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GONADAL PHYSIOLOGY AND DISEASE

Gene expression profiling of granulosa cells from PCOS patients following varying doses of human chorionic gonadotropin

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Abstract
Purpose Human chorionic gonadotrophin (hCG) has been used to induce ovulation and oocyte maturation. Although the most common dose of hCG used in IVF is 10,000 IU, there are reports that suggest 5,000 IU is sufficient to yield similar results. The objective of this study is to evaluate the dose dependent differences in gene expression of granulosa cells following various doses of hCG treatment.
Methods Patients with polycystic ovarian syndrome (PCOS) were stimulated for IVF treatment. The hCG injection was either withheld or given at 5,000 or 10,000 IU. Granulosa cells from the follicular fluids have been collected for RNA isolation and analyzed using Affymetrix genechip arrays.
Results Unsupervised hierarchical clustering based on whole gene expression revealed two distinct groups of patients in this experiment. All untreated patients were clustered together whereas hCG-treated patients separated to a different group regardless of the dose. A large number of the transcripts were similarly up- or down-regulated across both hCG doses (2229 and 1945 transcripts, respectively). However, we observed dose-dependent statistically significant differences in gene expression in only 15 transcripts.
Conclusions Although hCG injection caused a major change in the gene expression profile of granulosa cells, 10,000 IU hCG resulted in minimal changes in the gene expression profiles of granulosa cells as compared with 5,000 IU. Thus, based on our results, we suggest the use of 10,000 IU hCG should be reconsidered in PCOS patients.

Capsule Human chorionic gonadotrophin (hCG) gene expression of granulosa cells following various doses of hCG during IVF treatment.

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Keywords Granulosa cells · Gene expression · hCG · Microarray/PCOS

Introduction

Human chorionic gonadotrophin (hCG) has been routinely used to mimic the midcycle luteinizing hormone (LH) surge to induce final oocyte maturation and cumulus expansion following controlled ovarian stimulation. Several studies have employed varying doses of hCG to study the cycle outcome [23, 30, 37]. This has been particularly important for patients with polycystic ovary syndrome (PCOS) since they are at a greater risk of ovarian hyperstimulation syndrome (OHSS) following hCG injection. Although an earlier study showed a minimum dose of 5,000 IU to be enough

Table 1 Comparison of patients' age and stimulation characteristics among the different doses of hCG. Numbers are given mean ± standard deviation. Ranges are given in brackets

Number of cycles	No hCG 4	5000 IU 5	10000 IU 4	*P* value
Age	28±6 (19–32)	31±4 (26–37)	29±5 (24–36)	0.64
BMI	28±2 (27–30)	29±3 (25–34)	30±5 (22–34)	0.84
Baseline FSH	4±2 (3–6)	7±2 (5–10)	8±3 (4–10)	0.23
Number of hMG ampoules	20±8 (10–29)	23±8 (17–36)	29±10 (18–43)	0.44
Cycle day of hCG injection	NA	13.5 (12–18)	14 (11–16)	0.76
Number of follicles	60±25 (39–95)	53±14 (38–68)	41±14 (30–61)	0.35

for the optimal response in in-vitro fertilization (IVF) treatment [1], subsequent studies have indicated that even 2,500 and 3,300 IU hCG are also as effective as the higher doses [23, 30, 37]. However, 10,000 IU hCG remains the standard dose.

The injection of hCG induces major morphological, biochemical and functional changes in the preovulatory follicles. Stimulation of LH/hCG signals somatic cells of the follicle to undergo final follicular maturation and luteinization [35]. As the most abundant cell type inside the follicle, granulosa cells also undergo substantial differentiation, interaction with oocytes, and mediation of the effect of gonadotrophins on the follicular maturation [7]. Therefore, granulosa cells are considered as easily accessible specimen for studying the overall quality of the follicles in response to gonadotrophins [20].

Recent advances in microarray technology have allowed researchers to examine gene expression profiling in order to gain insight into the molecular changes that occur in ovarian cells [10, 19]. Transcriptional profiling is an important tool for understanding the underlying molecular mechanisms of various physiological and drug-induced biological processes. Gene expression profiling of granulosa cells was used to investigate a patient with recurrent empty follicle syndrome and apoptotic pathways have been implicated in the disappearance of oocytes [17]. Others have reported that the changes detected by microarray analyses may predict the competence of granulosa cells in supporting the development of the oocytes from women with either normal or diminished ovarian reserve [10]. Similarly, quantitative real-time RT-PCR analysis of genes from human cumulus cells was predictive of the quality of their enclosed oocytes [12, 29, 46]. These observations were further supported by subsequent studies that measured global gene expression of cumulus cells, which was concluded to be a non-invasive test for oocyte/embryo quality [2, 3, 15, 41]. Granulosa and cumulus cell gene expression profiling has also been used to compare the effects of recombinant FSH vs. human menopausal gonadotrophin (hMG) [6, 14], floating vs. cumulus granulosa cells [22], cumulus cells from lean vs. overweight-obese PCOS patients [21], and ovarian reserve status in young women with diminished ovarian reserve [39].

The aim of the current study is to investigate the dose dependent differences in gene expression profiles of granulosa cells obtained after the administration of varying doses of hCG in patients with PCOS.

Materials and methods

Patients

A total of 13 patients with PCOS were included in the study. Pituitary down regulation was performed by administering gonadotrophin releasing hormone (GnRH) agonist long (11 cycles) or short (2 cycles—1 in no hCG and 1 in 10,000 hCG group) protocols. In the long protocol, patients were given 3.75 mg Lupron Depot (Abbott Laboratories, Chicago, IL) during the early follicular phase of the menstrual cycle. Once the ovarian suppression was observed about 3 weeks later, at which time, ovarian stimulation with hMG (Menegon®, Ferring, Germany) was started. For the short protocol, Suprefact S.C. injection (400 μg, Hoechst UK Limited, Middlesex, UK) was given with hMG injections and continued until the day of hCG administration. The dose of hMG was adjusted according to patient's response with close monitoring via transvaginal ultrasound scans. hCG (Pregnyl, Organon, Oss, Holland) were administered IM whenever at least three mature follicles (≥16 mm) were present. The standard dose of hCG is 10,000 IU in our clinic and 4 patients in the study were administered 10,000 IU hCG. In 5 cycles, the hCG dose was reduced to 5,000 IU due to high risk of ovarian hyperstimulation syndrome (prior history and/or large ovarian volume). In the remaining four cycles, one patient was not given hCG due to high risk of ovarian hyperstimulation and the cycle was converted to IVM without hCG injection and one patient forgot to take her hCG injection. The other two cycles were the stimulated cycles without hCG injection due to known history of spontaneous oocyte activation. Patients were counseled about the study and a signed IRB-approved informed consent form was obtained. Oocyte pick-up was performed with transvaginal ultrasound guidance using an

