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Dual effect of insulin resistance and cadmium on human granulosa cells - *In vitro* study

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ABSTRACT

Combined exposure of cadmium (Cd) and insulin resistance (IR) might be responsible for subfertility. In the present study, we investigated the effects of Cd *in vitro* in IR human granulosa cells. Isolated human granulosa cells from control and polycystic ovary syndrome (PCOS) follicular fluid samples were confirmed for IR by decrease in protein expression of insulin receptor-β. Control and IR human granulosa cells were then incubated with or without 32 μM Cd. The combined effect of IR with 32 μM Cd in granulosa cells demonstrated significant decrease in expression of StAR, CYP11A1, CYP19A1, 17β-HSD, 3β-HSD, FSH-R and LH-R. Decrease was also observed in progesterone and estradiol concentrations as compared to control. Additionally, increase in protein expression of cleaved PARP-F2, active caspase-3 and a positive staining for Annexin V and PI indicated apoptosis as the mode of increased cell death ultimately leading to decreased steroidogenesis, as observed through the combined exposure. Taken together the results suggest decrease in steroidogenesis ultimately leading to abnormal development of the follicle thus compromising fertility at the level of preconception.

1. Introduction

Reproductive disorders are important health issues. Many couples desire children but cannot achieve pregnancy through natural means. The causative factors of infertility may be identified in either of the partner, however, the cause of subfertility remains unknown in about 50% of these cases. It is now believed that a combination of environmental and endocrine factors may be responsible for the same.

In our previous studies we have discussed the effect of both cadmium and insulin resistance in isolation and combination as cited from various animal studies with respect to HPO axis and the results clearly demonstrate that Cd and IR together mediate more deleterious effects at different levels of reproduction affecting granulosa cell receptors, steroidogenesis, higher rate of apoptosis and dysregulation from hypothalamus in the form of decreased GnRH and gonadotropins (Belani Muskaan et al., 2014). We further wanted to study the combined effect of IR and Cd in *in vitro* condition in human model for which we chose human luteinized granulosa cells.

The presence of insulin receptor (INSR-β) in human luteinized granulosa cells (hLGC's) identifies ovary as a target of insulin activity (Poretsky et al., 1999). Alterations in insulin functioning due to sedentary life style and diet rich in carbohydrate leads to IR which is associated with abnormalities leading to infertility. Amongst several endocrine factors, IR is more prevalent and is present in 50–70% of PCOS women contributing to its pathogenesis (Mukherjee and Maitra, 2010). Increase in the fatty acid content during IR affects granulosa cells and developmental competence of oocyte possibly by influencing its lipid metabolism ultimately leading to poor ovulations, poor pregnancy outcomes and frequent miscarriages (Jakubowicz and Sharma, 2007). *In vitro* experiments with hLGC's from anovulatory PCOS subjects have demonstrated increased steroid accumulation with physiologically high levels of insulin accompanied by gonadotropins (Willis et al., 1996). On the contrary, resistance has been observed to insulin dependent glucose metabolism in human granulosa-luteal cells from anovulatory women with polycystic ovaries indicating inhibition of the metabolic activities (Rice et al., 2003). Cd is one of the reproductive toxicants exposed usually as a result of waste from human activities in the environment (Frydman et al., 2010). Its long half-life in vivo facilitates its bioaccumulation and results in bio magnification in the food chains, thereby exposing females through diet (Satarug et al., 2011). Cadmium is known to induce oxidative damage and apoptosis in cultured granulosa cells from chicken ovarian follicles (Jia et al., 2011). Exposure of Cd during their developmental age decreases the expression of StAR in granulosa cells of *F*1 generation rats (Pillai et al., 2010). Decrease in gene expression of CYP19A1 due to Cd exposure has been established in carp ovarian follicle (D DSaM, 2013). Presence of Cd has been identified

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in follicular fluid as well as oocytes of female smokers undergoing *in vitro* fertilization (IVF) therapy leading to reduction in fertility and fecundity (Zenzes et al., 1995; Thompson and Bannigan, 2008; Jackson et al., 2011). Studies have demonstrated increased accumulation of Cd in the ovary with an increase in age thus leading to failure of progression of oocyte development and ovulation (Frydman et al., 2010). *In vitro* administration of Cd to cultured human ovarian granulosa cells decreased preovulatory LH surge and progesterone secretion (Paksy et al., 1997). Cd accumulation in gonads of foetus decreases the number of germ cells and in the embryos it leads to degeneration, apoptosis and breakdown in cell adhesion thus inhibiting its progression to the blastocyst stage (Frydman et al., 2010).

Nowadays the risk of infertility is enhanced by a variety of environmental as well as lifestyle factors. Owing to this there has been an increase in the process of *in vitro* fertilization for the couples willing to have a child. hLGC's obtained from IVF patients after controlled stimulation and retrieved from the follicles during aspiration of the ovulated egg represent a homogeneous population of luteinized cells. Several such layers of cumulus and mural granulosa cells are generally discarded at the time of IVF. These granulosa luteal cells being the most abundant cell type inside the follicle undergo substantial differentiation, interact with oocytes, mediate the effect of gonadotropins on the follicular maturation and are easily accessible for studying the overall quality of the follicles in response to gonadotropins (Albertini, 2004). Hence these discarded granulosa luteal cells are used for extensive research purpose (Tripathi et al., 2013).

In the present paper, based on our prior work, we made an effort to understand *in vitro* effect of combined exposure of heavy metal Cd and IR in human luteinized granulosa cells (hLGC's) isolated from follicular fluid of PCOS and non-PCOS patients undergoing IVF. PCOS patients were classified as IR and NIR based on a novel molecular marker that is insulin receptor-β (INSR-β) expression on granulosa cells. We investigated protein expression of INSR-β in granulosa cells and then classified them as IR and NIR. We then exposed control and IR hLGC's to 32 μM Cd that has been used to define environmental, occupational and smoking risk factors in female reproductive life span (Paksy et al., 1997; Varga et al., 1993). The study is novel as there are no results for effect of Cd on naturally IR human luteinized granulosa cells mimicking today's scenario. Thus in the present study the combined effect of IR and Cd was evaluated on granulosa cell death parameters along with other physiological, biochemical and molecular parameters such as mRNA and protein expression of StAR for cholesterol transport and CYP11A1, 3β-HSD, CYP19A1 and 17β-HSD responsible for steroid synthesis.

2. Materials and methods

2.1. Hormones and reagents

Histopaque, Hyaluronidase, DMEM/F12, penstrep, amphotericin and trypan blue were from Sigma Chemical Co. (USA). Human estradiol and progesterone ELISA kits were procured from Diametra. Rabbit polyclonal antibody against INSR-β, CYP19A1 and β-actin was purchased from Cell Signaling and Goat polyclonal antibody against CYP11A1 was purchased from Santa Cruz. Rabbit polyclonal antibodies against 3β-HSD, 17β-HSD were generous gift from Dr. Vann Luu-The (CHUL Research Center and Laval University, Canada) and StAR from Dr. Douglas M. Stocco (Department of Cell Biology and Biochemistry, Texas Tech University, Lubbock, Texas, USA).

2.2. Human follicular fluid collection

Human follicular fluid samples were collected after informed consent from patients undergoing IVF/ ICSI over the course of 32 months at Nova Pulse IVI Fertility, Ahmedabad, India from 2012 August to 2015 April. All the controls and patients were non-smokers (according to hospital data) and underwent controlled ovarian hyperstimulation (COH) using flexible antagonist protocol. Recombinant FSH &/or urinary human menopausal gonadotropin (hMG) was started from 2nd day of period followed by the antagonist-cetrorelix acetate. Final oocyte maturity was triggered with recombinant human chorionic gonadotropin (hCG) or gonadotropin releasing hormone (GnRH) agonist. Follicles were aspirated by transvaginal ultrasound retrieval after 35 h of trigger injection. Follicular fluid was sent in embryology laboratory for oocyte identification & oocytes were separated out for IVF/ICSI. All the controls and patients received a GnRH analog (GnRH-a) in combination with FSH or human menopausal gonadotropin (hMG), followed by administration of human chorionic gonadotropin (hCG). The follicular fluid devoid of oocyte was collected for the experiments on the day of oocyte retrieval.

Inclusion criteria: The diagnosis included donors and PCOS with an age ranging from 20 to 40 years.

Exclusion criteria: Patients with endometriosis and poor ovarian response were excluded from the study.

The study was approved by the Institutional Ethics Committee for Human Research (IECHR), Faculty of Science, The M. S. University of Baroda, Vadodara (Ethical Approval Number FS/IECHR/BC/SG2).

