATP content in HGrC1 cells, but did not affect the cell number. Fluorescence microscopy analyses showed that the EDC mixture did not affect the number of active mitochondria, with the intensity of MitoTracker Deep Red being similar in control and experimental cells. However, the EDC mixture affected the structure of mitochondria in HGrC1 cells. Mitochondria were detected as individual, slightly elongated organelles in control cells, but were interconnected and formed extensive networks in cells treated with the EDC mixture. Formation of such mitochondrial networks is part of the continual mitochondrial fusion and fission processes termed "mitochondrial dynamics" or "mitochondrial homeostasis" [32,33]. Alterations in mitochondrial fission lead to the formation of extensive, hyperfused mitochondrial networks, whereas disruption of mitochondrial fusion maintains mitochondria as small bean-shaped, individual organelles

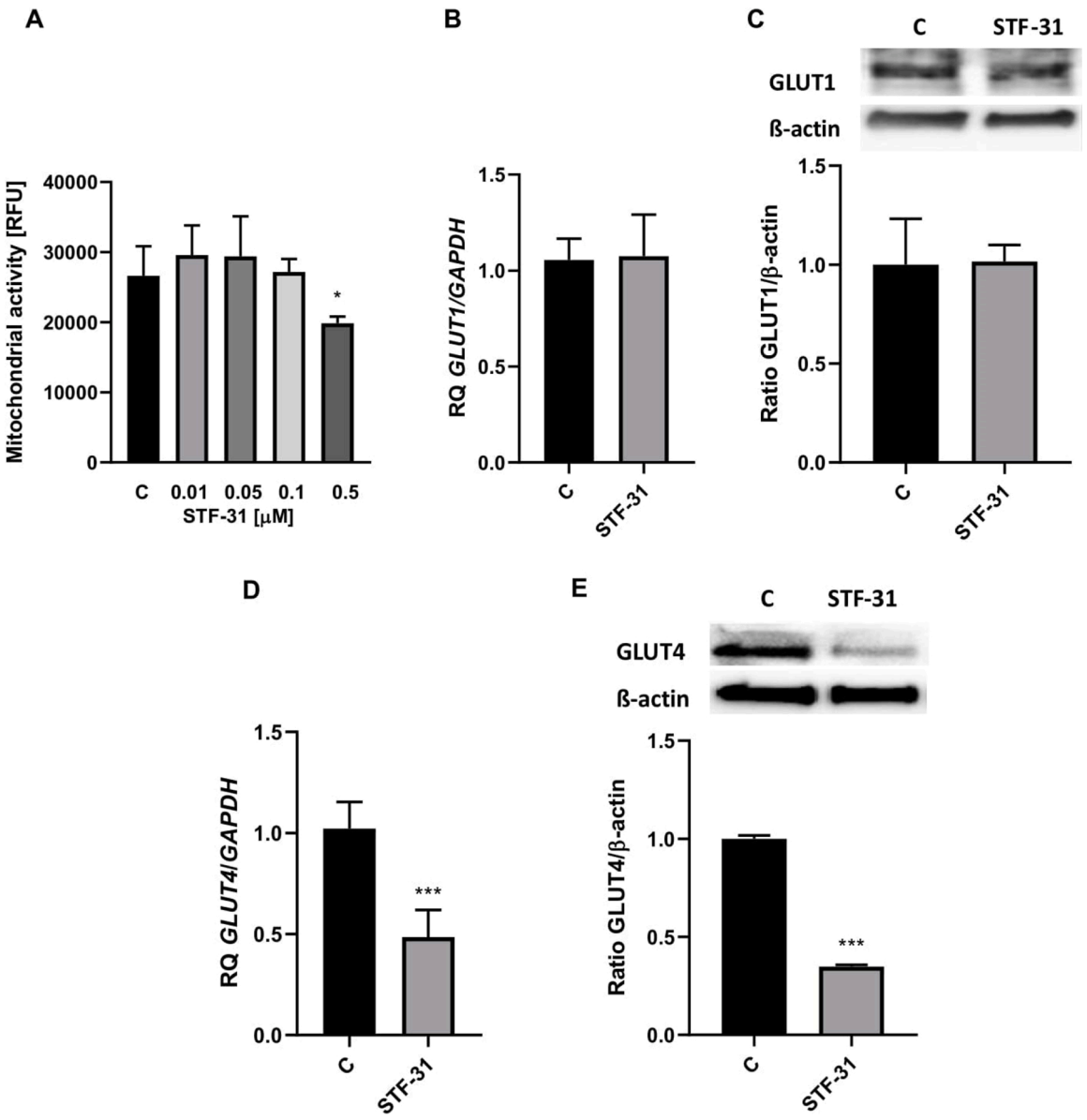

Fig. 6. Effects of STF-31 (0.01, 0.05, 0.1, and 0.5 µM) on (A) mitochondrial activity, (B) mRNA and (C) protein expression of GLUT1, and (D) mRNA and (E) protein expression of GLUT4 in HGrC1 cells after 24 h (gene expression) or 48 h (protein expression and mitochondrial activity). mRNA expression of GLUT1 and GLUT4 in vehicle-treated cells was set to 1.0. RQ, relative quantity. Each bar represents the mean ± SEM of three independent experiments. C, control (0.01 % DMSO). RFU, relative fluorescence units. $^{*}P < 0.05$ and $^{***}P < 0.001$.

[32,33]. Thus, the morphology of mitochondria in a given cell depends on the balance between these two opposed processes. Our analyses suggest that the EDC mixture disrupts the balance between mitochondrial fusion and fission, leading to excessive formation of mitochondrial networks. Active and inactive mitochondria are indistinguishable using standard techniques. Therefore, we performed JC-1 staining to detect changes in mitochondrial activity. Our results strongly indicate that the EDC mixture increased the inner mitochondrial membrane potential in a dose-dependent manner.

