5

Role of Isolated Phytocomponents of Non-polar fraction of *Aloe vera* Gel Extract in PCO-like ovarian cellular models

5.1. Rationale:

Data from previous chapters have clearly demonstrated isolation, characterization and also exhibited the bioactive potential of partially purified non-polar phytocompounds from *Aloe vera* gel. Amongst the five partially purified isolates, LP1 and LP3 were found to possess better pharmacokinetic properties in rats and also steroidogenic and metabolic regulatory properties when they were incubated with the KGN cell-line. The results obtained so far has evidently demonstrated that LP1 and LP3 can effectively modulate the hormone secretion, and therefore, suggest to be a potent drug molecule/s for management of endocrinopathies like PCOS, which is characterized by dysregulated steroidogenesis as well metabolic abnormalities.

The pathological aetiologies of PCOS are highly complicated, since there is a connection between intrinsic and extrinsic variables, leading to hyperandrogenaemia and hyperinsulinemia complications (Rosenfield and Ehrmann, 2016). Women with PCOS are said to have selective insulin resistance, meaning that their insulin signalling is disrupted as a result of increased insulin receptor and insulin receptor substrate-1 (IRS-1) serine phosphorylation (Diamanti-Kandarakis and Dunaif, 2012) .Additionally, these women produce too much insulin as a result of excessive Luteinizing Hormone (LH) release, which stimulates insulin resistance and the insulin-like growth factor 1 receptor (IGFR1) expression, causing disturbed ovarian steroidogenesis (Poretsky et al., 1988). The circulating levels of insulin are increased in PCOS women (Polak et al., 2017) along with an increase in the follicular fluid (Li et al., 2018). The physiological concentration of insulin in follicular fluid was found to be 2- 65.4 μIU/ml (Diamond et al., 1985). However, the concentration of follicular fluid insulin in case of PCOS was found to be around 0.25-1.0 mIU/ml. (Phy et al., 2004). Hyperinsulinemia boosts local insulin-like growth factor (IGF)-1 activity in the ovary, causing theca cells to become

androgenic (Rojas et al., 2014). The ovulation problem in PCOS patients appears to be caused by elevated IGF-I activity as well as a high androgenic milieu (Hasegawa et al., 2017**).**

Modulation of LH and overexpression of Luteinizing Hormone Receptors/Human Chorionic Gonadotropin Receptor (LHR/ hCGR) further renders them hypersensitive to LH stimuli, resulting in hypersecretion of androgens in theca cells, which is another significant cause of hyperandrogenism in PCOS (Ehrman et al., 1995). Furthermore, progesterone's reduced suppression of Gonadotropin Releasing Hormone (GnRH) pulse frequency supports the development of the PCOS phenotype (Goodarzi et al., 2011). Despite having normal serum androgen levels, around 65 percent of PCOS patients had elevated androgen titres, a decreased estrogen-to-testosterone (E2/T) ratio, and insulin resistance in the follicular fluid, according to latest findings (Li et al., 2018). The normal physiological androgen levels in follicular fluid are 5- 20 ng/mL (Costa et al., 2004), but clinical samples of PCOS patients had androgen levels in the follicular fluid of 10-100 ng/mL (Roe et al., 2013). The ovarian microenvironment has a significant impact on follicular growth and development, however it's still unknown how hyperandrogenaemia and hyperinsulinemia in the PCOS ovarian microenvironment work together to induce ovarian dysfunction. As a result, it would be fascinating to construct a *"in vitro"* model in which insulin and androgen are co-exposed, allowing us to examine both independent and cross-talk activities at the cellular level. The objective of this chapter was to create a *"in vitro"* ovarian microenvironment model of the PCO phenotype in two different types of cells: i) primary culture of rodent derived granulosa luteal cell culture and ii) human derived-KGN cell-line.

Both the cultures have biological differences, precisely the rodent derived primary culture of granulosa luteal cells is a mixture of theca and granulosa cells, therefore, it provides a comprehensive model to study 2 cell-2 gonadotropin-based steroidogenesis. On the other hand, KGN cell line is a steroidogenic human ovarian granulose-like tumour cell line that has been used to study steroidogenesis, cell proliferation, and FSHR-coupled signalling in human granulosa cells (Havelock, 2004). Furthermore, KGN cell are capable of secreting estrogen and progesterone (Nishi et al., 2001). The ovarian microenvironment plays a very important role in regulation of the ovarian structure-function (Veikkolainen et al., 2020). As discussed earlier, PCOS is characterized by hyperandrogenism and hyperinsulinemia, both at systemic as well as ovarian level. Hence, it would be interesting to understand the impact of high levels of insulin and androgens towards the transformation of granulosa cells and its associated steroidogenic

targets. The identification of the molecular targets will set up the framework to elucidate the bioactivity of partially purified non-polar phytochemicals (PPNPP) isolated from *Aloe vera* gel in PCO-like condition.

5.2. Materials and methods

All the materials and methods used in this study has been elaborately explained in Chapter 2. In short, *"in-vitro"* model of PCO was developed and validated in primary culture of rodent derived granulosa luteal cell culture and human derived-KGN cell-line. After successful validation of the model, the steroidogenic modulatory potential of the isolated phytocompounds- LP1 and LP3 were evaluated. The grouping of cultures for the bioassay was as follows:

Group I (Control group)- comprised of untreated KGN cell-line

Group II (PCO group)- comprised of hyper insulin (2mIU/mL- insulin) and hyper androgen (100ng/mL- DHT) treated KGN cell-line

Group III (PCO+DMSO group)- comprised of PCO group treated with 0.1% DMSO. Served as vehicle control

Group IV (PCO+ PE group)- comprised of PCO group treated with 70 ng/mL of petroleum ether extract of *Aloe vera* gel

Group V (PCO+ LP1 group)- comprised of PCO group treated with 25ng/mL of LP1

Group VI (PCO+HA group)- comprised of PCO group treated with 25ng/mL of n-Hexadecanoic acid

Group VII (PCO+LP3 group)- comprised of PCO group treated with 9 ng/mL of LP3

Group VIII (PCO+CA group)-comprised of PCO group treated with 9 ng/mL of Campesterol acetate

The doses of the phytochemicals were decided based upon their IC_{50} values as mentioned in the previous chapter. Given below are the results obtained.

5.3. Results

5.3.1. Development of *"in-vitro"* PCO- like model using rodent derived granulosa luteal cell primary culture

5.3.1.1. 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 analysed upto 72 hours. Cell viability assay by MTT demonstrated that around 50% of luteinized granulosa cells (LGCs) were viable up to 72 hours (Figure 5.1). 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.

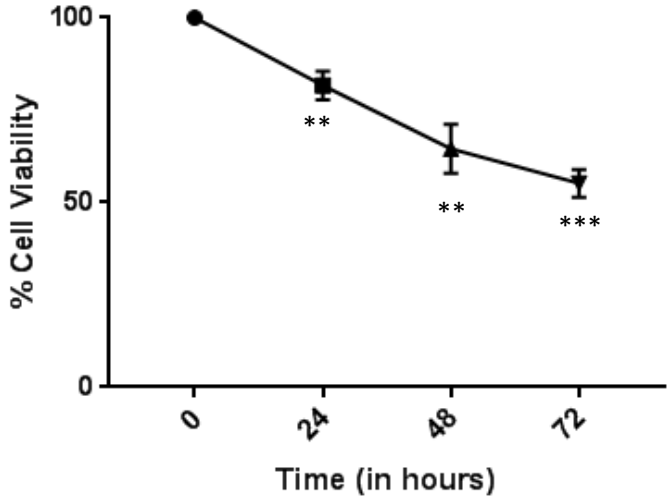


Figure 5.1: Cell viability of isolated luteinized granulosa cells in a time –dependent manner when grown in modified serum free media by Trypan blue exclusion assay. The values are represented as Mean± SEM. N=6. **p<0.01, ***p<0.0001 as compared to 0 hour.

Further, characterization of the isolated LGCs (Figure 5.2) 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* and *Star* from 48 hours onwards, suggesting that the isolated granulosa 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.