2.3. Human granulosa-luteal cell isolation

DMEM/F12 with 10% FBS and Penicillin-G/Streptomycin (100 IU/ml/100 mg/ml) was used as the basal medium for human luteal granulosa cell (hLGC) preparation and culture. Granulosa cells from follicular aspirates of individual patients $n = 30$ control and $n = 39$ PCOS were isolated using the protocol in the literature (Földesi et al., 1998). The follicular aspirates were centrifuged at 300 *g*, room temperature for harvesting the cells. The harvested cells were resuspended in plain DMEM/F12 media and layered on Histopaque to form a gradient followed by centrifugation at 400 *g* at room temperature to remove red blood cells and white blood cells. The middle layer of cells containing LGCs was collected and suspended in 10 ml volume of DMEM/F12 medium and washed thrice with DMEM/F12 by a further 5 min centrifugation. The hLGCs pellet was then incubated with 0.1% hyaluronidase in DMEM/F12 medium without FBS for 30 min at 37 °C with constant shaking and gentle repeated pipetting to obtain single cell suspension. The enzymatic reaction was stopped by addition of DMEM/F12 with FBS followed by centrifugation at 300 *g* at room temperature. The cell pellet was finally suspended in 1 ml of DMEM/F12 with FBS. The viable cell count by trypan blue dye exclusion was observed to be 90%. 0.5×10^6 cells from each patient were aliquoted for protein expression of INSR-β. Rest of the cells were cryopreserved for further use.

2.4. Immunocytochemistry

Isolated cells from follicular fluid samples were characterized by immunocytochemistry for granulosa cells. Cells were grown on glass slides in plastic tissue culture dishes. After their adherence, the cells were fixed with ice chilled methanol or 4% paraformaldehyde at 4 °C for 10 min followed by 2 washes with PBS. The cells were permeabilized with 0.1% Triton X-100 for 3–4 min at 4 °C followed by 2 washes with PBS. Incubation was then done with blocking buffer (0.5% BSA + 0.5% FBS in PBS) for 40–45 min at RT. The cells were then incubated overnight with primary antibody (CYP19A1, 17β-HSD and 3β-HSD) at a dilution of 1:100 at 4 °C. After 5 washes with washing buffer (PBS + 0.1% Tween 20) the cells were incubated in secondary antibody (goat anti-rabbit IgG-FITC) at a dilution of 1:250 for 1 h at R.T. After 5 washes with washing buffer nuclear staining was done with 100 ng/ml DAPI for 5 min. The cells were washed with PBS and then glass slide was mounted on a slide in mounting medium and then observed under Zeiss Laser scanning confocal microscope-710. The excitation/emission spectrum for DAPI complexes was 358 nm/461 nm. The excitation/

emission spectrum for Cy3 complexes was 550 nm/570 nm. The images were analysed by LSM browser/ZEN 2010 software.

2.5. Optimization of Cd concentration by MTT assay

Granulosa cells were seeded in a 96-well plate at a density of 1×10^{-4}/200 μl/well in DMEM/F12 with 10% FBS for 48 h. Cd acetate was added at different concentrations ranging from 0, 8, 16, 32 and 64 μM for 24 and 48 h in triplicates. This was followed by addition of 10 μl of 5 mg/mL MTT to each well for 4 h. After incubation with MTT, the formazon crystals formed inside the cells were dissolved in DMSO and then the measurement of the purple solution at an optical density of 490 nm by using a Multiskan microtiter plate reader from Thermo Scientific. Experiment was repeated independently 3 times.

2.6. Protein expression of INSR-β in hLGC's

Follicular fluid samples availed from infertility clinic were clinically not defined for IR condition. Hence to segregate IR samples from NIR ones, the expression of INSR-β was determined by western blotting in individual samples of hLGC's from donors, tubal factor's, male factor and PCOS. The cells were suspended in cell lysis solution [62.5 mM Tris–HCl, pH 6.8, 6 M urea, 10% (*v*/*v*) glycerol, 2% (*w*/*v*) SDS, 0.00125% (*w*/*v*) bromophenol blue and freshly added 5% (*v*/*v*) β-mercaptoethanol] followed by sonication at 40% amplitude with 2 s pulse on and off for 20 s on ice. Total protein content of the cell lysates were quantified using Bradford assay (Bio-Rad Bradford Solution, USA). 20 μg protein was loaded on 10% SDS-polyacrylamide gel electrophoresis under reducing conditions, along with pre-stained molecular weight markers. The separated proteins were transferred onto a nitrocellulose membrane (GE Healthcare) by a wet method (Bio Rad, USA). The transfer was performed at a constant voltage (100 V) for 90 min in a buffer consisting of 25 mM Tris, 192 mM glycine and 20% methanol. The membrane was then incubated for 1 h at room temperature in blocking buffer (PBS - containing 5% skimmed milk and 0.1% Tween-20) followed by overnight incubation at 4 °C with anti INSR-β antibody (4B-8) (cell signaling-3025) at a dilution of 1:800 in PBS - containing 5% skimmed milk and 0.1% Tween-20. The membrane was washed in PBS-0.1% Tween-20 for 5 times, incubated for 1 h at room temperature with a horseradish peroxidase-conjugated anti-rabbit at a dilution of (1:2500) from Bangalore Genei in PBS - containing 5% skimmed milk and 0.1% Tween-20. They were again washed 5 times with PBS-0.1% Tween-20 followed by 2 washes in PBS. The signal was detected by ultra-sensitive enhanced chemiluminescence reagent (Millipore, USA) and image captured by Alliance 4.7 UVI Tec chemidoc.

2.7. In vitro culture of hLGC with Cd

After confirming for the presence of INSR-β in the hLGC's isolated from the follicular fluid aspirates of donors, male factor and tubal factor infertility, the cells were categorized to be non-insulin resistant and were pooled to get an adequate number for all the experiments and were demarcated as the control group. Whereas luteinized granulosa cells isolated from follicular fluid aspirates of PCOS that showed down regulation of INSR-β as against control cells were termed as IR and pooled. Control and PCOS-IR granulosa cells were cultured in DMEM/F12 with 10% FBS for 48 h and then incubated in fresh medium with or without 32 μM of Cd acetate for 24 h forming four different groups: control, control + Cd, PCOS-IR and PCOS-IR + Cd. The supernatant was collected and frozen in −80 °C for analysis of steroid hormones and cells harvested and proceeded for expression of genes, proteins, cell death parameters and enzyme activity as required. The flow chart of the study is shown in Fig. 1.

2.8. Total RNA extraction and RT-PCR

RNA was isolated from cultured granulosa cells by using TRIzol, (Sigma-Aldrich, USA). Purity of RNA was confirmed by *A*260/280 ratio and checked for integrity. 2 μg of total RNA was reverse transcribed into first strand cDNA. cDNA for gonadotropin receptors was subjected to qRT PCR. For real time analysis, c-DNA was amplified for 40 cycles using 5 μl Power SYBR-Green master mix, 10 pM of each forward and reverse primers and 100 ng cDNA (1/20th of total cDNA preparation) in Applied Biosystems, 7500-Real-time PCR Sequence Detection System and the results were normalized to the level of β-actin determined in parallel reaction mixtures to correct any differences in RNA input. The steroidogenic genes were analysed in a 10 μl of total reaction containing 5 μl Taqman FAST master mix, 0.5 μl of 20× predesigned Taqman gene expression assays and 100 ng cDNA (1/20th of total cDNA preparation) in Applied Biosystems, 12K FLEX Quantstudio Real Time PCR. The qRT-PCR results for human steroidogenic genes were normalized to the level of 18S rRNA determined in parallel reaction mixtures to correct any differences in RNA input. mRNA expression of gonadotropin receptors were analysed by real time PCR (Applied-Biosystem 7500-Real-time PCR Sequence Detection System) and for steroidogenic genes by predesigned from TaqMan gene expression assays by real time PCR (Applied Biosystem 7500 FAST Real Time PCR Sequence Detection System). Fold changes in qRT-PCR gene expression were analysed using 7500 Real time PCR software V.2.0.6 and Data assist software (Applied Biosystems, Inc.) which led to a possible estimation of the actual fold change. The qPCR results are expressed as mean ± S.E.M. of RQ values versus target gene. Negative RT was performed with untranscribed RNA. For primers refer to Tables 1 and 2.

2.9. Expression of proteins involved in cell death and steroidogenesis

Protein expression of PARP-F2, caspase-3, StAR, CYP11A1, CYP19A1, 3β-HSD and 17β-HSD from cultured human luteinized granulosa cells were determined as described earlier in Section 2.4. Details of antibodies are presented in Table 3.

