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Synergistic Interplay of Hyperandrogenism and Hyperinsulinism on Primary Culture of Luteinized Granulosa Cells – an "*in-vitro*" Model Mimicking Ovarian Microenvironment of Poly-Cystic Ovary Syndrome (PCOS)

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Abstract

Poly-Cystic Ovary Syndrome (PCOS) is the most prevalent endocrine disorder, characterized by hyperandrogenism and hyperinsulinemia, both at systemic and ovarian levels. This study investigated the synergistic effect of hyperinsulinemia and hyperandrogenism on the regulatory mechanism of ovarian steroidogenesis using Luteinized Granulosa Cells (LGCs). LGCs were isolated from 40 weaning female Charles Foster rats by superovulation by PMSG and characterized for purity and stability in modified DMEM: F12 media. The isolated cells were divided into following groups- control, hyperinsulinic group (0.1-2 mIU/mL of insulin), excess androgen (10-100 ng/mL of DHT) and combination of both. One-way ANOVA was performed with a Bonferroni post-hoc test. Results demonstrate that the LGCs exhibit reduced expression of FSHR and CYP19A and increased expression of LHR, StAR and CYP17A1 at 72 hours. There was reduction in cell viability of LGCs when induced with hyperinsulin and hyperandrogen doses individually or in combination.0.1 mIU/mL of insulin and 50 ng/mL of DHT in combination were the minimum effective dose in inducing PCO like ovarian microenvironment in the primary culture of LGCs. There was exaggerated androgen biosynthesis, reduced progesterone secretion and non-significant change is estradiol levels in the LGCs. The abnormal steroidogenesis can be attributed to upregulation of key genes such as StAR, CYP17A1, AMH and SREBP1-c and down-regulation of genes like CYP19A1, HSD3B2, IGFBP1 and SHBG. This model can be used to study downstream signaling pathways involved with dysregulated ovarian microenvironment as observed in PCOS at cellular level and for screening of drug targets for such pathological conditions.

Keywords: Hyperinsulinemia, Hyperandrogenemia, Luteinized Granulosa Cells, Polycystic Ovary Syndrome, Primary Culture, Steroidogenesis

1. Introduction

Poly-Cystic Ovary Syndrome (PCOS) is defined by the presence of polycystic ovaries, chronic anovulation and hyperandrogenism, leading to metabolic syndrome and infertility. Pathological etiologies of this syndrome are extremely complex as there is link between the intrinsic and extrinsic factors further leading to complication of hyperandrogenemia and hyperinsulinemia[1]. It is reported that women suffering from PCOS exhibit selective insulin resistance, i.e., they have defective insulin signaling due to enhanced insulin receptor (IR) and insulin receptor substrate-1(IRS-1) serine phosphorylation[2]. Also, there is excess insulin production in these women

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due to hyper secretion of Luteinizing Hormone (LH), thereby, stimulating IR and Insulin like growth factor 1 receptor (IGFR1) further leading to perturbed ovarian steroidogenesis[3]. The serum levels of insulin are increased in PCOS women[4] along with an increase in the follicular fluid[5]. Hyperinsulinemia reinforces local insulin-like growth factor (IGF)-I activity in the ovary and, as a result, this induces an androgenic composition in theca cells[6]. Both increased IGF-I activity and a high androgenic environment appear to be involved in the ovulation disorder in PCOS patients[7].

Another important cause of hyperandrogenism in PCOS is modulation of LH and over expression of Luteinizing Hormone Receptors /Human Chorionic Gonadotropin Receptor (LHR/hCGR), further rendering them to be hypersensitive to LH stimuli along with hypersecretion of androgens in theca cells[8]. In addition to this, reduction in inhibition of Gonadotropin Releasing Hormone (GnRH) pulse frequency by progesterone, promotes the development of the PCOS phenotype[9]. Recent studies have demonstrated that 65% of PCOS patients exhibit high titres of androgens, reduced estrogen-to-testosterone (E2/T) ratio as well as insulin resistance in the follicular fluid in spite of having normal serum androgen levels[10]. Normal physiologica androgen levels in follicular fluid is found to be 5-20 ng/mL[11], whereas clinical samples of PCOS patients are known to have 10-100 ng/mL androgen levels in the follicular fluid[12]. The microenvironment of the ovary critically influences the follicular development and hence, it is still unclear as to how hyperandrogenemia and hyperinsulinemia present in the PCOS ovarian microenviroment synergistically causes ovarian dysfunction associated with PCOS. Therefore, it would be interesting to develop an *"in vitro"* model wherein, there is co-exposure of insulin and androgen, which would allow us to study independent as well as cross-talk events at cellular levels. The present study has attempted to develop an *"in vitro"* ovarian microenvironment model of PCO phenotype.