aspiration needle (Swemed Lab, Billdal, Sweden) under I.V. sedation 36 h after hCG injection or when appropriate in stimulated IVM cycles all the accessible follicles that were present in the ovary were aspirated. The follicular aspirate was poured into 60-mm Falcon dishes (Beckton Dickinson Labware, Franklin Lakes, NJ) and cumulus-oocyte complexes were collected for use in assisted reproduction. Remaining follicular fluid from the same patient was pooled and filtered over a 70 μm cell strainer (Beckton Dickinson Labware) and granulosa cells remaining over the strainer were collected for an immediate RNA isolation. RNA was kept at −80 °C until it was analyzed. Samples from each patient were run on a separate chip.

Microarray analysis

The transcriptional profiles of the samples were measured using the Affymetrix HGU 133 Plus 2 chips according to previously described protocols for total RNA purification, cDNA synthesis, in-vitro transcription reaction for production of biotin-labeled cRNA, hybridization of cRNA with Affymetrix gene chips, and scanning of image output files [17]. Briefly, total RNA was isolated by using blood RNA extraction kit from Qiagen (Valencia, CA). We then used 5.0 μg of total RNA for cDNA synthesis by reverse transcriptase to generate the first strand, and followed by RNA-seH nicking and DNA polymerase I to generate the second strand. Labeled cRNA then was generated by in-vitro transcription with biotinylated UTP and CTP using the GeneChip Expression 3′-amplification reagents for IVT Labeling kit (Affymetrix, CA). Next, 40.0 μg of biotinylated cRNA was fragmented to lengths ranging from 50 to 150 nucleotides, and then hybridized overnight onto the Affymetrix Human Genome U133 Plus 2.0 array. The chips were subsequently washed, stained with streptavidin-phycoerythrin, and then scanned to determine gene expression of the arrayed elements. The scanned array images were analyzed by dChip [25]. dChip is more robust than Affymetrix software Microarray Analysis Suite (MAS) 5.0 in signal calculation for about 60 % of genes [4]. In the dChip analysis, a smoothing spline normalization method was applied prior to obtaining model-based gene expression indices, which is referred to as signal values in dCHip. There were no outliers identified by dChip, hence all samples were used for subsequent analyses.

A hierarchical clustering technique was used to construct an unweighted pair group method with arithmetic-mean tree using Pearson's correlation as the distance measure [40]. Samples were clustered using the normalized and modeled expression values that were obtained from the dChip analysis. Expression data matrix was row-normalized for each gene prior to the application of average linkage clustering.

When comparing two groups of samples to identify modulated genes in a given group, we used the lower confidence bound (LCB) of the fold change (FC) between the two groups as the cut-off criterion. If 90 % LCB of FC between the two groups was above 1.5, then the corresponding gene was considered to be differentially expressed. LCB is a stringent estimate of the FC and has been shown to be a better measure for ranking [26]. dChip's LCB method for assessing differentially expressed genes has been shown to be superior to other commonly used approaches, such as MAS 5.0 and Robust Multiarray Average (RMA) based methods [18, 38].

By the use of LCB, we can be 90 % confident that the actual FC is some value above the reported LCB. Using custom arrays and quantitative reverse transcriptase real-time PCR (QRTPCR), it has been suggested that Affymetrix chips may underestimate differences in gene expression [43]. In regard to their work, and by [34], a criterion of selecting genes that have a LCB above 1.2 most likely corresponds to genes with an "actual" fold change of at least 3 in gene expression.

Once differentially expressed genes between the various groups were identified, we determined the gene ontology (GO) categories for each of these genes [45]. For each category, we used Expression Analysis Systematic Explorer (EASE) to identify the category's degree of over-representation in the set [16]. EASE identifies GO categories in the input gene list that are overrepresented using jackknife iterative resampling of Fisher exact probabilities, with Bonferroni multiple testing correction. We chose an EASE value of 0.05 to assess if a given category is significantly over-represented and therefore may be of further interest. Principal Components Analysis (PCA) was used to project samples onto three dimensional space, which was further visualized to see the constellation of all samples using all the genes measured on the chips

Microarray confirmation

The original samples used for microarray analysis were not available for further qRT-PCR analysis. In order to validate our microarray results, two different strategies were utilized. First, we checked to see if genes that are represented by more than one probe set yield same degree and direction of regulation (up or down) for each probe set. Second, we collected six new samples (three in 5,000 and three in 10,000 IU group) and isolated RNA as described above to utilize as independent samples in real-time (quantitative) RT-PCR (qRT-PCR) experiments by using the ABI 7500 Sequence Detection System (ABI, Foster City, CA, USA). First, total RNA (50 ng) procured from the samples was transcribed into complementary DNA using SensiScript Kit from Qiagen (QIAGEN Inc., Valencia, CA, USA) under the

following conditions: 25 °C for 10 min, 42 °C for 2 h, and 70 °C for 15 min in a total volume of 20 ml. Five genes which were known to be regulated by hCG in granulosa cells [amphiregulin (*AREG*), epiregulin (*EREG*), FSH receptor (*FSHR*), cytochrome P450, family 19, subfamily A, polypeptide 1 (*CYP19A*), and steroidogenic acute regulator (*StAR*)] were selected and primers were designed using Primer3 software. After the primer optimization, the PCR assays were performed in 6 μl of the cDNA using the QIAGEN QuantiTect SYBR Green Kit, employing *GAPDH* as the control gene. All reactions were conducted in duplicates and the data was analyzed using the delta delta C_T method [27].

Results

Patients' characteristics were similar in terms of age, number of hMG ampoules injected, the cycleday when hCG was administered, and the number of follicles (Table 1). None of the patients developed ovarian hyperstimulation. The average presence call of chips was approximately 57 % (range 52.0–60.6 %). The hCG injection caused dramatic changes in granulosa cell gene expression and unsupervised hierarchical clustering analysis based on all genes showed two distinct groups among the 13 samples. All samples from patients who did not receive hCG injections were clustered into one group, while the remaining samples were clustered into a second group (Fig. 1a). A supervised clustering based on 4,174 significantly differentially expressed genes between no hCG and hCG groups showed a clear distinction between these two groups, as expected (Fig. 1b). Furthermore, principal component analysis using all genes placed the hCG-free vs. hCG samples into two distinct spaces, while 5,000 vs. 10,000 IU hCG were in the same vicinity (Fig. 2).

