

Optimizing the Luteinization Timeline: A Comparative Study of Human Mature and Immature Granulosa Cells *In Vitro*

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During luteinization, the granulosa cells (GC) undergo extensive expressional changes. This transition, prompted by an increase in gonadotrophins, transforms GC into granulosa-lutein cells of the corpus luteum. However, the timeline of the transition has currently not been adequately explored. In this study, human GC (mature GC) from preovulatory follicles collected through in vitro fertilization (IVF) and GC (immature GC) from antral follicles, collected through our ovarian tissue cryopreservation (OTC) program, are compared to establish the *in vitro* timeline for luteinization of early and late-stage GC. During cell culture both media and GC were collected every 12 hours, to investigate changes in gene expression and hormone levels.

GC was donated by six women (n=57) aged 27-40 years undergoing fertility treatment or preservation. The expression of STAR, CYP11A1, CYP19A1, and 17 β HSD genes, along with hormone assay for estradiol and progesterone, was utilized to analyze luteinization.

The mature GCs displayed minor differences in gene expression and estradiol production between the control and androstenedione (100 nm) groups. There was observed a significant difference ($p \leq 0.05$), between the treatment groups in the expression of CYP11A1 after 72 hours ($p=0.03$) and STAR after 84 hours ($p=0.02$). Furthermore, within the androstenedione treatment group, a significant variation was noted in the expression of the gene 17 β HSD between the time points 72 hours and 96 hours ($p=0.04$). Estradiol levels demonstrated no time-dependent difference within or between the treatment groups.

The collection of immature GCs is ongoing; preliminary data indicate a gene-level trend wherein the expression of luteal cell markers, STAR and CYP11A1, increases 8-10-fold over 48 hours, while the expression of GC-specific gene markers, CYP19A1 and 17 β HSD, exhibits a decrease ranging from

1.2- to 2.6-fold over the same period. Additionally, estradiol levels increase 2-fold after 24 hours post-initiation of *in vitro* culture, parallel with a marked increase in progesterone for each time point resulting in a 9-fold increase after 48 hours.

Preliminary observations suggest that the majority of mature GCs harvested from IVF procedures exhibit characteristics of luteinization, with a peak in luteal gene expression observed approximately 48 hours following the onset of *in vitro* culture, and appears unaffected by the presence of androgen supplementation. This pattern raises concerns regarding their suitability as a model for *in vitro* studies of luteinization. Conversely, immature GCs demonstrate an upregulation of luteal gene expression approximately 24 hours post-culture initiation. This is accompanied by an increase in estradiol levels up to the 24-hour timepoint, followed by a pronounced surge in progesterone. These findings may imply that GCs from antral follicles undergo luteinization between 24-48 hours after the onset of *in vitro* culture. Additional time points and patients will be included over the following months to specify and confirm these preliminary conclusions.