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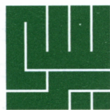

Primordial Follicles Development of Immature Mice Ovary after FSH and Ovary Cutting Treatments

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KEYWORDS *ovary; primordial follicles; in vitro culture; development*

ABSTRACT *The aims of the study were to investigate the influence of FSH and the ovary cutting treatments on the development of primordial follicles into preantral and antral follicles. Ovaries were grouped as whole ovary (without cutting); partially cut ovary and completely cut ovary (hemi ovary). All ovary groups were cultured in Dubellco's Modified Eagle Medium (DMEM) containing 5μg/ml insulin, 10μ g/ml transferrin, 5μg/ml selenium (ITS), 5% FBS, 50 μg/ml gentamycin with and without 100 μIU/ml Follicle Stimulating Hormone (FSH). Cultures were done in 5% CO_2 incubator at 37°C. Results showed that after 8 days in vitro cultured in DMEM containing FSH, the average number of preantral follicles isolated from each whole, partially-cut and completely-cut ovaries were 16.1 $\pm$ 3.3, 26.8 $\pm$ 7.7 and 12.3 $\pm$ 1.9, respectively. On the other hand, those cultured in DMEM without FSH they were 17.3 $\pm$ 3.8, 23.3 $\pm$ 5.2 and 17.8 $\pm$ 2.8, respectively. After additional cultured for 8 days, the percentage of preantral follicles developing into the antral follicles in DMEM with and without FSH were 26.7 $\pm$ 5.7 and 11.7 $\pm$ 2.9, respectively. In conclusion, the supplementation of FSH in the culture medium did not increase the number of preantral follicles, but significantly increased the number of antral follicles. The ovary cutting treatment significantly increased the average number of collected preantral follicles.*

Ovary is the primary organ in female reproductive system and has two major physiological roles, the first is for the development and release of a matured oocyte for fertilization (McGee & Hsueh, 2000) and the second is for synthesizing and secreting hormones that are essential for follicles development, estrous cyclicity and maintenance of the reproductive tract and its function (Hirshfield, 1991). Mammalian ovary contains a large number of follicles that are in various developmental stages. The resting primordial follicles are the earliest development stage which is the most abundant, followed by the preantral and early antral follicles and the smallest number are the fully antral follicles (Katska-Ksiqzkiewicz, 2006). Throughout the female reproductive lifespan only a small portion of the primordial follicles undergo development and will produce oocytes that competent to undergo successfully maturation and ovulation. While the rest of the ovarian oocytes undergo

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atresia. Therefore, rescuing some of these follicles will give great practical benefit for increasing the utilization of reproductive potential for further application in livestock production and reproduction in human and rare or endangered species (Gunasena *et al.*, 1998; Hovatta *et al.*, 1999; Wright *et al.*, 1999).

Efforts have been done by multiple ovulation and *in vitro* embryo production through *in vitro* culture of oocytes. However, this procedures only utilized a small portion of the available tertiary early antral and antral follicles. Therefore, to provide additional sources of oocytes, utilization of the earlier development stages of follicles (the resting primordial follicles) and development of the simple and efficient technologies for developing these follicles into antral follicles and producing developmental competence oocytes is considerable interest.

Two main methods have been used for developing the resting primordial follicles, that are the transplantation of ovarian tissue grafts and *in vitro* culture of primordial follicles in the ovarian tissue. The control of primordial activation requires complex bidirectional signalling between the oocytes and the surrounding somatic cells, involving specific cytokines and growth factors (Skinner 2005; Mclaughlin & McLver 2009). Since, the oocytes-granulosa cell complexes from mice younger than 6 days has been reported could not successfully cultured and developed to the later stages (Eppig & O'Brien 1996), the development of primordial follicles should be done in organ culture system. However, the obstacles in organ culture system is the size of the samples being cultured will faced the oxygen and nutrient exchange problems.

Two types of *in vitro* culture systems have been reported that are the nonspherical (two dimensional) and spherical (three-dimensional) have been developed to support the preantral and antral follicle growth and development (Xu *et al.*, 2006). The two-dimensional culture system have been developed to culture enzymatically isolated granulosa-oocyte complexes and the preantral follicles mechanically isolated from mice (O'Brien *et al.*, 2003; Lenie *et al.*, 2004).

