Doctoral Thesis

Study on Mitochondria Functions in Granulosa Cells during Follicular Development Process

(Summary)

S. A. Masudul Hoque

Graduate School of Biosphere Science
Hiroshima University

September 2019

The quality, as well as the number of ovulated oocytes is essential for *in vitro* production (IVP) of embryos. Moreover, successful *in vivo* fertilization is also dependent on the successful follicular development and oocyte competence that is decedent from healthy follicles. During the follicular development process, the proliferation and differentiation of granulosa cells (GCs) are essential where the mitochondria functions are critical. Therefore, the present study was aimed to investigate the mitochondria functions in GCs during the follicular development process and the subsequent events of female reproduction. To achieve the aim, firstly, the mitochondrial central dogma and protein turnover mechanism were investigated. After that, generation of relative oxygen species (ROS) with their negative effects on GCs, oocytes quality, and fertility was studied. Finally, the technique of how to minimize the ROS associated damages and to improve the ovarian health and fertility was investigated.

1. Mitochondrial central dogma is activated during the follicular development process

Mitochondrial biogenesis is essential to induce mitochondrial activity and ATP production. In Experiment 1, it was hypothesized that activation of mitochondrial central dogma is essential for mitochondrial activity and ATP production in GCs during the follicular development process. To clear the hypothesis, the mitochondrial gene expression, protein synthesis, and the replication of mtDNA in GCs were studied using both *in vivo* superovulation and *in vitro* culture model. For *in vivo* study, GCs were collected from immature (23-days-old) mice ovaries before and after 24/48 h of eCG injection. It was observed that the mitochondrial activity and ATP production were dramatically induced with a significant increase in the expression of mitochondrial genes coding the polypeptides in the ETC complexes (*Nd1-6*, *Cytb*, *Atpase6,8*) and synthesis of mitochondrial proteins (MT-ND1 and MT-ND6) in a time-dependent manner up to 48 h of eCG injection. Moreover, a significant time-dependent increase in the expression of the gene coding nuclear enzymes and proteins that regulate the mtDNA replication (*Polg1* and *Polg2*) and transcription (*Polrmt*, *Tfam*, *Tfb2m*), along with mtDNA copy number was observed. For more clarification, an *in vitro* culture of GCs was performed in serum-supplemented DMEM/F12 medium added with or without FSH+testosterone for 24 h. It was also observed that the expression of mitochondrial genes and the genes controlling the replication and transcription factors were also significantly increased. The dramatic increase in the mitochondrial gene expression, protein synthesis, and mtDNA replication observed in this experiment revealed the activation of mitochondrial central dogma during the follicular development process. This is the first report regarding the underlying mechanisms of mitochondrial biogenesis in GCs during the follicular development process.

2. Mitochondrial protein turnover is critical for the proliferation and differentiation of granulosa cells during the follicular development process

Mitochondrial ATP production in the ETC-complexes involves a number of proteins, among which 13 are encoded by mtDNA, the rests are by the nucleus. Moreover, various mitochondrial membrane proteins are essential for mitochondrial biogenesis. In Experiment 2, it was hypothesized that mitochondrial protein turnover is critical for the proliferation and differentiation of GCs. To clear this hypothesis, mitochondrial protein synthesis was inhibited by D-chloramphenicol (CRP) treatment. For this, an *in vitro* culture of GCs collected from eCG-primed immature mice ovaries was conducted in serum-supplemented DMEM medium added with or without FSH + testosterone + CRP for 24 h. It was observed that CRP not only suppressed the FSH-induced mitochondrial protein synthesis but also dramatically reduced the ATP production, mitochondrial activity and GCs proliferation along with the

expression of genes marker for GCs functions, that were previously increased by FSH stimulation. Since the follicular fluid contains a lower level of glucose compared to the serum, GCs were cultured in the serum-supplemented DMEM media containing high glucose, low glucose, and glucose free. It was observed that the GCs proliferation and ATP production were independent of glucose concentration, suggesting that mitochondrial ATP production is the primary source of ATP supplies to GCs during the follicular development process. These findings highlight the physiological importance of mitochondrial protein turnover mechanisms and ATP production in GCs proliferation and differentiation during the follicular development process.

