Chapter 3

Materials and methods

3.1 Hormones and reagents

Pregnant Mare Serum Gonadotrophin hormone (PMSG), Histopaque, Hyaluronidase, DMEM/F12, penstrep, amphotericin and trypan blue were from Sigma Chemical Co. (USA). All the other chemicals used were of analytical grade. Glucose estimation kit Glucose oxidase-peroxidase (GOD-POD) was from Reckon Diagnostics. Rat Insulin ELISA kit was procured from mercodia, human insulin, estradiol, progesterone and testosterone ELISA kits were procured from Diametra,

3.2 Antibodies

Rabbit polyclonal antibody against CYP19A1 and β-actin were purchased from Cell Signaling and Goat polyclonal antibody against CYP11A1 was purchased from Santa Cruz. Rabbit polyclonal antibodies against 3β-HSD, 17β-HSD and StAR protein were generous gift from Dr. Vann Luu-The (CHUL Research Center and Laval University, Canada) and Dr. Douglas M. Stocco (Department of Cell Biology and Biochemistry, Texas Tech University, Lubbock, Texas, USA) respectively. Rabbit antibody against PKC ζ, P38 MAPK and ERK ½ MAPK were a generous gift from Prof. R. K. Rao (University of Tennessee, Memphis, USA). Antibody against IR-β, p-IRS (ser-307), PPAR-γ, PI (3)K, p IRS(ser 307),p AKT, PKC ζ and β-actin were from cell signaling. For details refer Table 2.

3.3 Animals and Experimental Design

Adult virgin female *Charles Foster* rats weighing 180–220 g were used for this study. Animals were maintained under standard laboratory conditions (temperature: 24±2°C; light: 12-h light/12-h dark). They were given standard pellet diet and water ad libitum throughout the experimental period and were monitored regularly for each stage of estrous cycle. Animals were divided into four groups with 6 animals in each group. The dosage of cadmium (0.05mg/kg b.w) and dexamethasone (3mg/kg b.w) was selected on the basis of previous reports (Pillai et al. 2002; Nampoothiri and Gupta 2006; Gao et al. 2007). After 28 days of daily dexamethasone treatment, animals were monitored for IR status by FIRI. Those animals showing IR were chosen for the study and sacrificed at proestrus stage after superovulation induction with pregnant mare

serum gonadotropin (PMSG) - 10 IU. and human chorionic gonadotrophin (hCG -100 IU.). The experimental study was approved by the Animal Ethical Committee of the Department of Biochemistry, The M.S. University of Baroda, and was in accordance with the CPCSEA norms.

3.4 Selection of IR animals and confirmation of IR

Standard oral glucose tolerance test was performed after 28 days of dexamethasone injection to monitor the development of hyperglycemia in the animals. After 12 hour fasting 300µl of blood was collected from retro orbital sinus for glucose and immunoreactive insulin measurement. For oral glucose tolerance test (OGTT) 2 gm/kg body weight glucose was administered and 100 µl blood was collected at 30, 60, 90, and 120 min. The blood was subjected to 4000 rpm for 10 min and serum was separated. Glucose was estimated from serum using GOD-POD kit as per the manufacturers instructions. Fasting serum was then proceeded for determining insulin levels using Rat Insulin ELISA kit according to manufacturers protocol (Mercodia, Germany). Further to monitor IR in these hypergycemic animals, Fasting insulin resistance index (FIRI), a measure of the insulin sensitivity was calculated according to the formula,

$$\text{FIRI} = \frac{\text{Fasting serum insulin (µIU/ml)} \times \text{Fasting serum glucose (mmol/L)}}{25}$$

FIRI has been shown to have a better correlation with insulin sensitivity values than the fasting glucose/insulin ratio giving a reference index value as shown in Table 1 (Duncan MH 1995).

3.5 Estrous cyclicity

Estrous cyclicity was monitored throughout the dosage schedule. Vaginal lavages from female rats were obtained and viewed under a microscope daily (between 0900-1000 h) for atleast three consecutive cycles. A normal estrous cycle was defined as exhibiting vaginal cytology that was leukocytic (Diestrus) for 2 days followed by nucleated (Proestrus) for1 day, cornified (Estrus) for 1 day and mixed cells (Metestrus) for 1 day.

3.6 Histological analysis

One ovary from each group was removed and stored in 10% formaldehyde and then proceeded for sectioning and hematoxylin and eosin staining. Histological observations were made microscopically. The slides were observed for mature follicle, fibrosis, and other morphological changes.

3.7 Granulosa cell isolation from rat

Granulosa cells were isolated at proestrus stage from the rat ovary as explained earlier (Campbell 1979). Briefly, ovaries were removed from animals and kept in Hanks Balanced Salt Solution (HBSS) and centrifuged at 1000rpm, 4°C to remove all the fat. The ovaries were then incubated in EGTA-BSA solution for 15 min at 37°C followed by centrifugation at 1000 rpm for 5 min. Samples were then incubated in hypertonic sucrose solution for 5 min at 4°C and then centrifuged at 1500rpm. The granulosa cells were expressed from ovary in HBSS by blunt spatula and then washed three times with HBSS-EGTA by centrifugation at 1500rpm for 5 min. The viability of cells was analysed at final stage by trypan blue exclusion dye method.