The observations that mitochondrial network formation and mitochondrial activity increased in parallel with 3βHSD expression support the hypothesis that mitochondria are the target site of the EDC mixture. To confirm this hypothesis, we analyzed the effect of the EDC mixture on glucose uptake. Glucose is an important energy substrate for generation of ATP for the metabolic and physiological functions of the ovary. It is a hydrophilic molecule that cannot be absorbed directly by cells. Consequently, its uptake is facilitated by members of the glucose transporter family (GLUTs) [34]. GLUT1, GLUT2, GLUT3, and GLUT4 are expressed in ovarian tissues and their expression patterns differ considerably between species and tissues [35–38]. HGrC1 cells expressed GLUT1 and GLUT4. The EDC mixture decreased GLUT4 expression but did not affect GLUT1 expression in these cells. Moreover, the EDC mixture directly reduced glucose availability in HGrC1 cells. To the best of our knowledge, the direct actions of EDC mixtures on GLUT expression and glucose uptake in human GCs have not been previously investigated *in vivo* or *in vitro*. However, some studies showed that these chemicals affect GLUT expression when administered individually. PFOA reduces GLUT4 expression in adipose tissue of mice [39], whereas PCB153 does not affect GLUT1 or GLUT4 expression in 3T3-L1 cells [40]. Taken together, these findings suggest that downregulation of GLUT4 expression is involved in the slight dysfunction of glucose uptake observed in EDC mixture-treated human GCs.

