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The adiponectin agonist, AdipoRon, inhibits steroidogenesis and cell proliferation in human luteinized granulosa cells

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author statement

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Joëlle Dupont Funding acquisition; Validation; review & editing

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Abstract

During obesity, excess body weight is not only associated with an increased risk of type 2-diabetes, but also several other pathological processes, such as infertility. Adipose tissue is the largest endocrine organ of the body that produces adipokines, including adiponectin. Adiponectin has been reported to control fertility through the hypothalamic–pituitary–gonadal axis, and folliculogenesis in the ovaries. In this study, we focused on a recent adiponectin-like synthetic agonist called AdipoRon, and its action in human luteinized granulosa cells.

We demonstrated that AdipoRon activated the adenosine monophosphate-activated protein kinase (AMPK) and peroxisome proliferator-activated receptor alpha (PPAR) signalling pathways in human luteinized granulosa cells. A 25 μ M AdipoRon stimulation reduced granulosa cell proliferation by inducing cell cycle arrest in G₁, associated with PTEN and p53 pathway activation. In addition, AdipoRon perturbed cell metabolism by decreasing mitochondrial activity and ATP production. In human luteinized granulosa cells, AdipoRon increased phosphodiesterase activity, leading to a drop in cyclic adenosine monophosphate (cAMP) production, aromatase expression and oestrogens secretion.

In conclusion, AdipoRon impacted folliculogenesis by altering human luteinized granulosa cell function, via steroid production and cell proliferation. This agonist may have applications for improving ovarian function in metabolic disorders or granulosa cancers.

1. Introduction

Excess fatness is associated not only with an increased risk of type 2-diabetes, but also with several pathological processes, such as atherosclerosis, inflammation, cancer or infertility (Park et al., 2011). The obese state accelerates ovarian follicular development, decreases oocyte quality, and contributes to polycystic ovary syndrome (PCOS) (Gelsomino et al., 2019).

It is accepted that adipose tissue is a fat storage organelle, but in the past 20 years, adipose tissue has been shown to secrete numerous hormones, therefore its role in the endocrine system is unequivocal. The hormones secreted by adipocytes are called adipokines, and include leptin and adiponectin (Ahima and Lazar, 2008). Adiponectin is a 30 kDa multimeric protein, comprising 244 amino acid residues (Nishida et al., 2007), and is the most abundant secretory product of white adipose tissue (Kadowaki et al., 2006). It binds two seven-transmembrane domain receptors, called AdipoR1 and AdipoR2, and activates the adenosine monophosphate-activated protein kinase (AMPK) pathway, and via AdipoR2, the peroxisome proliferator-activated receptor alpha (PPAR α) pathway (Yamauchi and Kadowaki, 2008; Okada-Iwabu et al., 2013; Wang et al., 2017).

AdipoRon, a recently developed adiponectin receptor agonist, binds and activates both AdipoR1 and AdipoR2. In mice fed a high-fat diet, AdipoRon improves insulin resistance and glucose intolerance, via AMPK activation (Okada-Iwabu et al., 2013). Similar to adiponectin, it also improves many metabolic processes, including glucose metabolism, insulin sensitivity, lipid metabolism (Choi et al., 2018), attenuates postischaemic cardiomyocyte apoptosis (Zhang et al., 2015), and tumour suppression both *in vitro* and *in vivo*, through adiponectin receptor-dependent mechanisms (Akimoto et al., 2018; Malih and Najafi, 2015).

As described, metabolic disorders that lead to alterations in fertility, impact severely on granulosa cells. Indeed, in growing mammalian follicles, the oocyte is surrounded by several layers of granulosa and thecal cells. Granulosa cells communicate to oocytes via gap junctions, and provide environmental status and various energy substrates to nurse oocytes (Eppig, 2004; Su et al., 2009). Moreover, granulosa cells produce steroids such as oestrogens, which are important sex steroid hormones.

In the last decade, several studies have shown that adiponectin signals may affect ovarian activity (Barbe et al., 2019; Chabrolle et al., 2009; Lagaly et al., 2008). Both receptors are expressed by ovarian cells (Maillard et al., 2010), and adiponectin enhances the secretion of progesterone and estradiol in the presence of follicle-stimulating hormone (FSH) or insulin growth factor-1 (IGF-1) (Chabrolle et al., 2009; Pierre et al., 2009). In mice, mutated adiponectin alters steroid production and follicular development (Cheng et al., 2016).

The adiponectin concentrations in follicular fluids (FF) have been found significantly lower in both lean and obese women with polycystic ovary syndrome when compared to non-PCOS counterparts (Artimani et al., 2016). These data raise the hypothesis of the use of this new pharmaceutical drug, AdipoRon, in the treatment of female infertility associated to metabolic perturbation. Recently, it was shown that AdipoRon exerts anti-proliferative and pro-apoptotic effects on specific human high grade epithelial ovarian cancer cell lines (Ramzan et al., 2019). However, none or little information are available about the action of this kind of drug in granulosa cells, which are the cells which communicate directly with oocyte and help in its maturation..

In this study, we investigated the role of AdipoRon on human luteinized granulosa cells (cell line and primary cells), and performed different assay to evaluate its impact on viability, cell proliferation, metabolism and in particular on steroidogenesis, which is an important role of granulosa cells to control fertility.

2. Materials and Methods

2.1. Isolation of human primary luteinized granulosa cells

Human luteinized granulosa cells were collected from pre-ovulatory follicles during oocyte retrieval for *in vitro* fertilisation (IVF). The ovarian stimulation procedures used here were previously reported by (Roche et al., 2016). Ethical approval for this study was obtained from the Research Ethics Board of the “Centre Hospitalier Régional et Universitaire” (CHRU) Bretonneau, and written informed consent was obtained from all patients. No patients received any monetary compensation for participating in the study, and all patients provided written informed consent before use of their cells. Primary human granulosa cell preparations were donated by women (36 ± 4 years) undergoing IVF. The causes of infertility were mechanical or male factor infertility, without any known endocrinopathy, such as PCOS or hyperprolactinaemia. The PCOS diagnosis was established according to the Rotterdam criteria (oligomenorrhea and/or anovulation, clinical and/or biochemical signs of hyperandrogenism, and polycystic ovaries on ultrasonography scans, and exclusion of other aetiologies that mimic the PCOS phenotype) (Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group, 2004).

The cell recovery protocol was described by (Roche et al., 2016). Cells were maintained in *in vitro* cultures in McCoy 5A medium (Sigma-Aldrich, Saint-Quentin Fallavier, France) supplemented with 20 mmol/L Hepes, penicillin (100 IU/ml), streptomycin (100 mg/ml), L-glutamine (3 mM), 0.01% bovine serum albumin (BSA), 0.1 μ mol/L androstenedione, 5 mg/L transferrin, 20 μ g/L selenium, and 5% foetal calf serum (FCS).

2.2. Cell culture

The KGN cell line is a human cell line, derived from an ovarian carcinoma of granulosa cells, from a 63-year-old Japanese menopausal woman in 1994 (Nishi et al., 2001). KGN has preserved some differentiation criteria, such as the presence of the Follicle Stimulating Hormone (FSH) receptor, sensitivity to FSH, and secretion of steroids. A mutant KGN cell line expressing DsRed was created after infection with a lentivirus (pLV-tTRKRAB-red was a gift from Didier Trono (Addgene plasmid # 12250 ; <http://n2t.net/addgene:12250> ; RRID:Addgene_12250, Watertown, USA) (Wiznerowicz and Trono, 2003).

Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) F12 medium (Sigma, l'Isle d'Abeau Chesnes, France), supplemented with L-Glutamine (2 mM final), antibiotics (100 IU/ml Streptomycin/Penicillin, Sigma, Saint-Louis, USA), and (10% FCS), at 37°C in 5% CO₂. Cells were cultured at the following concentrations; 1×10^6 cells/T75 flask, 0.5×10^6 cells/T25 flask for western blots or qRT-

PCR analysis, or for 96-well plates, 5×10^3 cells/well for immunocytochemistry, and 1×10^4 cells/well for assays). The medium was changed every 48 h.

Cells were exposed to AdipoRon (Sigma Saint-Louis, USA) at two concentrations: 2.5 μ M and 25 μ M or Nutlin (5 μ M, Sigma Saint-Louis, USA) to inhibit the proliferation or a recombinant human adiponectin (1 μ g/ml, Sigma Saint-Louis, USA), or the PPAR ligand (rosiglitazone, Sigma Saint-Louis, USA). AdipoRon at 25 μ M generates significant effects on the adiponectin pathway (Fairaq et al., 2017). Cell surfaces were analysed using ImageJ software (NIH, Bethesda, Maryland, USA).

2.3 Transient transfection of granulosa cells

After 24 h culture, KGN cells were transfected with 250 ng/well of a vector expressing PPAR Response Elements (PPREs), driven by the firefly luciferase gene (PPRE-Luc) (Blanquart et al., 2002), and 25 ng renilla luciferase plasmid to normalise transfection efficiencies (Promega, Madison, USA). Transfections were performed using polyethylenimine (PEI) transfection reagent (Sigma Saint-Louis, USA) as described previously (Raymond et al., 2011). The transfection medium was removed after 3 h, and cells were incubated in fresh medium with appropriate AdipoRon concentrations for 60 h. Then, cells were lysed according to manufacturer's instructions (Luciferase assay protocol, Promega, Madison, USA), and both firefly and renilla luciferase activities were quantified on a luminometer (TD-20/20, Turner Designs, Sunnyvale, CA, USA). Each treatment combination was assayed in triplicate, from three independent cultures.

2.4. Colony formation assay

1.5×10^3 KGN cells were seeded into each well of a 6-well plate, and treated with AdipoRon (2.5 and 25 μ M). The cells were allowed to form colonies for up to two weeks, with media replaced every 48 h. Colonies were stained (1% crystal violet in 35% methanol), and colony surfaces were quantified using ImageJ software (NIH, USA).

2.5. Wound-healing assay

KGN cells were seeded at 5×10^5 cells per well into 12-well culture dishes, and were grown to confluence. Then using a sterile tip, a linear scratch was generated in the cell monolayer. The cells were washed three times with PBS to remove floating cells, and medium was changed and supplemented with 2.5 or 25 μ M AdipoRon. Three different representative fields of the initial scratch, and at three different times: 4h, 8h and 24h were recorded. The distances of cell migration were calculated by subtracting the distance between the wound edges at 4h, 8h, and 24 h from the distances measured at 0 h. The data were analysed using NIH Image J (NIH, USA). Experiments were performed three times for each sample.