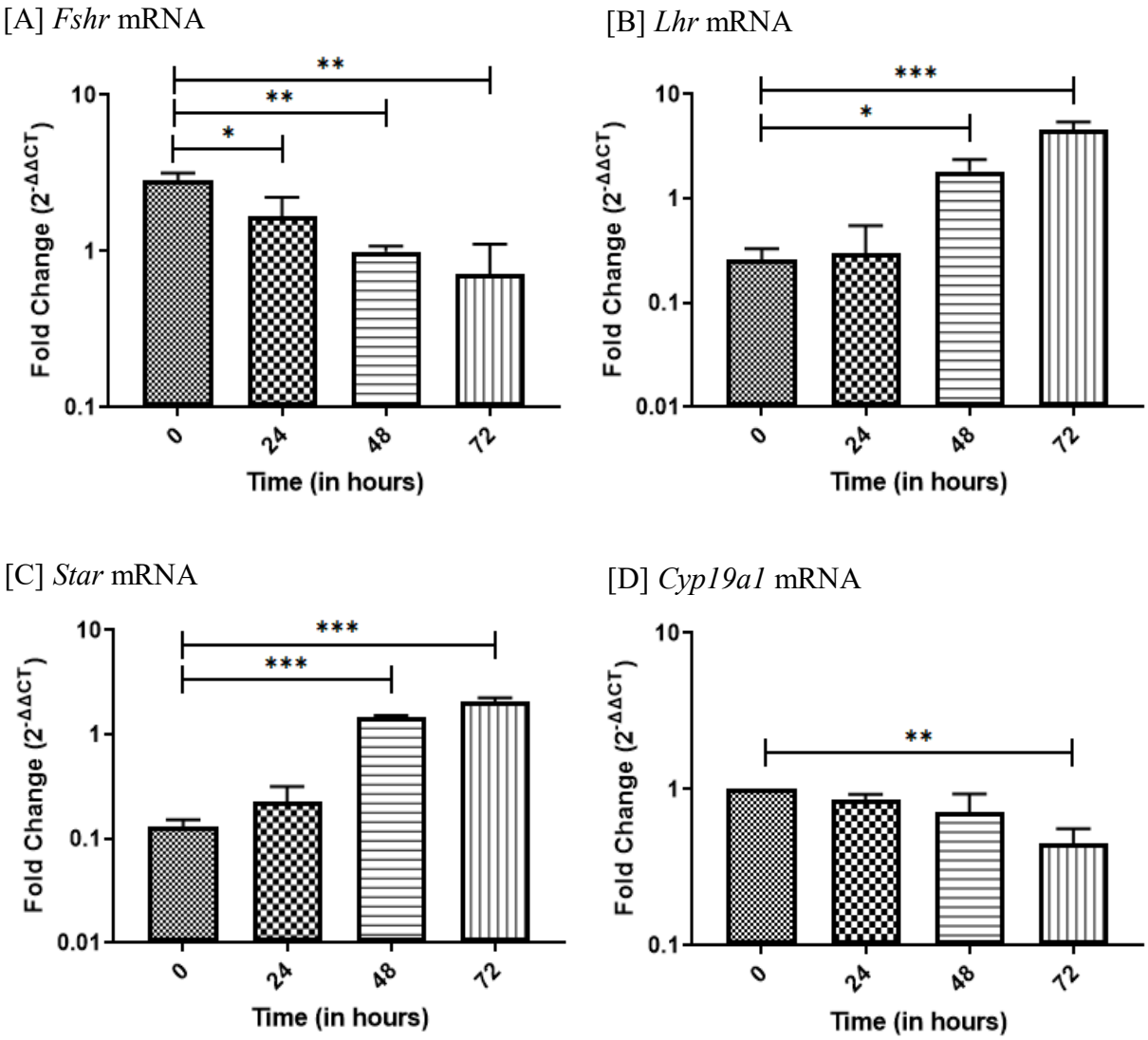


Figure 5.2: Characterization of isolated luteinized granulose cell markers [A] *Fshr*, [B] *Lhr*, [C]*Star* and [D] *Cyp19a1*by estimating their transcript abundance. Values are mean fold changes in gene expression. Error bars represent SEM; N=6. *P<0.05, **P<0.01, ***P<0.001 as compared to 0 hour.

5.3.1.2. 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 5.3A). The dose of 0.1 mIU/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.

5.3.1.3. Effect of Hyperandrogenaemia 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 5.3B). It is important to note that results were not 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 (Yoshizawa et al., 2000). Therefore, all the four doses (i.e., 10, 25, 50 and 100 ng/mL) of DHT were selected for the further co-administration studies.

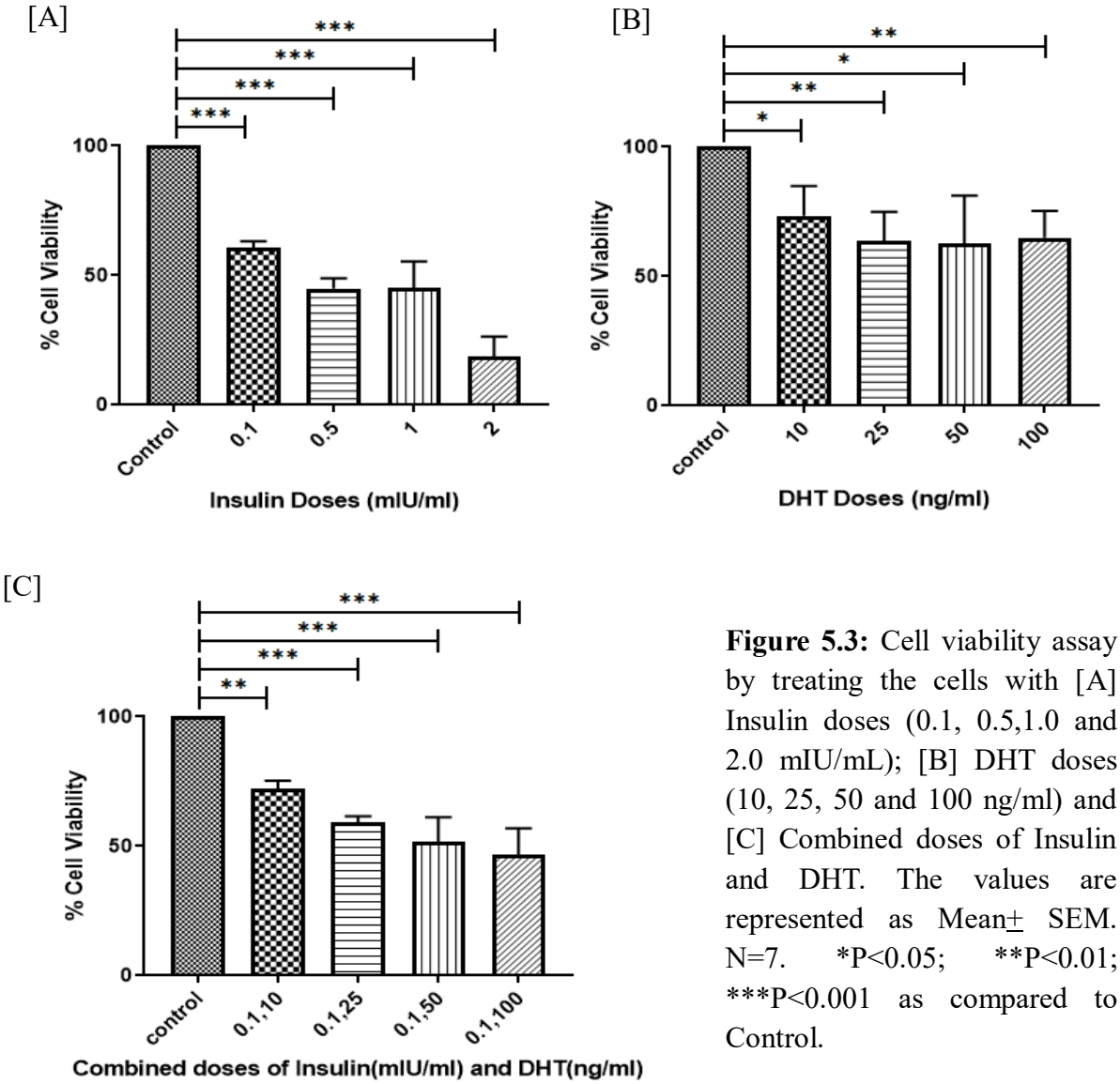
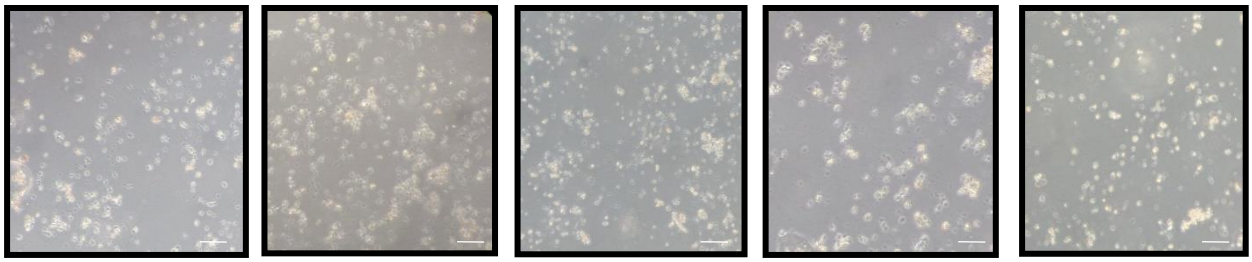


Figure 5.3: Cell viability assay by treating the cells with [A] Insulin doses (0.1, 0.5,1.0 and 2.0 mIU/mL); [B] DHT doses (10, 25, 50 and 100 ng/ml) and [C] Combined doses of Insulin and DHT. The values are represented as Mean± SEM. N=7. *P<0.05; **P<0.01; ***P<0.001 as compared to Control.

5.3.1.4. Synergistic Effect of Hyperinsulinemia and Hyperandrogenaemia on Cell Viability and Morphology of Primary Culture of Luteinized Granulosa Cells

Insulin, at 0.1 mIU/mL, 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 hyperandrogenaemia in the ovarian microenvironment. Results from MTT assay demonstrated that all the treatment groups exhibited greater than 50% cell viability (Figure 5.3C) suggesting that the doses are non-toxic to the cells. The cells were observed under inverted light microscope at 40X magnification and it was observed that the primary culture of granulosa cells had normal and healthy morphology in all the treatment groups (Figure 5.4).



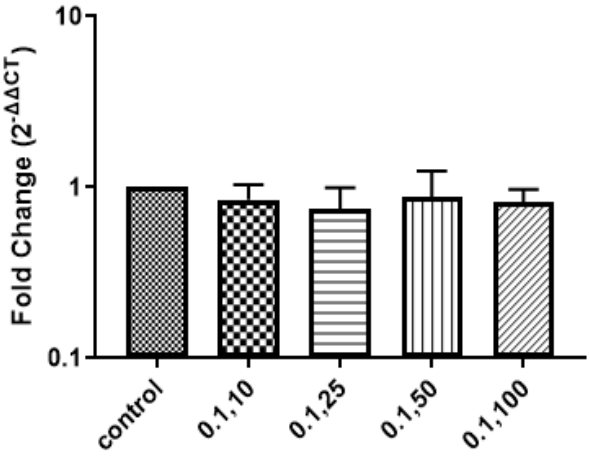
[A] [B] [C] [D] [E]

Figure 5.4: The morphology of the cells as observed under 40X magnification of inverted light microscope. [A] control; [B] 0.1 mIU/mL Insulin,10ng/mL DHT; [C] 0.1 mIU/mL Insulin,25ng/mL DHT; [D] 0.1 mIU/mL Insulin,50 ng/mL DHT; [E] 0.1 mIU/mL Insulin,100 ng/mL DHT.