3.8 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 IVF Clinic, Ahmedabad, India from 2012 August TO 2015 April. All the controls and patients underwent controlled ovarian hyper stimulation (COH) using flexible antagonist protocol. Recombinant FSH & / or urinary human menopausal gonadotropin (hMG) was started from second day of period followed by the antagonist (cetrorelix acetate). Final oocyte maturity was triggered with recombinant hCG or GnRH agonist. Oocyte retrieval was performed after 35 hours of trigger injection. Follicular fluid was sent in embryology laboratory for oocyte identification & oocytes were separated out for IVF/ICSI. The follicular fluid devoid of oocyte was collected for the experiments. 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 was collected on the day of oocyte retrieval.

Inclusion criteria: The diagnosis included donors, male factor infertility, tubal factor infertility and PCOS with an age ranging from 20–40 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).

3.9 Human granulosa-luteal cell isolation and culture

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. Follicular aspirates from individual patients n= 30 control and n= 40 PCOS were centrifuged at 300 g at room temperature. Human granulosa cell were isolated using the protocol in the literature (Földesi et al. 1998). Briefly, blood contaminants were removed from GCs by Histopaque gradient centrifugation at 400 g at room temperature. The middle layer of cells was then collected and resuspended in 10 ml volume of medium and washed twice by a further 5 min centrifugation. The GC pellet was then incubated with 0.1% hyaluronidase in DMEM/F12 medium without FBS for 30 mins at 37 deg c with constant shaking and gentle repeated pipetting to break up cellular clumps. The enzymatic reaction was stopped by addition of DMEM/F12 with FBS and centrifugation at 300 g at room temperature. The cell stock was finally resuspended in 1 ml of DMEM/F12 with FBS. The viable cell count by trypan blue dye exclusion was observed to be 90%. 0.5 x 10^6 cells from each patient were aliquoted for protein expression of INSR-β. Rest of the cells were cryopreserved for further use.

3.10 Immunocytochemistry

Isolated cells from follicular fluid samples were characterized by immunocytochemistry. 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 mins 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 over night with primary antibody (17β-HSD and 3β-HSD) at a dilution of 1:100 at 4°C. After 5 washes with washing buffer the cells were incubated in secondary antibody (Goat anti-rabbit IgG-FITC) at a dilution of 1:250 for 1 hr at R.T. After 5 washes with washing buffer nuclear staining was done with 100ng/ml DAPI for 5min. 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.

3.11 Atomic absorption spectroscopy (AAS) for Cd analysis

The levels of Cd in follicular fluid of patients and controls were analysed by AAS. Total 5 samples including 2 control and 3 PCOS were outsourced to Vasu Health Care Ltd, Baroda for the estimation. For validation of the experiment two follicular fluid samples were spiked with known concentration of Cd.

3.12 Measurement of Cd toxicity

The enzyme succinate dehydrogenase synthesized by the mitochondria of live cells reduces 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) and converts it into yellow formazan. Therefore, the production of formazan is proportional to the number of viable cells, which was used to represent granulosa cell viability in the present study. Granulosa cells were seeded in 96-well plate at a density of 1x 10^4/200µl /well in DMEM/F12 with 10% FBS for 48 hrs. Following addition of Cd acetate at a concentration ranging from 0-64µM for 24, 10 µL of MTT solution containing 5 mg/mL thiazolyl blue MTT was added to each well. The cells were cultured for a further 4 h and the optical density (OD) of the yellow color was measured at 490 nm by using a Multiskan microtiter plate reader from Thermo Scientific. Each treatment was repeated by 3 triplicate wells, and then repeated by 3 independent experiments.

3.13 *In vitro* culture of hLGC with Cd

After confirming for the presence of INR-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 adequate number of them for all the experiments and were demarcated as control group. Whereas luteinized granulosa cells isolated from follicular fluid aspirates of PCOS that showed down regulation of INR-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 hr and then incubated in fresh medium with or without 32 µM of Cd acetate for 24 hrs. Thus for chapter 5 we made four different groups: control, control + Cd, PCOS-IR and PCOS-IR+Cd. The supernatant was collected and freezed 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.

3.14 Human granulosa cell culturing with bioactives

hLGC's were isolated from n=8 control, PCOS-IR and PCOS-NIR and were pooled in respective groups. The cells were initially cultured at a density of 0.5 x 10^6 cells in 3ml of DMEM/F12 culture medium supplemented with 10% FBS in a 6-well plate at 37 ° C and 5% CO_2 in a humidified incubator for 48 h with no other treatment. After this, the media was removed and cells washed with PBS to remove any traces of red blood cells and the cells were then incubated in serum free culture medium with or without swertiamarin (66 μM), curcumin (33 μM) and positive control- metformin (1mM) for additional 72 hrs. The doses were selected based upon the studies in literature (Sonntag et al. 2005; Patel 2015; Qin et al. 2015). The supernatant was collected for hormone analysis and cells harvested for gene and protein expression and enzyme activity.