2.6. Cell cycle analysis using flow cytometry

KGN cells were cultured for two days, followed by serum starvation for 6 h to synchronise cells, and then subjected to treatments for 48 h. After treatment, cells were harvested, washed in 1 x phosphate buffered saline (PBS), centrifuged and incubated in buffer that lysed cells and marked DNA containing 0.01% trisodium citrate, 1% Nonidet P-40, 16 µg/ml propidium iodide and 250 µg/ml RNase A. Cells were analysed using flow cytometry (MoFlo Astrios^{EQ}, Beckman Coulter, USA). Twenty thousand events were collected per sample, and the percentage of cells in each cell cycle phase was assessed using MultiCycle software for Windows (Phoenix, Flow System, USA).

2.7. Immunofluorescence

Analysis of Bromodeoxyuridine (BrdU) incorporation, and mitotracker intensity were performed using the High Content Screening (HCS) imaging system.

For BrdU analysis, cells were labelled for the last 24 h of culture in 10 µM BrdU (Sigma, Saint-Louis, USA). Cells were fixed in 4% Paraformaldehyde (PFA)/PBS, and BrdU positive cells were immunostained after indirect immunofluorescence, as described by (Migliorini et al., 2002). For the mitotracker, cells were incubated for 30 min at 37°C with 200 nM mitotracker Orange CM-H2TMRos (Invitrogen, Fisher Scientific, Illkirch, France).

Briefly, fixed cells were incubated in 1 x PBS/0.1 M glycine for 15 min at room temperature. This was to saturate aldehyde groups. Cells were then permeabilised in 0.1% Triton X-100 (w/v) in 1 x PBS for 15 min, and nonspecific binding sites were blocked in 2% BSA/1 x PBS for 15 min. Cells were incubated for 60 min at room temperature with an antibody against BrdU (clone Bu33, Sigma, l'Isle d'Abeau Chesnes, France) or p53 (clone 1C12, Cell Signalling Technologies) or rabbit or mouse IgG used as negative controls and purchased from Sigma. All primary antibodies were diluted at 1:100 in 1% BSA/1 x PBS. After primary antibody incubation, cells were washed three times in PBS and incubated for 45 min at room temperature with goat anti-rabbit or anti-mouse IgG Alexa Fluor® 488 antibodies (diluted at 1:500 in 1% BSA/1 x PBS, Invitrogen, Fisher Scientific, Illkirch, France). Cells were counterstained with Hoechst 33342 (Sigma), and examined using standard immunofluorescence microscopy (phalloidin staining) on the ArrayScan® VTI HCS Reader (Thermo Fisher Scientific - Cellomics, Pittsburgh, PA, USA). Analyses were performed in triplicate, on three different cultures. A minimum of 3×10^3 cells was analysed per well.

2.8 Cell viability

Cell viability was evaluated using the Cell Counting Kit-8 (CCK8, Sigma Aldrich, Saint Quentin-Fallavier, France). Cells were previously dispensed into sterile 96 wells cell culture plats to have about 4,000

cells per well. Twenty-four hours later, cells adhering to the plat were stimulated with increasing concentrations of ligands for at least 24 h. Supernatant was then removed and replaced with DMEM medium containing 100 μ L of CCK8 10%. After 3.5 h incubation at 37°C in water-saturated atmospheres with 5% CO₂, the absorbance at 450 nm was measured using a microplate reader. The optic density obtained were then related to a standard range previously defined.

2.9. Cell apoptosis

Cell apoptosis was assessed by caspase-3 cleavage activity. Caspase activity measurements were performed using the commercially available Caspase-Glo™ 3/7 assay kit (Promega, Madison, USA), according to manufacturer's instructions. Cells were seeded in a white 96-well culture plate at a density of 1×10^4 cells per well, and treated with different concentrations of AdipoRon (2.5 or 25 μ M) for 48 h. Then, a volume of Caspase-Glo® 3/7 assay reagent equal to the volume in each well was added, and incubated for 30 min in the dark to stabilise the luminescent signal. The plate was assayed on a Luminoskan Ascent microplate reader, (VWR International, France) to record luminescence.

2.10. Oxidative stress analysis

The ROS-Glo™ H₂O₂ assay kit (Promega, Madison, USA) was used to analyse oxidative stress in cells after 48 h exposure to different concentrations of AdipoRon (2.5 and 25 μ M). Assays were performed according to manufacturer's instructions. Briefly, after treatment, cells were stressed with hydrogen peroxide (H₂O₂) substrate solution for 3 h, then incubated for 20 min with ROS-Glo™ detection solution in the dark to stabilise the luminescent signal. The plate was assay on a Luminoskan Ascent microplate reader, (VWR International) to record luminescence

2.11. Metabolites

ATP and cAMP concentrations were measured using the CellTiter-Glo™ ATP assay kit and cAMP-Glo™ assay kit (Promega, Madison, USA), respectively, according to manufacturer's instructions. Cells were seeded in a white 96-well culture plates at a density of 1×10^4 cells per well, and treated with different concentrations of AdipoRon (2.5 or 25 μ M) for 48 h.

Lactate and pyruvate concentrations in the culture medium were measured using Lactate Assay Kit (Sigma Saint-Louis, USA) and Pyruvate Assay Kit (Cliniscience, Nanterre, France) respectively, according to manufacturer's instructions. (Sigma, Clinisciences, Nanterre, France). Cells were seeded in a white 96-well culture plates at a density of 1×10^4 cells per well, and treated with different concentrations of AdipoRon (2.5 or 25 μ M) for 48 h. The results of each assay were normalised per 1×10^4 cells.

2.12. Glucose uptake assay

Glucose uptake was measured using the Glucose Uptake-Glo™ assay (Promega, Madison, USA) according to manufacturer's instructions. Cells were seeded in white 96-well culture plates at a density of 1×10^4 cells per well, and treated with different concentrations of AdipoRon (2.5 or 25 μ M) for 48 h. After treatment, the medium was removed and cells were incubated in 2-deoxyglucose (2-DG) for 10 min. Cells were further incubated with 2DG6P detection reagent for 60 min in dark. 2-DG uptake was measured on the Luminoskan Ascent microplate reader, (VWR International) to record luminescence.

2.13. Phosphodiesterase assay

Cyclic nucleotide phosphodiesterase activity measurements were performed using the luminescent *PDE-Glo™* phosphodiesterase assay (Promega, Madison, USA) according to manufacturer's instructions. Experiments were performed on KGN protein extracts, incubated with increasing AdipoRon concentrations for 15 min.

2.14. Steroid analysis

Estradiol levels were determined by enzyme-linked immunosorbent assay (ELISA) kit according to manufacturer's instructions (No. 501890, Cayman Chemical Company, Ann Arbor, MI, USA). Other steroids (testosterone and dihydrotestosterone) levels were determined by liquid chromatography–mass spectrometry (LC-MS/MS), as described by Meunier *et al.* (Meunier *et al.*, 2015).

2.15. Western immunoblotting

KGN or primary granulosa cells were lysed and exposed to three repeated freeze/thaw cycles in lysis buffer. Protein concentrations in supernatants were determined using a calorimetric assay kit (DC assay kit; Uptima Interchim, Montluçon, France), and equal protein concentrations (80 μ g) were electrophoresed on SDS-PAGE under reducing conditions, and transferred to nitrocellulose membranes (Schleicher & Schuell, Ecquevilly, France), as described (Tartarin *et al.*, 2012). Membranes were incubated at 4°C overnight with the following antibodies: phospho- α AMPK (Thr172), proliferating cell nuclear antigen (PCNA), phospho-akt (ser473), Akt, PTEN, p53, Cyclin D3 (antibodies #2535, #2586, #4058, #9272, #9559, #2524, #2936, Cell Signalling Technologies), 3 β hydroxysteroid dehydrogenase (3 β HSD), p21 and p27 (antibodies #37-2, #F-5 and #F-8, Santa Cruz, CA, USA), vinculin (clone hVIN-1, Sigma), steroidogenic acute regulatory protein (StAR) and aromatase antibodies (ab58013, ab18995, Abcam, Cambridge, UK). All antibodies were used at a 1:1000 dilution, except 3 β HSD antibodies which were diluted at 1:500. Signals were detected by enhanced chemiluminescence (Amersham Pharmacia Biotech, Orsay France) and band densities were quantified using

ImageJ software (NIH, USA). Intensity signals were expressed in arbitrary units after normalisation to an internal standard (total protein or vinculin), corresponding to the average of three different experiments.

2.16. Mitochondrial DNA analysis

The relative number of mitochondrial DNA (mtDNA) copies in KGN cells was assessed using quantitative real-time PCR (qPCR). Nuclear DNA was used as a standard. For quantification, primers for mtDNA, and primers for nuclear DNA (ACTB, EEF1A1, GAPDH), were used (Supplemental Table). Each reaction was performed in duplicate and included a negative control (without DNA). Cycle threshold (Ct) values were calculated automatically, and analysed using CFX Manager™ Software (version 3.1). Relative mtDNA copy numbers were calculated using the geometric means of ACTB, EEF1A1 and GAPDH signals (Bijak et al., 2017; Vandesompele et al., 2002).

2.17. Imaging of ex-vivo organs

Leg muscle from embryonic chicks at day 15 were injected with 2×10^6 KGN-DsRed cells in PBS, in addition to 2.5 or 25 μ M AdipoRon. Muscles were cultured in inserts (porosity: 0.4 μ m) (VWR, Radnor, USA) at 37°C in 5% CO₂ for 72 h. After this period, injected muscles were analysed with a "In Vivo Imaging System" (IVIS Spectrum, Perkin Elmer, Waltham, USA), with auto exposure epi-illumination settings to record images, according to manufacturer's instructions. Living Image 4.5.2 software (Perkin Elmer) facilitated apparatus set up, data acquisition and data analysis.

2.18. Statistical analysis

The GraphPad Prism® software (Version 8, La Jolla, CA, USA) was used for all analyses. Data were tested for homogeneity of variance by Bartlett's test and for normal distribution by Shapiro-Wilk test. One-way ANOVA was performed with Tukey-Kramer multiple comparisons tests. Mann-Whitney tests were performed where variances were unequal. Values were deemed significant when *p < 0.05, **p < 0.01 and ***p < 0.001.

3. Results

3.1. Activation of AMPK and PPAR by AdipoRon in human luteinized granulosa cells

Under our experimental conditions, AdipoRon significantly increased AMPK phosphorylation in primary human granulosa cells (Figure 1A, $p < 0.01$). In KGN cells transfected with a PPRE reporter vector, AdipoRon (25 μM) significantly enhanced PPRE promoter activity at the same level as recombinant human adiponectin (1 $\mu\text{g/ml}$), or rosiglitazone after 24 h stimulation (Figure 1B, $p < 0.05$).