Furthermore, impairment of glucose uptake by Mix 1 did not influence the glycolytic rate. Moreover, the EDC mixture did not affect

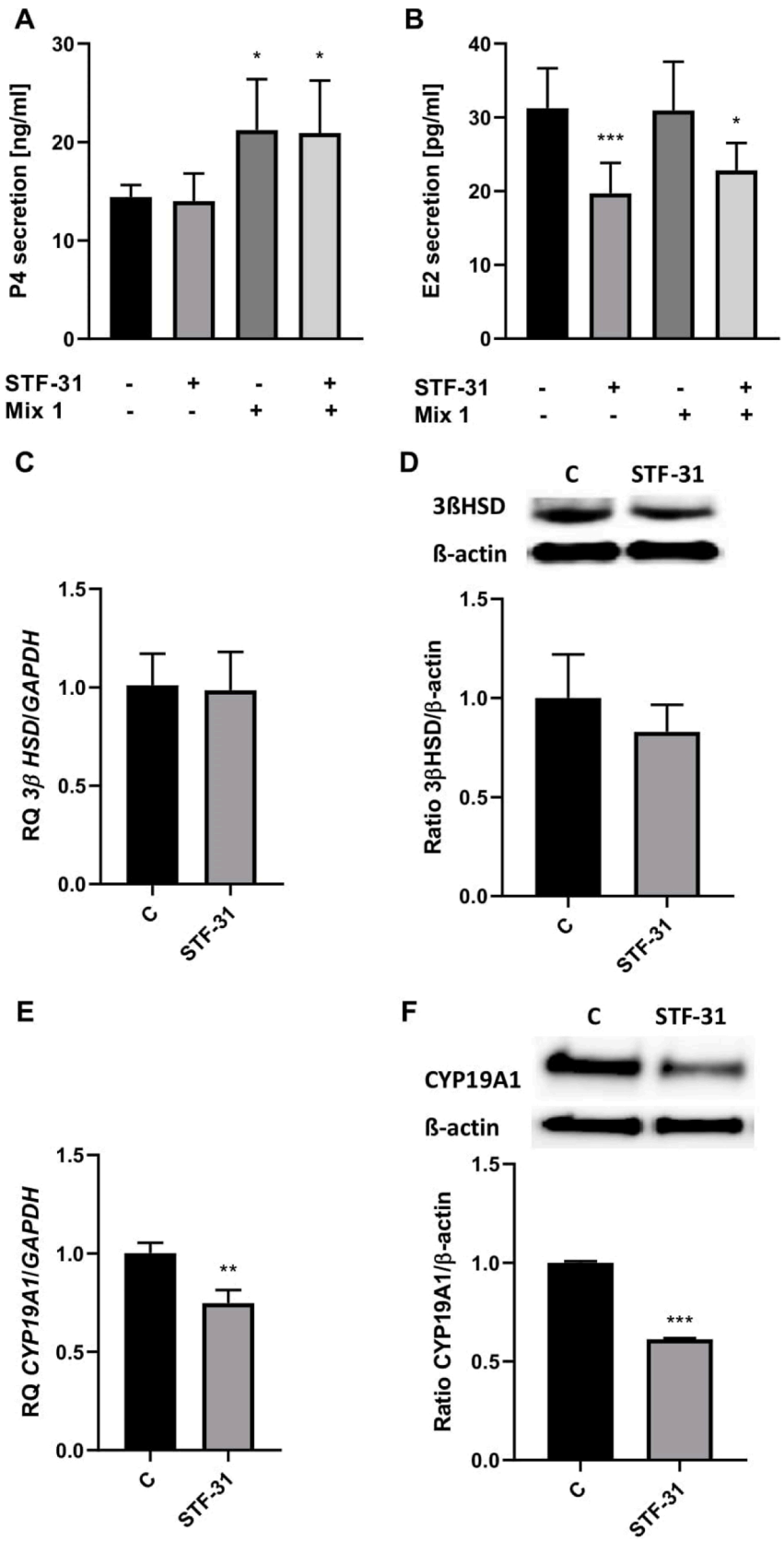

Fig. 7. Effects of Mix 1 in combination with STF-31 (0.1 μM) on (A) P4 secretion and (B) androstenedione-stimulated E2 secretion after 48 h. Effects of STF-31 on (C) mRNA and (D) protein expression of 3βHSD and (E) mRNA and (F) protein expression of CYP19A1 in HGrC1 cells after 24 h (gene expression) or 48 h (protein expression). mRNA expression of *3βHSD* and *CYP19A1* in vehicle-treated cells was set to 1.0. RQ, relative quantity. Each bar represents the mean ± SEM of three independent experiments. C, control (0.01 % DMSO). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