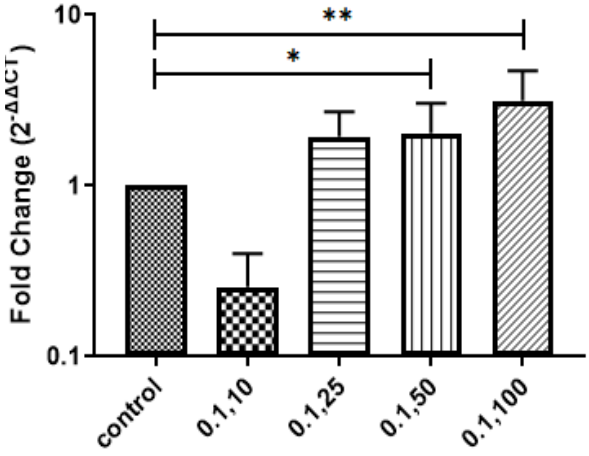
5.3.1.5. Synergistic Effect of Hyperinsulinemia and Hyperandrogenaemia on the Transcript Levels of Key Steroidogenic Targets in Primary Culture of Luteinized Granulosa Cells

Results showed that gene expression of *Lhr*, *Star*, *Cyp17a1*, *Ar* and *Amh* increased significantly when the primary LGCs were co-administered with 0.1 mIU/mL of insulin along with 50 and 100 ng/mL of DHT. However, a significant decrease in the gene expression of *Cyp19a1*, *Hsd3b1*, was observed when primary LGCs were co-administered with 0.1 mIU/mL insulin and 50 ng/mL DHT (Figure 5.5). Therefore, 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.

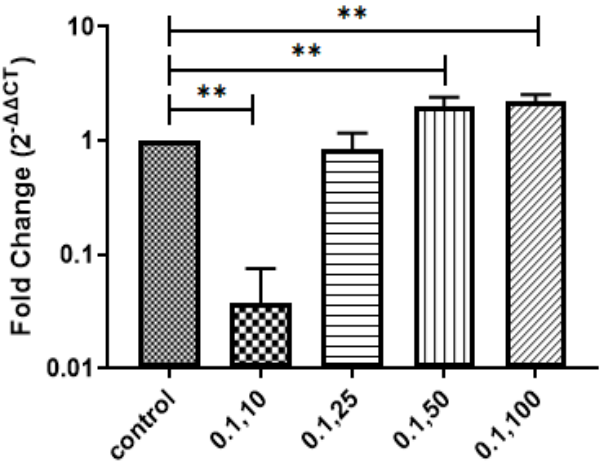
[A] *Fshr* mRNA



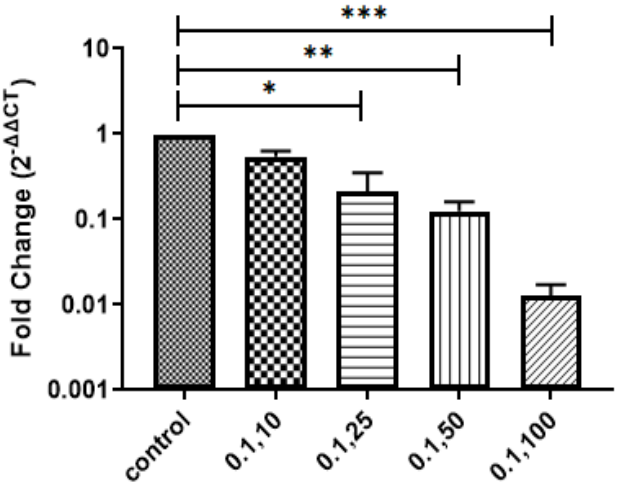
[B] *Lhr* mRNA



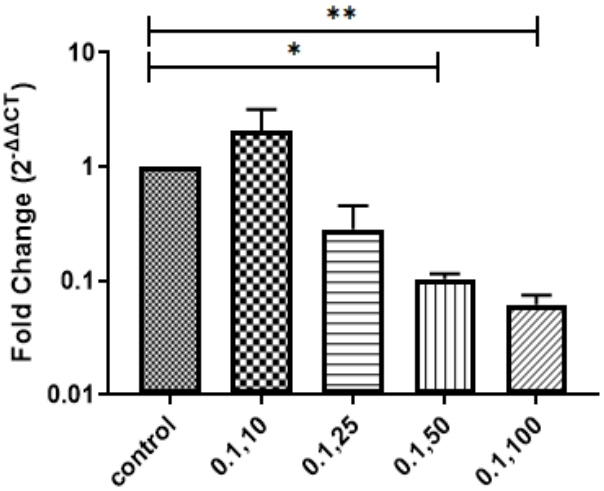
[C] *Star* mRNA



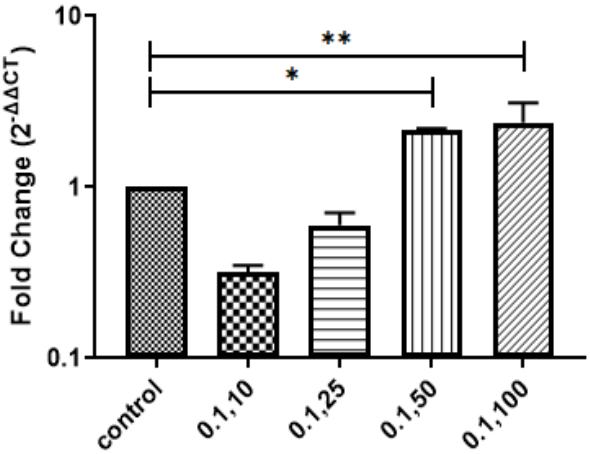
[D] *Cyp19a1* mRNA



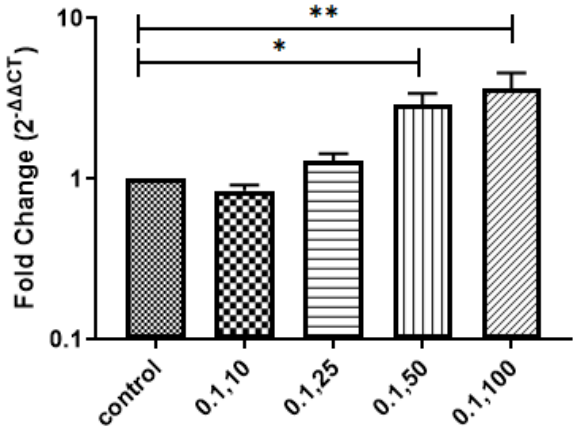
[E] *Hsd3b1* mRNA



[F] *Cyp17a1* mRNA



[G] *Amh* mRNA



[H] *Ar* mRNA

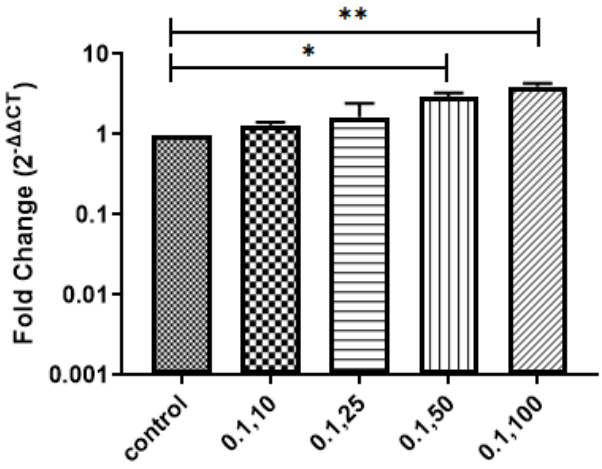


Figure 5.5: Synergistic effect of hyperinsulinemia and hyperandrogenaemia on the transcript levels of key steroidogenic targets [A] *Fshr*, [B] *Lhr*, [C] *Star*, [D] *Cyp19a1*, [E] *Hsd3b1*, [F] *Cyp17a1*, [G] *Amh* and [H] *Ar*. Values are mean fold changes in gene expression. Error bars represent SEM; N=6. *P<0.05, **P<0.01, ***P<0.001 as compared to control.

5.3.1.6. Synergistic Effect of Hyperinsulinemia and Hyperandrogenaemia on the Hormone Secretion by Primary Culture of Luteinized Granulosa Cells

Table 5.1 reveals that there was an increase in the secretion of testosterone by the primary culture of LGCs, when they are subjected to hyper insulin (0.1 mIU/mL) and hyperandrogenic (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 with 0.1 mIU/mL of insulin and 50 and 100 ng/mL of androgen. 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 were 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.

Table 5.1: Hormone analysis of the spent media collected from primary luteinized granulosa cells treated with hyper insulin and hyperandrogenic conditions.

	Control	0.1,10 (mIU/mL, ng/mL)	0.1,25 (mIU/mL, ng/mL)	0.1,50 (mIU/mL, ng/mL)	0.1,100 (mIU/mL, ng/mL)
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. **P<0.01, ***P<0.001 as compared to control.

5.3.2. Development of *"in-vitro"* PCO- like model using Human derived KGN cell-line

5.3.2.1. Effect of Hyperinsulinemia on Cell Viability of KGN cell-line

Results from MTT assay demonstrated that KGN cell-line exhibited significant dose-dependent decline in cell viability when incubated with insulin (Figure 5.6A). The dose of 2.0 mIU/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.