3.2. AdipoRon reduces human luteinized granulosa cell viability

The first phenotype observed in KGN cells with optic microscope was a modification of the cells morphology associated with reduction in cell surface of KGN cells exposed to 25 μM AdipoRon (approximately 50%, $p < 0.001$), in comparison to untreated cells (Figure 2A, $p < 0.001$). Also, KGN granulosa cell viability were assessed by CCK-8, TUNEL and Annexin V assay. A 2.5 μM AdipoRon exposure for 48 h was enough to significantly decrease cell viability (Supplemental Data 1, $p < 0.05$). The caspase 3 cleavage activity, which is a marker associated with cell death, was also analyzed and shown that activity was significantly increased at 2.5 μM and 25 μM AdipoRon concentrations in KGN (Figure 2B, $p < 0.05$) and primary granulosa cells (Figure 2B, $p < 0.01$). In addition, we observed a significantly higher oxidative status, due to elevated Reactive Oxygen Species (ROS) production in primary human luteinized granulosa cells, at both AdipoRon concentrations (Figure 2C, $p < 0.05$).

3.3. AdipoRon impacts on different cell characteristics in both cell types

AdipoRon effects on cell proliferation were evaluated by BrdU incorporation, colony formation and scratch assay. In KGN cells, AdipoRon (25 μM) reduced BrdU incorporation to approximately 48%, and increased the p53-positive cells (Figure 3A, $p < 0.01$, Supplemental Data 1). In primary human luteinized granulosa cells, cell proliferation was decreased to approximately 27% after 25 μM AdipoRon exposure, with 11% BrdU positive cells, when compared to 15% BrdU positive cells in untreated controls (Figure 3B, $p < 0.05$). In parallel, KGN cells were seeded at 1.5×10^3 cells/well in plate 6 wells and the colonization of KGN was measured after 2 weeks of treatment. The surface of KGN cell colony seeded at low density was reduced to approximately 20% for 2.5 μM ($p < 0.05$) and 40% for 25 μM ($p < 0.001$, Figure 3C).

The speed of repair process in case of a wound healing test was slower after 25 μM AdipoRon supplementation, with a speed of 95 $\mu\text{m}/24$ h after 25 μM AdipoRon exposure versus 160 $\mu\text{m}/24$ h for untreated cells (Supplemental Data 2). Similarly, in an *ex-vivo* experiment, 25 μM AdipoRon limit the volume of tumour progression induced by injection of Ds-Red KGN cells into chick embryo muscle (Supplemental Data 3). To determine how AdipoRon impacted cell proliferation, we investigated cell cycle progression by

flow cytometry. We observed a significant accumulation of KGN cells in G₁ phase upon 25µM AdipoRon treatment ($p < 0.001$, Figure 4), accompanied by decreases in cells in G₂ phase ($p < 0.001$, Figure 4). However, the number of KGN cells in S phase did not vary according to the treatments (Figure 4).

Cell cycle arrest was also associated with decreased expression (approximately 40%) of the cell cycle progression proteins, Cyclin D3 and PCNA by western blotting, (2.5 µM AdipoRon, $p < 0.05$ and 25 µM AdipoRon, $p < 0.01$, Figure 5), and an increase in expression of the CDK inhibitors, p21 and p27 (25 µM AdipoRon, Figure 5, $p < 0.05$).

Because Akt is involved in proliferation and steroid production in granulosa cells (Brandmaier et al., 2017; Vara et al., 2004), we analysed the active form of Akt and its phosphatase PTEN by immunohistochemistry and western blotting. We observed that KGN cells exhibited a decrease in Akt phosphorylation (Ser 473) ($p < 0.001$, Figure 6A), which was associated with higher levels of phosphatase PTEN after a 48 h (25 µM) AdipoRon exposure ($p < 0.001$, Figure 6B).

3.4. AdipoRon affects mitochondria activity

Because AdipoRon was developed to control different metabolic processes, and to improve insulin sensitivity (Okada-Iwabu et al., 2013), we observed increased glucose uptake in primary human luteinized granulosa cells, after AdipoRon exposure (KGN: $p = 0.06$, primary cells: $p < 0.05$, Figure 7A). In KGN cells, the analysis of mtDNA content has allowed to show a lower mtDNA content after 25µM AdipoRon exposure showing a reduced mitochondrial activity ($p < 0.01$, Figure 7B). Moreover, mitochondrial activity (represented by mitotracker intensity) was decreased in staining intensity after 25 µM AdipoRon exposure, when compared to untreated cells ($p < 0.05$, Figure 7C). Additionally, ATP production was significantly reduced ($p < 0.01$, Figure 7D). In contrast, lactate production was higher after 25 µM AdipoRon treatment, in comparison to untreated cells (KGN: $p < 0.05$, primary luteinized cells: $p < 0.01$, Figure 7E).

3.5. AdipoRon reduces steroid production

In granulosa cells, a direct consequence of dysfunctional mitochondria activity is the production of steroid hormones. To investigate steroidogenesis, we investigated StAR expression, a cholesterol transporter, and two enzymes involved in steroid production, 3β-HSD and aromatase, in human primary granulosa cells. StAR expression was not affected at the protein level by AdipoRon (Figure 8). However, a decrease in 3β-HSD and aromatase protein levels was observed (3β-HSD: $p = 0.08$, aromatase: $p < 0.001$, Figure 8) and led to a decrease in androgen precursors and estradiol secretion measured after adding dehydroepiandrosterone

(DHEA) (testosterone: $p < 0.05$, DHT: $p < 0.01$, estradiol: $p < 0.01$) (Figure 9) showing a decrease in steroid production.

Decreased enzyme expression was associated with lower cAMP levels after AdipoRon (25 μM) treatment (Supplemental Data 4A). This was due to increased phosphodiesterase activity which transformed cyclic adenosine monophosphate (cAMP) to 5' adenosine monophosphate (5'AMP) (Supplemental Data 4B). 5'AMP is an important metabolite that activates the protein kinase A (PKA) pathway and is involved in steroid enzyme expression (Gyles et al., 2001).

4. Discussion

We sought to understand the effects of the adiponectin synthetic agonist, AdipoRon on two main functions in human luteinized granulosa cells, i.e. steroidogenesis and cell proliferation, which are involved in folliculogenesis (Drummond, 2006). For the first time, we have shown that AdipoRon reduced estradiol and androgen secretion via the expression of enzymes involved in steroid production like aromatase or 3 β -HSD, in human granulosa cells. Moreover, in both our cell models, AdipoRon induced a cell cycle arrest in G₁ phase, through the Akt/PTEN pathway which is involved in granulosa cell functions (Makker et al., 2014) (Figure 10).

Human primary luteinized granulosa cells appear to be more sensitive to AdipoRon compared to KGN cells. AdipoRon 2.5 μ M is sufficient to decrease cell proliferation, ROS production and metabolic change as measured by ATP content and glucose uptake. Despite a similar phenotype described with the KGN granulosa cell line, this requires a 10 times more concentrated dose of Adiporon (25 μ M) to obtain the same results.

AdipoRon was developed to treat diabetes and obesity (Okada-Iwabu et al., 2013), by activating AdipoR1, AdipoR2 and key proteins involved in their signalling, the AMPK kinase and PPAR pathways playing an important role in cells such as cell survival and energy production. Adiponectin receptors, AMPK and PPARs are expressed in ovarian cells including granulosa cells, in different species (Chabrolle et al., 2007; Komar et al., 2001; Pierre et al., 2009; Tosca et al., 2005). In human luteinized granulosa cells, we observed that AMPK and PPAR pathways were activated by AdipoRon (2.5 and 25 μ M), suggesting that both pathways are stimulated by this drug, such as adiponectin.

AdipoRon exerts anti-proliferative and pro-apoptotic effects on different cancer cell lines (Kim et al., 2010; Ramzan et al., 2019), however these models are mainly epithelial in nature, thus they do not have the specificity of granulosa cells to switch from proliferative cells, to highly differentiated, steroid hormone secreting cells. In terms of cell proliferation, we observed similar effects from BrdU analyses, migration, and colony assay experiments even with human primary luteinized granulosa cells which are low proliferative cells. Moreover, AdipoRon administration reduced the volume occupied by human granulosa cancer cells, when injected into embryonic chick muscle tissue. By observing a strong effect on cell proliferation, we focused on a few proteins linked to the cell cycle. Consequently, for granulosa cells, AdipoRon treatment

induced reductions in Cyclin D3, p53 and the cyclin dependent kinase inhibitors, p21 and p27. In other type cancer such as breast cancer models, AdipoRon has been shown to have an effect on stopping the cell cycle by downregulating Cyclin D (Fairaq et al., 2017; Ramzan et al., 2019). This cell cycle arrest induced by p21 and p27 expression was in agreement with other studies (Gelsomino et al., 2019; Huang et al., 2018).

We demonstrated that AdipoRon increased phosphatase PTEN, which dephosphorylates and inactivates Akt kinase (Vara et al., 2004). The PI3K/Akt/PTEN pathway is a key component in granulosa mediated steroidogenesis and cell proliferation. PTEN dephosphorylates Akt and inhibits G₁ phase progression (Brandmaier et al., 2017; Furnari et al., 1997; Vara et al., 2004), by inducing the expression of p27 (Li and Sun, 1998). Interestingly, Akt phosphorylation levels were increased in mutant mice deficient for adiponectin, and its (adiponectin) overexpression reduced high levels of phosphorylated Akt (Fujishima et al., 2014). In the ovaries, mutated PTEN in granulosa cells upregulated Akt phosphorylation, leading to the development of granulosa cell tumours in 7% of their mouse model (Laguë et al., 2008). To validate this, the authors analysed PTEN and phospho-Akt in human and equine tumours and demonstrated that several granulosa cell tumours also presented abnormal nuclear or perinuclear localisation of phospho-Akt.