expression of HK2, which is involved in the first step of glycolysis, or LDHA, which converts the glycolytic product pyruvate into lactate. These findings suggest that glycolysis is not the main target of these compounds in human GCs. Our findings are partly consistent with the previous observation that genes encoding glycolytic pathway proteins are poorly expressed in mouse [41] and human [42] GCs. Interestingly, we observed a shift toward mitochondrial OXPHOS in Mix 1-treated HGrC1 cells. This observation may explain why treatment with Mix 1 increased the ATP content of these cells. Moreover, our results are partly consistent with the previous observation that ATP production in mouse GCs is not dependent on the glucose content [43]. Taken together, these findings suggest that reduced GLUT4 expression perturbs glucose uptake in EDC mixture-treated human GCs, but this effect is insufficient to influence the glycolytic rate. We cannot rule out the possibility that exposure to the EDC mixture for a longer duration may affect the glycolytic rate in HGrC1 cells.

The glucose level might influence steroid production [8,9]. Thus, we further analyzed if the change in steroid secretion is connected to decreased glucose uptake upon Mix 1 treatment. We utilized the GLUT1 inhibitor STF-31, which binds directly to GLUT1 and blocks glucose uptake. Unexpectedly, STF-31 inhibited GLUT4 expression at both the gene and protein levels in HGrC1 cells. To the best of our knowledge, there is no information regarding the effect of STF-31 on GLUT4 expression or crosstalk between GLUT1 and GLUT4 expression. However, our observation may be partly explained by the previous finding that glucose deprivation decreases the GLUT4 mRNA level in 3T3L1 adipocytes [44]. Interestingly, STF-31 decreased E2 production and CYP19A1 expression, but not P4 secretion, in basal and Mix 1-treated conditions. Our results suggest that glucose availability plays a role in steroid secretion by GCs. Most studies demonstrating the differential effects of glucose on steroidogenesis used rodent and bovine ovaries [9, 45,46]. A study of mouse ovaries indicated that P4 production is elevated in response to 2-DG (a pharmacological agent that inhibits glucose metabolism) [9], whereas GLUT1 inhibitors (cytochalasin B and STF-31) decrease P4 production in early and mid-luteal cells of bovine ovaries. Treatment with glucose (10 g/L) decreases P4 and E2 secretion as well as steroidogenic enzyme expression in rat primary GCs [46]. Overall, our results and those of others indicate that glucose homeostasis plays a role in steroid production by GCs.

Taken together, our results indicate that a mixture of persistent organic pollutants present in human FF has major detrimental effects on a human GC line. Our data provide evidence that the EDC mixture targets mitochondria because it induced formation of mitochondrial networks, increased mitochondrial activity, upregulated expression of 3βHSD, which is associated with the inner mitochondrial membrane, and disrupted steroidogenesis. These important alterations in steroidogenesis are associated with ovarian dysfunction, which negatively affects female fertility. Moreover, our data suggest that exposure to the EDC mixture induced a shift from aerobic glycolysis to OXPHOS. Further investigations are required to determine whether these endocrinological mechanisms are directly related to the effects of the EDC mixture on OXPHOS; however, this study suggests that EDC mixtures modulate mitochondrial functions.

Funding

This work was supported by grants DS/D.WB.IZiBB/6/2019 and 446.31150.24.2020 from the Faculty of Biology, Jagiellonian University, Poland.

Conflict of Interest

The authors declare no conflict of interest.

Declaration of Competing Interest

The authors report no declarations of interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.reprotox.2021.07.009.