5.3.2.2. Effect of Hyperandrogenaemia on Cell Viability of KGN cell-line

Results revealed that 50, and 100 ng/mL of DHT dose significantly decreased the cell viability when compared to the control (Figure 5.6B).

5.3.2.3. Synergistic Effect of Hyperinsulinemia and Hyperandrogenaemia on Cell Viability and Morphology of KGN cell-line

Insulin, at 2.0 mIU/mL, 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 hyperandrogenaemia in KGN cell-line. Results from MTT assay demonstrated that all the treatment groups exhibited greater than 50% cell viability (Figure 5.6C) suggesting that the doses are non-toxic to the cells. The cells were observed under inverted light

microscope at 10 X magnification and it was observed that the KGN cells had normal and healthy morphology in all the treatment groups (Figure 5.7).

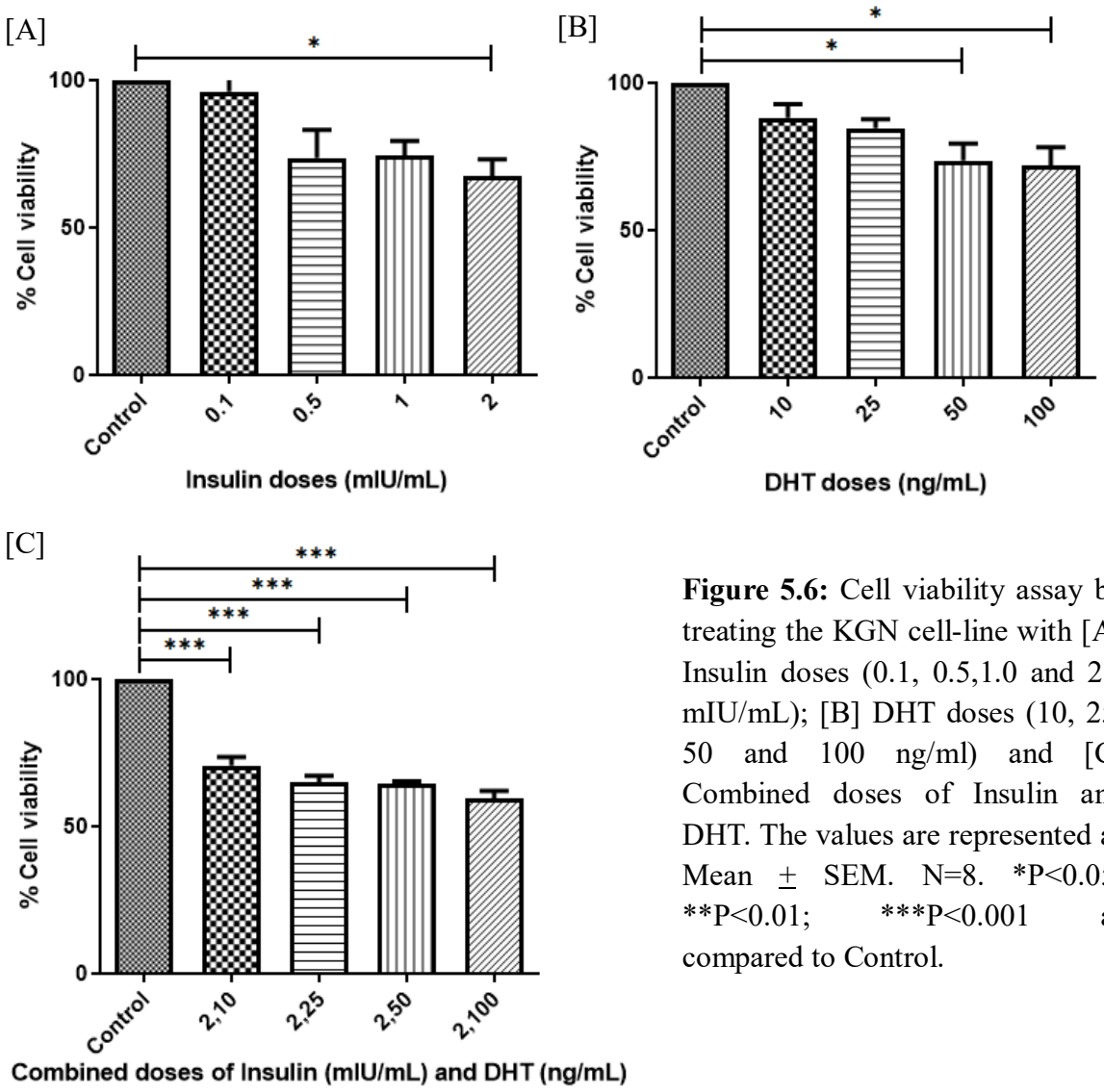
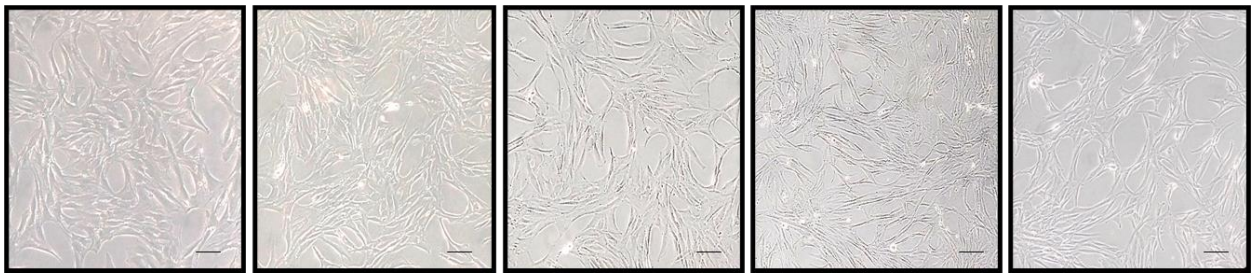


Figure 5.6: Cell viability assay by treating the KGN cell-line with [A] Insulin doses (0.1, 0.5,1.0 and 2.0 mIU/mL); [B] DHT doses (10, 25, 50 and 100 ng/ml) and [C] Combined doses of Insulin and DHT. The values are represented as Mean $\pm$ SEM. N=8. *P<0.05; **P<0.01; ***P<0.001 as compared to Control.



[A] [B] [C] [D] [E]

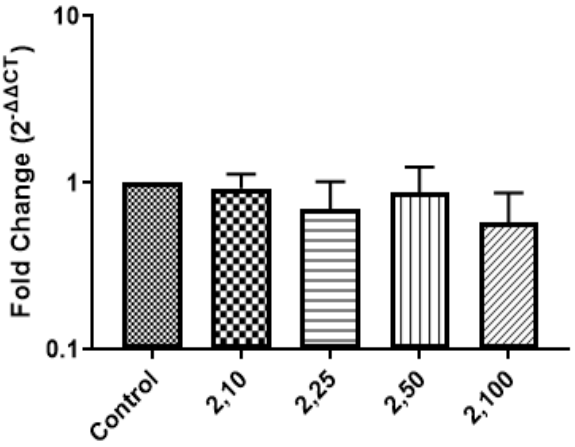
Figure 5.7: The morphology of KGN cell-line as observed under 10X magnification of inverted light microscope. [A] control; [B] 2.0 mIU/mL Insulin,10 ng/mL DHT; [C] 2.0

mIU/mL Insulin,25 ng/mL DHT; [D] 2.0 mIU/mL Insulin,50 ng/mL DHT; [E] 2.0 mIU/mL Insulin,100 ng/mL DHT.

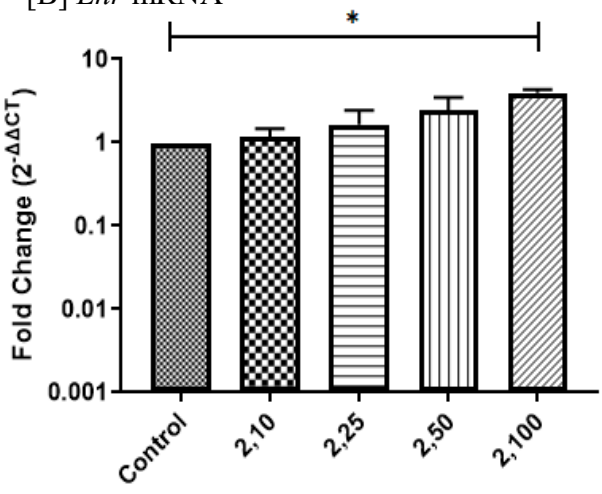
5.3.2.4. Synergistic Effect of Hyperinsulinemia and Hyperandrogenaemia on the Transcript Levels of Key Steroidogenic Targets in KGN cell-line

Results showed that gene expression of *Lhr*, *Star*, *Cyp17a1*, *Ar* and *Amh* increased significantly when the KGN cells were co-administered with 2.0 mIU/mL of insulin along with 100 ng/mL of DHT. However, a significant decrease in the gene expression of *Cyp19a1*, *Hsd3b1*, was observed when the cells were co-administered with 2.0 mIU/mL insulin and 100 ng/mL DHT (Figure 5.8). Therefore, minimum effective dose of insulin and DHT to mimic ovarian microenvironment present in PCO in KGN cell-line was found to be a combined dose of 2.0 mIU/mL and 100 ng/mL, respectively. Further validation of the same can be done on the basis of hormone secretion by the KGN cells.