In primary rat granulosa cells, human recombinant adiponectin had no effect on steroidogenesis in the presence or absence of FSH (Chabrolle et al., 2007). However, the exposure of human primary granulosa cells to adiponectin decreased P450 aromatase expression and estradiol synthesis (Tao et al., 2019). The use of short hairpin RNA against adiponectin receptors has allowed the unravelling of pathways and functions of each receptor in cell viability and steroid production. Thus, in KGN cells, AdipoR1 appears to be involved in cell survival, whereas AdipoR2, through MAPK ERK1/2 activation, may be involved in the regulation of steroid production. (Pierre et al., 2009). Elevated lactate production by human granulosa cells could be a factor that decreases estradiol production, via modulation of genes involved in folliculo-luteal transition, such as aromatase which is known as the enzyme which converts testosterone to estradiol as described by (Baufeld and Vanselow, 2018). In our study, lactate was more elevated associated with a dysregulation of steroid production like aromatase, when compared to untreated cells. In addition, steroid enzyme and steroid production dysregulation can disrupt follicle development. On the other hand, several cancers are sensitive to oestrogens (breast cancers, genital tract and ovarian cancers (François et al., 2015)). Granulosa cell tumours comprise approximately 2%-5%, of ovarian cancers, but present the specificity to secrete abnormally hormones (oestrogens, inhibin and Müllerian inhibiting substances). The risk factors for developing granulosa cell tumours include nulliparity, fatness, steroids in oral contraceptives and family cancer history (Li et al.,

2018). Approximately 70% of granulosa cell tumour patients present elevated circulating estradiol due to aromatase overexpression, which is known to increase tumour growth and progression in a number of cancers cases (Fleming et al., 2010). The main therapeutic issue with granulosa cell tumours (approximately 20% of patients die from tumour consequences) is the limited success of chemotherapy, therefore surgery remains the main therapeutic approach (Ottolenghi et al., 2007). Thus, in our model, a drop in oestrogen production, associated with reductions in cell proliferation, both *in vitro* and *ex-vivo* induced by AdipoRon, may suggest a therapeutic approach for folliculogenesis, or to limit factor of invasiveness in case of cancer sensitive to steroids.

We observed increased glucose uptake by primary luteinized granulosa cells, but not KGN cells. However, we also observed decreased mitochondrial activity and increased lactate production, suggesting a switch to glycolysis. *In vitro*, it has been described that glucose consumption and lactate production rates are gradually increased with follicle development and granulosa differentiation, suggesting that AdipoRon could stimulate differentiation (Boland et al., 1993; Harris et al., 2007). However, in granulosa and cumulus cells, in the case of low ovarian reserves or in aged ovaries, lactate production is increased when compared with young women with normal ovarian reserves (Pacella et al., 2012). Granulosa cells provide nutrients for the development and maturation of follicles and oocytes (Alam and Miyano, 2020). Mitochondria in ovarian granulosa cells generate energy and provide an environment conducive to the synthesis of lipids and steroids, such as androgens and oestrogen, leading to folliculogenesis and good oocytes maturation (Chowdhury et al., 2016; Morohaku et al., 2013; Zhao et al., 2019). In our study, AdipoRon reduced mitochondrial activity probably by decreasing mitochondrial cell numbers, and mitochondrial respiratory activity, leading to a drop in ATP production and ROS accumulation (Dröse et al., 2014; Wang and Hekimi, 2015; Zhao et al., 2019).

AdipoRon, exhibits antiproliferative and steroid-lowering effects in primary human luteinized granulosa cells. AdipoRon may function by limiting oestrogen production and increasing glucose uptake sensitivity, and could be relevant for treating pathological PCOS issues, or cell proliferation in some cancers that are oestrogen-sensitive. Hence, AdipoRon which has an elevated half-life when compared to endogenous adiponectin, a large molecule with a half-life of 45 to 60 min in the mouse (Halberg et al., 2009), could be more easily adopted as a viable therapeutic strategy to stimulate granulosa cell differentiation for infertility management (Bagnjuk et al., 2019; Khan et al., 2013).

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Legends

Figure 1. AdipoRon activates AMPK and PPAR pathways. (A) Western blot analysis of phospho-AMPK-alpha (Thr172) in primary granulosa cells after 1h of AdipoRon exposure (0, 2.5, 25 μ M). Quantification of proteins was shown on the side of the western-blot. Results are representative of 3 independent experiments. (B) KGN human granulosa cells were transfected with the PPRE-Luc plasmid as described in *Materials and Methods*. After transfections, granulosa cells were stimulated for 24 h with AdipoRon (2.5, 25 μ M), positive controls were human adiponectin 1 μ g/ml and 10 μ M of rosiglitazone (n = 6 independent experiments). Luciferase activities were measured in cell lysates and values were expressed in percentage \pm SEM of control, the activity of the unstimulated control being taken as 100%. Values are the mean \pm S.E.M. *P < 0.05, **P < 0.01, ***P < 0.001, significant differences from control conditions.

Figure 2. Reduction in viability of granulosa cells after AdipoRon exposure. (A) Representative microscopic fields of KGN cells exposed or not with 25 μ M AdipoRon for 48h. Scale bar = 50 μ m. (right panel), Quantification of cell surface (μ m²) by ImageJ software (B) Quantification of the caspase-3/7 cleaved activity and (C) ROS content in KGN and primary human granulosa cells incubated with increasing concentration of AdipoRon for 48h (n = 3 independent experiments). Data are presented as mean \pm S.E.M. (n = 3 independent experiments). Values are the mean \pm S.E.M. *P < 0.05, **P < 0.01, ***P < 0.001, significant differences from control conditions.

Figure 3. AdipoRon decreased proliferation rate in granulosa cells (A) KGN cells were cultured for 48h in presence or absence of AdipoRon or nutlin (5 μ M) as a positive control. Cells were cultured in presence of 10 μ M BrdU during the last 24h of the culture. Percentage of BrdU positive cells was analysed by High Content Screening. Results are representative of 3 independent experiments in triplicate with at least 3000 cells counted per well. (B) The percentage of BrdU positive cells was analysed in primary human granulosa cells by High Content Screening. (C) For colony formation assay, cells were plated at a very low density (1.5 \times 10³ cells/well) and 2 weeks later surface occupied by foci were quantified (n = 3 independent experiments). Values are the mean \pm S.E.M. *P < 0.05, **P < 0.01, ***P < 0.001, significant differences from control conditions.

Figure 4. AdipoRon reduced KGN cell progression (A) Representative graphics of flow cytometry analysis to detect the effect of AdipoRon on G0/G1, G2/M phases of cell cycle. (B) Cells were stimulated for 48h with AdipoRon and were stained with propidium iodide. Percentage of cells in each cell cycle phases measured by flow cytometry. (n = 3 independent experiments). Values are the mean \pm S.E.M. ***P < 0.001, significant differences from control conditions.

Figure 5. AdipoRon stimulated expression of cell-cycle inhibitors. (A) Western blot analysis of PCNA, Cyclin D3, p21 and p27 in KGN cells after 48h of AdipoRon exposure. (B) Quantification of proteins. Results are representative of 3 independent experiments. Values are the mean \pm S.E.M. *P < 0.05, **P < 0.01, significant differences from control conditions.

Figure 6. AdipoRon inhibited Akt through activation of PTEN. (A) Immunostaining and quantification of the phospho-akt (ser473) and PTEN in KGN cells after 48h of AdipoRon exposure analysed by High Content Screening. Results are

representative of 3 independent experiments in triplicate with at least 3000 cells counted per well. IgG was used as negative control. Scale bar = 10 μ M. (B) Western blot analysis of phospho-akt (ser473) and PTEN in KGN granulosa cells. Results are representative of 3 independent experiments. Values are the mean \pm S.E.M. *P < 0.05, **P < 0.01, ***P < 0.001, significant differences from control conditions.

Figure 7. AdipoRon mediated alterations on cell metabolism. AdipoRon has changed cell metabolism. (A) Glucose uptake in KGN and human primary granulosa cells was quantified using the Glucose Uptake-Glo Assay after medium change and 30 min incubation with 2DG (2-DeoxyGlucose). (n = 3 independent experiments). (B) mtDNA mitochondria gene expression in KGN cells exposed to AdipoRon for 48h was analyzed by RT-qPCR (n = 6 independent experiments). (C) The mitochondrial membrane potential in KGN cells was analysed by mitotracker dye after 25 μ M AdipoRon exposure for 24h. Intensity of mitotracker was quantified by High Content Screening. (D) Concentration of ATP in granulosa cells (M) per 10 000 cells. (E) Lactate concentration was quantified in the conditioned medium of KGN and human primary granulosa cells after 48h of AdipoRon exposure per 10 000 cells. (n = 3 independent experiments). Results are representative of 4 independent experiments. Values are the mean \pm S.E.M. *p < 0.05, **p < 0.01, ***p < 0.001, significant differences from control conditions.

Figure 8. AdipoRon inhibited expression of proteins involved in steroidogenesis. StAR, 3 β -HSD and aromatase protein expression in primary granulosa cells exposed to AdipoRon for 48h were quantified and normalized to vinculin (n = 3 independent experiments). Values are the mean \pm S.E.M. **p < 0.01, ***p < 0.001.

Figure 9. Steroid production by primary human granulosa cells (A) Human primary granulosa cells were incubated for 48h with dehydroepiandrosterone (DHEA) and the following steroids were quantified by mass spectrometry: (A) testosterone (TS), (B) dihydrotestosterone (DHT). (C) Estradiol was measured by ELISA. Values are the mean \pm S.E.M. *P < 0.05, **P < 0.01, significant differences from control conditions.

Figure 10. AdipoRon activity in human granulosa cells: schematic summary of AdipoRon consequences on granulosa cell metabolism, steroid production and cell cycle. cAMP: Cyclic adenosine monophosphate, ATP: Adenosine triphosphate, AC: Adenylate cyclase, E2: estradiol, PDE: phosphodiesterase.