References

[1] E.M. Petro, J.L. Leroy, A. Covaci, E. Fransen, D. De Neubourg, A.C. Dirtu, I. De Pauw, P.E. Bols, Endocrine-disrupting chemicals in human follicular fluid impair in vitro oocyte developmental competence, Hum. Reprod. 27 (2012) 1025–1033, https://doi.org/10.1093/humrep/der448.

[2] E.M.L. Petro, W. D'Hollander, A. Covaci, L. Bervoets, E. Fransen, D. De Neubourg, I. De Pauw, J.L.M.R. Leroy, E.P.A. Jorssen, P.E.J. Bols, Perfluoroalkyl acid contamination of follicular fluid and its consequence for in vitro oocyte developmental competence, Sci. Total Environ. 496 (2014) 282–288, https://doi.org/10.1016/j.scitotenv.2014.07.028.

[3] C.C. Bach, A. Vested, K.T. Jørgensen, J.P. Bonde, T.B. Henriksen, G. Toft, Perfluoroalkyl and polyfluoroalkyl substances and measures of human fertility: a systematic review, Crit. Rev. Toxicol. 46 (2016) 735–755, https://doi.org/10.1080/10408444.2016.1182117.

[4] C. Chevrier, C. Warembourg, E. Gaudreau, C. Monfort, A. Le Blanc, L. Guldner, S. Cordier, Organochlorine pesticides, polychlorinated biphenyls, seafood consumption, and time-to-pregnancy, Epidemiology 24 (2013) 251–260, https://doi.org/10.1097/EDE.0b013e31827f53ec.

[5] R.D. Björvang, C. Gennings, P.I. Lin, G. Hussein, H. Kiviranta, P. Rantakokko, P. Ruokojärvi, C.H. Lindh, P. Damdimopoulou, C.G. Bornehag, Persistent organic pollutants, pre-pregnancy use of combined oral contraceptives, age, and time-to-pregnancy in the SELMA cohort, Environ. Health 19 (2020) 67, https://doi.org/10.1186/s12940-020-00608-8.

[6] M. Prasad, J.L. Thomas, R.M. Whittal, H.S. Bose, Mitochondrial 3β-hydroxysteroid dehydrogenase enzyme activity requires reversible pH-dependent conformational change at the intermembrane space, J. Biol. Chem. 287 (2012) 9534–9546, https://doi.org/10.1074/jbc.M111.333278.

[7] L. Qi, B. Liu, X. Chen, Q. Liu, W. Li, B. Lv, X. Xu, L. Wang, Q. Zeng, J. Xue, Z. Xue, Single-cell transcriptomic analysis reveals mitochondrial dynamics in oocytes of patients with polycystic ovary syndrome, Front. Genet. 11 (2020) 396, https://doi.org/10.3389/fgene.2020.00396.

[8] N.L. McGuire, A. Koh, G.E. Bentley, The direct response of the gonads to cues of stress in a temperate songbird species is season-dependent, PeerJ 1 (2013) e139, https://doi.org/10.7717/peerj.139.

[9] K. Wilsterman, A. Pepper, G.E. Bentley, Low glucose availability stimulates progesterone production by mouse ovaries in vitro, J. Exp. Biol. 220 (2017) 4583–4588, https://doi.org/10.1242/jeb.164384.

[10] T. Baba, H. Otake, T. Sato, K. Miyabayashi, Y. Shishido, C.Y. Wang, Y. Shima, H. Kimura, M. Yagi, Y. Ishihara, S. Hino, H. Ogawa, M. Nakao, T. Yamazaki, D. Kang, Y. Ohkawa, M. Suyama, B.C. Chung, K. Morohashi, Glycolytic genes are targets of the nuclear receptor Ad4BP/SF-1, Nat. Commun. 5 (2014) 3634, https://doi.org/10.1038/ncomms4634.