2. Materials and Methods

2.1 Chemicals

Pregnant Mare's Serum Gonadotropin (PMSG), Insulin (40IU), Bovine Serum Albumin (BSA), 5-Androsten-3b-ol-17-one sulfate sodium salt and Transferrin were procured from Sigma Aldrich India. DMEM: F12 (1:1) and Penstrep solution were procured from GIBCO®. Human Menopausal Gonadotrophin (HMG) [HUMOG®-150 IU] was procured from Bharat Serums and Vaccines Limited. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), EGTA [ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid], sucrose, trypan blue and di-methyl sulf-oxide (DMSO) were procured from HiMedia Laboratories Pvt. Ltd. Hank's Balanced Salt Solution (HBSS) and Phosphate Buffered Saline (PBS) were prepared manually in the laboratory using the standard recipe. Sterile cell culture plastic wares were procured from Corning Inc. RNAiso Plus was procured from Takara Inc. High-Capacity cDNA Reverse Transcription Kit was procured from Applied Biosystems. Primers for key steroidogenic and metabolic genes were designed by primer blast tool of NCBI and synthesized by INTEGRATED DNA TECHNOLOGIES (IDT). Hormones- testosterone, estradiol and progesterone were assayed using direct ELISA kits (DBC Canada).

2.2 Animals

Forty weaning Charles Foster female rats were chosen for the study which were housed in standard controlled animal care facility, in cages (four rats/cage), and maintained in a temperature-controlled room (22-25°C, 45% humidity) on a 12: 12-hour dark-light cycle. The animals were maintained under standard nutritional and environmental conditions throughout the experiment. All the experiments were carried out between 9:00 and 16:00 hours, at ambient temperature. The CPCSEA guidelines were strictly followed and all the studies were approved by the Institutional Animal Ethics Committee (IAEC), Department of Biochemistry, The M. S. University of Baroda, Vadodara (Ethical Committee Approval Number (MSU/BIOCHEMISTRY/IAEC/2018/06)).

2.3 Luteal Granulosa Cell (LGC) Isolation from Rat Ovaries

Forty weaning female rats were super-ovulated in batches of four animals, by injecting 10 I.U of pregnant mare's serum gonadotropin (PMSG) subcutaneously. After 48 hours, 50 I.U of Human Chorionic Gonadotropin (hCG) was injected to the animals intra-peritoneally (ip). After 24 hours, the ovaries from four super-ovulated rats were excised and processed for isolation of granulosa- lutein

cells by the Campbell method[13]. In this procedure, ovaries were initially punctured and then incubated in EGTA followed by hypertonic sucrose at 37°C. The ovaries were then gently squeezed to release the cells into Hank's Balanced Salt Solution (HBSS) and further centrifuged over 45% Percoll to remove RBCs. Luteal cells were isolated from the interface, washed 3-4 times with HBSS and resuspended in 1 mL of modified serum- free DMEM/F12 media (Media Composition: DMEM/ F12 (1:1), 0.1% BSA, Insulin (10 ng/mL), hMG (50 ng/mL), 5 µg/mL Transferrin, 1% Penstrep (100 U/mL Penicillin, 100 µg/mL Streptomycin) and the cell yield was estimated using trypan blue exclusion method[14].

2.4 Characterization of Isolated Luteinized Granulosa Cells

Cultures of luteinized granulosa cells were tested for cell type purity by analysis of the transcript abundance of key molecular targets such as Follicle-stimulating Hormone Receptor (FSHR), Luteinizing Hormone Receptor (LHR), Steroidogenic Acute Regulatory protein (StAR), Cytochrome P450 family 19 subfamily a member 1 (CYP19A1) and Cytochrome P450 Family 17 subfamily A member 1 (CYP17A1). The crude cell isolates were counted with a hemocytometer and diluted to 1×10^5 cells/ mL and cultured in 1.0 mL of modified media in 35-mm polystyrene tissue culture treated dishes. Cells were collected at different time intervals of 24 hours upto 72 hours and their RNA was extracted using RNAiso Plus reagent and reverse transcribed to form cDNA. The relative expression of the molecular targets mentioned above was studied by Real-time quantitative Polymerase Chain Reaction (qPCR). The details of RNA isolation, cDNA preparation, primers and Real time qPCR are explained below.

2.5 Culture and Development of PCOS Conditions in Luteinized Granulosa Cells Primary Culture

1×10^6 cells were cultured in modified serum- free DMEM/ F12 medium and the culture conditions consisted of humidified atmosphere of 5% CO2 and 95% air at 37^0C for 24 hours. Thereafter, the cells were incubated with insulin [0.1 – 2 IU/mL (0.7 – 13.9 nM)] and Di-Hydro-Testosterone (DHT) [10 – 100 ng/mL (34.4 – 344 µM)] treatments separately as well as in combinations for 36 hrs. The concentrations of insulin and DHT used in these experiments were significantly greater than the concentrations present in the normal women. However, the concentrations used in these experiments were within an order of magnitude of the concentrations present in the patients with severe insulin resistance[15]. In order to exclude the effect of conversion of testosterone to estradiol, we utilized DHT, instead of testosterone, as the androgen for assessment of the appropriate AR action. At the end of the treatment period, spent medium was collected at different time intervals and stored at -20°C until assayed for hormonal content. For gene expression analysis, treated and control cells were lyzed for RNA extraction.