2.10. Estimation of hydroxysteroid dehydrogenase activity

17β-HSD and 3β-HSD activities were estimated in granulosa cells of rats and humans following Shivanandappa and Venkatesh (1997). In brief, the assay system contained 0.1 M Tris–HCl (pH 7.8), 5 mM nicotinamide adenine dinucleotide (NAD), 1 mM estradiol/dehydroepiandrostenedione (DHEA), and 0.4 mM 2-p-iodophenyl-3-p-nitrophenyl-5-phenyl tetrazolium chloride (INT) and 50 μl of granulosa cell lysate containing enzyme in a total volume of 3 ml, which was incubated for 1 h at 37 °C. The reaction was terminated using 50 mM potassium phthalate buffer, and absorbance was measured at 490 nm.

2.11. Annexin V binding assay

The Annexin V-FITC antibody (BD) detects the externalisation of phosphatidylserine on the cell membrane, which is one of the typical markers for early apoptosis. The isolated granulosa cells from all the experimental groups were washed once with cold PBS at 300 *g* for 5 min. The pellet was then reconstituted in cold 1× binding buffer, 2 μl of Annexin V-FITC and 10 μl of 20 μg/ml propidium iodide (PI) was added and incubated at room temperature for 15 min in dark. After the incubation, cells were washed once with 1× binding buffer at 1000 rpm for 5 min and resuspended in 100 μl 1× binding buffer. 20 μl of cell suspension was mixed with equal amount of mounting media, coverslip was applied and visualized under laser scanning confocal microscopy. The cells were qualitatively analysed for presence of green and red fluorescence. Presence of green fluorescence of Annexin V-FITC and absence of red fluorescence of PI together indicated cells to

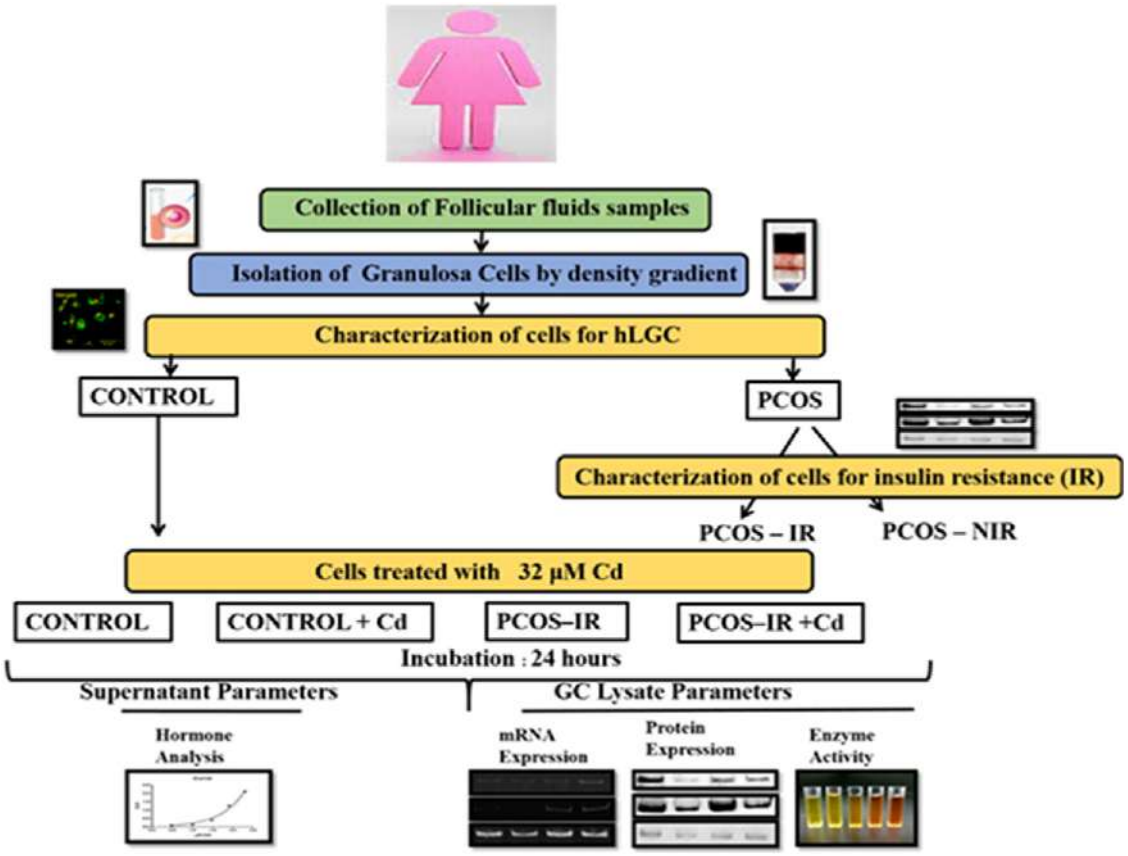

Fig. 1. Flow chart of the study describing collection of follicular fluid samples, isolation of granulosa cells, characterization for IR, treatment with Cd followed by analytical parameters.

be in early apoptotic stage while the cells positive for both Annexin V-FITC and PI fluorescence indicated loss of membrane integrity characterizing apoptotic death.

2.12. Steroid hormone analysis

The steroid hormones were measured in the culture medium by enzyme-linked immunosorbent assay (Diametra; Italy), according to the manufacturer's instructions. The standard curve for E_2 and P_4 ranged from 0 to 2000 pg/ml and 0 to 40 ng/ml respectively. The supernatants were diluted to 1:1000 for E_2 and 1:250 for P_4 in PBS to ensure that the final value fell within the detection range of the standard curve. Each sample was assayed in duplicate, and the E_2 and P_4 concentration was calculated by multiplying the end value by the dilution factor. The assay sensitivity range was 8.68 pg/ml for E_2 and 0.05 ng/ml for P_4.

2.13. Statistical analysis

The results are presented as mean ± standard error mean. The data were statistically analysed by employing one-way analysis of variance followed by Newman Keuls Multiple Comparison Test (GraphPad Prism; Graph Pad Software, Inc., La Jolla, CA). The minimum level of significance ($P < 0.05$) was considered.

3. Results

Follicular fluid sample from IVF patients were collected as per the inclusion and exclusion criteria and classified as control and PCOS based on hospital record.

3.1. Characterization of cells isolated from follicular fluid

Granulosa cells were isolated from follicular fluid sample of IVF patients with and without PCOS. These cells were characterized by immunostaining with antibodies against steroidogenic enzymes exclusively present in LGCs. Isolated cells were positive for CYP19A1, 17β-HSD and 3 β-HSD with DAPI as the nuclear stain (Fig. 2) confirming that they were LGCs.

3.2. INSR-β expression in hLGC's

Individual analysis of all samples for protein expression of INSR-β employing β-actin as an internal control was done in triplicate to

Table 1
List of Taqman gene expression probes (human).

S.·no	Name	ID	Cat. no	Amplicon length
1	StAR - Steroidogenic Acute Regulatory protein	Hs00986558_g1	4448892	68
2	CYP11A1 - cytochrome P450 side chain cleavage	Hs00897322_g1	4448892	90
3	CYP19A1 - aromatase	Hs00903410_m1	4448892	88
4	HSD3B2 (3-beta hydroxy steroid dehydrogenase type-2)	Hs01080264_g1	4448892	77
5	HSD17B1 (17-beta hydroxy steroid dehydrogenase type-1)	Hs00907289_g1	4448892	93

Table 2
List of primers sequences (human) with its amplicon size.

Gene	Accession number	Sequence (5′ → 3′)	Product size	Annealing TEMPERATURE
FSH-R (H)	NM_000145.3	F: TTTCAAGAACAAGGATCCATTCC R: CCTGGCCCTCAGCTTCTTAA	336	60
LH-R (H)	NM_000233.3	F: TTCAATGGGACGACACTGACTT R: TGTGCATCTTCTCCAGATGTACGT	234	60

distinguish IR from NIR. The β-subunit of INSR was detected in granulosa cell lysates of all donors, tubal factors and male factors with no significant difference amongst themselves thus grouping them as control. Protein expression of INSR-β was revealed in 14 PCOS samples with no significant difference when compared to control. Granulosa cell lysate of 25 PCOS samples also demonstrated protein expression of INSR- β, however the expression was significantly less ($P < 0.05$) when compared to control and other 14 PCOS samples indicating down-regulation of INSR. Based on these densitometric data, 14 PCOS samples were grouped under PCOS-NIR and other 25 PCOS samples were grouped under PCOS-NIR giving the final percentage of PCOS-IR as 64% and PCOS-NIR as 36% (Fig. 3).

3.3. Dose-time dependency of Cd effect

Treatment of hLGC's with 8 μM and 16 μM Cd demonstrated a non-significant decrease in the % cell viability during 24 h exposure time. The response was marked and became significant ($P < 0.01$) at 32 μM and ($P < 0.001$) at 64 μM for 24 h exposure time. A profound inhibition in cell viability was observed during 48 h exposure time at 16 μM ($P < 0.01$) and increased drastically at 32 μM and 64 μM ($P < 0.001$). However, 32 μM being the lowest concentration showing significant decrease in cell viability at 24 h was taken into consideration for the study.