This research is aim to compare the development of primordial follicles to the preantral follicles in whole, partially-cut and completely-cut ovaries of 5 days old mice using two dimentional *in vitro* culture system in microdrop Dubellco's Minimal Eagle Medium (DMEM) medium with and without Follicle Stimulating Hormone (FSH) supplementation.

MATERIALS AND METHODS

The reseach was conducted in two steps experiment i.e: (I) to examine the effect of FSH and ovary cutting on the number of preantral follicles developed in whole, partially-cut and completely-cut ovaries in medium with and without Follicle Stimulating Hormone (FSH); and (II) to examine the effect of FSH supplementation on the number of antral follicles and oocytes using the preantral follicles from the experiment I. Ovaries of 5 days old mice were grouped as whole ovary (without cuting), partially-cut ovary and completely-cut ovary (hemi or half ovary).

In Vitro Culture of 5 Days Old Mice Ovaries: Experiment I

Five days old mice (DDY strain) ovaries were excised from the ovarian bursa and grouped into three that were whole, partially-cut and and completely-cut ovaries. The cutting of ovaries were using insulin gauge needle. Ovaries were washed three times in modified Phosphate Buffered Saline (PBS) containing 0.5% Fetal Calf Serum (FCS) and 50 μg/ml gentamycine, followed by two times washing in culture medium DMEM

containing 10% FCS, 5µg/ml insuline, 10µg/ml tranferrin, 5µg/ml selenium (Boehringer Mannheim, Germany), 50 µg/ml gentamycine supplemented with or without 100mIU/ml FSH (Denka Pharmaceutical, Japan). Whole, partially-cut and and completely-cut ovaries were cultured individually in 100 microliter drops of culture medium under mineral oil in plastic culture dish, in 5% CO_2 incubator at 37°C for 8 days. Every 48 hours, half of the culture medium from each drop was replaced by fresh medium.

Isolation of Preantral Follicles

After 8 days *in vitro* cultured, the whole, partially-cut and and completely-cut ovaries were further cultured in DMEM containing 0,5 mg/ml collagenase II (Sigma, USA) for 1 hour. The preantral follicles were isolated from whole, partially-cut and and completely-cut ovaries enzymaticall using collagenase followed by mechanically using insulin gauge neddle under wide field light inverted microscope. Only intact preantral follicles (defined as follicle with enclosed oocytes and surrounded by granulosa cells, basal membrane and theca cells) will further culture to develop to the antral follicles.

***In Vitro* Culture of Isolated Preantral Follicles: Experiment II**

The intact isolated preantral follicles from experiment I were further cultured individually in 30µl drop of the same medium of experiment I under mineral oil. Culture were done in 5% CO_2 incubator at 37°C for 8 days. Every 48 hours, half of the culture medium from each drop was replaced by fresh medium.

Oocytes Isolation and *In Vitro* Maturation

Oocytes were released from the antral follicles and after three times washing in culture medium, oocyted were matured in 50 microliters drops Tissue Culture Medium (TCM) 199 containing 10% FCS, 50 µg/ml gentamycine and 100mIU/ml Follicle Stimulating Hormone (FSH) (Denka Pharmaceutical, Japan) under mineral oil. Culture were done in 5% CO_2 incubator at 37°C for 24 hours. Oocytes were mechanically denuded to examine the presence of first polar body.

Data Analysis

Experiment I and II were conducted in three independent cultures. In experiment I, each repeat consisted of 6 ovaries for each treatment; and in experiment II each repeat consisted of 10 multilayered secondary follicles for each treatment. All data were analyzed using one way Analysis of Variance (ANOVA) followed by Duncan's multiple range test. Statistical significance was established at the $P<0.05$ level. The statistical analysis was performed on the SAS System Program.