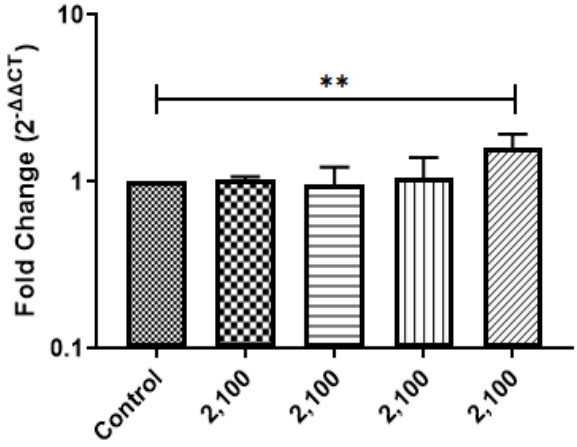
[A] *Fshr* mRNA



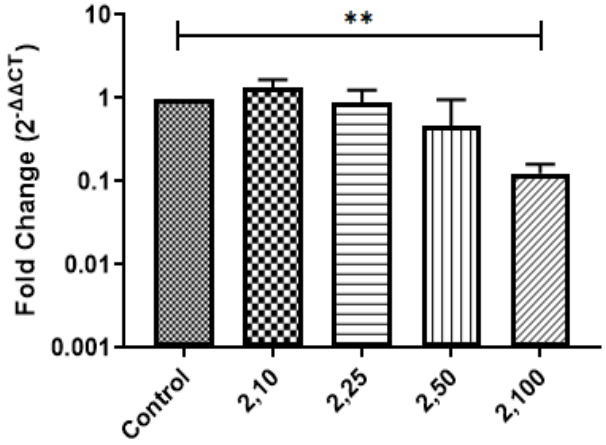
[B] *Lhr* mRNA



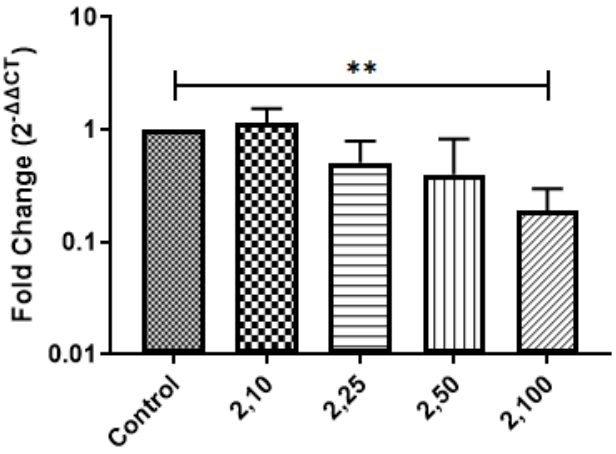
[C] *Star* mRNA



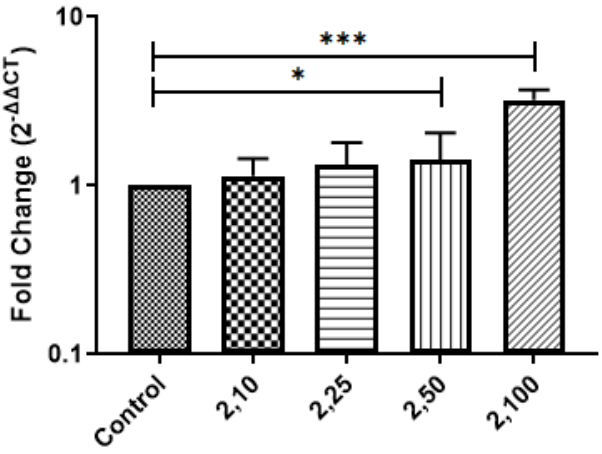
[D] *Cyp19a1* mRNA



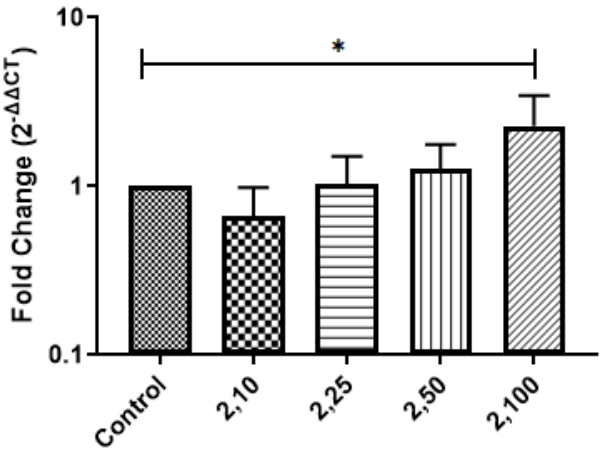
[E] *Hsd3b1* mRNA



[F] *Cyp17a1* mRNA



[G] *Amh* mRNA



[H] *Ar* mRNA

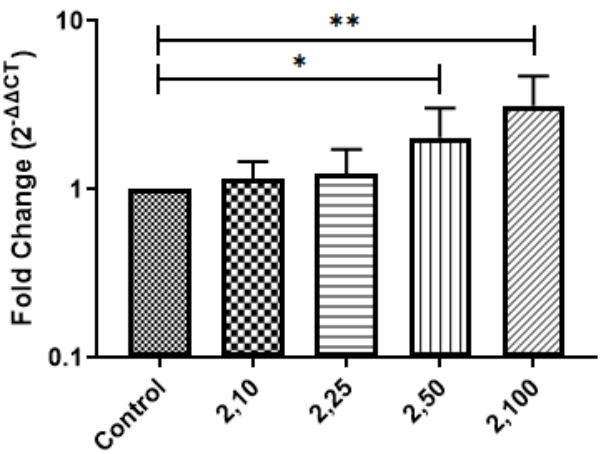


Figure 5.8: Synergistic effect of hyperinsulinemia and hyperandrogenaemia on the transcript levels of key steroidogenic targets [A] *Fshr*, [B] *Lhr*, [C] *Star*, [D] *Cyp19a1*, [E] *Hsd3b1*, [F] *Cyp17a1*, [G] *Amh* and [H] *Ar* in KGN cell-line. Values are mean fold changes in gene expression. Error bars represent SEM; N=6. *P<0.05, **P<0.01, ***P<0.001 as compared to Control.

5.3.2.5. Synergistic Effect of Hyperinsulinemia and Hyperandrogenaemia on the Hormone Secretion by KGN cell-line

Data from Table 5.2 suggests that there was an increase in the secretion of testosterone by the KGN cells, when they are subjected to hyper insulin (2.0 mIU/mL) and hyperandrogenic (100 ng/mL of DHT) conditions (P<0.01). On the other hand, there was a significant decrease in the secretion of progesterone by the cells upon co-administering with 2.0 mIU/mL of insulin and 100 ng/mL of DHT (P<0.001). However, no significant difference was found in the levels of secreted estradiol. Data suggests that there is altered steroidogenesis by the KGN cell-line when

they are subjected to *"in-vitro"* PCO-like ovarian microenvironment. Thus, we can conclude that co-administration of 2.0 mIU/mL of insulin and 100 ng/mL of DHT is the minimum effective dose for developing *"in-vitro"* PCO-like condition in KGN cell-line.

Table 5.2: Hormone analysis of the spent media collected from KGN cells treated with hyper insulin and hyperandrogenic conditions.

	Control	2.0,10 (mIU/mL, ng/mL)	2.0,25 (mIU/mL, ng/mL)	2.0,50 (mIU/mL, ng/mL)	2.0,100 (mIU/mL, ng/mL)
Testosterone (ng/mL)	2.625±0.452	2.943±0.637	3.125± 0.69	4.036±0.832	5.131±0.325 **
Estradiol (pg/mL)	0.283±0.063	0.312±0.033	0.259±0.048	0.194±0.058	0.103±0.054
Progesterone (ng/mL)	0.193±0.02	0.227±0.09	0.137±0.076	0.103+0.09	0.083±0.006 ***

The values are represented as Mean± SEM. N=6. **P<0.01, ***P<0.001 as compared to Control.

An *"in-vitro"* steroidogenic modulatory bioassay was done after the establishment of a *"in-vitro"* PCO-like ovarian milieu in both primary granulosa luteal cell culture and KGN cell-line. Both the models revealed dysregulated steroidogenesis, similar to that found in PCOS, and might be used to investigate precise downstream cellular signalling and evaluate treatment options for ovarian dysfunctions such as PCOS. The cell yield of rodent-derived granulosa luteal cells, on the other hand, was relatively low. Large numbers of animals had to be sacrificed in order to acquire enough cells to complete the bioassay, which was unethical. As a result, we decided to evaluate the bioactivity of the most potent partially purified non-polar phytocompounds isolated from *Aloe vera* gel- LP1 and LP3 using solely the hyper androgen and hyper insulin generated *"in-vitro"* PCO model of the KGN cell-line. The results for which are described below.