3.4. Effect of Cd exposure on viability of control and PCOS-IR hLGC's

Control and PCOS-IR hLGC's were treated with or without 32 μM Cd acetate for 24 h. Cell viability was highly compromised in alone Cd group ($P < 0.01$) as well as PCOS-IR + Cd group ($P < 0.001$) with respect to control when observed by trypan blue exclusion dye after 24 h of *in vitro* incubation in optimum culture condition. However, decrease in the viability was also observed in PCOS-IR group ($P < 0.01$) as compared to control indicating the adverse effects of IR on granulosa cells. Moreover, decreased viability in PCOS-IR group indicated that the survival of hLGCs was negatively affected with Cd treatment during insulin resistant condition (Fig. 4).

Table 3
List of antibodies for western blotting.

Name of antibody	Company and catalog no.	Mono/poly clonal	Mol. weight (kDa)	Isotype
Insulin Receptor β (INSR-β)	Cell Signaling #3025	Mono	85	Rabbit
StAR	gifted by Prof. Stocco	Poly	30	Rabbit
CYP11A1	Santacruz	Poly	60	Goat
3β-HSD	Gifted by Prof. Van Luu THE	Poly	35	Rabbit
CYP19A1	Cell Signaling	Poly	58	Rabbit
17β-HSD	Gifted by Prof. Van Luu THE	Poly	35	Rabbit
Caspase-3	Thermo scientific	Poly	32	Rabbit
PARP (46D11)	Cell Signaling #9532	Mono	116,89	Rabbit
β Actin	Thermo Scientific #MAI-91399	Mono	43	Mouse

3.5. Effect of Cd and IR on cell death parameters in hLGC's

To assess the effects of Cd, IR and their co-exposure on the genes involved in cell death, protein expression of cleaved PARP-F2, activated caspase-3 and binding of AnnexinV/PI in hLGC's was analysed by immunoblotting and confocal microscopy respectively. There was a significant increase in protein expression of activated caspase-3 in PCOS-IR ($P < 0.05$) and PCOS-IR + Cd ($P < 0.01$) as compared to control and alone Cd group. Within the groups, significant increase ($P < 0.05$) was observed in PCOS-IR + Cd group as compared to PCOS-IR, ($P < 0.05$) in PCOS-IR and ($P < 0.01$) in PCOS-IR + Cd when compared to Cd, whereas alone Cd did not show any significant increase when compared to control. Similar results were obtained in the protein expression of cleaved (activated) PARP F2 where there was a remarkable increase in activated PARP F2 in PCOS-IR ($P < 0.05$) and PCOS-IR + Cd ($P < 0.01$). An increase in PARP F2 was also observed in alone Cd group, however it was non-significant when compared to control, whereas PCOS-IR + Cd did show significant increase ($P < 0.05$) when compared to alone Cd. Qualitative analysis of granulosa cell images of C + Cd, PCOS-IR and PCOS-IR + Cd captured by confocal microscopy exhibited increased fluorescence of Annexin V (green color) and PI (red color) as compared to control cells indicating apoptosis as the type of cell death mechanism. (Figs. 5 and 6).

3.6. Effect of Cd and IR on steroid concentrations in hLGC's

Estradiol and progesterone concentration was estimated in cell culture supernatant by ELISA. Significant decrease in estradiol concentration was observed in alone Cd ($P < 0.01$), PCOS-IR ($P < 0.01$) and PCOS-IR + Cd ($P < 0.05$) group as compared to control. Progesterone also demonstrated significant decrease in alone Cd ($P < 0.001$), PCOS-IR ($P < 0.001$) and PCOS-IR + Cd ($P < 0.01$) as compared to control (Fig. 7). However, estradiol as well as progesterone did not demonstrate any significant difference within the groups.

3.7. Effect of Cd and IR on expression of steroidogenic genes and proteins in hLGC's

Gene and protein expression of StAR, CYP11A1, 3β-HSD, CYP19A1, and 17β-HSD involved in steroidogenic machinery was assessed in hLGC's from control and PCOS-IR treated with or without Cd. mRNA expression of StAR revealed significant decrease ($P < 0.05$) in alone Cd group as compared to control, whereas it did not reveal any significant difference in PCOS-IR and PCOS-IR + Cd when compared to control and also within the groups. The protein expression of StAR demonstrated significant decrease ($P < 0.001$) in all the three groups as compared to control. However it did not demonstrate any significant difference within the group. mRNA and protein expression of CYP11A1 revealed a considerable decrease in C + Cd ($P < 0.001$) group with respect to control. To our surprise mRNA and protein expression of CYP11A1 showed a drastic increase ($P < 0.001$) in PCOS-IR which was significantly decreased in the presence of Cd ($P < 0.01$) when compared to control. A noteworthy decrease ($P < 0.001$ and $P < 0.001$) was observed in CYP11A1 protein expression in C + Cd and IR + Cd group respectively as compared to control. mRNA and protein expression of 3β-HSD, CYP19A1 and 17β-HSD showed significant decrease in all the three

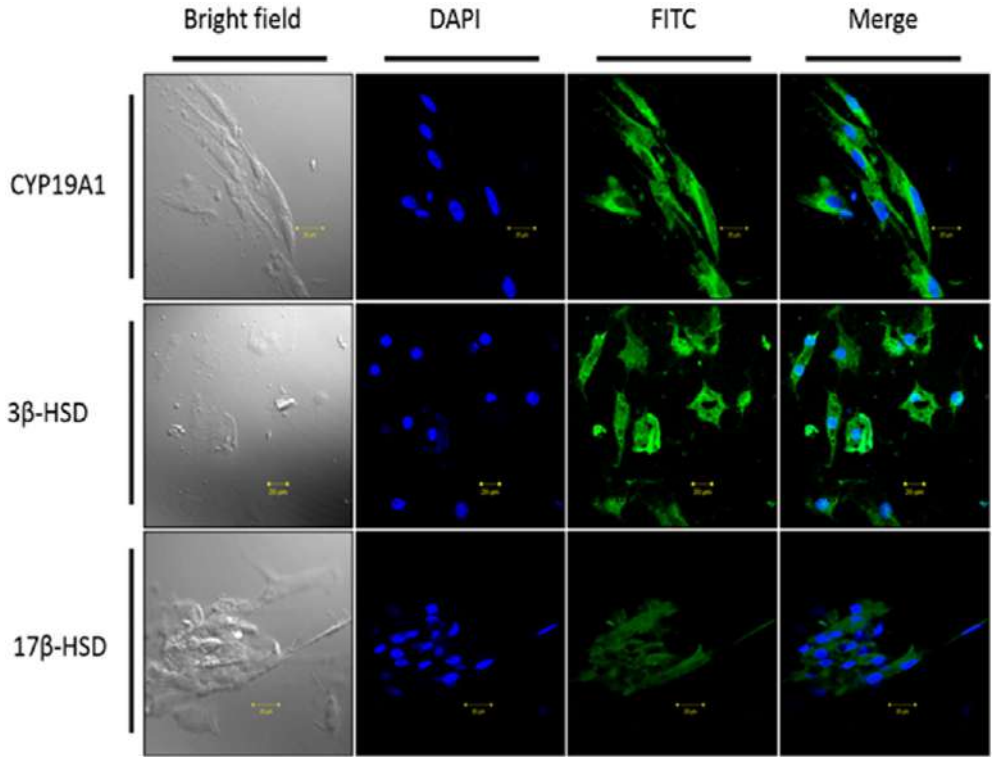

Fig. 2. Characterization of hLGC's isolated from follicular fluid samples. Blue color indicates presence of nucleus by DAPI staining and green color reflects presence of the CYP19A1, 17β-HSD and 3 β-HSD. (For interpretation of the references to colors in this figure legend, the reader is referred to the web version of this article.)

groups as compared to control with no significance within the groups (Fig. 8).