When compared to the hCG-free group, the injection of 5,000 IU hCG resulted in both up- and down-regulation of 3339 and 2748 genes, respectively. On the other hand, 10,000 IU hCG resulted in 2858 up-regulated while 3005 down-regulated transcripts compare to no hCG. Lists of significantly differentially expressed genes between all three groups are provided in the supplement 1. Comparative analysis was performed to determine the distribution of transcripts among different groups by obtaining gene expression patterns of interest (Fig. 3). Lists of genes in each pattern depicted in Fig. 3 are provided in the supplement 2. A statistically significant, dose-dependent difference in gene regulation between patients who received 5,000 IU and 10,000 IU hCG was only observed in 4 up- and 11 down-regulated transcripts, respectively (Patterns 1 and 2, Fig. 3 and Table 2). The majority of the transcripts were similarly up- or down-regulated (2229 and 1945 transcripts, respectively) in 5,000 IU hCG and 10,000 IU hCG (Patterns 3 and 4, Fig. 3).

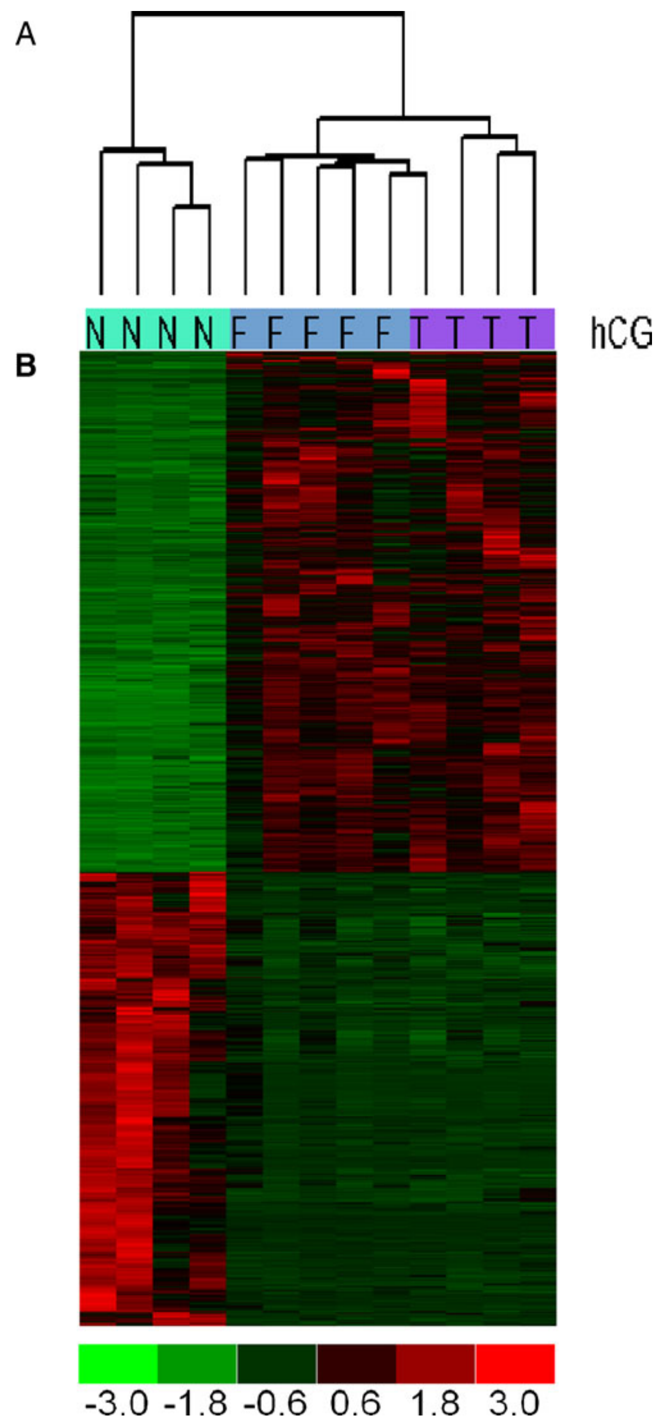

Fig. 1 Hierarchical cluster analysis of gene expression patterns using a centroid linkage algorithm with correlation distance measure. **a** Clustering of samples based on complete gene expression in granulosa cells from patients treated with zero hCG (N), five thousand units of hCG (F) and ten thousand units of hCG (T). This unsupervised clustering approach rendered two distinctive groups (normal and treated). **b** Supervised hierarchical clustering based on differentially expressed genes. The differential expression data are taken from the pairwise comparison analysis of normal and different treatments