5.3.3. Effect of PPNPPs isolated from *Aloe vera* gel on the steroidogenic targets in an *"in-vitro"* PCO- like model

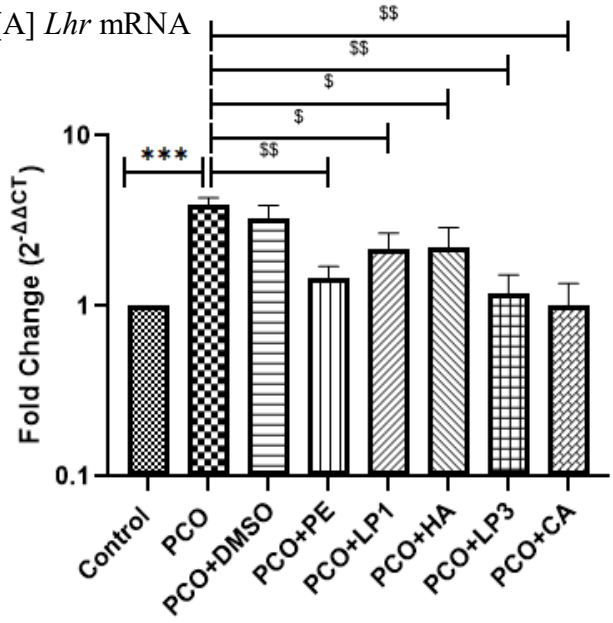
5.3.3.1. Effect of PPNPPs isolated from *Aloe vera* gel on the Transcript Levels of Key Steroidogenic Targets in an *"in-vitro"* PCO- like model

To evaluate the effect of partially purified non-polar phytocompounds isolated from *Aloe vera* gel on the transcript levels of steroidogenic targets in an *"in-vitro"* PCO model of the KGN cell-line, real time PCR was performed using specific primers for *Lhr, Fshr, Star, Cyp19a1, Hsd3b1, Cyp17a1, Amh, Ar, Esr-1, Esr-2* and *Pgr*. Results from Figure 5.9A demonstrates that all the phytochemicals, petroleum ether extract of *Aloe vera* gel ($P<0.01$), LP1 ($P<0.05$), HA ($P<0.05$), LP3 ($P<0.01$) and CA ($P<0.01$) significantly reduced the mRNA expression of *Lhr* gene upon incubation with the *"in-vitro"* PCO-like model. On the other hand, incubation with LP3 ($P<0.01$) and CA ($P<0.01$) significantly elevated the transcript levels of *Cyp19a1* (Figure 5.9D) and *Pgr* (Figure 5.9K). However, in both the cases, the response observed was comparatively lower as compared to petroleum ether extract of *Aloe vera* gel ($P<0.001$), suggesting that the potential of PE to modulate steroidogenesis is a synergistic effect. LP3 ($P<0.01$) and CA ($P<0.05$) was found to significantly decrease the mRNA levels of *Star* gene (Figure 5.9C). Interestingly, gene expression of *Ar* (Figure 5.9H) was significantly decreased when *"in-vitro"* PCO-like model was incubated with LP1 ($P<0.05$), HA ($P<0.01$) and petroleum ether extract of *Aloe vera* gel ($P<0.05$), suggesting anti-androgenic effect of these phytochemicals. However, there was no significant change observed in the transcript levels of *Fshr, Hsd3b1, Cyp17a1, Amh, Esr-1* and *Esr-2* (Figure 5.9B, E, F, G, I and J respectively), when *"in-vitro"* PCO-like model was incubated with the phytochemicals.

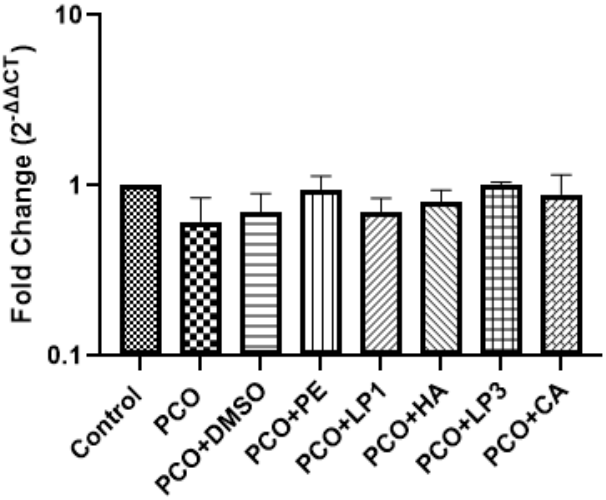
5.3.3.2. Effect of PPNPPs isolated from *Aloe vera* gel on the Hormone secretion in an *"in-vitro"* PCO- like model

Data mentioned above has clearly demonstrated that the PPNPPs of *Aloe vera* gel have the potential to modulate steroidogenic targets in *"in-vitro"* PCO-like model. Next, it was interesting to evaluate the effect of these bioactive phytochemicals on the hormone secretion by the KGN cell-line. Results from Figure 5.10 have shown that both LP3 and CA could improve the estradiol ($P<0.05$) and progesterone ($P<0.01$; $P<0.05$) secretion by the *"in-vitro"* PCO-like KGN cells. The results demonstrate that both-LP3 and CA are potent stimulatory modulators for estradiol and progesterone biosynthesis. Similar effects could be seen when the PCO-like KGN cells were treated with petroleum ether extract of *Aloe vera* gel ($P<0.05$). Surprisingly, the PPNPPs isolated from *Aloe vera* gel, when incubated with the PCO model could not modulate the secretion of testosterone. On the contrary, incubation with PE significantly reduced the testosterone levels ($P<0.05$) in the spent media, again suggesting synergistic influence.