3.8. Effect of Cd and IR on hydroxy steroid dehydrogenase activity in hLGC's

Control and PCOS-IR hLGC's were treated with 32 μM Cd for 24 h followed by which 17β-HSD and 3β-HSD enzyme activity were analysed from the granulosa cell lysate. hLGC from PCOS-IR + Cd, PCOS-IR and C + Cd group showed significant decrease ($P < 0.001$,

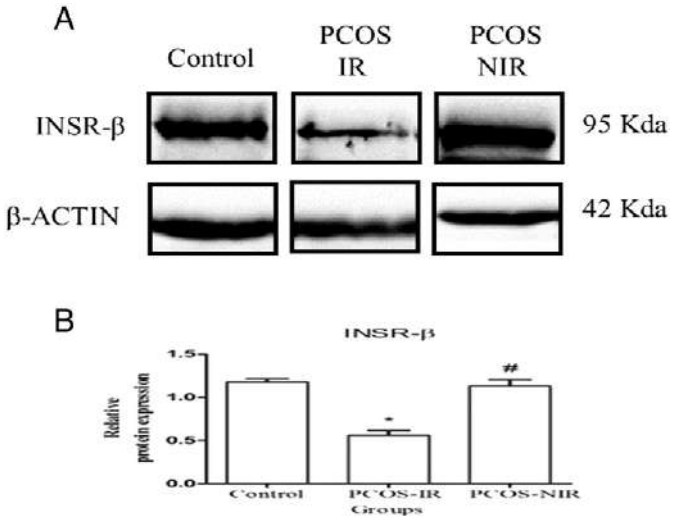

Fig. 3. INSR-β expression in hLGC's. hLGC's from all samples (donor, male factor, tubal factor, PCOS) individually were subjected to protein expression A) Western blot of INSR-β using β-actin as endogenous control. B) Densitometry analysis. Data represented as mean ± SEM of $n = 30$ control, $n = 25$ PCOS-IR and $n = 14$ PCOS-NIR. Protein expression of INSR-β was observed to be significantly decreased in PCOS-IR group as compared to control and PCOS-NIR ($^{*}P < 0.05$ and $^{\#}P < 0.05$) (20 μg protein).

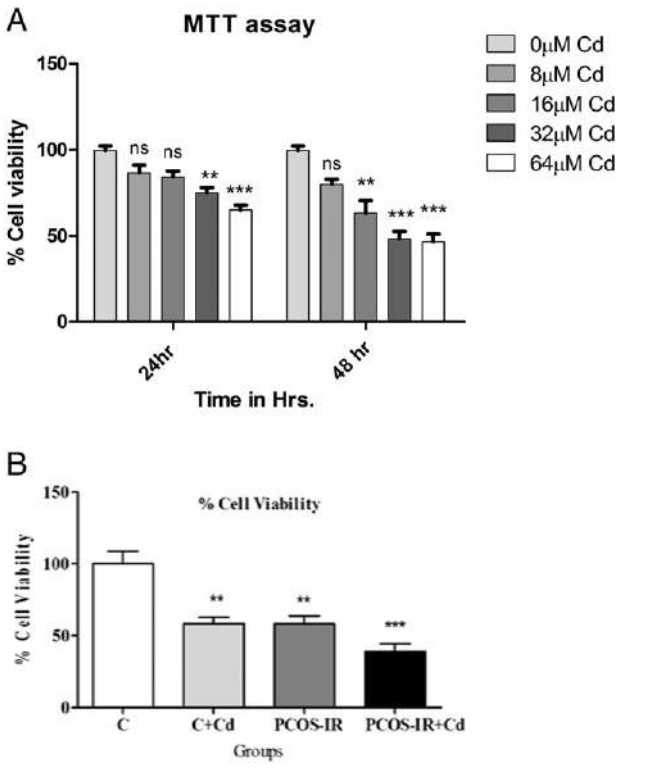

Fig. 4. A. Dose dependent study of Cd on hLGC's viability. 0, 8, 16, 32 and 64 μM concentrations of Cd were incubated for 24 and 48 h in hLGC followed by MTT assay. Decrease in % cell viability was observed at 32 μM for 24 h as compared to 0 μM ($^{**}P < 0.01$) B. Effect of IR and Cd either alone or in combination on hLGC's viability. Data represented as mean ± SEM of 3 independent experiments. % cell viability was significantly decreased in C + Cd, PCOS-IR and PCOS-IR group as compared to control ($^{***}P < 0.001$, $^{**}P < 0.01$). C = control.

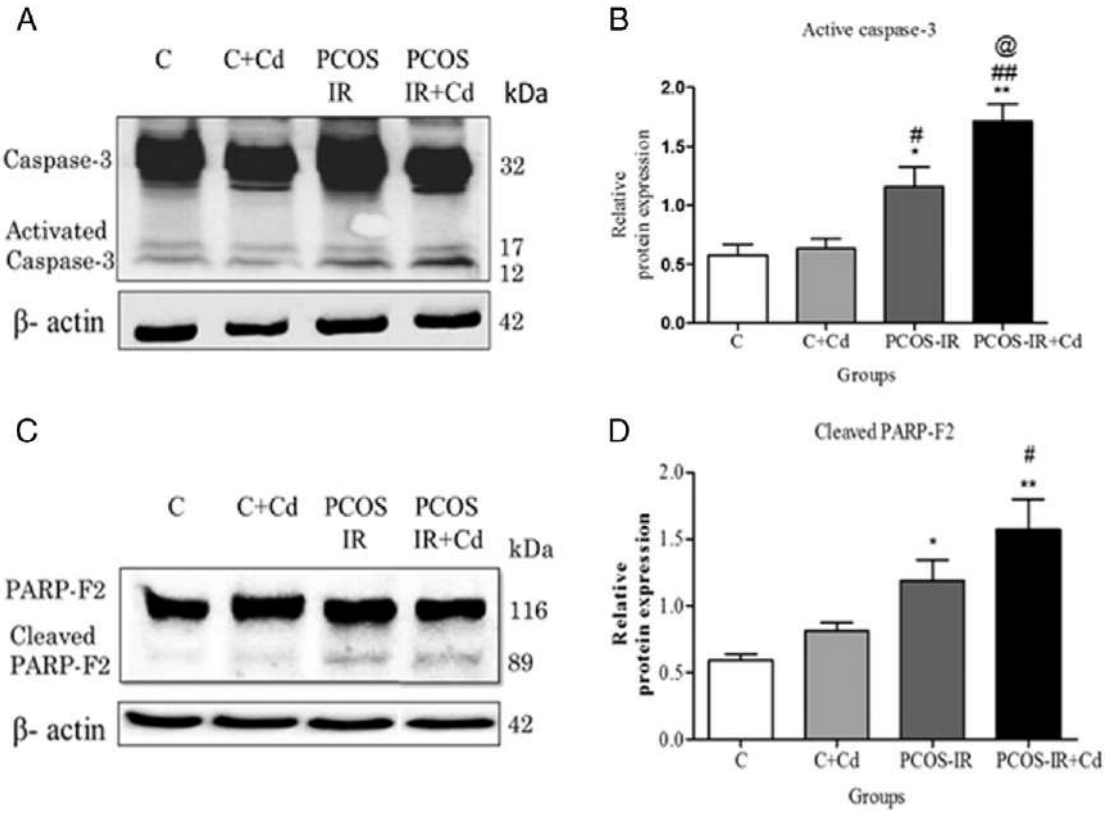

Fig. 5. Effect of 32 μM Cd on protein expression of cell death parameters in control and PCOS-IR granulosa cells for 24 h. A). Active caspase-3. B). Densitometry for active caspase-3. Significant increase in active caspase-3 was observed in PCOS-IR and PCOS-IR + Cd as compared to control and Cd ($^{*}P < 0.05$, $^{**}P < 0.01$ vs C and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs C + Cd). Within the groups PCOS-IR + Cd showed significant increase as compared to PCOS-IR ($^{@}P < 0.05$). C). PARP-F2. D) Densitometry for cleaved PARP-F2. Significant increase in cleaved PARP-F2 was observed in PCOS-IR and PCOS-IR + Cd as compared to control and Cd ($^{*}P < 0.05$, $^{**}P < 0.01$ vs C and $^{\#}P < 0.05$ vs. C + Cd). Data represented as mean ± SEM of 3 independent experiments (20 μg protein).