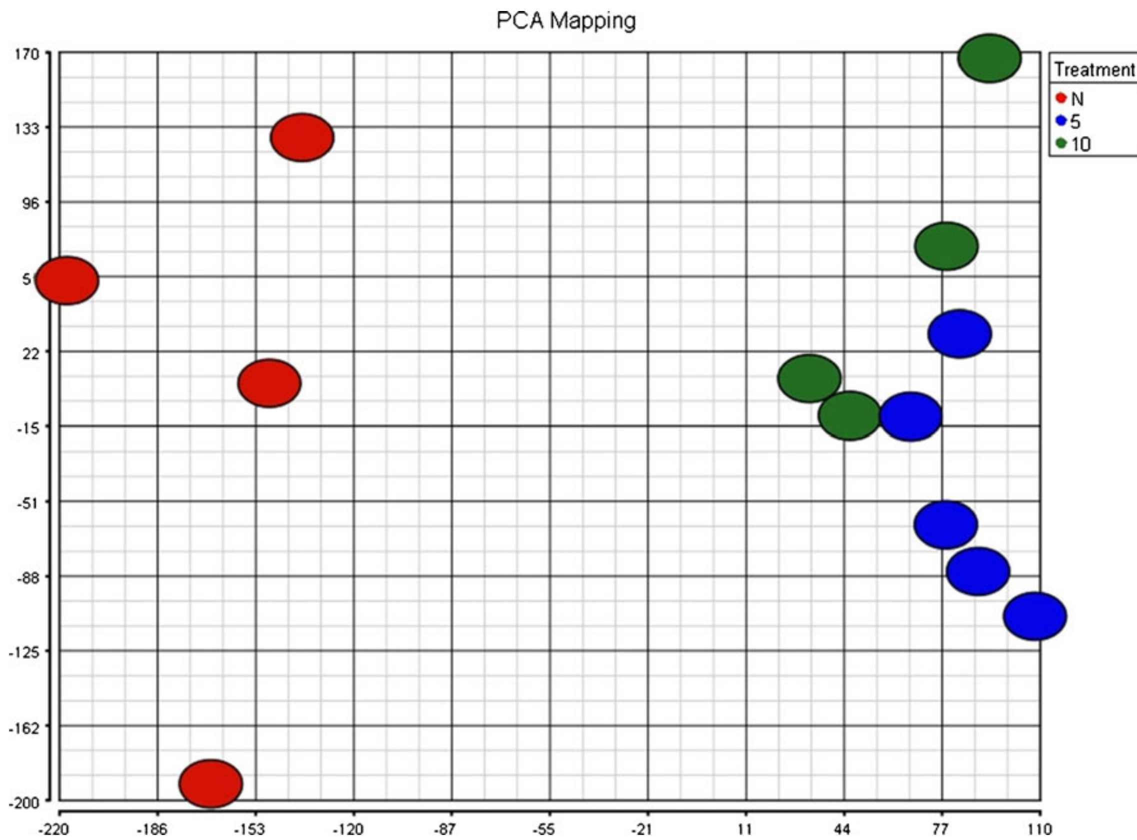

Fig. 2 Principal component analysis of entire gene expression levels of all samples on hCG treated and untreated samples. Principal component analysis (PCA) is performed on the entire expression levels of genes in each sample. The two axes in the figure are the first two principal components calculated by linearly transforming the original gene expression levels. As shown in the figure, samples belonging to the same groups are well separated from others. The distance between samples reflects their approximate degree of correlation. As it is seen in this figure the control samples (*blue*) were grouped into one distinctive group and with high distance, however, the treated samples (*red*=five thousand units, *green*=ten thousand units) were grouped into a space with crossing distance from each other

Since the variation between 5,000 IU and 10,000 IU hCG was minimal and the dose dependent differences were limited to few genes (Table 2), we used 4174 genes that were differentially expressed in both 5,000 IU and 10,000 IU hCG-treated samples to compare with hCG-free samples in the gene ontology analysis. We found 45 biologically significant processes to be involved in granulosa response to hCG. Table 3 shows the selected GO categories with the number of significantly altered gene with samples of up- or down-regulated important genes in these categories.

To confirm the microarray results by another method, first genes that are represented by more than one probe set in the microarray were checked for the concordance analysis to see whether they yield the same degree and direction of regulation (up or down) for each probe set. Percent of genes that show concordance among different probe sets representing the gene were above 95 % for each level of multi-probe sets. The transcriptional levels of AREG, EREG, FSHR, CYP19A, and STAR genes were investigated by using qRT-PCR on independently collected samples from patients who received either 5,000 or 10,000 IU hCG. FSHR was highly down-regulated (14.7 folds, this was 6.77 fold down regulation in the microarray experiments) in samples from 10,000 IU as compared to 5,000 IU in the qRT-PCR. The expression levels of other genes were similar between the two doses of hCG in both microarray and qRT-PCR analyses hence indicating the reliability of our gene expression findings.

Discussion

The results of this study demonstrated major changes in the gene expression profile of granulosa cells following hCG injections. However, the difference between 5,000 IU and 10,000 IU hCG was minimal in these PCOS patients. Gonadotrophin treatments have been previously reported to modulate gene expression in cultured granulosa cells [36]. Rapid effects of LH on murine granulosa cells included 60 genes to be differentially expressed within 1 h following the