[11] Bayasula, A. Iwase, T. Kiyono, S. Takikawa, M. Goto, T. Nakamura, Y. Nagatomo, T. Nakahara, T. Kotani, H. Kobayashi, M. Kondo, S. Manabe, F. Kikkawa, Establishment of a human nonluteinized granulosa cell line that transitions from the gonadotropin-independent to the gonadotropin-dependent status, Endocrinology 153 (2012) 2851–2860, https://doi.org/10.1210/en.2011-1810.

[12] J.E. Springer, R.D. Azbill, S.L. Carlson, A rapid and sensitive assay for measuring mitochondrial metabolic activity in isolated neural tissue. Brain research, Brain Res. Protoc. 2 (1998) 259–263, https://doi.org/10.1016/s1385-299x(97)00045-7.

[13] J. Gogola, M. Hoffmann, A. Ptak, Persistent endocrine-disrupting chemicals found in human follicular fluid stimulate the proliferation of granulosa tumor spheroids via GPR30 and IGF1R but not via the classic estrogen receptors, Chemosphere 217 (2019) 100–110, https://doi.org/10.1016/j.chemosphere.2018.11.018.

[14] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using realtime quantitative PCR and the 2(−Delta Delta C(T)) method, Methods 25 (2001) 402–408, https://doi.org/10.1006/meth.2001.1262.

[15] M. Jamnongjit, S.R. Hammes, Ovarian steroids: the good, the bad, and the signals that raise them, Cell Cycle 5 (2006) 1178–11803, https://doi.org/10.4161/cc.5.11.2803.

[16] O. Oktem, N. Akin, G. Bildik, K. Yakin, E. Alper, B. Balaban, B. Urman, FSH Stimulation promotes progesterone synthesis and output from human granulosa cells without luteinization, Hum. Reprod. 32 (2017) 643–652, https://doi.org/10.1093/humrep/dex010.

[17] I.Y. Järvelä, S. Pelkonen, O. Uimari, K. Mäkikallio, K. Puukka, A. Ruokonen, A. Tekay, H. Martikainen, Controlled ovarian hyperstimulation leads to high progesterone and estradiol levels during early pregnancy, Hum. Reprod. 29 (2014) 2393–2401, https://doi.org/10.1093/humrep/deu223.

[18] T.B. Hatler, S.H. Hayes, L.F. Laranja da Fonseca, W.J. Silvia, Relationship between endogenous progesterone and follicular dynamics in lactating dairy cows with ovarian follicular cysts, Biol. Reprod. 69 (2003) 218–223, https://doi.org/10.1095/biolreprod.102.012179.