RESULTS

***In Vitro* Culture of 5 Days Old Mice Ovaries**

In the experiment I, all ovaries treatments were cultured in microdrop medium layered with mineral oil in plastic nunclon culture dish as two-dimensional culture system for developing follicles in all ovaries treatments. The growth of the 5 days old mice whole ovaries began with the attachment of ovarian tissue at the hilus part to the bottom of the plastic culture dish after 24 hours cultured; and the attachment were more prominent in partially-cut and completely-cut ovaries. The outgrowth forming monolayer surrounding the ovary were seen after 48 hours of cultured in all treatments (Fig 1A). After 8 days of cultured, the round shape of the ovary become slightly flattened and prominent preantral follicles in various size seperated each other were seen in the outer part of the cortex (Figure 1B). However, further cultured until days 12

caused the ovary becoming more flattened and lead to the extrusion of the preantral follicles from the ovary boundary and disruption of the follicle basal membrane, especially in the hemi ovaries (Fig 1C). Therefore, preantral isolation was done by 8 days of cultured through enzimatically combined with mechanically method. The isolated preantral follicles consisted oocytes surrounded by more than 2 layers of granulosa cells, basal membrane and theca cells.

Influenced of the Cutting Treatments of Ovary on the Number of Preantral Follicles

After 8 days *in vitro* cultured (estimated as 12 days old mice ovary), the ovaries were incubated for 1 h in DMEM medium containing 0.5 mg/ml collagenese followed by mechanically isolation of the preantral follicles under the microdissected stereo microscope. The average number of preantral follicles isolated from each whole, partially-cut and 2-hemi ovaries were 16.1 ± 3,3, 26.8 ± 7,7 and 24.6 ± 3.8, respectively in DMEM medium containing FSH; and 17.3 ± 3,8, 23.3 ± 5.2 and 35.6 ± 5.6, respectively in medium not containing FSH (Tabel 1). The supplementation of FSH in the culture medium did not influence ($P>0.05$) the average number of the preantral follicles in all treatments (whole, partially-cut and hemi ovaries). However, the treatment of cutting ovaries (whole, partially-cut and completely-cut) significantly influenced the average number of collected preantral follicles. The average number of preantral follicles isolated from 1 whole and 1 hemi ovaries were not significantly different (16.1 ± 3,3 and 12.3 ± 1.9); this indicated that the number of preantral follicles collected from one hemi ovaries (altogether become whole ovary) was almost twice as from the whole ovary. This was confirmed by the number of preantral follicles collected from the partially-cut ovaries was 26.8 ± 7,7, significantly different with those from whole and hemi ovaries . The average diameter of the preantral follicles collected from all treatments ovaries was 96.9 ± 10.9 µm.

***In Vitro* Culture of Isolated Preantral Follicles**

The preantral follicles (Fig 2A) were cultured using the same system as employed for the ovarian culture. After 8 days cultured, the percentage of preantral follices developed into the antral follicles (Fig 2B) in DMEM with and without FSH were 26.7 ± 5.7 higher in DMEM with FSH compared to 11.7 ± 2.9 in DMEM without FSH, $P < 0.05$ (Table 2). Only the oocytes from the antral follicles cultured in DMEM with FSH that could reach maturation stage (metaphase-II) (16.7%) (Fig 2C-D) (Tabel 2).

Tabel 1. Average number of preantral isolated from each whole, partially-cut and hemi ovaries after *in vitro* culture in DMEM

Ovary size	Average number of preantral follicles per piece ovary	
	FSH+	FSH-
Whole	16.1 ± 3,3[a]	17.3 ± 3,8[a]
Partially-Cut	26,8 ± 7,7[b]	23.3 ± 5.2[b]
Completly-cut	24.4 ± 3.8[a]	35,6 ± 5,6[a]

Coloumn and row a,b significantly different P<0.05

Table 2. The influenced of FSH on the preantral follicles development and oocytes maturation

Medium	Number of preantral follicles cultured	Average number of follicles developed to antral follicles (%)	Average number of oocytes matured to metaphase-II (%)
DMEM + FSH	30	5.7 ± 0.6 (26.7 ± 5.7)[b]	3.3 ± 0.6 (16.7 ± 2.9)
DMEM - FSH	30	2.3 ± 0.6 (11.7 ± 2.9)[a]	0.00