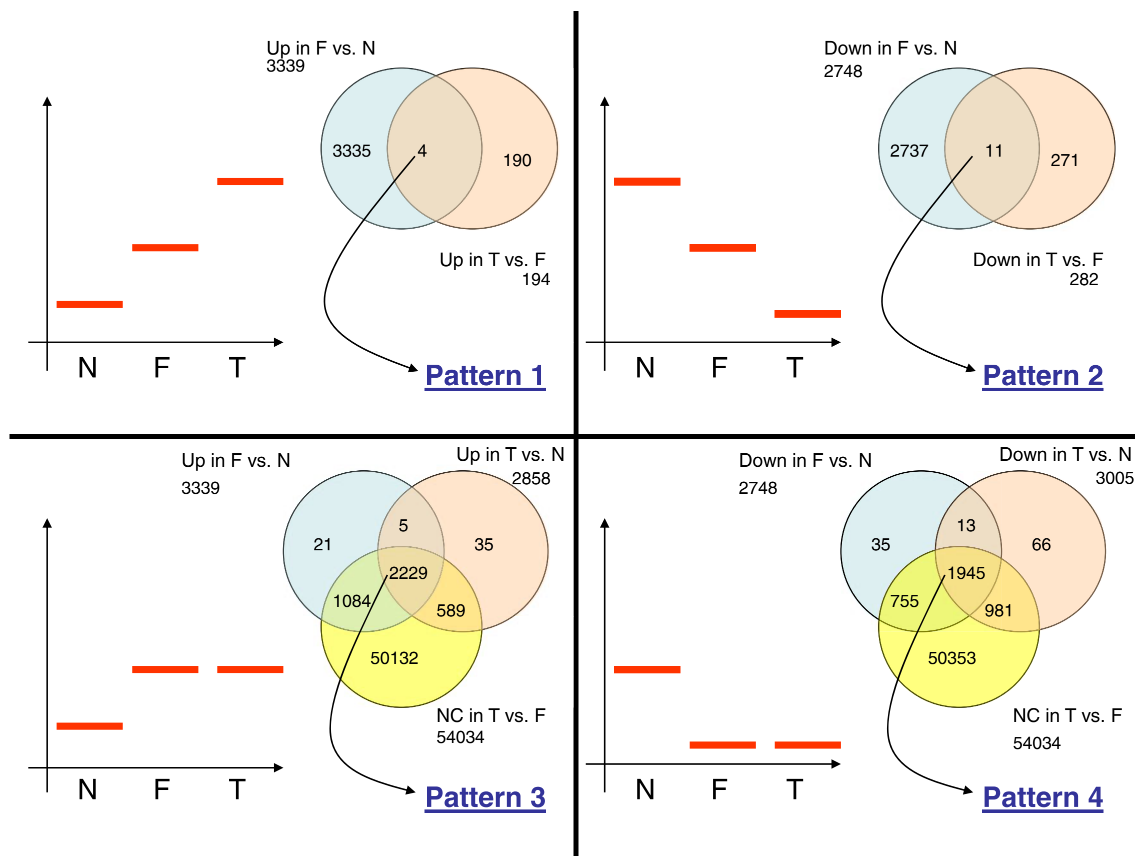

Fig. 3 A comparator analysis was done to enrich the genes further dis-regulated with different doses. In this comparative analysis, we seek genes up-regulated in five thousands unit treatment (compared to no-treatment), which are further up-regulated in ten thousands unit treatment (compared to five thousands unit treatment). We found only 4 genes that fall into this upward dose dependency pattern (Pattern 1). In contrast to up-regulation, there were 11 genes down regulated in a dose dependent manner (Pattern 2). Venn diagrams show the status of gene expression in comparison to different treatments. The line graphs show the up- or down-pattern in comparison to different treatments. (N: no hCG; F: 5,000 IU hCG; T: 10,000 IU hCG; NC no change)

Table 2 The transcripts those were up- or down-regulated in a dose dependent manner. The numbers were obtained by dividing the expression levels between different doses (F/N and T/N) and minus sign shows down regulation. (N no hCG; F 5,000 IU hCG; T 10,000 IU hCG)

Gene	Fold change (F/N)	Fold change (T/N)
Elastase 2A	29.34	2.87
LETM1 domain containing 1	2.1	2.11
MLX interacting protein-like	2.57	2.07
Chromosome 4 open reading frame 7	8.41	5.6
Heterogeneous nuclear ribonucleoprotein H1 (H)	−4.22	−2.11
ATPase, Class VI, type 11A	−7.79	−1.88
F-box protein 28	−3.62	−2.31
Calpain 5	−2.52	−1.94
HMG-box transcription factor 1	−2.18	−2.22
Follicle stimulating hormone receptor	−16.09	−6.77
Taxilin alpha	−3.9	−2.16
GNAS complex locus	−7.9	−3.33
Prune homolog (Drosophila)	−4.41	−2.11
Nucleosomal binding protein 1	−2.46	−2.47
Mediator of RNA polymerase II transcription, subunit 25 homolog (S. cerevisiae)	−2.97	−2.36

Table 3 Selected list of up- and down-regulated genes in different GO categories in response to hCG. 4174 genes were differentially expressed in both 5,000 IU and 10,000 IU hCG-treated samples to compare with hCG-free samples in the gene ontology analysis. Number in brackets shows the number of significantly altered transcript for each category

GO category	Upregulated	Downregulated
Cellular process (1901)	Adducin 1; amphiregulin; angiotensin I converting enzyme 2; axin 2; calmodulin 1; calpain 3; calponin 2; carboxypeptidase M; caveolin 2; CD24 molecule; claudin 2; collagen, type VI, alpha 3; complement factor H; cytosolic ovarian carcinoma antigen 1; discoidin; elastase 2A; ephrin-B2; epithelial membrane protein 2; fibroblast growth factor 12; Fibronectin 1; Glucagons; growth differentiation factor 15; Insulin receptor substrate 2; laminin, beta 3; lumican; Nuclear factor I/B; placental growth factor; prostaglandin F receptor; protocadherin 19; renin; septin 6; somatostatin receptor 1; Supervillin; synapsin I; syntaxin 2; titin;	Bone morphogenetic protein receptor, type II; calmodulin 3; calnexin; carboxypeptidase D; connective tissue growth factor; desmoglein 2; discoidin; docking protein 4; dual specificity phosphatase 1; dystonin; endothelial differentiation, sphingolipid G-protein-coupled receptor, 3; ephrin-B1; epithelial cell transforming sequence 2 oncogene; inhibitor of growth family, member 2; keratin 18; Kruppel-like factor 4; MAD1 mitotic arrest deficient-like 1; Nibrin; Paxillin; pituitary tumor-transforming 1; plexin C1; ras homolog gene family, member A; RAS-like, family 11, member B; replication factor C; secernin 3; secretory carrier membrane protein 1; sortilin 1; TIMP metallopeptidase inhibitor 3; transferrin receptor; translin; transportin 2; tumor protein p53 binding protein, 1
Metabolic process (1361)	Acyl-CoA synthetase long-chain family member 4; acyl-Coenzyme A dehydrogenase; adenosine deaminase; aldehyde oxidase 1; aminoadipate-semialdehyde synthase; apolipoprotein C-I; arachidonate 15-lipoxygenase, type B; argininosuccinate lyase; arylacetamide deacetylase (esterase); branched chain aminotransferase 1, cytosolic; carbonic anhydrase XII; carboxypeptidase M; carnitine O-octanoyltransferase; choline kinase beta; complement factor H; Cullin 1; fatty acid desaturase 3; fucosyltransferase 4; glutamate dehydrogenase 1; GPI deacylase; Heparanase; histidine decarboxylase; histone deacetylase 4; lactase; lactate dehydrogenase D; N-acetyltransferase 9; N-myristoyltransferase 2; phosphofructokinase, platelet; phospholipase D2; septin 11; steroid receptor RNA activator 1; titin; UDP-glucose dehydrogenase	Aldehyde dehydrogenase 18 family, member A1; ATPase, Class VI, type 11A; cathepsin L2; Dimethyladenosine transferase; Ethanolamine kinase 1; fumarate hydratase; galactose-1-phosphate uridylyltransferase; galactosylceramidase; gamma-glutamyltransferase-like 3; glutaminase; cytochrome P450, family 19, subfamily A, polypeptide 1; glutathione reductase; hect domain and RLD 5; Keratinocyte associated protein 2; lecithin retinol acyltransferase; lipoprotein lipase; MAX dimerization protein 3; palmitoyl-protein thioesterase 2; phosphatase, orphan 1; ribonuclease H2, large subunit; ring finger and CHY zinc finger domain containing 1; secernin 3; spermine oxidase; sulfatase 2; transcription elongation factor A (SII)-like 8; tripeptidyl peptidase I and II vesicle-associated membrane protein 3
Nucleobase, nucleoside, nucleotide and nucleic acid metabolic process (644)	Adenosine deaminase; Dihydropyrimidinase-like 2 and 3; Dihydropyrimidine dehydrogenase; Malate dehydrogenase 1, NAD (soluble); threonyl-tRNA synthetase-like 2; thymine-DNA glycosylase; UDP-glucose dehydrogenase; UDP-glucose pyrophosphorylase 2; valyl-tRNA synthetase	2′,5′-oligoadenylate synthetase 1; 2′-5′-oligoadenylate synthetase 3, 100 kDa; arginyl-tRNA synthetase-like; Dimethyladenosine transferase; exonuclease 1; flap structure-specific endonuclease 1; Heterogeneous nuclear ribonucleoprotein U; nei endonuclease VIII-like 3; nudix; pinin, desmosome associated protein; poly(A) polymerase alpha; ribonuclease H2, large subunit; senataxin; thymidylate synthetase
Signal transduction (551)	Calmodulin; ADP-ribosylation factor GTPase activating protein 31; CAP, adenylate cyclase-associated protein 1; G protein-coupled rece ptor 143; Janus kinase 1; GTPase activating Rap/RanGAP domain-like 3; Centaurin, gamma 1; adenylate cyclase 2; c-mer proto-oncogene tyrosine kinase; EPH receptor B1; G protein-coupled receptor 98, 115, 133 and 176; G protein-coupled receptor, family C, group 5, member A; growth factor receptor-bound protein 7; guanine nucleotide binding protein (G protein), gamma 8 Inositol 1,4,5-triphosphate receptor, type 1; inositol 1,4,5-trisphosphate 3-kinase A; Insulin receptor substrate 2; interleukin 1 receptor-like 1; interleukin-1 receptor-associated kinase 3; Membrane associated guanylate kinase; phosphatidylinositol transfer protein; phosphoinositide-3-kinase, class 2, beta	Plexin C1; endothelin receptor type A; protein tyrosine phosphatase, non-receptor type 11; mitogen-activated protein kinase kinase kinase kinase 5; epidermal growth factor receptor; glutamate receptor; protein tyrosine phosphatase, receptor type, F; guanine nucleotide binding protein (G protein), alpha 13; phosphatidylinositol-4-phosphate 5-kinase, type I, alpha; Ran GTPase activating protein 1; G protein-coupled receptor 20, 125 and 171; estrogen receptor 2; calcium/calmodulin-dependent protein kinase kinase 2, beta; regulator of G-protein signalling 3; phosphoinositide-3-kinase, regulatory subunit 1; G-protein signalling modulator 2; T cell receptor associated transmembrane adaptor 1; phosphoinositide-3-kinase, class 2, alpha polypeptide; colony stimulating factor 1 receptor; protein kinase, AMP-activated, beta 1 non-catalytic