[19] D. Apter, I. Räisänen, P. Ylöstalo, R. Vihko, Follicular growth in relation to serum hormonal patterns in adolescent compared with adult menstrual cycles, Fertil. Ster. 47 (1987) 82–88, https://doi.org/10.1016/S0015-0282(16)49940-1.
[20] H. Ishikawa, K. Ishi, V.A. Serna, R. Kakazu, S.E. Bulun, T. Kurita, Progesterone is essential for maintenance and growth of uterine leiomyoma, Endocrinology 151 (2010) 2433–2442, https://doi.org/10.1210/en.2009-1225.
[21] A.J. Poole, Li, Y.Y. Kim, S.J. Lin, W. Lee, E.Y. Lee, Prevention of Brca1-Mediated mammary tumorigenesis in mice by a progesterone antagonist, Science 314 (2006) 1467–1470, https://doi.org/10.1126/science.1130471.
[22] E.Ł. Gregoraszczuk, A. Ptak, A. Rak-Mardyła, J. Falandysz, Differential accumulation of HCBz and PeCBz in porcine ovarian follicles and their opposing actions on steroid secretion and CYP11, CYP17, 17β-HSD and CYP19 protein expression. A tissue culture approach, Reprod. Toxicol. 31 (2011) 494–499, https://doi.org/10.1016/j.reprotox.2011.01.006.
[23] E.L. Gregoraszczuk, M. Sowa, M. Kajta, A. Ptak, A. Wójtowicz, Effect of PCB 126 and PCB 153 on incidence of apoptosis in cultured theca and granulosa cells collected from small, medium and large preovulatory follicles, Reprod. Toxicol. 17 (2003) 465–471, https://doi.org/10.1016/s0890-6238(03)00042-x.
[24] A.K. Wójtowicz, M. Kajta, E.Ł. Gregoraszczuk, DDT- and DDE-induced disruption of ovarian steroidogenesis in prepubertal porcine ovarian follicles: a possible interaction with the main steroidogenic enzymes and estrogen receptor beta, J. Physiol. Pharmacol. 58 (4) (2007) 873–885.
[25] E.V. Younglai, A.C. Holloway, G.E. Lim, W.G. Foster, Synergistic effects between FSH and 1,1-dichloro-2,2-bis(P-chlorophenyl)ethylene (P,P'-DDE) on human granulosa cell aromatase activity, Hum. Reprod. 19 (2004) 1089–1093, https://doi.org/10.1093/humrep/deh252.
[26] N.K. Crellin, M.R. Rodway, C.L. Swan, C. Gillio-Meina, P.J. Chedrese, Dichlorodiphenyldichloroethylene potentiates the effect of protein kinase A pathway activators on progesterone synthesis in cultured porcine granulosa cells, Biol. Reprod. 61 (1999) 1099–1103, https://doi.org/10.1095/biolreprod61.4.1099.
[27] H. Nejaty, M. Lacey, S.A. Whitehead, Differing effects of endocrine-disrupting chemicals on basal and FSH-stimulated progesterone production in rat granulosa-luteal cells, Exp. Biol. Med. 226 (2001) 570–576, https://doi.org/10.1177/153537020122600610.
[28] A. Chaparro-Ortega, M. Betancourt, P. Rosas, F.G. Vázquez-Cuevas, R. Chavira, E. Bonilla, E. Casas, Y. Ducolomb, Endocrine disruptor effect of perfluorooctane sulfonic acid (PFOS) and perfluorooctanoic acid (PFOA) on porcine ovarian cell steroidogenesis, Toxicol. Vitr. 46 (2018) 86–93, https://doi.org/10.1016/j.tiv.2017.09.030.
[29] N. Zhang, W.S. Wang, W.J. Li, C. Liu, Y. Wang, K. Sun, Reduction of progesterone, estradiol and hCG secretion by perfluorooctane sulfonate via induction of apoptosis in human placental syncytiotrophoblasts, Placenta 36 (2015) 575–580, https://doi.org/10.1016/j.placenta.2015.02.008.
[30] W.L. Miller, Steroid hormone synthesis in mitochondria, Mol. Cell. Endocrinol. 379 (2013) 62–73, https://doi.org/10.1016/j.mce.2013.04.014.
[31] W.L. Miller, Disorders in the initial steps of steroid hormone synthesis, J. Steroid Biochem. Mol. Biol. 165 (2017) 18–37, https://doi.org/10.1016/j.jsbmb.2016.03.009.
[32] B. Westermann, Mitochondrial fusion and fission in cell life and death, Nat. Rev. Mol. Cell Biol. 11 (2010) 872–884, https://doi.org/10.1038/nrm3013.