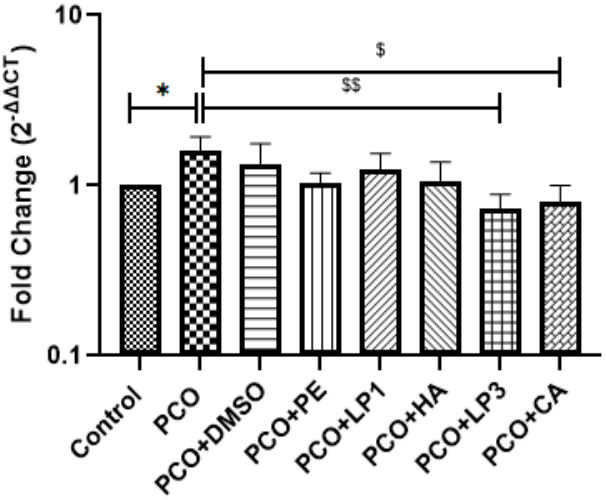
[A] *Lhr* mRNA



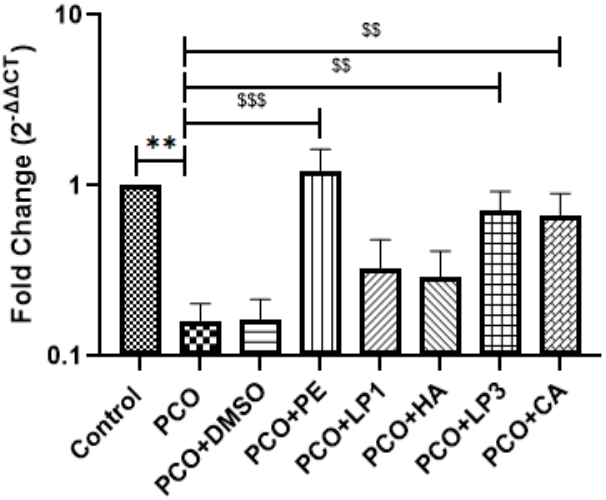
[B] *Fshr* mRNA



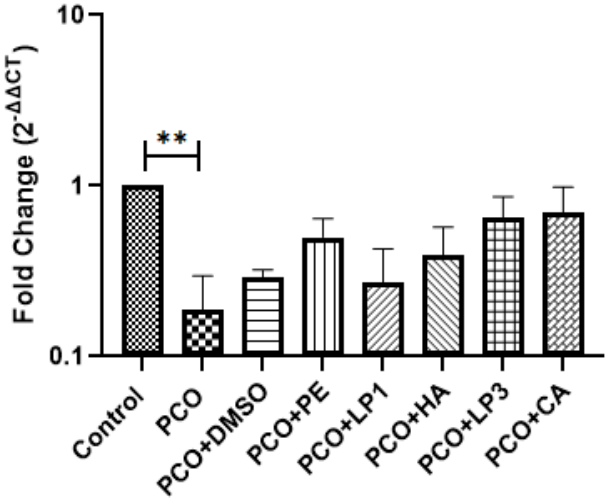
[C] *Star* mRNA



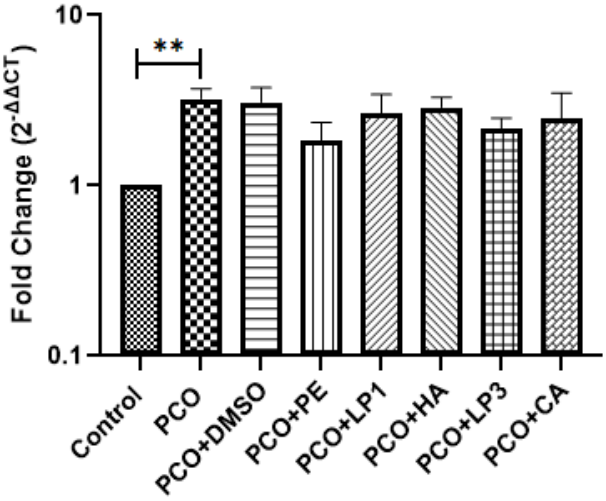
[D] *Cyp19a1* mRNA



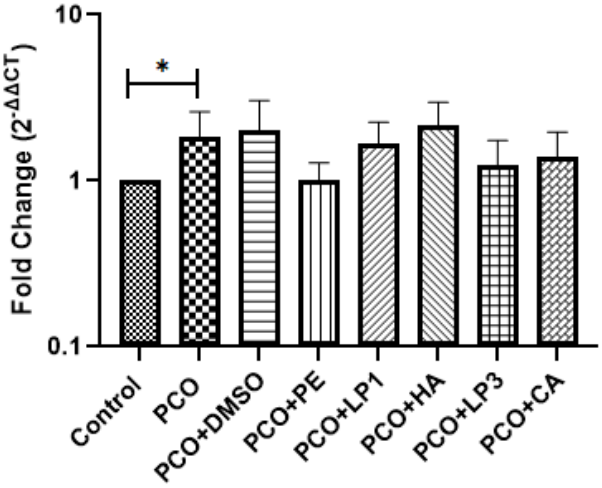
[E] *Hsd3b1* mRNA



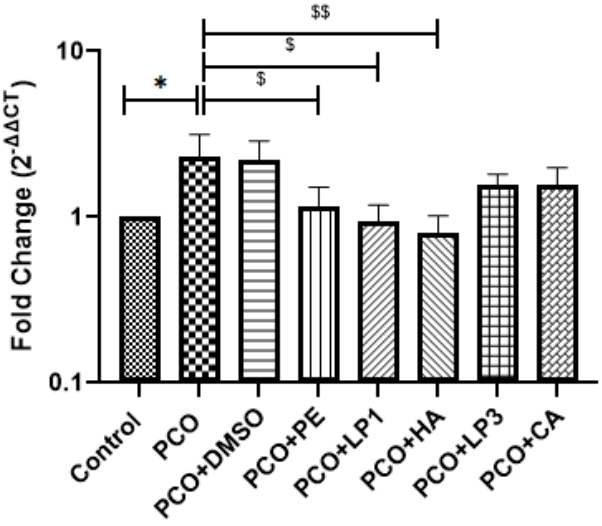
[F] *Cyp17a1* mRNA



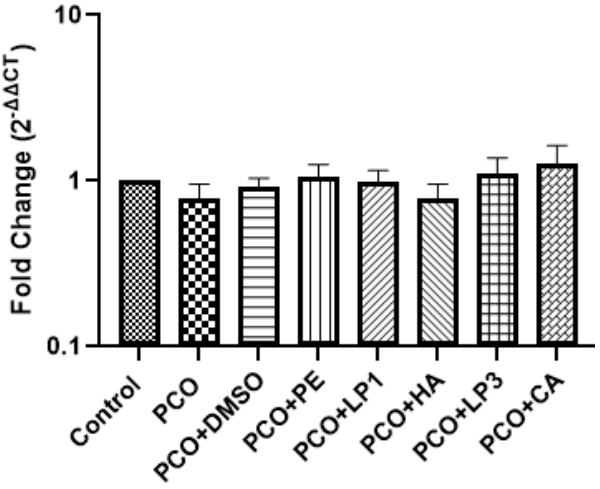
[G] *Amh* mRNA



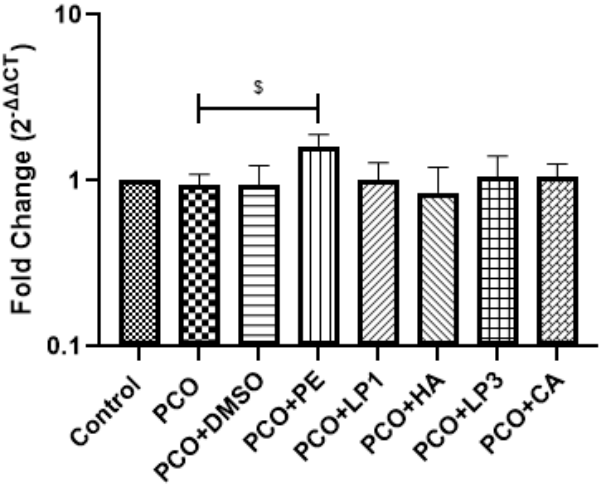
[H] *Ar* mRNA



[I] *Esr-1* mRNA



[J] *Esr-2* mRNA



[K] *Pgr* mRNA

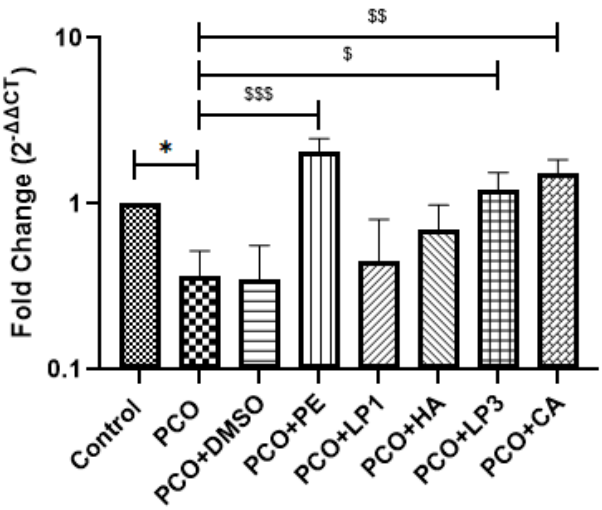


Figure 5.9: Effect of PPNPP of *Aloe vera* gel on the gene expression of steroidogenic targets [A] *Lhr*, [B] *Fshr*, [C] *Star*, [D] *Cyp19a1*, [E] *Hsd3b1*, [F] *Cyp17a1*, [G] *Amh*, [H] *Ar*, [I] *Esr-1*, [J] *Esr-2*, [K] *Pgr* in an *"in-vitro"* PCO-like model. Values are mean fold changes in gene expression. Error bars represent SEM; N=6. *P<0.05, **P<0.01, ***P<0.001 as compared to Control; $ P<0.05, $$P<0.01, $$$P<0.001 as compared to PCO group.

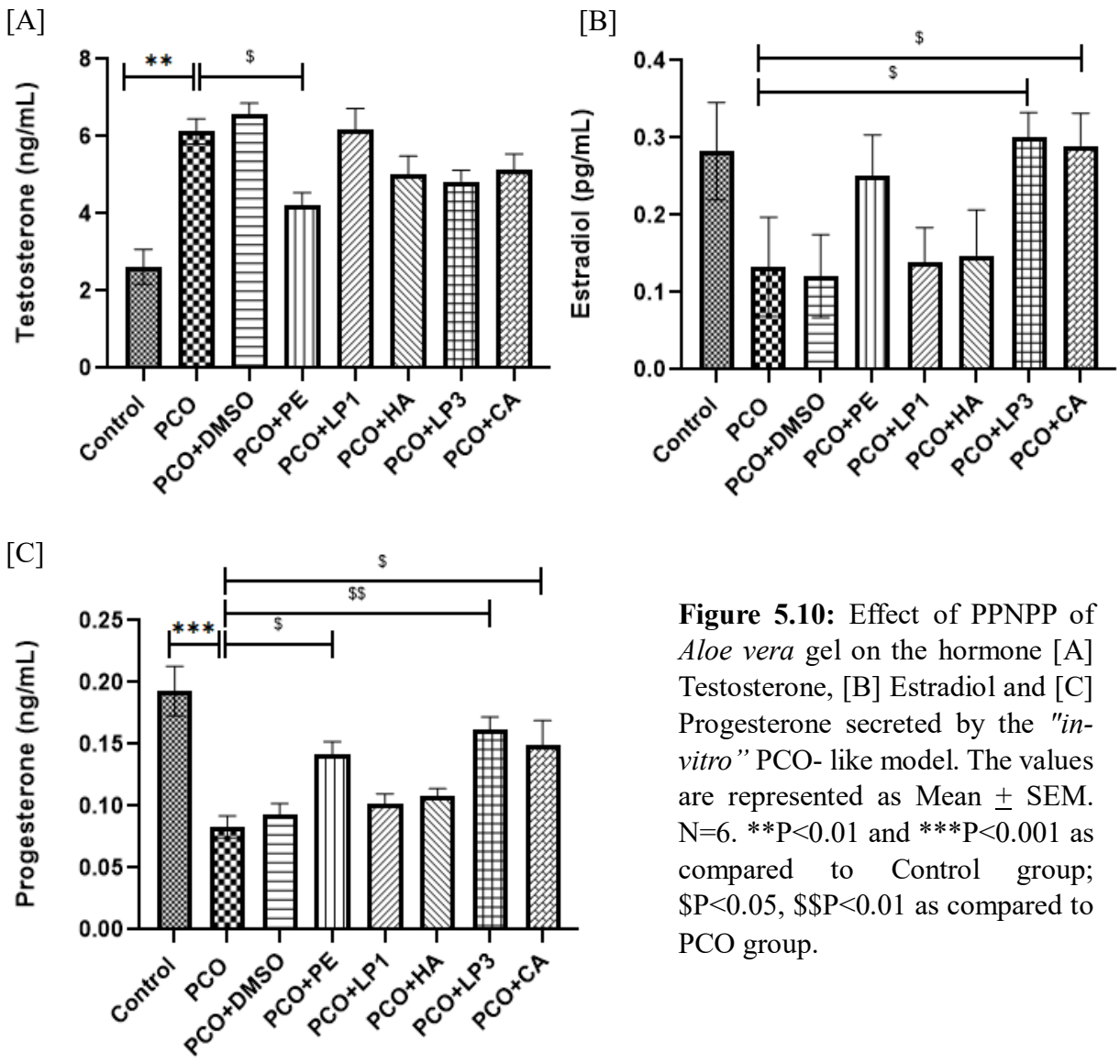


Figure 5.10: Effect of PPNPP of *Aloe vera* gel on the hormone [A] Testosterone, [B] Estradiol and [C] Progesterone secreted by the *"in-vitro"* PCO- like model. The values are represented as Mean ± SEM. N=6. **P<0.01 and ***P<0.001 as compared to Control group; $P<0.05, $$P<0.01 as compared to PCO group.

5.4. Discussion

It has long been disputed whether ovarian dysfunction in PCOS is caused by excessive levels of insulin and androgens in the bloodstream (Schüring et al., 2008). However, recent studies by Kinnear et al., (2020) have revealed that the ovarian microenvironment is critical for maintaining ovarian dynamics. As a result, any change in the ovarian microenvironment will cause follicular development, oogenesis, ovulation, and steroidogenesis to be disrupted. The major aim of this study was to create a *"in-vitro"* model of PCO that mimicked the ovarian milieu and investigate its effects on rat derived granulosa luteal cell primary culture and human derived-KGN cell-line cultures. For developing an *"in-vitro"* model of PCO using rat derived granulosa luteinized cells, the granulosa cells were collected by superovulation from weaning female rats and evaluated for purity using molecular markers such as *Fshr*, *Lhr*, *Star*, and

Cyp19a1. Enabling mice to super-ovulate with PMSG and hCG resulted in a large output of LGCs, according to several studies. Granulosa cells have previously been isolated from rats (Belani et al., 2014), mice (Arcos et al., 2017), rabbits (Piquette, 1990), buffaloes (Gutierrez et al., 1997), and pigs (Cai et al., 2015) using similar methods, showing that this is the most frequent granulosa cell isolation approach.