2. 6 Cell Viability Assay by MTT

Cells were seeded in a 96-well flat-bottom microtiter plate at a density of 1×10^4 cells/well and allowed to adhere for 24 hours at 37°C in a CO2 incubator. After 24 hours of incubation, the culture medium was replaced with a fresh medium. Cells were then treated with various concentrations of insulin and DHT for 36 hours at 37°C in a CO2 incubator. After 36 hours of incubation, the culture medium was replaced with a fresh medium and 10µL of MTT working solution (5 mg/mL in phosphate buffer solution) was added to each well and the plate was incubated for 2-3 hours at 37°C in a CO2 incubator. The medium was then aspirated, and the formed formazan crystals were solubilized by adding 100µL of DMSO per well for 30 min at 37°C in a CO2 incubator. Finally, the intensity of the dissolved formazan crystals (purple color) was quantified using the ELISA plate reader at 570 nm and 620nm.

2.7 RNA Isolation and Real time qPCR

Total RNA was obtained using RNAiso Plus reagent as per the manufacturer's instructions. The quantification was performed using NanoVue Plus spectrophotometer (GE Healthcare Life Sciences) at a wavelength of 260nm. RNA integrity was assessed by electrophoresis in a 1.2% agarose gel stained with ethidium bromide. Purity was assessed through absorption rate OD260/OD280 and samples showing a value less than 1.8 were discarded. The reverse transcription reaction to cDNA was performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to manufactures instructions. Real-time qPCR was performed using

QuantStudio 5 Real Time PCR system using SYBR Green (Power SYBR Green PCR Master Mix - Life Technologies, USA). All samples were run in triplicate and accompanied by a non-template control. Thermal cycling conditions included initial denaturation in one cycle of 2 min at 95°C, followed by 40 cycles of 15s at 95°C, 1 min at 60°C and 1 min at 72°C. After amplification, the melting curves were analyzed to verify the amplification of only one product. The relative mRNA expression and fold change was calculated based on the amplification of the reference gene beta act in (ACTB). The primers used for the amplification are given in Table 1. The fold changes in expression levels of less than 0.5 and greater than 2 were considered to be biologically significant.

2.8 Measurement of Hormone Levels

Spent media were collected after giving treatments and lyophilized and then dissolved in Phosphate buffered saline. It was used as samples to estimate the testosterone, estradiol and progesterone levels using kit-based direct ELISA, procured from Diagnostics Biochem Canada (DBC) as per the manufacturer's instructions. Each sample was assayed in duplicate. Sensitivity of the kits was 0.022 ng/mL, 10 pg/mL and 0.1 ng/mL for testosterone, estradiol and progesterone kits respectively. The working range was 0.08 to 116.7 ng/mL, 20 to 3200 pg/mL and 0.3 to 60 ng/mL of testosterone, estradiol and progesterone respectively. The intra-assay Coefficient of Variation (CV) was between 6.6% and 9.6%, 4.6% and 9.3% and 10.2% and 10.6% for testosterone, estradiol and progesterone kits respectively. The inter-assay CV was between 6.1% and 7.3%, 6.2% and 10.1% and 10.2% and 12.6%, for testosterone, estradiol and progesterone kits respectively. The recovery range was between 80.5% and 110.1%, 90.3% and 116.2% and 78% and 124% for testosterone, estradiol and progesterone kits respectively.

2.9 Statistical Analysis

The values are presented as mean ± standard error of mean in all the experiments. Statistical analysis was done using one-way analysis of variance followed by Bonferroni multiple comparison test (GraphPad Prism 5 software, La Jolla, CA). The p values when less than 0.05 were considered to be statistically significant at 95% confidence limit.

Table 1. List of primers used in the study along with the amplicon size.

Gene	Accession Number	Sequence (5' →3')	Product Size	Annealing Tempera-ture
FSHR	NM_199237.1	F:ACGCCATTGAACTGAGGTTT	148	58
		R:TTGGGTAGGTTGGAGAACAC		
CYP19A1	NM_017085.2	F:CCTGGCAAGCACTCCTTATC	199	58
		R:CCACGTCTCTCAGCGAAAAT		
CYP17A1	NM_012753.2	F: ATGATCCAAAACTGACCGCC	132	60
		R:AACCCTTTATCACCTCCAAGCC		
HSD3B2	NM_017265.4	F:GGATCCTTTGCAGAGACCAG	147	58
		R:TGGAGATGCTCAGCCACA		
SHBG	NM_012650.1	F:TATTCTGAGCCACTGGGT	153	60
		R:GAGCACTCTGGATAGGGTCAAT		
SREBF1-c	NM_001276708.1	F: TCAGTTCCAGCATGGCTACC	174	60
		R:TGGGAAGGGGTCTCTCAGTT		
IGFBP1	NM_013144.1	F:CACAGCAAACAGTGCGAGAC	162	60
		R:GGAGGGAGGAAACAACTTTCAG		
StAR	NM_031558.3	F:AGTGACCAGGAGCTGTCCTA	216	58
		R:GCGGTCCACCAGTTCTTCATA		
ACTB	NM_007393	F: ACTGTCGAGTCGCGTCC	88	60
		R:TCATCCATGGCGAACTGGT		