[33] R.J. Youle, A.M. van der Bliek, Mitochondrial fission, fusion, and stress, Science 337 (2012) 1062–1065, https://doi.org/10.1126/science.1219855.
[34] I. Wood, P. Trayhurn, Glucose transporters (GLUT and SGLT): expanded families of sugar transport proteins, Br. J. Nutr. 89 (2003) 3–9, https://doi.org/10.1079/BJN2002763.
[35] S.A. Williams, D. Blache, G.B. Martin, R. Foot, M.A. Blackberry, R.J. Scaramuzz, Effect of nutritional supplementation on quantities of glucose transporters 1 and 4 in sheep granulosa and theca cells, Reproduction 122 (2001) 947–956, https://doi.org/10.1530/rep.0.1220947.
[36] H. Nishimoto, R. Matsutani, S. Yamamoto, T. Takahashi, K. Hayashi, A. Miyamoto, S. Hamano, M. Tetsuka, Gene expression of glucose transporter (GLUT) 1, 3 and 4 in bovine follicle and corpus luteum, J. Endocrinol. 188 (2006) 111–119, https://doi.org/10.1677/joe.1.06210.
[37] P.H. Kodaman, H.R. Behrman, Hormone-regulated and glucose-sensitive transport of dehydroascorbic acid in immature rat granulosa cells, Endocrinology 140 (1999) 3659–3665, https://doi.org/10.1210/endo.140.8.6938.
[38] J. Zhu, K. Liu, L. Pei, X. Hu, Y. Cai, J. Ding, D. Li, X. Han, J. Wu, The mechanisms of mitochondrial dysfunction and glucose intake decrease induced by Microcystin-LR in ovarian granulosa cells, Ecotoxicol. Environ. Saf. 212 (2021), 111931, https://doi.org/10.1016/j.ecoenv.2021.111931.
[39] G. Du, J. Sun, Y. Zhang, Perfluorooctanoic acid impaired glucose homeostasis through affecting adipose AKT pathway, Cytotechnology 70 (2018) 479–487, https://doi.org/10.1007/s10616-017-0164-6.
[40] P. May, P. Bremond, C. Sauzet, P. Piccerelle, F. Grimaldi, S. Champion, P. H. Villard, In vitro cocktail effects of PCB-DL (PCB118) and bulky PCB (PCB153) with BaP on adipogenesis and on expression of genes involved in the establishment of a pro-inflammatory state, Int. J. Mol. Sci. 19 (2018) 841, https://doi.org/10.3390/ijms19030841.
[41] K. Sugiura, F.L. Pendola, J.J. Eppig, Oocyte control of metabolic cooperativity between oocytes and companion granulosa cells: energy metabolism, Dev. Biol. 279 (2005) 20–30, https://doi.org/10.1016/j.ydbio.2004.11.027.
[42] D.J. Gillott, A. Eldib, E. Iammarrone, K.Y. Leung, A.R. Thornhill, J.G. Grudzinskas, Glycolytic enzyme expression in human granulosa cells, Fertil. Ster. 90 (2008) 1405–1410, https://doi.org/10.1016/j.fertnstert.2007.08.053.
[43] S. Hoque, T. Kawai, Z. Zhu, M. Shimada, Mitochondrial protein turnover is critical for granulosa cell proliferation and differentiation in Antral Follicles, J. Endocr. Soc. 3 (2019) 324–339, https://doi.org/10.1210/js.2018-00329.
[44] K.M. Tordjman, K.A. Leingang, M. Mueckler, Differential regulation of the HepG2 and adipocyte/muscle glucose transporters in 3T3L1 adipocytes. Effect of chronic glucose deprivation, Biochem. J. 271 (1990) 201–207, https://doi.org/10.1042/bj2710201.
[45] R. Nishimura, H. Hasegawa, M. Yamashita, N. Ito, Y. Okamoto, T. Takeuchi, T. Kubo, K. Iga, K. Kimura, M. Hishinuma, K. Okuda, Hypoxia increases glucose transporter 1 expression in bovine corpus luteum at the early luteal stage, J. Vet. Med. Sci. 79 (2017) 1878–1883, https://doi.org/10.1292/jvms.17-0284.
[46] C. Chabrolle, E. Jeanpierre, L. Tosca, C. Ramé, J. Dupont, Effects of high levels of glucose on the steroidogenesis and the expression of adiponectin receptors in rat ovarian cells, Reprod. Biol. Endocrinol. 6 (2008) 11, https://doi.org/10.1186/1477-7827-6-11.