Figure 1

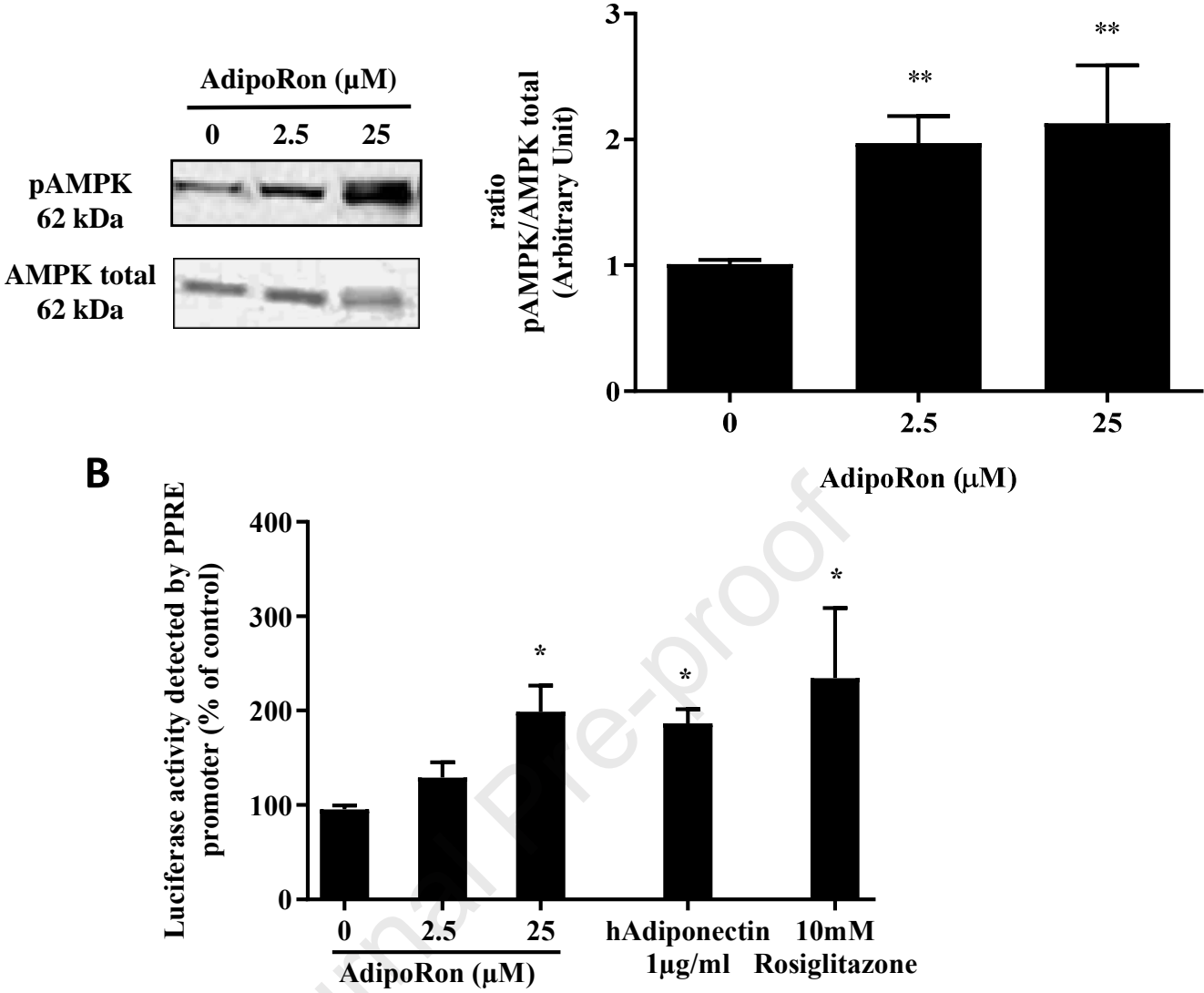


Figure 2

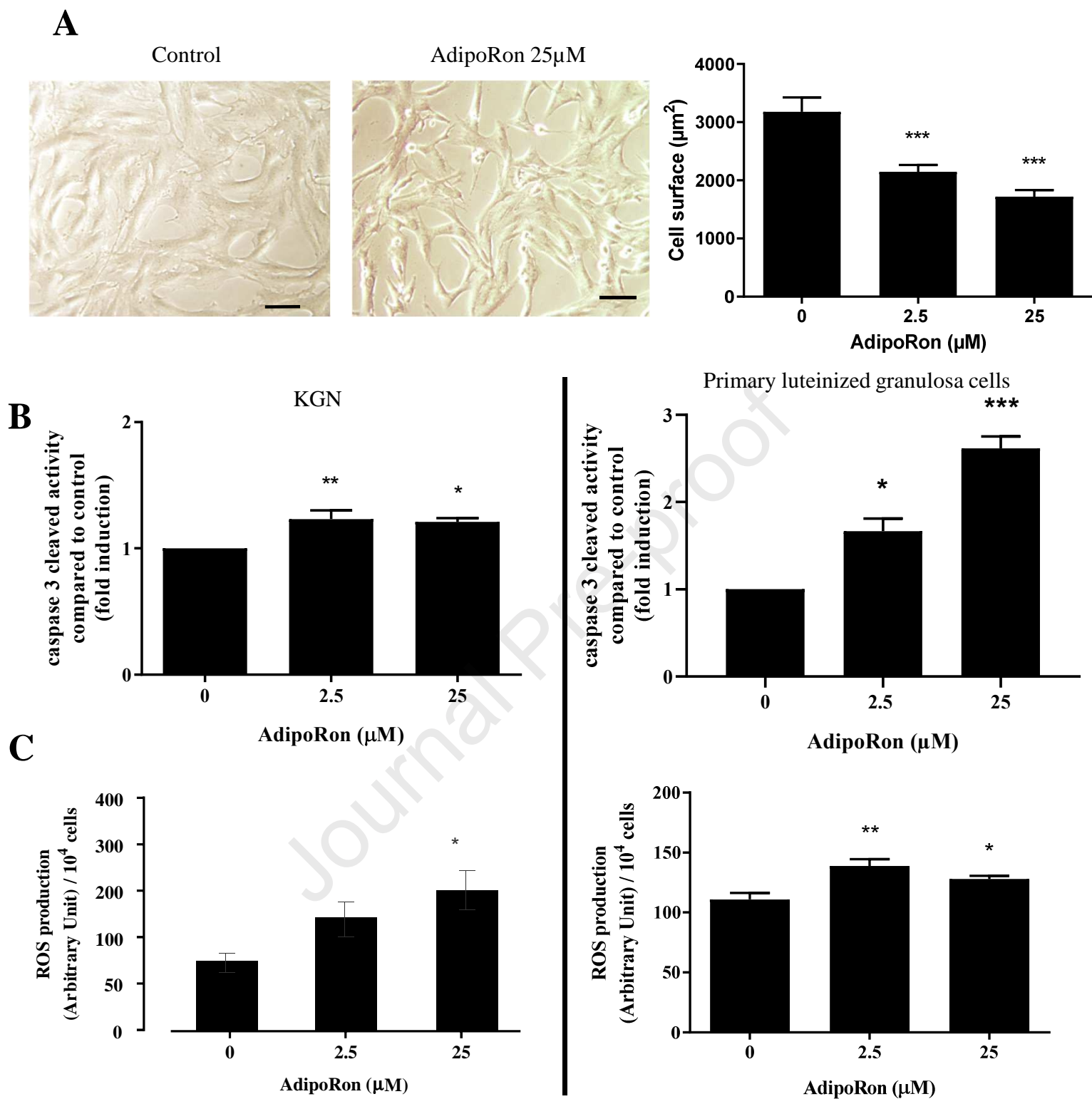


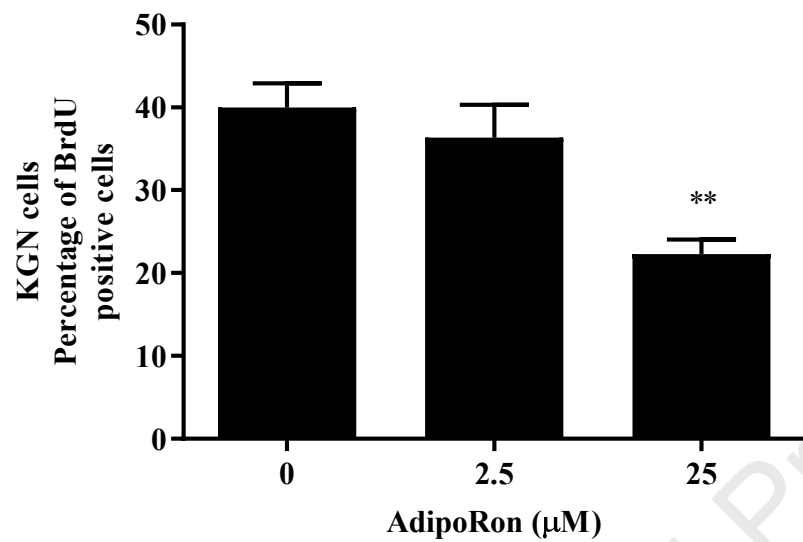
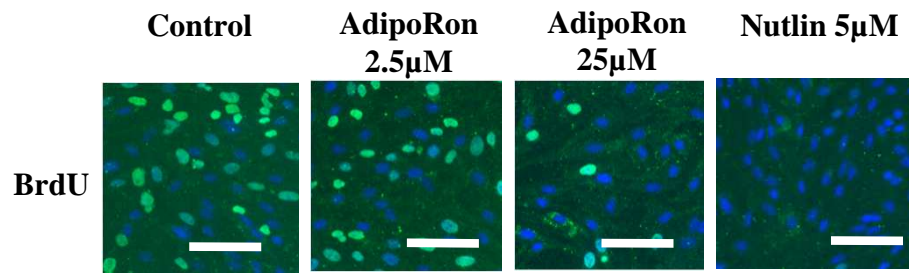
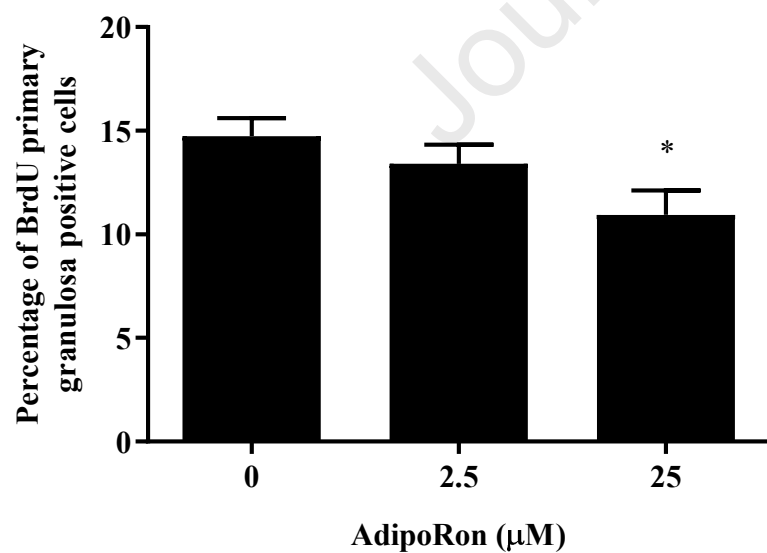
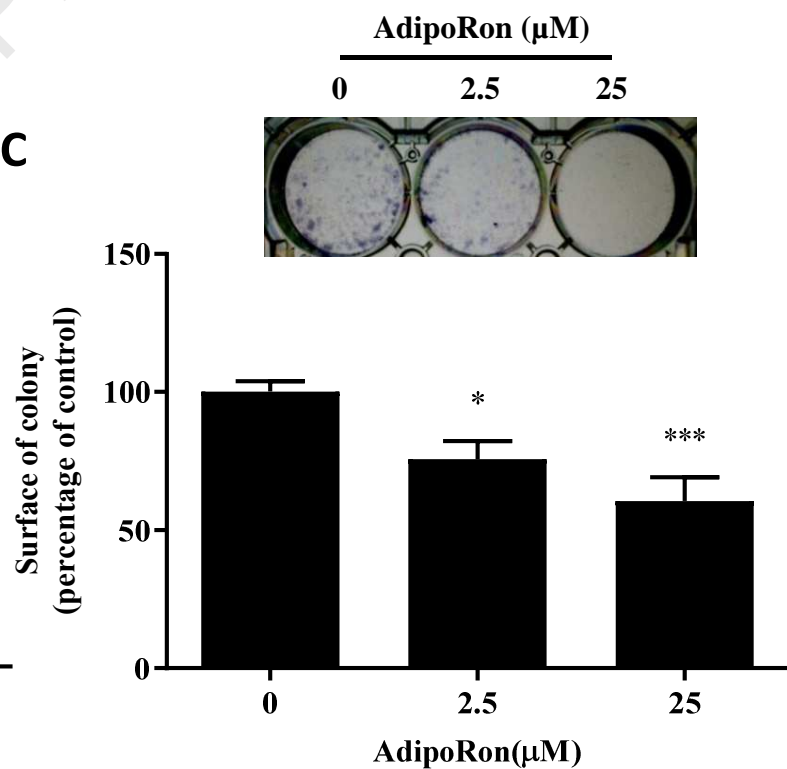
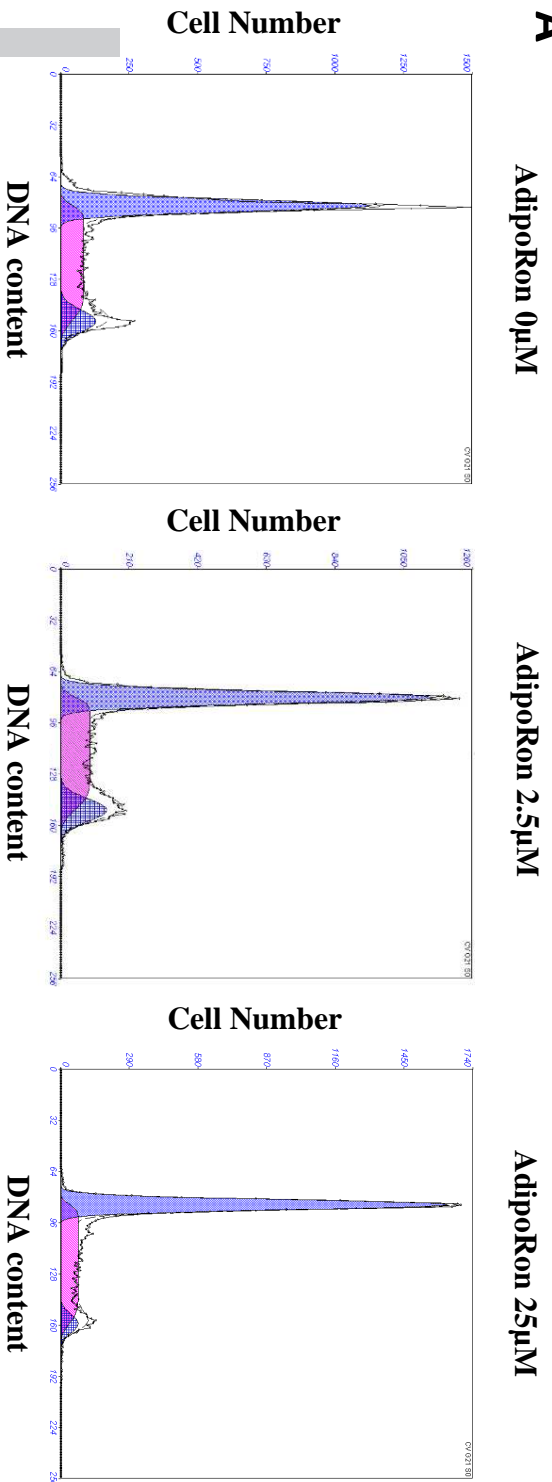
Figure 3**A****B****C**