3. Results

3.1 Isolation and Characterization of Luteinized Granulosa Cells

Results demonstrate that $10.94\times10^6 \pm 0.7708$ cells were obtained when ovaries from four animals were pooled. The isolated cells were further cultured for 72 hours and their characteristics such as cell viability and molecular markers were analyzed upto 72 hours. Cell viability assay by MTT demonstrated that around 50% of luteinized granulosa cells (LGCs) were viable up to 72 hours (Figure 1). There was a time-dependent decline in the cell viability which was not perfectly linear. The results were further confirmed using trypan blue exclusion assay. Characterization of the isolated LGCs (Figure 2) demonstrated the presence of FSHR and CYP19A1 in the isolated cells up to 72 hours suggesting that the isolated cells were indeed granulosa cells. However, the expression of FSHR decreased over time, which might be attributed to the fact that the cells might be undergoing luteinization. In addition to this, there was an increase in the expression of LHR, StAR and CYP17A1 from 48 hours onwards, suggesting that the isolated granulose cells have undergone luteinization. The LGCs are essential for normal oocyte development and steroid hormone production. Therefore, they are a good model for the current study.

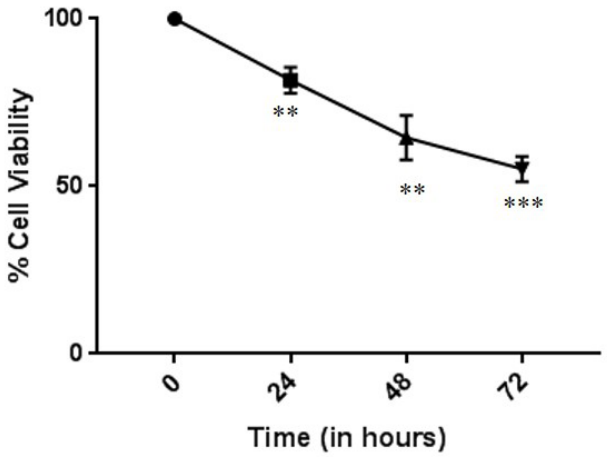

Figure 1. Cell viability of isolated luteinized granulosa cells in a time –dependent manner when grown in modified serum free media by Trypan blue exclusion assay. The values are represented as Mean plus/minus SEM. N=6-8. **p<0.01, ***p<0.0001 as compared to 0 hour.

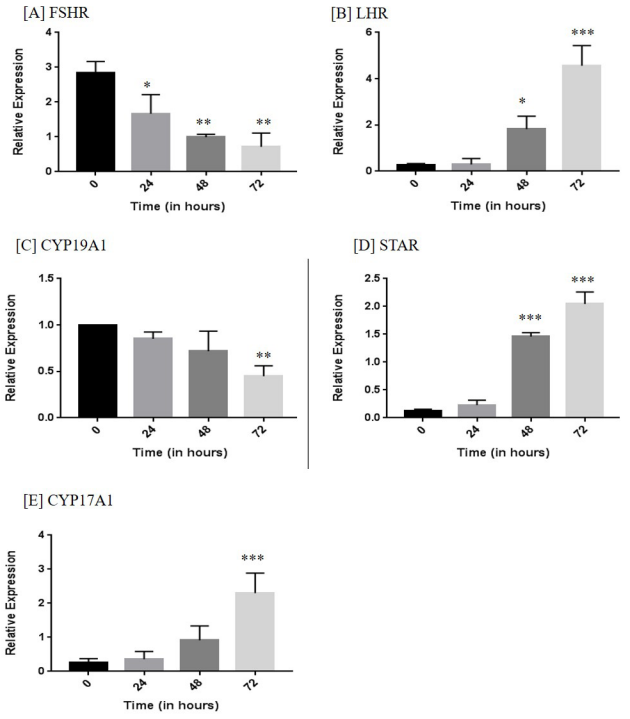

Figure 2. Characterization of isolated luteinized granulosa cells markers [**A**] *FSHR*, [**B**] *LHR*, [**C**] *CYP19A1*, [**D**] *STAR* and [**E**] *CYP17A1* by estimating their transcript abundance. The values are represented as Mean plus/minus SEM. N=6-8. *p<0.05, **p<0.01, ***p<0.0001 as compared to 0 hour.