Table 3 (continued)

GO category	Upregulated	Downregulated
		subunit; ADP-ribosylation factor-like 2 binding protein; ADP-ribosylation factor-like 5B; anti-Mullerian hormone receptor, type II; calmodulin 3; cAMP responsive element binding protein 1; citron; G protein-coupled receptor 132; G protein-coupled receptor kinase 4 and 5; GTP binding protein 2; guanylate cyclase 1, soluble, alpha 2; insulin receptor-related receptor; interleukin 6 signal transducer; Mitogen-activated protein kinase 12; nuclear receptor coactivator 2; phosphatidylinositol-4-phosphate 5-kinase, type II, beta; phospholipase D1; Prolactin receptor; protein kinase, AMP-activated, alpha 2 catalytic subunit; protein tyrosine phosphatase, receptor type, F; receptor-interacting serine-threonine kinase 2; serine/threonine kinase 4; tetraspanin 6; thyroid hormone receptor associated protein 6; TNF receptor-associated factor 4; tyrosine 3-monooxygenase
	polypeptide; phospholipase C, beta 1; prostaglandin E synthase; prostaglandin F receptor; ras homolog gene family, member U; Receptor tyrosine kinase-like; orphan receptor 1; Rho guanine nucleotide exchange factor (GEF) 4; somatostatin receptor 1	
Transcription (430)	Transcription factor AP-2 alpha; Kruppel-like factor 16; Nuclear factor I/B; AT-binding transcription factor 1; histone deacetylase 4; transducin (beta)-like 1X-linked; elongation factor, RNA polymerase II, 2; runt-related transcription factor 2	Nuclear receptor subfamily 4, group A, member 2; thymopoietin; primase; general transcription factor II; Sp1 transcription factor; transcription factor 8; MADS box transcription enhancer factor 2, polypeptide C; RNA binding motif protein 9; activating transcription factor 3; E2F transcription factor1 and2; BCL2-associated transcription factor 1; splicing factor, arginine/serine-rich 8; cAMP responsive element binding protein 1; transcription elongation factor A (SII), 3; pirin; nuclear receptor interacting protein 1; transcription factor Dp-1; activating transcription factor 1
Cell differentiation (315)	STAM binding protein; Cullin 1; Bone morphogenetic protein 6; caveolin 2; syntaxin 2; titin; dedicator of cytokinesis 1; filamin B, beta; laminin, alpha 3; tensin 4; ephrin-B2; p21 (CDKN1A)-activated kinase 2; follistatin; tumor necrosis factor receptor superfamily, member 11b and12A; Ataxin 1; pregnancy-associated plasma protein A; clusterin; gliomedin; Transforming growth factor, beta 2; actinin, alpha 2; Neuropilin 1; placental growth factor; hepatocyte growth factor; tumor necrosis factor superfamily, member 11	Sortilin 1; mitogen-activated protein kinase kinase kinase 1; bone morphogenetic protein 3; periphilin 1; connective tissue growth factor; Kruppel-like factor 6; senataxin; ephrin-B1; interferon-related developmental regulator 1; NGFI-A binding protein 1; myogenic differentiation 1
Intracellular signaling cascade (285)	STAM binding protein; Janus kinase 1; GTPase activating Rap; Signal transducer and activator of transcription 3; protein kinase C, alpha and eta; Tensin 1; growth factor receptor-bound protein 7; adenylate cyclase 2; Rho GTPase activating protein 6	Endothelin receptor type A; dual specificity phosphatase 1; guanine nucleotide binding protein (G protein), alpha 13; estrogen receptor 2 (ER beta); calcium/calmodulin-dependent protein kinase kinase 2, beta; regulator of G-protein signalling 3; phosphatidylinositol-specific phospholipase C, X domain containing 1; phospholipase D1, phosphatidylcholine-specific; natriuretic peptide receptor C; tetraspanin 6
Cell cycle (266)	M-phase phosphoprotein 6; quiescin Q6	Cell division cycle associated 5 and 8; cell division cycle 25A and 25C; checkpoint suppressor 1; spindling; karyopherin alpha 2; kinetochore associated 1; nuclear transcription factor Y, gamma; M-phase phosphoprotein 1; nibrin