Figure 4

A



E

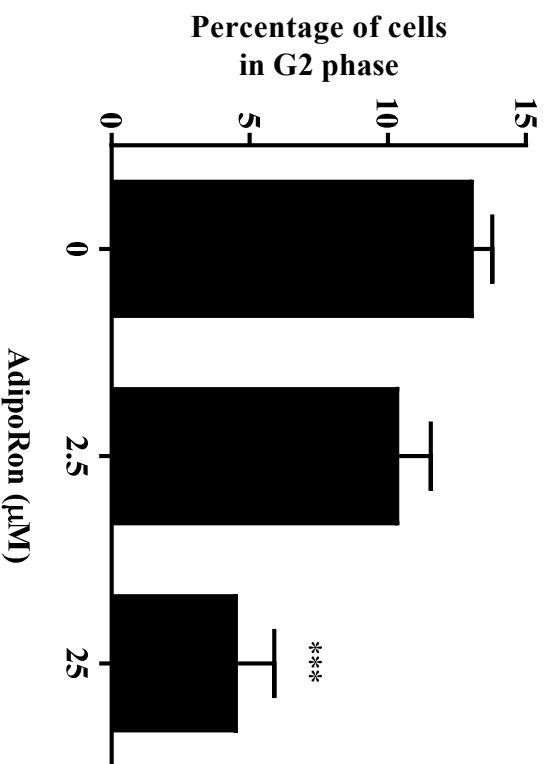
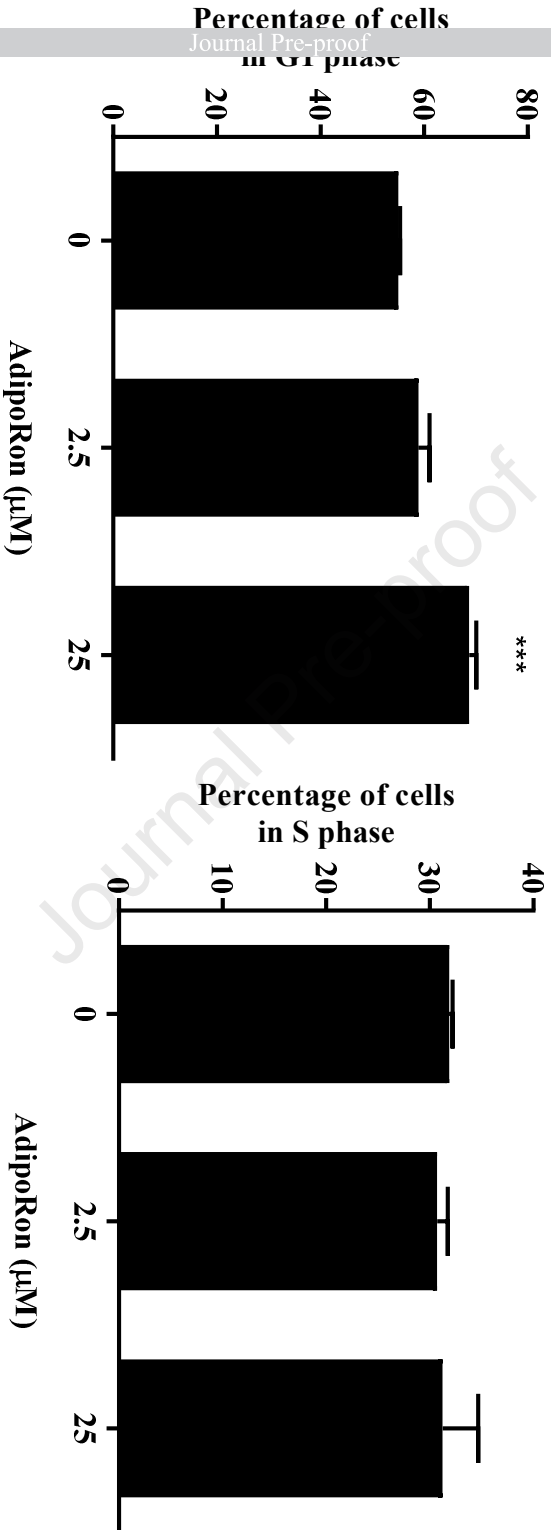


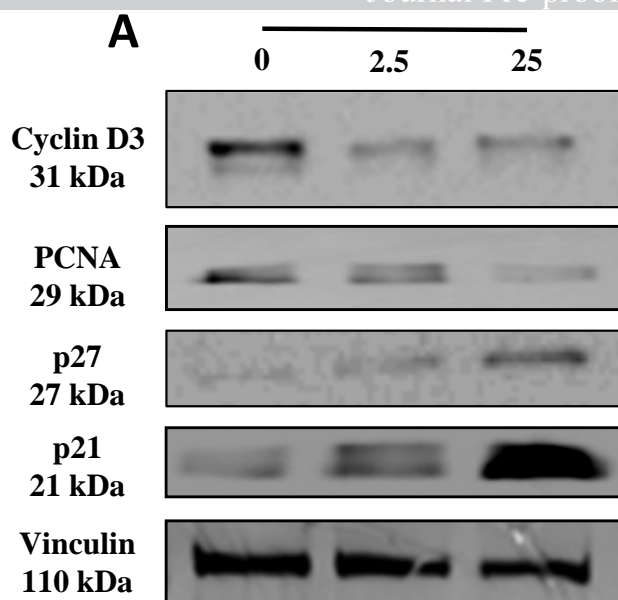
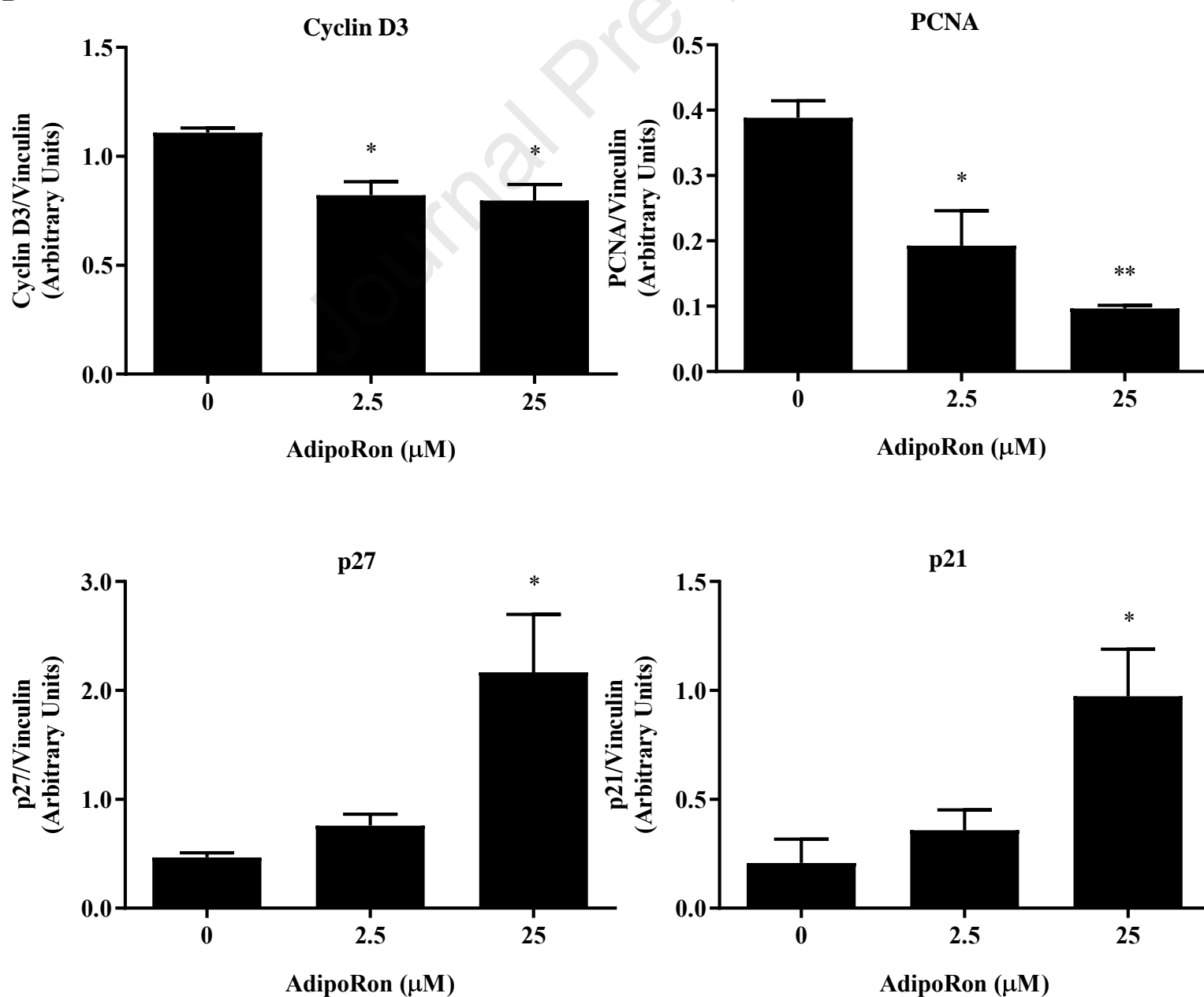
Figure 5**B**