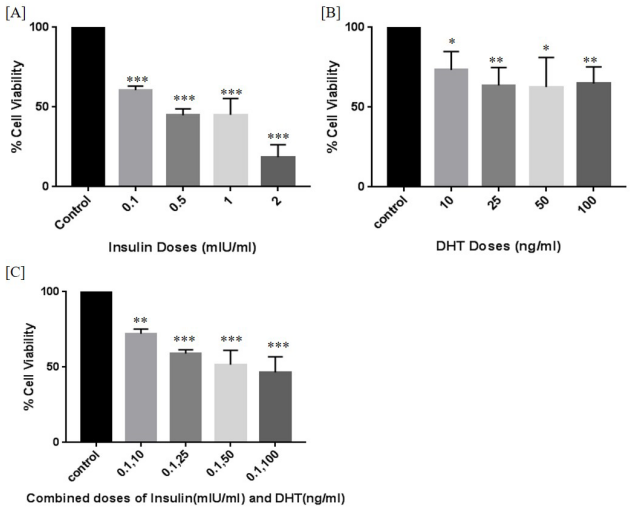

Figure 3. Cell viability assay by treating the isolated luteinized granulosa cells with [**A**] insulin doses (0.1, 0.5, 1.0 and 2.0 mIU/mL); [**B**] DHT doses (10, 25, 50 and 100 ng/mL) and [**C**] Combined doses of insulin and DHT. The values are represented as Mean plus/minus. N=7. *p<0.05, **p<0.01, ***p<0.001 as compared to control.

3.2 Effect of Hyperinsulinemia on Cell Viability of Luteinized Granulosa Cells

Results from MTT assay demonstrated that LGC exhibited significant dose- dependent decline in cell viability when incubated with insulin (Figure 3A). The dose of 0.1mIU/mL of insulin was found to be the minimum effective dose; therefore, it was used for further co-administration studies along with varying concentrations of androgen for development of "*in-vitro*" PCO condition.

3.3 Effect of Hyperandrogenemia on Cell Viability of Luteinized Granulosa Cells

Results revealed that all the treatment groups i.e. 10, 25, 50, and 100 ng/mL of DHT dose significantly decreased the cell viability when compared to the control (Figure 3B). It is important to note that results werenot significant as observed in case of hyperinsulinemia. Moreover, androgens are known to act differentially when they are present in high levels along with other growth factors like insulin or insulin like growth factor due to synergistic effects[16]. Therefore, all the four doses (i.e. 10, 25, 50 and 100 ng/mL) of DHT were selected for the further co-administration studies.

3.4 Synergistic Effect of Hyperinsulinemia and Hyperandrogenemia on Cell Viability and Morphology of Primary Culture of Luteinized Granulosa Cells

Insulin at 0.1 mIU/mL of insulin was considered as the optimum dose for inducing hyperinsulinemia along with varying concentrations of androgen (10, 25, 50 and 100 ng/ml of DHT) for induction of hyperandrogenemia in the ovarian microenvironment. Results from MTT assay demonstrated that all the treatment groups exhibited greater than 50% cell viability (Figure 3C) suggesting that the doses are non-toxic to the cells. The cells were observed under inverted light microscope at 20X magnification and it was observed that the primary culture of granulose cells had normal and healthy morphology in all the treatment groups (Figure 4).

3.5 Synergistic Effect Of Hyperinsulinemia and Hyperandrogenemia on the Transcript Levels of Key Steroidogenic Targets in Primary Culture of Luteinized Granulosa Cells

Results showed that, gene expression of *StAR, CYP17A1, SREBP1-c* and *AMH* increased significantly when the primary LGCs were co-administered with 0.1 mIU/mL of Insulin along with 50 and 100ng/mL of DHT. However, a significant decrease in the gene expression of *CYP19A1, HSD3B2, SHBG* and *IGFBP1* was observed when primary LGCs were co-administered with 0.1 mIU/mL Insulin and 50 ng/ml DHT (Figure 5). Thereby, minimum effective dose of Insulin and DHT to mimic ovarian microenvironment present in PCO using an "*in vitro*" primary culture of LGCs was found to be a combined dose of 0.1 mIU/mL and 50 ng/mL respectively. Further validation of the same can be done on the basis of hormone secretion by the primary culture of LGCs.

3.6 Synergistic Effect of Hyperinsulinemia and Hyperandrogenemia on the Hormones Secretion by Primary Culture of Luteinized Granulosa Cells

Table 2 reveals that there was an increase in the secretion of testosterone by the primary culture of LGCs, when they are subjected to hyperinsulin (0.1mIU/ml) and hyperandrogen (50 and 100 ng/mL) conditions ($p<0.05$ and $p<0.001$ respectively). On the other hand, there was a significant decrease in the secretion of progesterone by the LGCs upon co-administering them with 0.1mIU/mL of insulin and 50 and 100 ng/mL of androgen ($p\leq0.5$ and $p\leq0.05$). However, no significant difference was found in the levels of secreted estradiol. Data suggests that there is altered steroidogenesis by the primary culture of LGCs when they are subjected to "*in-vitro*" PCO like ovarian microenvironment. Thus, we can conclude that co-administration of 0.1 mIU/mL of insulin and 50 ng/mL of DHT is the minimum effective dose for developing "*in-vitro*" PCO like condition in LGCs.