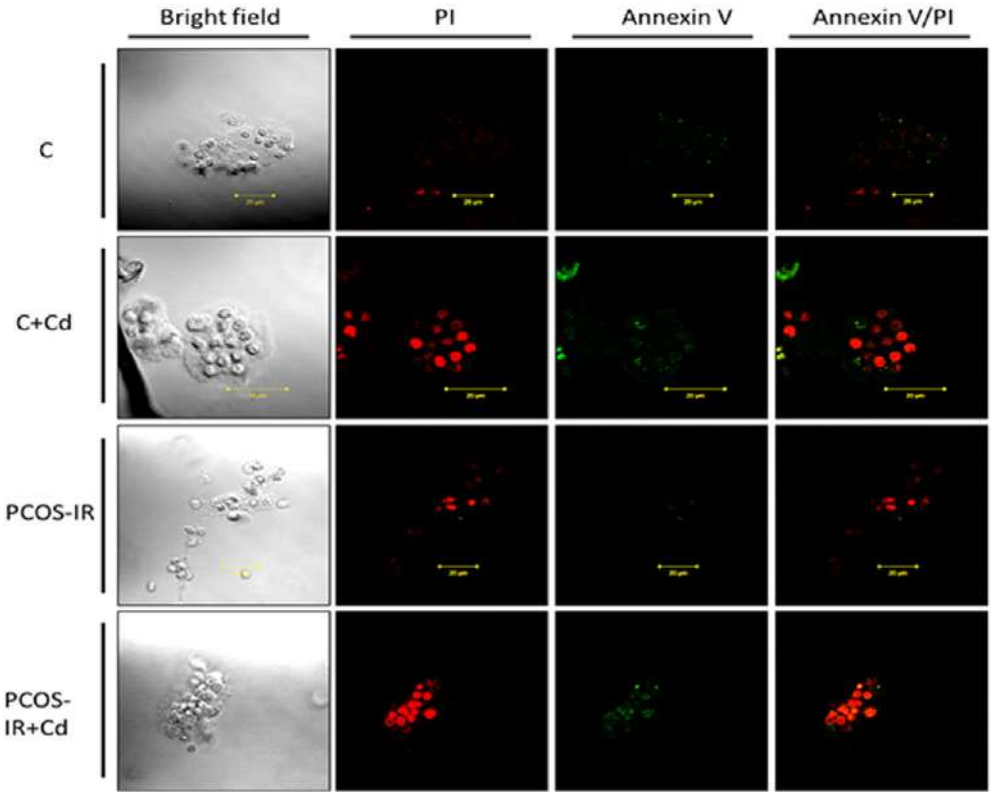

Fig. 6. Effect of 32 μM Cd on Annexin V and PI staining in control and PCOS-IR granulosa cells for 24 h. Positive staining of Annexin V-FITC indicated by green color and PI indicated by red color was observed in C + Cd, PCOS-IR and PCOS-IR + Cd groups. (For interpretation of the references to colors in this figure legend, the reader is referred to the web version of this article.)

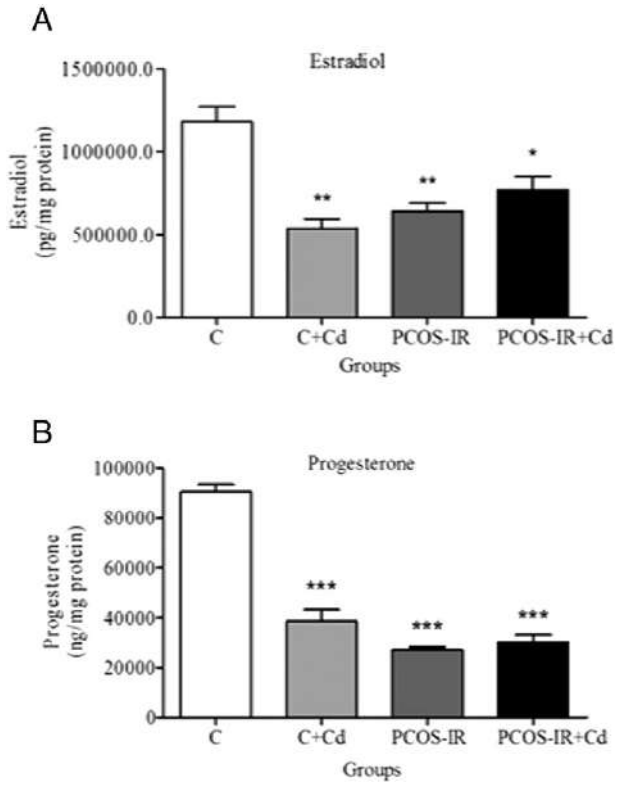

Fig. 7. Effect of 32 μM Cd on steroid hormone concentrations from supernatant of control and PCOS-IR granulosa cells for 24 h. A). estradiol. Significant increase in estradiol was observed in C + Cd, PCOS-IR and PCOS-IR + Cd as compared to control ($^{*}P < 0.05$, $^{**}P < 0.01$ vs C. B). Progesterone. Significant increase in progesterone was observed in C + Cd, PCOS-IR and PCOS-IR + Cd as compared to control ($^{***}P < 0.001$ vs C). Data represented as mean ± SEM of 3 independent experiments.

$P < 0.001$ and $P < 0.01$) in 17β-HSD activity as compared to control and ($P < 0.01$) when compared to alone Cd group with no significant difference between PCOS-IR and PCOS-IR + Cd. Similarly C + Cd, PCOS-IR and PCOS-IR + Cd groups demonstrated a significant decrease ($P < 0.01$) in the activity of 3β-HSD as compared to control with no significant difference within the groups (Fig. 9).

3.9. Effect of IR and Cd on expression of gonadotropin receptors genes in hLGC's

Gene expression of FSH-R and LH-R were analysed by quantitative real time PCR in control and PCOS-IR granulosa cells treated with Cd for 24 h. Real time analysis revealed significant decrease ($P < 0.001$) in mRNA expression of FSH-R and LH-R in C + Cd as compared to control. mRNA levels of FSH-R and LH-R were significantly higher in PCOS-IR ($P < 0.001$) as compared to control. However treatment with Cd demonstrated an antagonistic effect by significantly decreasing ($P < 0.001$) FSH-R and LH-R mRNA levels as compared to PCOS-IR. Within the groups PCOS-IR demonstrated a significant increase ($P < 0.001$) in mRNA expression of FSH-R and LH-R when compared to alone Cd. PCOS-IR + Cd demonstrated a significant decrease ($P < 0.01$) in mRNA expression of FSH-R and LH-R when compared to PCOS-IR (Fig. 10).

4. Discussion

Although as an individual agents Cd, an environmental disruptor and IR have been explored for their negative effect on the female fertility, their combination which might be more deleterious has not been studied. In our previous study, we demonstrated that combined effect of IR and Cd in animal model caused varied effects at different levels ultimately leading to disruption in steroidogenesis (Belani Muskaan et al., 2014). To make the study clinically more relevant, it was further studied in hLGC's isolated from follicular fluid during *in vitro* fertilization. Positive staining for 17β-HSD and 3β-HSD indicated the presence of