The collected luteinized granulosa cells were cultured in serum-free conditions in modified DMEM/F12 media for at least 72 hours and were found to be stable in the culture. In this context, studies have demonstrated that when cultured in serum-free circumstances with FSH (Monga et al., 2011), luteinized granulosa cells remain stable for up to 8 days. As a result, luteinized granulosa cells provide an excellent and robust *"in-vitro"* model for studying steroidogenesis, folliculogenesis, and ovulation signalling cascades. During the experiment, the isolated LGCs revealed the presence of *Fshr* and *Cyp19a1*. However, after 24 hours, the relative expression of these genes started to drop. After 48 hours of culture, however, gene expression of *Lhr* and *Star* increased, indicating that the cells were luteinizing. When granulosa cells isolated from human-derived follicular fluid were grown in serum-free conditions for an extended period of time, similar effects were observed (Belani et al., 2018). After establishing the conditions for luteinized granulosa cell primary culture, the cells were administered an induction of hyper androgen and hyper insulin. Similar treatments were provided to the KGN cell-line.

When treated with 0.1 mIU/mL-2mIU/mL insulin and 10-100 ng DHT separately, both primary LGCs and the KGN cell-line showed a dose-dependent decrease in cell viability. In the primary culture of LGCs and the KGN cell-line, the minimum effective dosage for inducing hyperinsulinemia was determined to be 0.1 mIU/mL and 2.0mIU/mL of insulin, respectively. The presence of variable amounts of androgens in the follicular fluid of PCOS patients has long been a source of debate (Naessen et al., 2010). As a result, for rat derived primary culture of LGCs and KGN cell-line, dose-dependent study was carried out by changing DHT concentrations in conjunction with 0.1 mIU/mL and 2.0mIU/mL of insulin, respectively. The co-administration of hyper insulin and hyper androgen circumstances resulted in a dose-dependent reduction in cell viability in cultured LGCs (Dey et al., 2021) and KGN cell-line. Studies like this have never been reported earlier. In certain isolated investigations, however, it has been demonstrated that incubation granulosa cells with IGF1 and/or DHT causes the cells to develop hyperinsulinemia and hyperandrogenic conditions (Mani et al., 2010; Doblado et al., 2020).

The ovarian structure-function is influenced by the ovarian microenvironment. PCOS patients' follicular fluid contains elevated androgen and insulin titres, as well as disrupted steroidogenesis (Magoffin, 2006). Anovulation in PCOS (Jonard and Dewailly, 2004) might be caused by intra-ovarian hyperandrogenism. Hypersecretion of insulin has also been linked to excess androgen synthesis in theca interna cells due to defective tyrosine kinase Ser-phosphorylation of inositol glycan (Cadagan et al., 2016) and decreased hepatic formation of sex hormone binding globulin, resulting in greater levels of free androgens in circulation (Jayagopal et al., 2003).In this context, it would be interesting to investigate the influence of both hyperandrogenic and hyperinsulinemic conditions on the granulosa cells, which are key cells in the ovaries. Our study demonstrates that when primary culture of LGCs (Dey et al., 2020) as well as KGN cell-line were induced with a *"in-vitro"* PCO-like microenvironment, there was an alteration in the markers of follicular development, steroidogenesis, and luteinization, with upregulation of key genes like *Lhr, Star, Cyp17a1,* and *Amh* and downregulation of genes like *Cyp19a1* and *Hsd3b1*. Because increased synthesis or concentration of StAR may result in aberrant steroidogenesis observed in PCOS (Aghaie et al., 2018), it has been established that *Star* expression strongly corresponds with steroidogenic activity in the ovary. Evidence shows that PCOS patients have altered androgen production, with *Star* being one prospective target. Kashar-Miller et al. (1999) postulated that the elevated ovarian and adrenal androgen observed in certain PCOS patients might be due to increased StAR production or concentration. Differential activity of the cytochrome p450 17-hydroxylase (*Cyp17a1)* gene promoters has also been reported in PCOS theca cells (Wickenheisser et al., 2012; Comim et al., 2013), in addition to *Star*. Several studies have found that *Cyp17a1*expression is greater in the ovaries of PCOS women at both the transcriptional and post-transcriptional levels, and that it is partly responsible for the disrupted steroidogenesis (Wickenheisser et al., 2005). In the theca interna cells of human polycystic ovaries, *Cyp17a1* promoter activity was also shown to be 4-fold higher (Wickenheisser et al., 2000).

Anti-Mullerian hormone is another essential regulator of ovarian function (AMH). It is very important for follicular recruitment (Tokmak et al., 2016; Nardo et al., 2009). Hyperandrogenism and hyperinsulinemia regulate *Amh*, resulting in aberrant follicular dynamics in PCOS pathology (Pigny et al., 2003; Dewailly et al., 2003), according to recent studies. PCOS patients have two to three times the amount of AMH in their blood than normal women (Wiweko et al., 2014). It's startling to learn that the polycystic ovary's granulosa cells

produce 75 times more AMH than healthy women (Pellatt et al., 2007). Furthermore, histological examination of PCOS patients' ovaries revealed higher *Amh* expression in the granulosa cells, which corresponded to a 2–3-fold increase in the number of pre-antral and small antral follicles compared to normal ovaries (Karkanaki et al., 2011). Elevated levels of *Amh* appear to play a key role in the long-term alteration of ovarian physiology in women with PCOS (Pierre et al., 2013), with higher AMH concentrations related to poor reproductive outcomes (Chang and Cook-Andersen, 2013).

The increased androgen production and reduced progesterone secretion can be attributed to the upregulation of *Lhr, Cyp17a1, Star, Amh, Ar* and downregulation *Cyp19a1* and *Hsd3b1* (Dey et al., 2021). Reduced estradiol and progesterone synthesis is also caused by excessive amounts of LH and hyperactive theca cells, as well as altered granulosa cell activity (Chaves et al., 2012). Aromatase is a steroidogenesis enzyme that converts androgens (androstenedione and testosterone) to estrogens (estrone and estradiol). It is encoded by the *Cyp19a1* gene. Insulin increases aromatase activity and causes the formation of antral follicles because granulosa cells are more sensitive to FSH (Franks et al., 2008). There is evidence that elevated insulin concentrations cause premature granulosa cell differentiation and follicular arrest (Franks et al., 1998). Follicular hyperandrogenism also suppresses aromatase in PCOS women's luteinized granulosa cells (Yang et al., 2015). Clinical studies show that luteinizing granulosa cells from PCOS patients' follicles have a lower capacity to manufacture progesterone *"in-vitro"* due to lower*Hsd3b1*gene expression (Doldi et al., 2000). Furthermore, mutations in *Hsd3b1*is responsible for excessive androgen production in PCO patients' ovaries (Dadachanji et al., 2018). These findings clearly indicate that a PCO-like phenotype has formed in the primary culture of LGCs (Dey et al., 2021) as well as KGN cell-line as a result of the co-administration of hyperandrogenic and hyper insulin conditions.

During initial screening of the drug candidates, researchers prefer *"in-vitro"* disease models over time-consuming pre-clinical and/or clinical trials that often involve high levels of individual variation and ethical concerns. *"In-vitro"* models also facilitate better understanding of the molecular signalling associated with the disease (Arantes-Rodrigues et al., 2013). In the present study, successful development of an *"in-vitro"* model, which mimics the ovarian microenvironment of PCO is a milestone achievement, as there are scanty reports on *"in-vitro"* models of PCOS (Belani et al., 2016; 2018; Allemand et al., 2005; 2009). The developed *"in-vitro"* PCO-like model was utilized to test the bioactivity of partially purified isolates of *Aloe*

vera gel-LP1 and LP3 towards steroidogenic modulation. Studies involving screening of phytochemicals for evaluating their steroidogenic modulatory potential in case of PCO-like ovarian microenvironment has not been performed earlier. However, few studies have highlighted the role of phytochemicals in regulation of steroidogenic targets in general using different *"in-vitro"* cultures (Banerjee et al., 2019; Santini et al., 2009; Hasegawa et al., 2013). More detailed description of such phytochemicals has been discussed elaborately in the previous chapters. The present study has emphasized on the effect of LP1 and LP3 on the modulation of steroidogenic targets and hormone secretion by the PCO-like KGN cells. LP1 and LP3, along with their respective standards could effectively modulate expression of steroidogenic targets like *Lhr*, *Star*, *Cyp19a1*, *Ar*, and *Pgr*. The observed results highlight on the anti-androgenic, estrogenic and progestogenic potential of these bioactive molecules isolated from non-polar extract of *Aloe vera* gel.

5.5. 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 hyperandrogenaemia in the primary culture of luteinized granulosa cells isolated from rat ovaries (Dey et al., 2021) as well as human derived KGN cell-line. Co-administration of 0.1 mIU/mL of insulin & 50ng/mL of DHT and 2.0 mIU/mL of insulin and 100ng/mL of DHT to the primary culture of LGCs and KGN cell line respectively 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 signalling as well as screening drug targets for ovarian dysfunctions like PCOS, ovarian cancer and infertility. Further, the bioactivity of LP1 and LP3 was evaluated in the PCO-like model and they were found to elicit anti-androgenic, estrogenic and progestogenic potential along with modulation of key genes associated with the dysregulated ovarian microenvironment, as observed in case of PCOS.