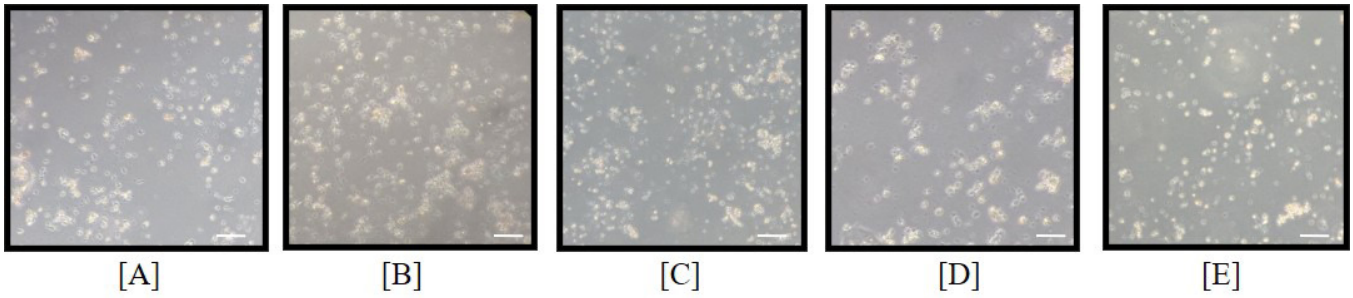

Figure 4. The morphology of the isolated luteinized granulosa cells as observed at 40x magnification of inverted light microscope. [**A**] control; [**B**] 0.1 mIU/mL Insulin,10 ng/mL DHT; [**C**] 0.1 mIU/mL insulin, 25 ng/mL DHT; [D] 0.1 mIU/mL insulin, 50 ng/mL DHT; [E] 0.1 mIU/mL insulin, 100 ng/mL DHT.

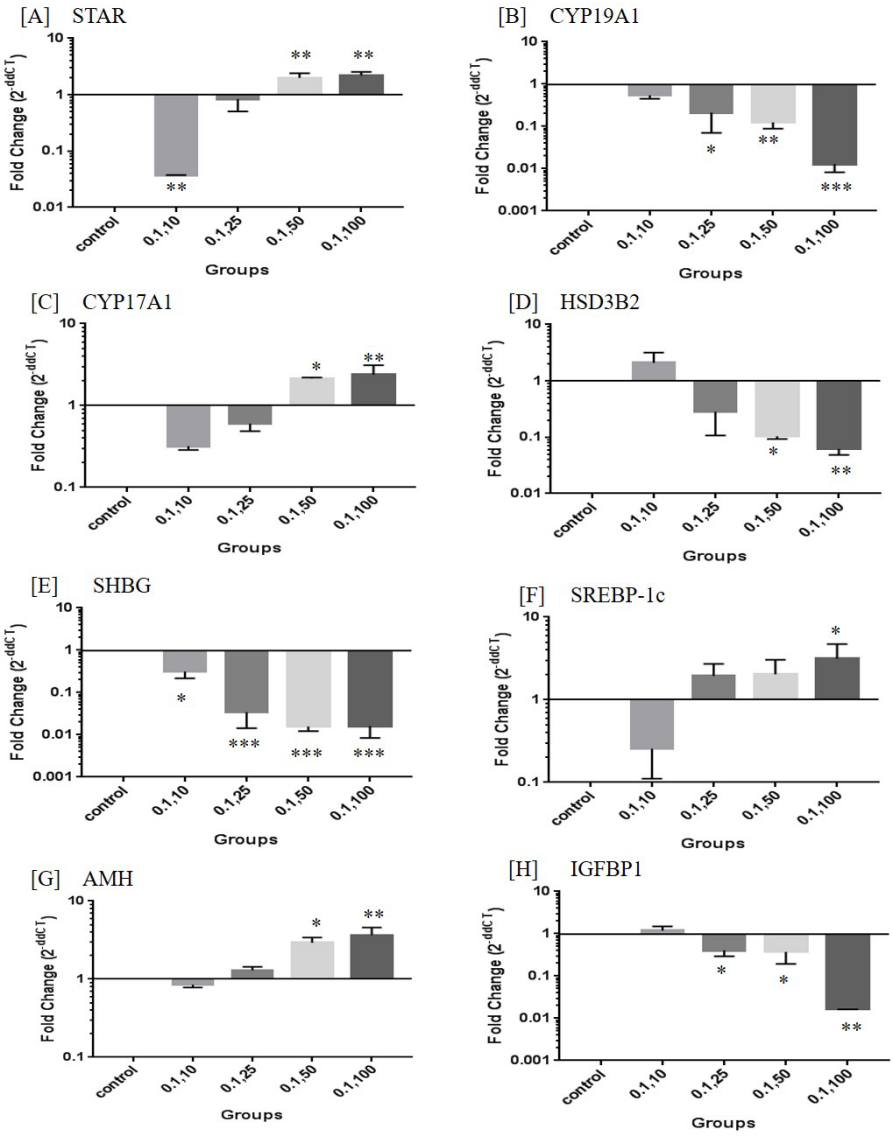

Figure 5. Synergistic effect of hyperinsulinemia and hyperandrogenemia on the transcript levels of key steroidogenic targets [**A**] STAR, [**B**] CYP19A1, [**C**] CYP17A1, [**D**] HSD3B2, [**E**] SHBG, [**F**] SREBP1-c, [**G**] AMH and [**H**] IGFBP1 of isolated luteinized granulosa cells. The values are represented as Mean plus/minus SEM. N=6-8. *p<0.05, **p<0.01, ***p<0.0001 as compared to control.