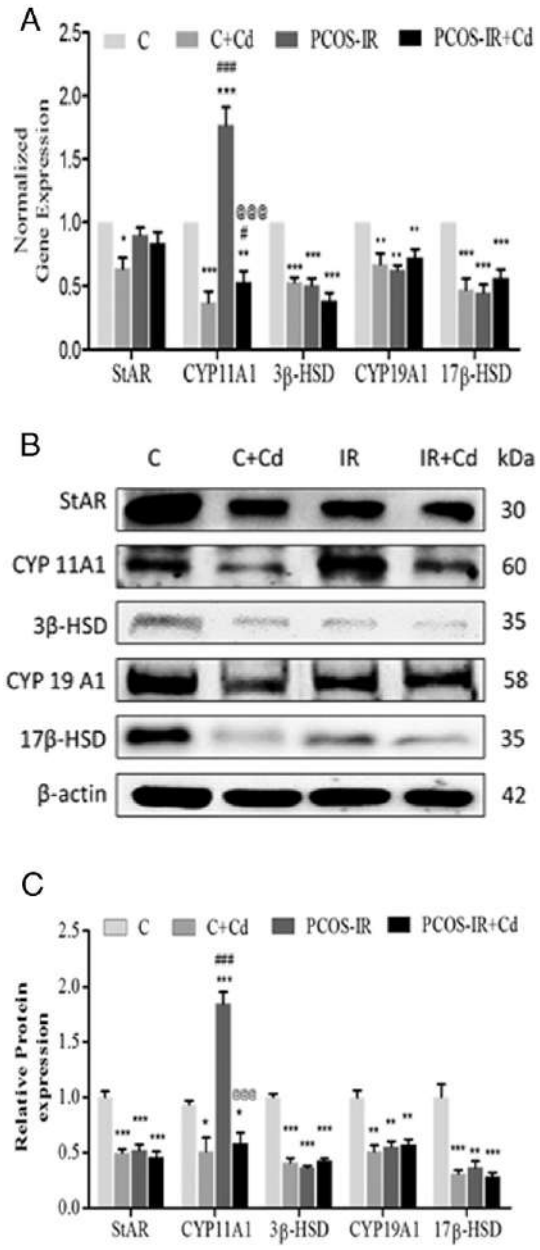

Fig. 8. Effect of 32 μM Cd on mRNA and protein expression of genes involved in steroidogenesis in control and PCOS-IR granulosa cells for 24 h. (A) mRNA expression of genes involved in steroidogenesis. Significant decrease was observed in mRNA expression of 3β-HSD, CYP19A1 and 17β-HSD in C + Cd, PCOS-IR and PCOS-IR + Cd group ($^{**}P < 0.01$, $^{***}P < 0.001$ vs. C) with no change within the group. Significant decrease was observed in mRNA expression of CYP11A1 in C + Cd ($^{**}P < 0.001$) and PCOS-IR + Cd ($^{**}P < 0.01$) and increase in PCOS-IR ($^{***}P < 0.001$) when compared to control. Significant decrease was observed in mRNA expression of StAR in C + Cd group as compared to control ($^{*}P < 0.05$). (B) Protein expression of genes involved in steroidogenesis. (C) Densitometric analysis for protein. The normalized expression values are represented as mean ± SEM of three independent experiments. Significant decrease was observed in protein expression of 3β-HSD, CYP19A1 and 17β-HSD in C + Cd, PCOS-IR and PCOS-IR + Cd group ($^{**}P < 0.01$, $^{***}P < 0.001$ vs. C) with no change within the group. Significant decrease was observed in protein expression of CYP11A1 in C + Cd ($^{*}P < 0.05$) and PCOS-IR + Cd ($^{*}P < 0.05$) and increase in PCOS-IR ($^{***}P < 0.001$) when compared to control. Significant decrease was observed in protein expression of StAR in C + Cd, PCOS-IR and PCOS-IR + Cd group when compared to control ($^{***}P < 0.001$). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. Cd, $^{@}P < 0.05$, $^{@@}P < 0.01$, $^{@@@}P < 0.001$ vs. PCOS-IR.

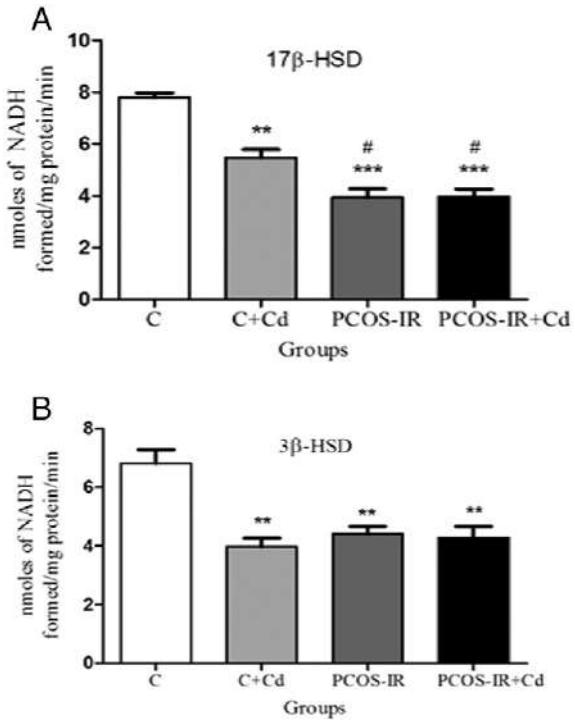

Fig. 9. Effect of 32 μM Cd on 17β-HSD and 3β-HSD activity in control and PCOS-IR granulosa cells for 24 h. A. 17β-HSD. Significant decrease was observed in 17β-HSD activity in C + Cd, PCOS-IR and PCOS-IR + Cd group ($^{**}P < 0.01$, $^{***}P < 0.001$) as compared to control and within the groups PCOS-IR and PCOS-IR + Cd ($^{\#}P < 0.05$) as compared to C + Cd. B. 3β-HSD. Significant decrease was observed in 3β-HSD activity in C + Cd, PCOS-IR and PCOS-IR + Cd group ($^{**}P < 0.01$) as compared to control. Data represented as mean ± SEM of 3 independent experiments.

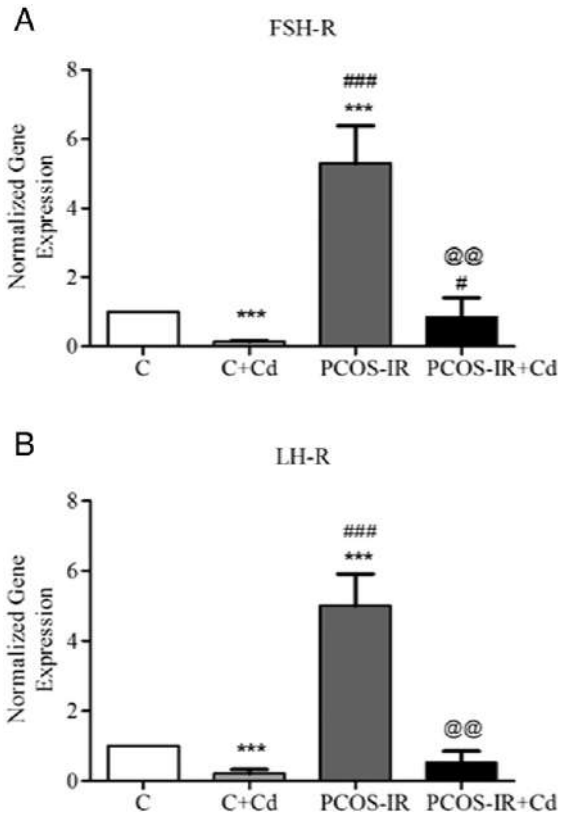

Fig. 10. Effect of 32 μM Cd on FSH-R and LH-R in control and PCOS-IR granulosa cells for 24 h. (A) mRNA expression of FSH-R. Significant decrease was observed in C + Cd ($^{***}P < 0.001$), increase in PCOS-IR ($^{***}P < 0.001$) when compared to control. Significant decrease was observed in PCOS-IR + Cd group as compared to PCOS-IR ($^{@@}P < 0.01$) and C + Cd ($^{\#}P < 0.01$). (B) mRNA expression of LH-R. Significant decrease was observed in C + Cd ($^{***}P < 0.001$), increase in PCOS-IR ($^{***}P < 0.001$) when compared to control. Significant decrease was observed in PCOS-IR + Cd group as compared to PCOS-IR ($^{@@}P < 0.01$). The normalized expression values are represented as mean ± SEM of three independent experiments.

steroidogenic proteins, thus characterizing the cells isolated from human follicular fluid as luteinized granulosa cells (Kossowska-Tomaszczuk et al., 2009).

Decrease in INSR protein during IR has been demonstrated in various insulin-target tissues such as liver, skeletal muscle, adipose tissue and kidney (Tiwari et al., 2007). The same has also been demonstrated in cultures of granulosa cells isolated from ovaries of PCOS-IR (Fedorcsak et al., 2000). In the present study, reduction in expression of INSR-β was observed in 64% of LGC's isolated from follicular fluid aspirates of PCOS whereas rest 36% did not show INSR-β reduction when compared to controls. This was in line with the literature claiming 60–80% of PCOS as IR and other PCOS as non insulin resistant (NIR) (Kaur et al., 2012). In light of our previous study with rats, IR and Cd demonstrated a deleterious effect (Belani Muskaan et al., 2014). Hence we intended to understand for the same condition on steroidogenesis and cell death parameters in hLGC's. Cd at micromolar concentrations has been observed to change the morphology and steroidogenesis of cultured human granulosa cells, in follicular fluid of cigarette smoking females and used to define environmental, occupational and smoking risk factors in female reproductive life span (Varga et al., 1993, Paksy et al., 1997). In the present study Cd at 32 μM concentration demonstrated significant decrease in granulosa cell viability in 24 h leading to its selection.

Amongst the somatic cells of the ovary, granulosa cells provide nutrients and maturation-enabling factors to ensure successful maturation, developmental competency and protection of oocytes (Tripathi et al., 2013). Along with our previous study in rat granulosa cells, other reports from literature have demonstrated decrease in granulosa cell number and change in its morphology with cadmium exposure (Paksy et al., 1997, Smida et al., 2004, Nampoothiri and Gupta, 2006). Hyperinsulinemia in PCOS implicated to decrease granulosa cell numbers relative to follicle size (Poretsky et al., 1999). Our data for reduced cell viability implies that Cd exposure along with IR alters granulosa cell viability more deleteriously in the co-exposed group.

Cleavage of PARP-1 into two fragments by execution caspase-3 is an important terminal event and is strongly implicated in the process of follicular atresia. Moreover increased reactivity of cleaved caspase-3 has been observed in the granulosa cells from ovarian cysts in the PCOS patients (Banu et al., 2011, Wei and Shi, 2013). These findings from the literature confirm the basal expression of activated caspase-3 in luteinized granulosa cells from control group in the present study. Cd induces apoptosis through activation of caspase-3 and cleaved PARP F2 the phenomenon which is mainly dependent on dose, exposure time, species specificity, type of primary cultures and cell lines (Zhao et al., 2015). The Cd dose and exposure time in the present manuscript might be insufficient for activating caspase-3 at a significant level thus setting rationale for no significant increase in cleaved caspase-3 in Cd group as compared to control. Insulin mediated PI3K signaling is an essential regulator of apoptotic pathways (Du et al., 2004; Banu et al., 2011). Hence during IR, reduced activation of insulin mediated PI3K leads to activation of caspase-3 in PCOS-IR and PCOS-IR + Cd groups terminating into cell death. This was also evident from the positive staining of Annexin V and PI in granulosa cells of C + Cd, PCOS-IR and PCOS-IR + Cd which correlated with activation of p38MAPK, ERK1/2, and c-Jun N-terminal kinase signaling. These kinases are known to induce proinflammatory cytokines such as TNF leading to apoptosis in PCOS ovaries (Jansen et al., 2004).