Table 3 (continued)

GO category	Upregulated	Downregulated
Response to stress (208)	Titin; heat shock 27 kDa protein 1 and 3; Interleukin 17D; growth arrest and DNA-damage-inducible, alpha; clusterin; hypoxia-inducible protein 2; Fibronectin 1; hepatocyte growth factor; interleukin 1 receptor, type I	Heat shock 60 kDa protein 1; heat shock transcription factor 2; thrombomodulin; replication factor C; heat shock protein B6; senataxin; mitogen-activated protein kinase 9
Cell proliferation (168)	Axin 2; platelet-derived growth factor alpha polypeptide; Hepatocyte growth factor-regulated tyrosine kinase substrate; cholecystokinin B receptor; Insulin receptor substrate 2; glucagons; Transforming growth factor, beta 2; somatostatin receptor 1; epiregulin; placental growth factor; amphiregulin	Insulin-like growth factor 2; epidermal growth factor receptor; vascular endothelial growth factor; fibroblast growth factor receptor-like 1; bone morphogenetic protein receptor, type II; platelet derived growth factor C; hepatoma-derived growth factor; ephrin-B1; proliferating cell nuclear antigen; androgen receptor; nibrin
Phosphorylation (152)	Janus kinase 1; cyclin-dependent kinase 3; AXL receptor tyrosine kinase; caveolin 2; neurofibromin 2, titin; protein kinase C, alpha and eta	Epidermal growth factor receptor; anti-Mullerian hormone receptor, type II; vaccinia related kinase 1; mitogen-activated protein kinase 9 and 12
Programmed cell death (150)	Programmed cell death 11; caspase 6; tensin 4; Apoptosis, caspase activation inhibitor; protein kinase C, alpha; tumor necrosis factor receptor superfamily, member 19; growth arrest and DNA-damage-inducible, alpha; osteoprotegerin; clusterin; Transforming growth factor, beta 2	Death-associated protein kinase 1; neurofibromin 1; BCL2-like 1 and 13; caspase 2; estrogen receptor 1; protein phosphatase 1; BRCA1 associated RING domain 1; TGF-beta induced apoptosis protein 2; apoptosis inhibitor 5; p53 target zinc finger protein; PRKC, apoptosis, WT1, regulator; B-cell translocation gene 1, anti-proliferative
Macromolecular complex assembly (103)	Caveolin 2; Histone 1, H1c, H2ad and H3d; histone 2, H2be; hemochromatosis	Paxillin; chromatin assembly factor 1
Ubiquitin cycle (101)	Ubiquitin specific peptidase 11, 13 and 32; ubiquitin-activating enzyme E1	Ubiquitin-conjugating enzyme E2C, E2S; ubiquitin specific peptidase 18, 34, 48 and 51; ubiquitination factor E4B
Mitosis (96)	Cyclin-dependent kinase 3; titin; cyclin A1	Cyclin-dependent kinase 2; aurora kinase A and B; MAD2; cyclin A2, B1, B2 and G2; cell division cycle associated 5; M-phase phosphoprotein 1; nibrin
DNA replication (79)	Ligase IV, DNA, ATP-dependent; Topoisomerase (DNA) I	Topoisomerase (DNA) II alpha; ribonucleotide reductase M2 polypeptide; replication initiator 1; polymerase (DNA directed), eta, epsilon beta and kappa; replication factor C; topoisomerase (DNA) III alpha; exonuclease 1; replication factor C; primase; geminin
Protein kinase cascade (78)	Neurofibromin 2; Hepatocyte growth factor-regulated tyrosine kinase substrate; protein kinase C, alpha	Calcium/calmodulin-dependent protein kinase kinase 2, beta; mitogen-activated protein kinase 1
DNA repair (66)	Ligase IV, DNA, ATP-dependent; Kinesin 2; cullin 4B	Polymerase (DNA directed), eta; chondroitin sulfate proteoglycan 6; senataxin; nibrin
Growth (63)	Bone morphogenetic protein 6; activin A receptor, type IB; discoidin; WNT1 inducible signaling pathway protein 1; Transforming growth factor, beta 2; epiregulin	Estrogen receptor 2; fibroblast growth factor receptor-like 1; tumor protein p53; connective tissue growth factor
Enzyme linked receptor protein signaling pathway (61)	Activin A receptor, type IB; protein kinase C, alpha; endothelin 2, follistatin, Insulin receptor substrate 2; growth differentiation factor 15; epiregulin; Fibronectin 1	Epidermal growth factor receptor; insulin-like growth factor 2; vascular endothelial growth factor; inhibin, beta A; colony stimulating factor 1 receptor; SMAD; bone morphogenetic protein receptor, type II; Prolactin receptor; connective tissue growth factor; insulin receptor-related receptor
Ras protein signal transduction (54)	Rho guanine nucleotide exchange factor (GEF) 10	Rho GTPase activating protein 1; neurofibromin 1; ras homolog gene family, member A and B
Cell growth (42)	Activin A receptor, type IB; discoidin; WNT1 inducible signaling pathway protein 1; Transforming growth factor, beta 2; sphingosine kinase 1; pregnancy-induced growth inhibitor	Estrogen receptor 2 (ER beta); fibroblast growth factor receptor-like 1; tumor protein p53; connective tissue growth factor; inhibitor of growth family, member 2; androgen receptor
Dephosphorylation (34)	Protein phosphatase 2C; tensin 3; myotubularin 1; protein tyrosine phosphatase, non-receptor type 1	Dual specificity phosphatase 1, 3, 5 and16; protein tyrosine phosphatase, non-receptor type 11 and 12; protein tyrosine phosphatase, receptor type, A and F;

Table 3 (continued)

GO category	Upregulated	Downregulated
	type 3; Protein tyrosine phosphatase, receptor type, G and M; protein phosphatase 1E and H	myotubularin related protein 1; protein phosphatase 5, catalytic subunit; protein phosphatase 1 K; protein tyrosine phosphatase type IVA
Nucleocytoplasmic transport (32)		Exportin 7; importin 8; transportin 2
Phosphoinositide-mediated signaling (26)	Endothelin 2; sphingosine kinase 1	Endothelin receptor type A; epidermal growth factor receptor; aurora kinase A; replication factor C
JNK cascade (18)	Mitogen-activated protein kinase kinase kinase 6 and 13; TRAF2 and NCK interacting kinase	Mitogen-activated protein kinase kinase kinase kinase 2 and 5; mitogen-activated protein kinase 9
Response to hypoxia (14)	Argininosuccinate lyase; Hypoxia-inducible factor 1	Vascular endothelial growth factor
Regulation of lipid biosynthetic process (5)	Steroidogenic acute regulator, protein kinase, AMP-activated, alpha 1 catalytic subunit	
Gonadotropin secretion (4)	Follistatin	Inhibin alpha and beta A
Oocyte maturation (4)	Epiregulin	

hCG injection [8]. Gilbert et al. [13] recently showed that over 3,000 transcripts were differentially expressed following LH surge in cows. Similarly, in the current study, hCG injections resulted in the modulation of thousands of genes.