3.15 Total RNA Extraction, RT-PCR and qRT-PCR

For the experiments in rat model total RNA was isolated from granulosa cells, hypothalamus and pituitary whereas for humans RNA was isolated from follicular fluid granulosa cells and cultured granulosa cells by using TRIzol, (Sigma-Aldrich, USA). Purity of RNA was confirmed by *A*260/280 ratio and checked for integrity. 2μg of total RNA was reverse transcribed into first strand cDNA. cDNA for different genes was subjected to PCR or qRT PCR. mRNA expression of rat steroidogenic genes and cell death parameters were analysed by RT-PCR, primers for the same mentioned in table: 5. mRNA expression of GnRH and CYP19A1 in hypothalamus and FSH-β and LH-β in pituitary were analysed by qRT-PCR, primers for the same mentioned in table 5. For RT-PCR, gradient PCR was performed with a range of annealing temperature from 51 to 60°C. cDNA was amplified for 35 cycles using Fermentas 2x master mix (1.5 unit Taq Polymerase, 2mM dNTP, 10x Tris, glycerol reaction buffer, 25mM$MgCl_2$) with 20 pM forward and reverse primer PCR products were separated on a 15 % polyacrylamide gels (Sigma-Aldrich, USA) and visualized and images were captured with Alpha Imager software (UVP Image Analysis Software Systems, USA) for densitometric analysis.

For human studies, mRNA expression of lipogenic genes (Table: 7), IGF system, gonadotropins and their receptors (Table: 8) 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 (Table: 6) by real time PCR (Applied Biosystem 7500 FAST Real Time PCR Sequence detection System) for steroidogenic gene expression.

For real time analysis, c-DNA was amplified for 40 cycles using 5µl Power SYBR-Green master mix, 10pM of each forward and reverse primers and 100ng cDNA (1/20th of total cDNA preparation) in (Applied Biosystems, 7500-Real-Time PCR Sequence detection System). All qRT-PCR results were normalized to the level of β-actin determined in parallel reaction mixtures to correct any differences in RNA input. For FAST, 10µl of total reaction volume containing 5µl Taqman FAST master mix, 0.5 µl of 20X predesigned Taqman gene expression assays (Applied Biosystems) using 100ng 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.

Fold changes in qRT-PCR gene expression was analyzed 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.

3.16 Western blot analysis

Granulosa cells isolated from rat, human follicular fluid and human granulosa cells from culture were suspended in 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 and subjected to sonication on ice. Total protein content was quantified using Bradford assay (Biorad 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 electrophoretically transferred onto a nitrocellulose membrane (GE Healthcare) by a wet method (Bio rad, USA). The transfer was performed at a constant voltage (100V) 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).Then, the membranes were incubated overnight at 4°C with appropriate antibody dilution (Table: 2) in PBS- containing 5% skimmed milk and 0.1% Tween-20. They were washed in PBS-0.1% Tween-20 for 5 times, incubated for 1 h at room temperature with a horseradish peroxidase-conjugated anti-rabbit or anti-goat or anti-mouse IgG from Bangalore Genei (Table: 2) in PBS- containing 5% skimmed milk and 0.1% Tween-20. They were again washed 5 times with PBC-0.1% Tweeen-20 followed by 2 washes in PBS. The signal was detected by Ultra-sensitive enhanced chemiluminiscence reagent (Millipore, USA) and image captured by Alliance 4.7 UVI Tec chemidoc.

3.17 Estimation of hydroxysteroid dehydrogenase activity

17β-HSD and 3β-HSD activities were estimated in granulosa cells of rats and humans following Shivanandappa &Venkatesh (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.

3.18 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 1000rpm for 5 min. The pellet was then reconstituted in cold 1X binding buffer, 2 µL of Annexin V-FITC and 10 µL of 20µg/ml propidium iodide (PI) was added and incubated at room temperature for 15 min in dark. After the incubation, cells were washed once with 1X binding buffer at 1000rpm for 5 mins and resuspended in 100 µL 1X 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.

3.19 Follicular fluid and hLGC culture hormone analysis

The steroid hormones were measured in the follicular fluid devoid of cells and in the culture medium by enzyme-linked immunosorbent assay (Diametra; Italy), according to the

manufacturer's instructions. The standard curve for E2, P4 and T ranged from 0 to 2000 pg/mL, 0 to 40 ng/ml and 0 to 16 ng/ml respectively. The supernatants were diluted to 1: 1000 for E2 and 1: 250 for P4 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 E2 and P4 concentration was calculated by multiplying the end value by the dilution factor. The assay sensitivity range was 8.68pg/ml for E2, 0.05ng/ml for P4 and 0.07ng/ml for testosterone.

3.20 Statistical Analysis

The results are presented as mean ± standard error mean. The data were statistically analyzed 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.

3.21 References:

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