Table 2. Synergistic effect of hyperinsulinemia and hyperandrogenemia on the hormone secretion by the primary culture of luteinized granulosa cells

	Control	0.1,10 (mIU/mL Insulin, ng/mL DHT)	0.1,25 (mIU/mL Insulin, ng/mL DHT)	0.1,50 (mIU/mL Insulin, ng/mL DHT)	0.1,100 (mIU/mL Insulin, ng/mL DHT)
Testosterone (ng/ml)	0.11+ 0.003	0.9+0.010	7.93+0.005**	28.47+ 0.93***	36.4+ 0.43***
Estradiol (pg/ml)	142+ 3.679	121+0.041	150+ 4.952	140+0.284	135+ 11.53
Progesterone (ng/ml)	58+4.75	42+9.10	20+2.46*	17.15+ 0 .62**	25.80+ 0.035**

The values are represented as Mean+ SEM. N=6-8. **p<0.01, ***p<0.0001 as compared to Control.

4. Discussion

PCOS is the most common endocrinopathy of women in reproductive age. Since quite long, it has been debated that the ovarian dysfunction in PCOS is a consequence of high levels of insulin and androgens in circulation[17]. However, recent reports have shown that ovarian microenvironment plays a crucial role in maintenance of the ovarian dynamics[18]. Therefore, any alteration in the ovarian microenvironment would lead to to perturbed follicular development, oogenesis, ovulation and steroidogenesis. Therefore, the present study was aimed at developing an *"in-vitro"* model of PCO that mimics their ovarian microenvironment and study its implication on the most important cells of the ovary- granulosa luteinized cells. In this context, the luteinized granulosa cells were isolated from weaning female rats by superovulation and characterized for their purity by studying the molecular markers like *FSHR, CYP19A1, LHR, StAR* and *CYP17A1*. Data showed that letting animals superovulate on treatment with PMSG and hCG, lead to high yield of LGCs. Similar procedures have also been performed in the past for obtaining granulosa cells from rats[19], mice[20], rabbits[21], buffaloes[22] and pigs[23], suggesting that this is the most standard protocol of granulosa cell isolation.

The isolated luteinized granulosa cells were grown in serum free conditions using modified DMEM/F12 medium and the cells were found to be stable in the culture for at least 72 hours. In this regard, results from literature have shown that luteinized granulosa cells are stable upto 8 days, when grown in serum free conditions containing FSH[24]. Therefore, the luteinized granulosa cells are a good and stable *"in-vitro"* model to study the signaling cascades involved in steroidogenesis, folliculogenesis and ovulation. The isolated LGCs demonstrated the presence of *FSHR* and *CYP19A1* during the course of experiment. However, there was a decline observed in the relative expression of these genes beyond 24 hours. On the other hand, there was an increase in the gene expression of *LHR, StAR and CYP17A1* after 48 hours of culture condition, suggesting that the cells might be undergoing luteinization. Similar results have been observed when granulosa cells isolated from human derived follicular fluid were cultured in serum free conditions for a prolonged time[25]. After establishment of the conditions for luteinized granulosa cell primary culture, an induction of hyperandrogen and hyperinsulin was given to cells.

The isolated LGCs demonstrated a dose dependent decline in the cell viability when treated with 0.1 mIU/mL-2 mIU/mL of Insulin and 10-100 ng of DHT independently. 0.1 mIU/mL of Insulin was found to be the minimum effective dose for induction of hyperinsulinemia in the primary culture of LGCs. There is a long debate regarding the varying levels of androgens present in the follicular fluid of PCOS patients[26]. Therefore, a dose dependent study was performed by varying DHT concentrations in combination with 0.1 mIU/mL of Insulin. It was observed that a dose dependent decrease in cell viability in the cultured LGCs upon co-administration of hyperinsulin and hyperandrogen conditions. Similar studies have not been performed in the past. However, in some isolated studies it has been shown that incubating granulosa cells with IGF1 and/or DHT lead to induction of hyperinsulinemia and hyperandrogenic condition in the cells[27,28].