Figure 6

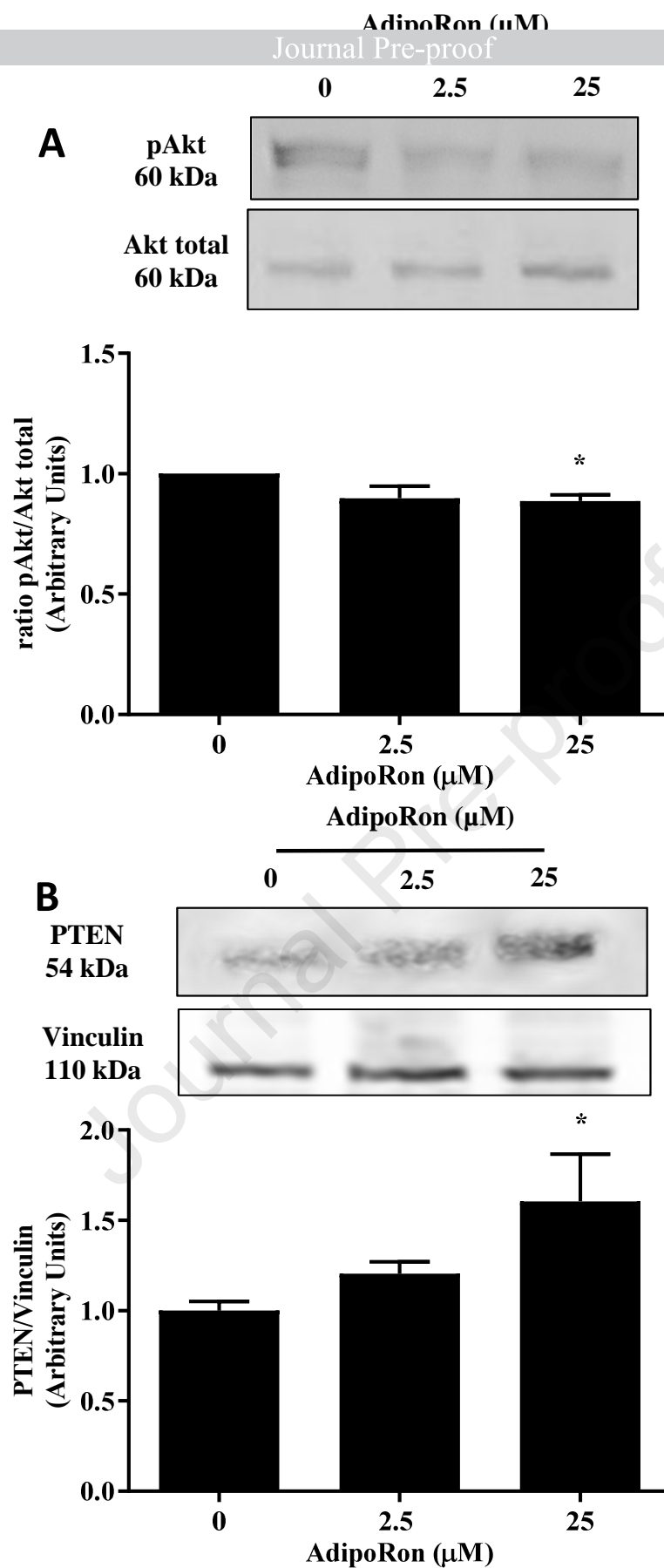


Figure 7**KCN**

Journal Pre-proof

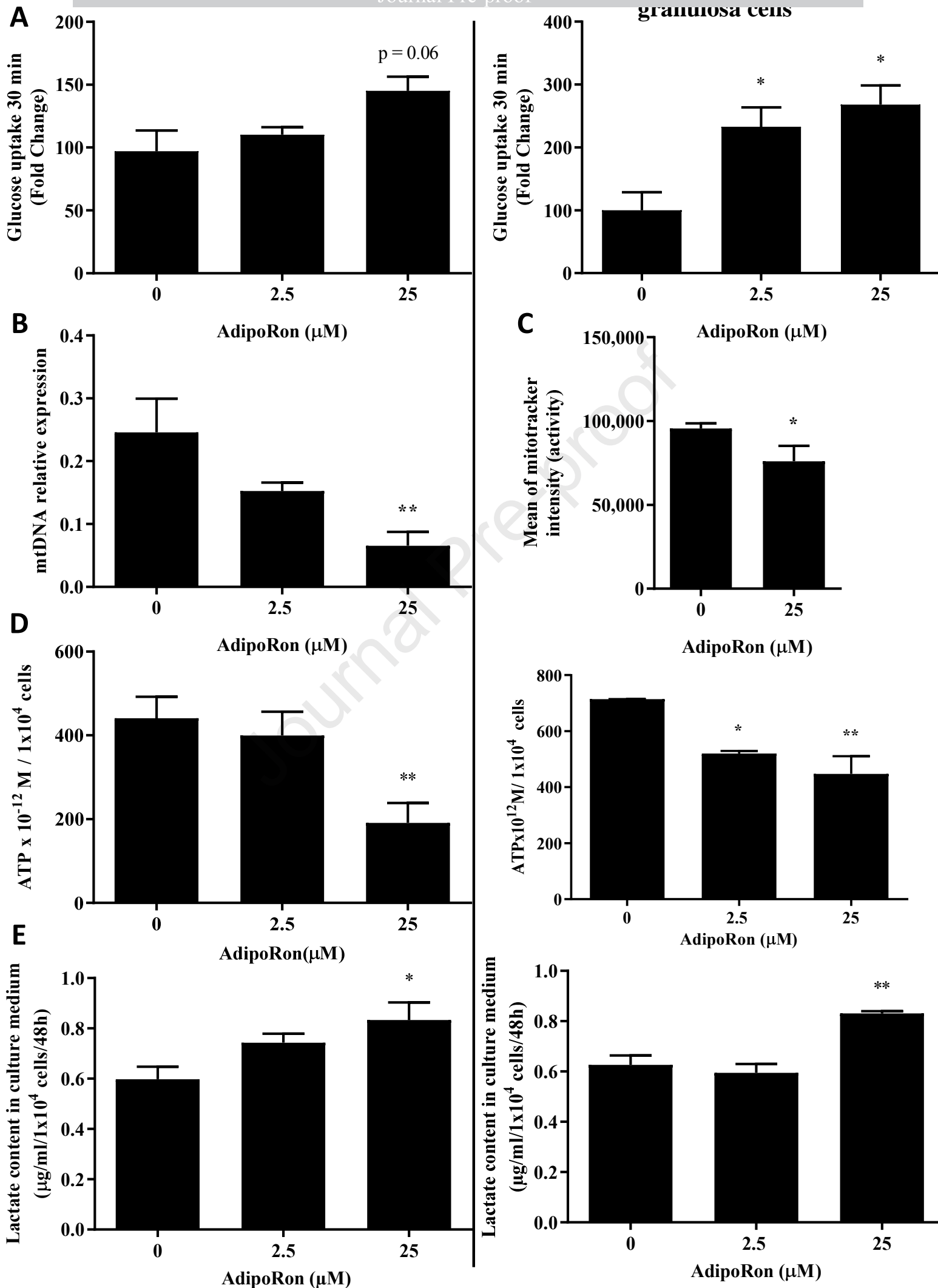
**Primary human luteinized
granulosa cells**