Although the differences in gene expression caused by hCG injection was substantial, the difference between 5,000 IU and 10,000 IU hCG was minimal. The similarity of gene expression between 5,000 and 10,000 IU hCG was apparent in both the clustering and principal component analyses. One can expect a drug to have a dose-dependent response up to the drug's saturation level. The results showed that thousands of genes were neither further up- nor down-regulated with the 10,000 IU hCG injection compared to 5,000 IU suggesting that this saturation might have been reached in a dose which is equal or lower than 5,000 IU in PCOS. In our study, only 15 genes responded in a dose-dependent manner. Among these 15 genes, only FSHR has been reported to have a known function in granulosa cells.

In the present study, the injection of hCG caused 16-fold reduction in FSHR gene expression and hCG has been earlier suggested as a down-regulator of FSHR [24]. PCOS patients have been reported to overexpress FSHR, as compared to healthy women, [9], which may explain the dose-dependent response of FSHR in the current study. FSHR is involved with the growth of antral follicles and its expression has been reported to be higher in granulosa cells obtained from small follicles than those from larger follicles in both PCOS and healthy women [9]. Furthermore, hCG has been shown to down-regulate FSHR gene expression in bovine dominant follicles [31], which is similar to the response observed in our study. Mutations and polymorphisms of FSHR gene has been linked to the development of OHSS [28]. It will be interesting to investigate the role of dose responsive regulation of FSHR in the development of OHSS in future studies since the low-dose hCG has been suggested to have preventive role on OHSS [30].

To further elucidate the changes in gene expression following hCG injection, gene ontology analysis was performed by comparing hCG-free vs. hCG-injected patients. The effect of hCG on a large number of biological processes is not surprising, as LH surges or hCG injections trigger a plethora of events that lead to the ovulation. These include vascular changes, rupture of the follicle wall, cumulus cells expansion, oocyte maturation and luteinization of granulosa cells [11]. The largest groups of differentially expressed genes (more than 500 genes) belong to regulation of cellular processes, signal transduction, cellular component organization, and biogenesis, which are in line with hCG's physiological functions.

Furthermore, we have looked into differentially expressed genes related to oocyte maturation since the maturity of oocytes is an important end point following hCG injection in assisted reproduction. Epidermal growth factor (EGF)-like growth factors have been previously reported to be involved in cumulus expansion and oocyte maturation [32]. Among those, amphiregulin and epiregulin have been found to be rapidly up-regulated in response to LH in periovulatory mouse granulosa cells [8] and increased the in vitro maturation rates of human and rhesus oocytes [5, 33, 44]. In the present study, amphiregulin and epiregulin were up-regulated around 30 and 9 fold in response to both doses hCG, respectively. Amphiregulin has been shown to be the most abundant EGF-like growth factor during the human peri-ovulatory period [44]. Although hCG injection up-regulated the amphiregulin and epiregulin gene expression, the difference between the two doses of hCG was not significantly different in both micro-array and RT-PCR analysis in the present study.

Recently, Xu et al. [42] studied ovarian follicular gene expression of rhesus monkeys before, and 12, 24 and 36 h following the hCG injection. Similar to the present study and the reports mentioned earlier [8, 44], amphiregulin and epiregulin were upregulated by hCG injection. They also analyzed the changes in mRNA level for gonadotropin receptors and steroidogenic enzymes, and showed that mRNA levels for FSHR, enzymes converting androgen to estrogen (CYP19A) declined following hCG injection [42]. We also found similar reductions in our study. Steroidogenic acute regulatory protein (StAR) which is important in luteinization showed an increased expression following hCG [42]. Similarly, StAR mRNA was up-regulated in our study. Again there were no differences in transcriptional levels between 5,000 and 10,000 IU hCG for all of the above mentioned genes in this study.

Similar to the molecular response demonstrated in our study, the clinical efficacies has been reported to be parallel among different doses of hCG in patients with PCOS. Abdalla et al. [1] showed that 5,000 IU and 10,000 IU resulted in similar numbers of successfully recovered oocytes and clinical outcome. A reduced dose of 3,300 IU of hCG also resulted in a similar proportion of mature eggs, and fertilization and pregnancy rates in high responder patients, as compared to patients who received higher doses of hCG [37]. Moreover, an hCG dose as low as 2,500 IU was not reported to have any adverse effects on IVF outcomes in PCOS patients [23]. This same low dose of hCG has been found to prevent the occurrence of OHSS, without affecting the IVF cycle outcome, in high risk women [30]. Similarly, none of the patients developed ovarian hyperstimulation in this study.

One of the drawbacks of this study is the selection of the patients. Although the patients in no hCG group do not demonstrate the normal situation, they were used as a reference to compare to hCG treated patients. They served the purpose of study since the difference between no hCG and hCG treated groups was a major one. Moreover, patients in 5,000 IU and 10,000 IU hCG groups were not selected prospectively; rather they were selected subjectively during the stimulation process according to history or ovarian volume. This might present a bias to the study; nonetheless, the difference in the response was very minimal which implicates that this bias might have been insignificant.

In conclusion, although hCG injection caused a major change in gene expression profiles of granulosa cells, 10,000 IU hCG resulted in very minimal changes in the gene expression levels of granulosa cells as compared with 5,000 IU. These results are in line with previously published reports that did not correlate hCG dosages with clinical outcomes. Thus, the use of 5,000 IU hCG may be sufficient to achieve final follicular maturation in PCOS patients. The use of 10,000 IU hCG may not provide any additional benefit to the patient as previously suggested in the clinical studies.

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