Coloumn a,b significantly different $P<0.05$

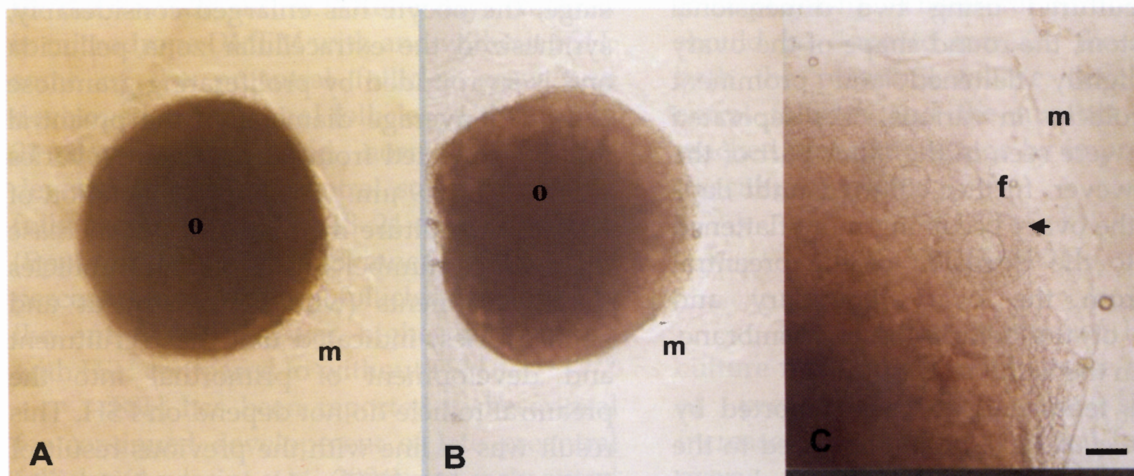

Figure 1. Development of mouse ovary after *in vitro* cultured for (A) 4 days; (B) 8 days and (C) 12 days. The ovarian tissue was attached to the substrate by the growth of monolayer cells (m) around the ovarian tissue (o) and the growth of the follicles (f) at the peripher of the ovarian tissue. Arrow head: the follicle basal membrane. Bar: 80 µm.

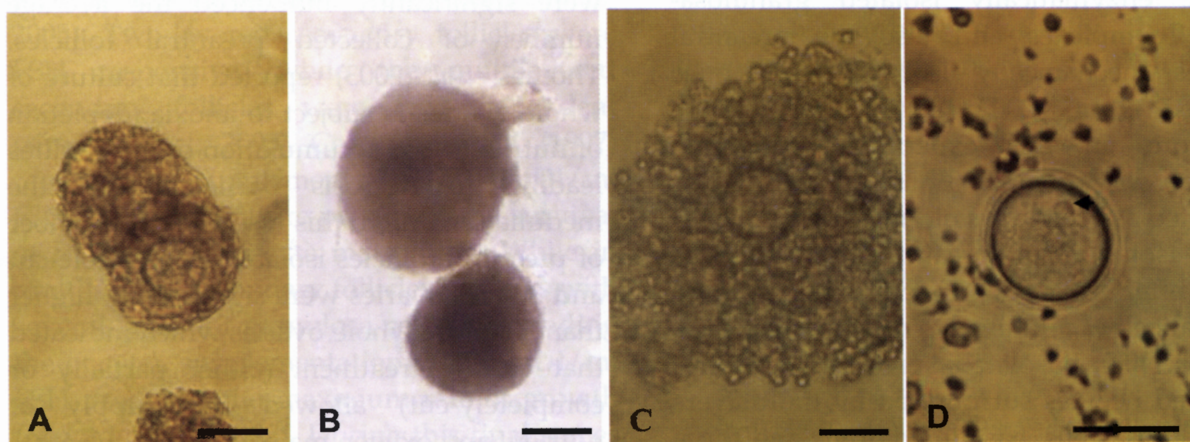

Figure 2. The development of preantral follicle into anthral follicle. A Preantral follicle (pf); B. Anthral follicle (af); C. Matured oocytes with cumulus expansion; D. Matured (metaphase II) oocytes with first polar body (arrow head). Bar: 80 µm.