Figure 8

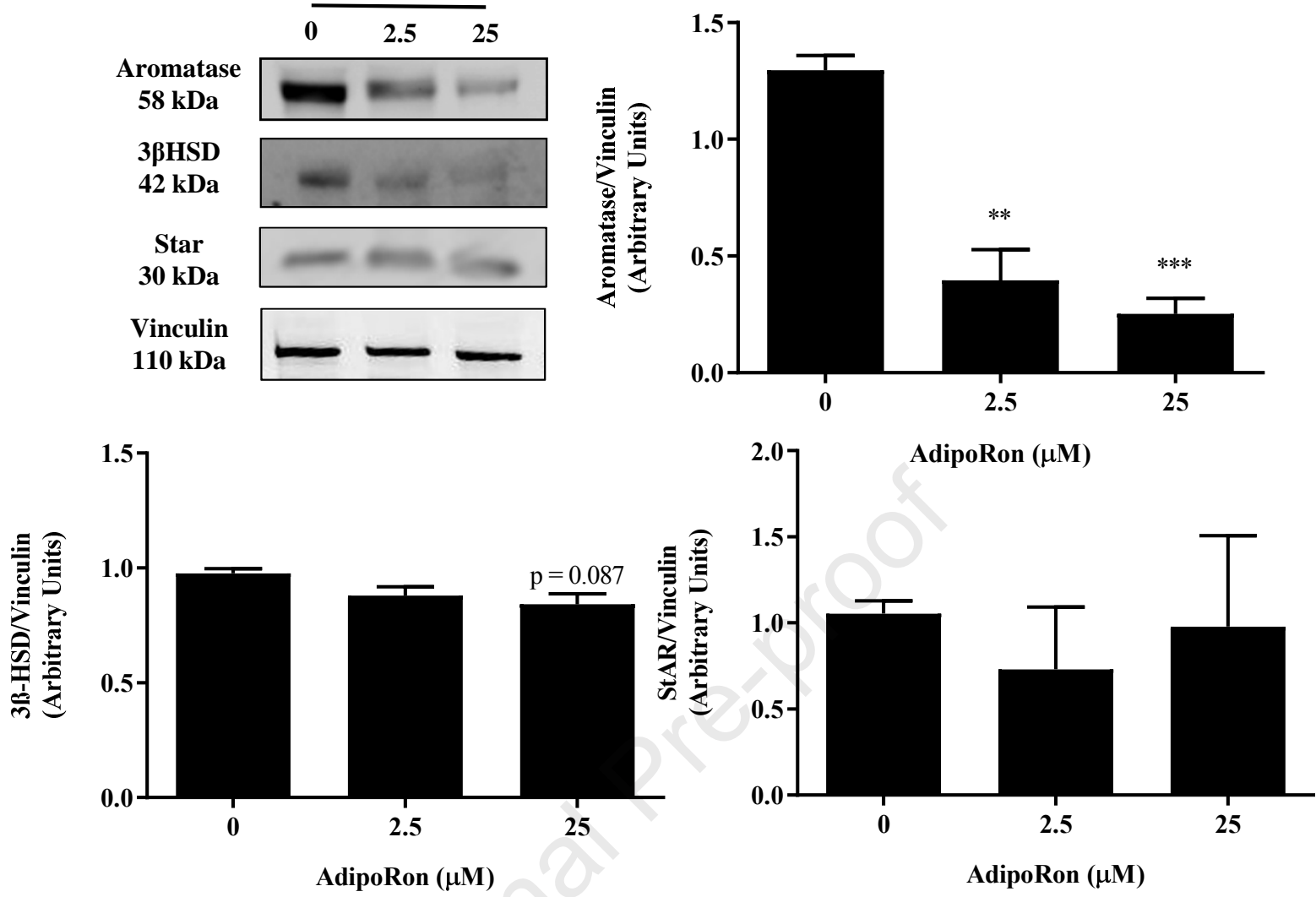
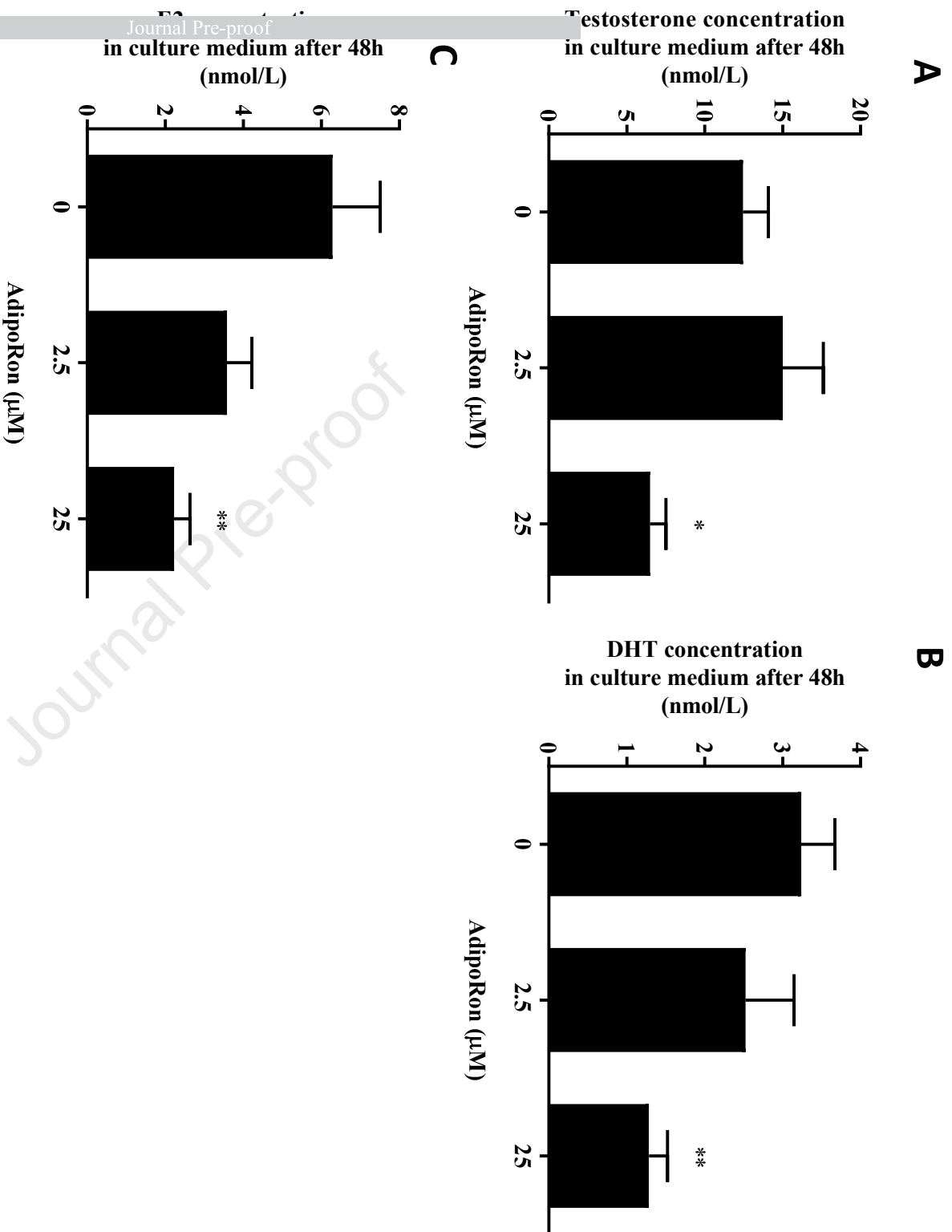
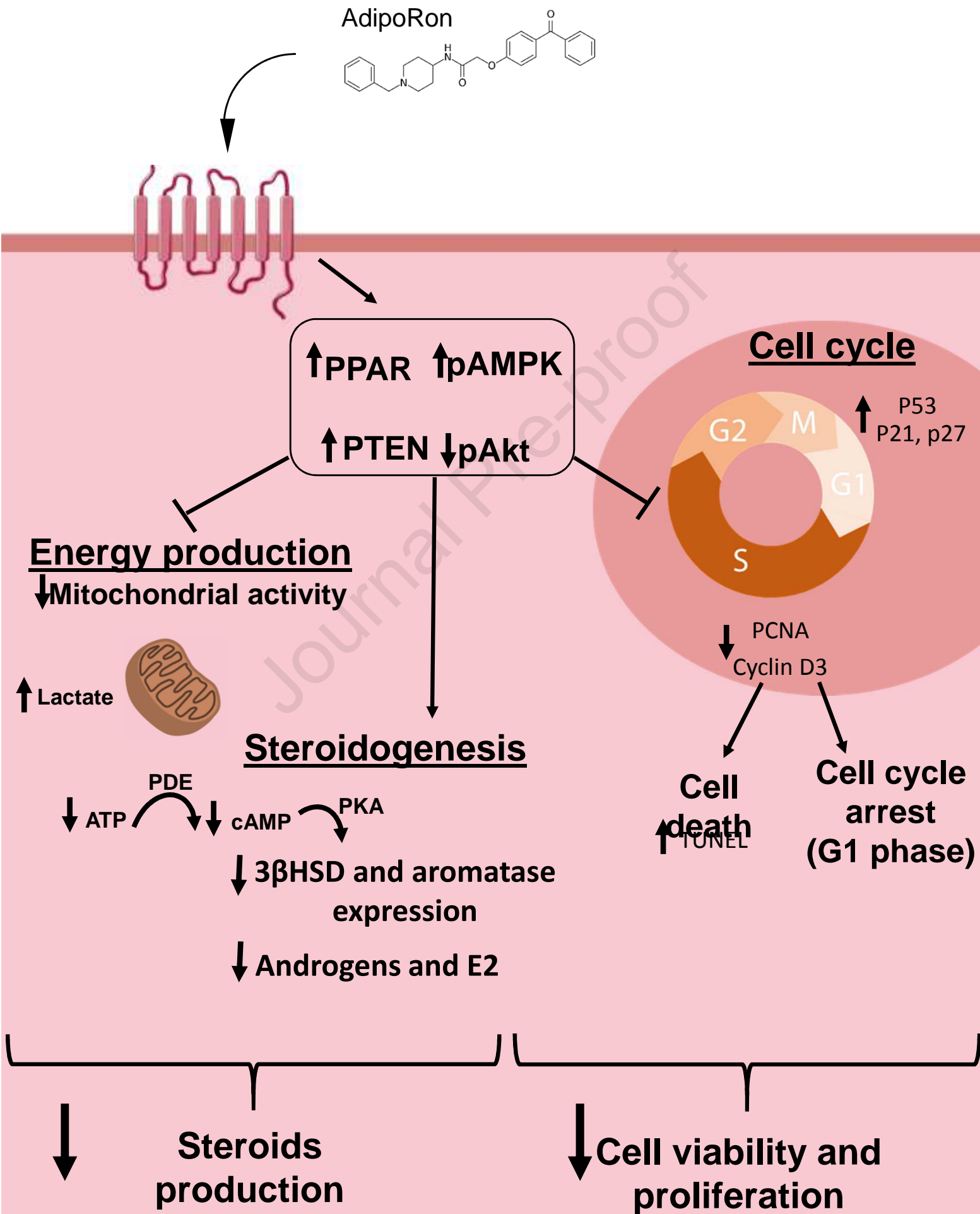


Figure 9





Highlights

The adiponectin pathway is downregulated in infertile women with polycystic ovary syndrome raising the hypothesis of the use of a new pharmaceutical drug called AdipoRon could be of interest in the treatment of female infertility. However, none or little information are available about the action of AdipoRon in granulosa cells, which are the cells nursering the oocyte. In this study, we investigated the role of AdipoRon on human luteinized granulosa cells (cell line and primary cells), and performed different assay to evaluate its impact on viability, cell proliferation, metabolism and in particular on steroidogenesis, which is an important role of granulosa cells to control fertility.