3. ROS generated in FSH-stimulated GCs during the follicular development process causes mitochondrial damages and apoptosis in GCs

Mitochondrial ATP is produced through a sequential redox reaction where ROS were produced as a by-product, which in excess quantity causes mitochondrial and cellular damage. Therefore, it was hypothesized in Experiment 3, that ROS associated damages occurred during *in vitro* FSH-induced GCs proliferation or eCG-stimulated follicular development process *in vivo*. Apparently, it is difficult to estimate the ROS associated damages. Therefore, the mice (*in vivo*) and GCs (*in vitro*) were treated with pyrroloquinoline quinone (PQQ) and hypothesized that if PQQ improved the mitochondria functions and/or GCs physiology, it would be evident that ROS damages occurred in FSH-stimulated GCs. In this experiment, PQQ treatment was applied in both *in vitro* culture of GCs (0-1000 nM PQQ in DMEM/F12 media) and *in vivo* superovulation treatment through drinking water (2 mg PQQ/Kg body weight). In results, it was observed in both *in vitro* and *in vivo* study that ATP production, mitochondrial membrane potential, genes expression, and protein synthesis were dramatically increased with a significant increase in ROS generation by FSH-stimulation. However, it was observed that PQQ treatment could further significantly increase the FSH-induced mitochondria attributes mentioned above while decreasing the ROS. The improved performance by reducing the ROS generation suggested that the ROS damages and/or inhibits the induction of mitochondria functions, gene expression, protein synthesis, and ATP production during FSH-induced GCs proliferation in the follicular development process. Moreover, significant damage in mtDNA was evident in FSH-stimulated GCs, which was recovered by PQQ treatment. In cellular level, it was observed in both *in vivo* and *in vitro* study that FSH significantly induced the cell cycle progression to S and G2/M phase and increased the viability of GCs with a significant reduction in apoptosis and death. However, these parameters were significantly improved by PQQ treatment. The performance gap between PQQ treated and the untreated group might be considered as the ROS damage and might be due to the damages in GCs mitochondria attributes. However, the ROS damages and the recovery role of PQQ provide a new story to ovarian biology.

4. PQQ improves the number of oocyte quality, ovarian health, and female fertility

Considering the results of the Experiment 3, it was hypothesized in Experiment 4, that PQQ not only improves the GCs physiology but also has an extended effect on the oocyte quality, follicular health, and fertility. To clear this hypothesis, the oocyte quality and ovarian morphology were studied in eCG-superovulated immature mice, and the fertility parameters were studied in natural mating condition using mature mice (8-weeks-old). In both cases, mice were treated with PQQ through drinking water (2 mg/Kg body weight). It was observed that PQQ treatment improves the ovarian morphology and increases the number of ovulatory follicles by reducing the follicular atresia during the follicular development process. Moreover, the serum estrogen was also increased dramatically during the follicular development process, but the synthesis of progesterone became adequate, during

luteinization and ovulation process. Furthermore, the number of ovulated oocytes at superovulation, along with the number of offspring per delivery was also increased by PQQ treatment. Nevertheless, there was no abnormality observed in the subsequent *in vitro* fertilization nor in the regularity of estrous and pregnancy. These findings will contribute to animal reproduction and also in the treatment of woman infertility.

Conclusion

In conclusion, the present study unveiled the mechanism of mitochondrial biogenesis through the induction of gene expression and protein synthesis that is essential to induce mitochondrial activity and ATP production during the follicular development process. This study also specified the mitochondria as the primary sources of ATP supplies to the GCs. Moreover, the generation of ROS and their adverse effects causing mitochondrial damages, apoptosis in GCs and follicular atresia, contributed a new story in the mammalian ovarian biology. Furthermore, the beneficial and/or regulatory role of exogenous mitochondria-specific antioxidants (such as, PQQ) in repairing the mitochondrial damages, rescuing GCs from apoptotic pathways and also in reducing the follicular atresia throughout the FSH-stimulated follicular development process that consequently, resulted in a significant increase in the number of ovulated oocytes at ovarian stimulation as well as the number of offspring per delivery in natural cyclic mice is a milestone in the mammalian reproduction and fertility. This is a novel technology unveiled in this study, which will contribute new insights to the ovarian biology. Moreover, this technique will be useful in developing ART for animal reproduction as well as could be applied in the diagnosis and treatment of woman clinical infertility.