Ovarian microenvironment influences the ovarian structure-function. The follicular fluid of PCOS patients contains high androgen and high insulin titers, along with disturbed steroidogenesis[29]. Intra-ovarian hyperandrogenism may be causatively linked with anovulation in PCOS[30]. It has also also studied that hypersecretion of insulin is responsible for excess production of androgens in theca interna cells by impaired tyrosine kinase Ser-phosphorylation of inositolglycan[31] and also reduced hepatic production of sex hormone binding globulin, resulting in higher concentrations of free androgens in circulation[32]. In this regard, it would be interesting to study the impact of hyperandrogenic and hyperinsulinemic condition in combination on the important cells of the ovaries- luteinized granulosa cells. Data demonstrates that there is an alteration in the markers of follicular development, steroidogenesis and luteinization, mainly upregulation of key genes such as *StAR*, *CYP17A1, SREBP1-c* and *AMH* and down-regulation of genes like *CYP19A1, HSD3B2, SHBG* and *IGFBP1* has been observed when primary culture of LGCs were induced with "*in-vitro*" PCO-like microenvironment. It has been demonstrated in the ovary that *StAR* expression highly correlates with steroidogenic activity, because the increased production or concentration of *StAR* may result in abnormal steroidogenesis found in PCOS[33]. Evidence suggests that patients with PCOS may have an abnormality in androgen biosynthesis, with StAR as one possible target of abnormality. Kashar-Miller, *et al.*[34] hypothesized that increased production or concentration of StAR may be responsible for the increased ovarian and adrenal androgen found in PCOS of some patients. In addition to StAR, differential activity of the cytochrome p450 17α-hydroxylase (CYP17A1) gene promoters have been observed in PCOS theca cells[35,36].

There are several reports in this regard, which show higher expression of *CYP17A1* at both transcriptional and post-transcriptional level in the ovaries of PCOS women and it is partly responsible for the perturbed steroidogenesis[37]. Also, 4-folds greater *CYP17A1* promoter activity was observed in the theca interna cells of human polycystic ovaries[38]. Recent studies have linked aberrant regulation of cholesterol mobilization in PCOS, through up-regulation of SREBP1-c, suggesting that this pathway might play a role in the manifestation of PCOS[39]. The gene expression of *SREBP1-c* has been found to significantly increase in the endometrium of women with PCOS and endometrial cancer compared with controls[40].

Another important regulator of ovarian activity is Anti-Mullerian Hormone (AMH). It plays plays a crucial role in the follicular recruitment[41,42]. Studies from the past decade have shown that AMH is modulated by hyperandrogenism and hyperinsulinemia, leading to abnormal follicular dynamics in PCOS pathology[43,44]. AMH levels in the serum of PCOS patients are two or three times higher than average women[45]. It is surprising to note that AMH production by granulosa cells in the polycystic ovary is 75 times higher compared to healthy women[46]. Additionally, histological analysis of ovaries of PCOS patients have shown an increased AMH expression in the granulosa cells, which coincide with an increased number of preantral and small antral follicles, 2–3 times greater than that found in normal ovaries[47]. In women with PCOS, elevated levels of AMH appears to play an important role in long term disruption of ovarian physiology[48], with greater AMH concentrations being linked to poor fertility outcomes[49].

The down-regulation of genes like *CYP19A1, HSD3B2, SHBG* and *IGFBP1* can be considered to be responsible for the elevated androgen biosynthesis and reduced progesterone secretion. Also, high levels of LH and hyperactive theca cells as well as altered granulosa cell activity results into decreased estradiol and progesterone production[50]. Aromatase is encoded by *CYP19A1* gene and is responsible for conversion of androgens (androstenedione and testosterone) to estrogens (estrone and estradiol) during steroidogenesis. Insulin stimulates aromatase activity and induces the development of antral follicles due to the greater sensitivity of granulosa cells to FSH[51]. There is evidence that high concentrations of insulin result in premature differentiation of the granulosa cells and follicular arrest[52]. Also, follicular hyperandrogenism downregulates aromatase in luteinized granulosa cells in PCOS women[53]. Clinical studies demonstrate that luteinizing granulosa cells from follicles of patients with PCOS have a reduced capacity to synthesize progesterone "*in-vitro*" due to reduced 3BHSD gene expression[54]. In addition to this, mutations in 3BHSD and SHBG are responsible for the excess androgen production in the ovaries of PCO patients[55]. Also, PCOS patients demonstrate low serum concentration of IGFBP1, which has been found to be associated with high concentrations of circulating androgens and a greater risk of endometrial hyperplasia and neoplasia[56]. These reports clearly suggest that PCO like phenotype has been developed in the primary culture of

LGCs induced by co-administration of hyperandrogen and hyperinsulin

5. Conclusion

In the present study, an "*in-vitro*" model of PCO that mimics the ovarian microenvironment has been successfully developed by co-induction of hyperinsulinemia and hyperandrogenemia in the primary culture of luteinized granulosa cells isolated from rat ovaries. Co-administration of 0.1 mIU/mL of Insulin and 50 ng/mL of DHT to the LGCs could effectively modulate the key genes involved in steroidogenesis, folliculogenesis and ovulation at molecular level along with abnormal steroid hormone secretion by the cells. The development of an "*in-vitro*" PCO model is novel and holds huge potential towards studying detailed downstream cellular signaling as well as screening drug targets for ovarian dysfunctions like PCOS, ovarian cancer and infertility.

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7. Conflict of Interest

The authors declare that they have no conflict of interest.

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