Luteinizing granulosa cells from PCOS ovaries lose the capacity of secreting progesterone and estradiol as compared to the normal ovaries

(Doldi et al., 1998; Chang, 2007). Cadmium is known to have endocrine disruptive effects on sexual steroid synthesis even at very low concentrations (Knazicka et al., 2015). In agreement with these studies we found a decrease in concentrations of estradiol and progesterone in cell culture supernatants in all the groups as compared to the control.

The StAR protein performs a critical function by delivering cholesterol to the inner mitochondrial membrane (Park et al., 2015). Free radicals generation by Cd could be a reason for decrease in expression of StAR as observed in the present study thus revealing anti-steroidogenic property of Cd (Gunnarsson et al., 2004; Gupta et al., 2004). Many studies have also demonstrated StAR as a potent target for Cd to bind and alter steroidogenesis (Paksy and Lázár, 1992, 1997; Zhang and Jia, 2007; Pillai et al., 2010). Although, not much difference was observed in gene expression of StAR, the decrease in StAR protein in IR condition seemed to be post-transcriptional, as observed by a lack of change in its mRNA. Further it indicated initiation of premature luteinization of granulosa cells as observed in polycystic ovaries (Willis et al., 1998; Jakimiuk et al., 2001; Petrescu et al., 2001). CYP11A1 and 3 β-HSD play a key role in the biosynthesis of progesterone. Cd decreases CYP11A1 and 3β-HSD by interfering with the DNA binding zinc finger motif through the substitution of Cd for Zn or by the cAMP-protein kinase A-dependent pathway which is the downstream pathway or by interfering with the phosphorylation of protein kinases (Kawai et al., 2002). Further, binding of Cd to the active site of enzyme decreases its enzyme activity (Sengupta, 2013), as observed in the present study. IR condition decreases the activity of enzymes involved in steroidogenesis (Belani Muskaan et al., 2014). Studies with human luteinized granulosa cells from PCOS have reported increased expression of CYP11A1 and decreased expression along with activity of 3 β-HSD (Doldi et al., 2000; Jakimiuk et al., 2001). All the above findings together indicate these as the possible mechanism for the decrease in progesterone synthetic pathway as observed in the PCOS-IR + Cd group in the present study.

CYP19A1 is a key enzyme for estradiol biosynthesis and for maintaining homeostasis balance between androgens and estrogens (Wang et al., 2011). 17β-HSD plays an important role in conversion of estrone to estradiol, the principle ovarian estrogen (Glister et al., 2012). Granulosa cells from PCOS begin to luteinize at premature stage of follicle development. These terminally differentiated cells lose their capacity to proliferate, thus leading to decreased expression of CYP19A1 as observed in the present study (Jakimiuk et al., 2001). Other than this presence of several proteins in PCOS follicular fluid such as high molecular weight FSH receptor binding inhibitor, inhibin-a subunit precursor, insulin-like growth factor binding proteins (IGFBPs), epidermal growth factor (EGF), tumour necrosis factor-a (TNF-a) and 5a-androstane-3,17-dione reflect that the physiological microenvironment is insufficient to induce the expression of CYP19A1 and 17β-HSD (Jakimiuk et al., 1998; Naessen et al., 2010). Moreover expression and activity of CYP19A1 and 17 β-HSD are found to be decreased in vivo in smokers and *in vitro* by exposure to Cd (Belani Muskaan et al., 2014). All these findings confirm the decreased expression of CYP19A1 and 17β-HSD as observed in PCOS-IR + Cd group in the present study.

FSH-R plays a major role during folliculogenesis by involving in the growth of antral follicles and LH-R plays a major role in ovulation for the release of oocyte. In the present study Cd concentration led to decrease in the gene expression of FSH-R and LH-R. Our data was in agreement with the previous studies demonstrating that GC's exposed to Cd showed decrease in the gene expression of FSH-R and LH-R along with the decrease in levels of cAMP in the testis of Cd treated rats (Gunnarsson et al., 2003; Yang et al., 2008; González-Fernández et al., 2011). Cd alters the gene expression and directly interferes with LH-R in a way that makes it non-functional or less functional than normal thus indicating more than one site for its drastic action (Gunnarsson et al., 2003). GC's from PCOS express high amounts of FSH-R and are highly responsive to FSH hormone in culture because of the amplification of the physiological phenomenon (Catteau-Jonard et al., 2008; González-Fernández et al., 2011). An increase in LH-R in GC's along with an exaggerated responsiveness to LH compared with granulosa cells in control follicles of similar diameter has also been observed in PCOS (Jakimiuk et al., 2001). These findings support our observation of an increase in gene expression of FSH-R and LH-R in PCOS GC's as

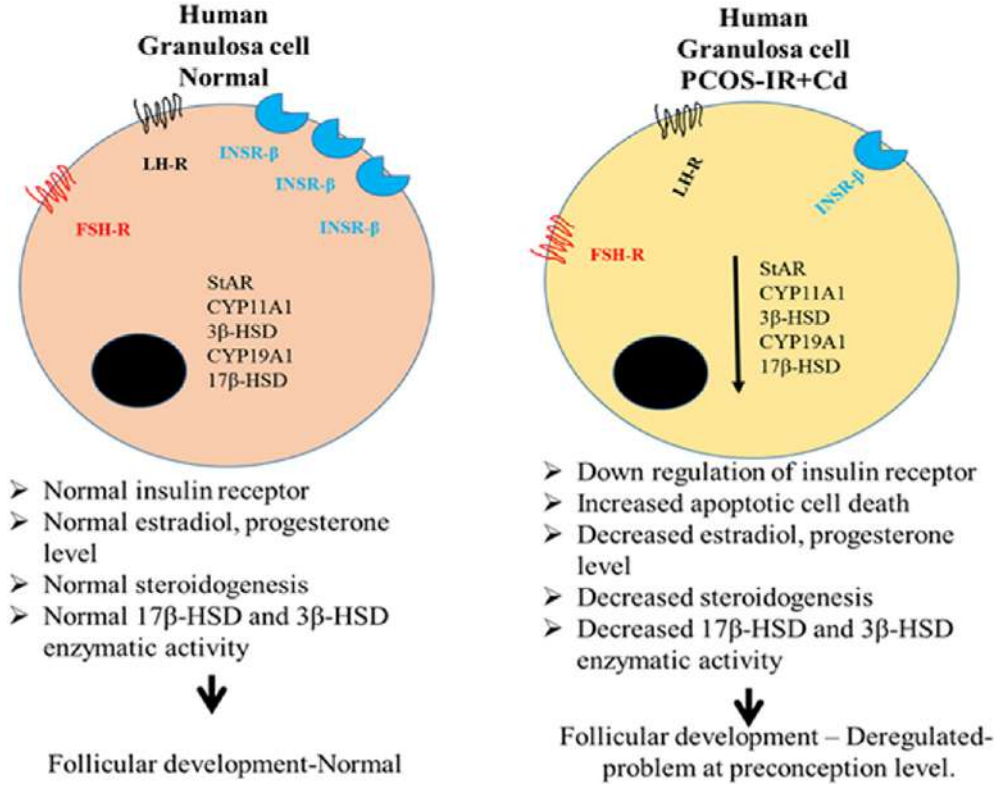

Fig. 11. Schematic diagram summarizing potential effect of Cd on human insulin resistant granulosa cells isolated from PCOS patients.

compared to control cells. Further decrease in gene expression of FSH-R and LH-R in the combined group as compared to IR group could be a result of more indicated adversity of Cd over IR.

Based on these studies, we propose that infertility observed is due to granulosa cell apoptosis with decreased steroidogenesis and deregulated responsiveness (Fig. 11). Thus IR and Cd both make PCOS patients more prone to follicular atresia leading to reduced rate of healthy oocyte production and thus fertilization.

Author's role

Belani. M - contributed towards conception and design, performance of the experiments, analysis and interpretation of the data and drafting of the manuscript. Shah. P and Banker. M - Contributed in providing follicular fluid samples and participated in discussion of the study. Gupta. S - Contributed towards conception and design, interpretation of the data and critical reviewing of the manuscript.

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Conflict of interest

The authors declare that there is no conflict of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

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