DISCUSSION

In this experiment, all ovaries were collected from 5 days old mice, since in rodents primordial follicles are detected 2-4 days post-birth (Hirshfield, 1991). The primordial follicles contain oocyte that is arrested in meiotic prophase I and only re-enters meiosis or germinal vesicle breakdown upon ovulation (Picton *et al.*, 2008). After 8 days of cultured using two dimensional culture system, the round shape of the ovary become slightly flattened and prominent preantral follicles in various sizes seperated each other were seen in the outer part of the cortex. However, further cultured until days 12 caused the ovary becoming more flattened and lead to the extrusion of the preantral follicles from the ovary boundary and disruption of the follicle basal membrane, especially in the hemi ovaries.

This fenomena was also reported by Demeestere *et al.* (2002) that compared to the spherical three dimensional, the non spherical two-dimentional cultured system has limitation and caused the disruption of follicle architecture. The two-dimensional culture system have been developed to culture enzymatically isolated granulosa-oocyte complexes and also the preantral follicles mechanically isolated from mice (O'Brien *et al.*, 2003; Lenie *et al.*, 2004). Therefore, in this experiment, preantral isolation was done by 8 days of cultured or estimated as 12-13 days old mice ovary.

Isolation of preantral follicles was done after 8 days of cultured through enzimatically combined with mechanically method. The isolated preantral follicles consisted oocytes surrounded by multilayers of granulosa cells, basal membrane and theca cells. In rodents primordial follicles begins development 5 days after birth (McGee & Shueh 2000). Eppig *et al.* (2002) reported that in 5 days old mice, ovary is a solid organ that consist of primordial follicles. The result above was in line with McGee and Shueh (2000) that within 10-12 days reaches the secondary follicle stage where the oocyte is in mid-growth stage and is surrounded by two to three layers of granulosa cells.

Primordial follicles are stimulated to grow and enter development into primary, secondary or antral follicles (Skinner, 2005). By this time follicle has reached the pre-antral stage, the oocyte has enlarged considerably, synthesized the extracellular zona pellucida and is surrounded by proliferating granulose cells. The average diameter of the preantral follicles collected from all treatments ovaries was 96.9 ± 10.9 µm. The supplementation of FSH in the culture medium did not increase the average number of the preantral follicles in all treatments (whole, partially-cut and hemi ovaries) indicated that the recruitment and development of primordial into the preantral follicle do not depend on FSH. This result was in line with the previous result of Kreeger *et al.* (2005) who reported that the development of primordial follicles into the preantral follicles did not depend on FSH.

However, the ovaries cutting treatments (partially and completely cut) were significantly influenced the average number of collected preantral follicles. Thomas *et al.* (2003) reported that culture of whole ovaries is subject to anoxia, depletion of nutrients and accumulation of metabolites leading to necrosis particulary in the medulla region. In this research, the number of preantral follicles isolated from whole-cut and 2 hemi ovaries were significantly higher than from the whole ovaries which indicated that cutting treatment (either partially or completely-cut) allowed the supply of nutrient and oxygen maintained sufficiently.

Follicles isolated enzimatically combined with mechanically method could maintained the intact preantral follicles structure consisted of oocytes surrounded by

multilayered granulosa cells, basal membrane and some theca cells. Demeestere *et al.* (2002) reported that using the same culture system the percentages of preantral follicles developed into the antral follicles were 24.1% from the enzimatically and 62.3% from the mechanically isolated follicles. The intact preantral follicles collected were further cultured using the same system as employed for the ovarian culture. After 8 days cultured in microdrops DMEM with and without FSH, some of the preantral follicles grew and developed to the antral follicles (estimated as 21 days ovary). And normally, follicles develop to the large antral stage containing fully grown oocytes by 18–24 days after birth (Eppig *et al.,* 2002; Skinner, 2005). The percentages of preantral follicles developed to the antral follicles was higher in medium with FSH compared to without FSH.

FSH has been reported to be critical for continued development of late preantral follicles (Andreint *et al.,* 2004) and early antral follicles (Spears *et al.,* 1998). In serum supplemented cultured of two layered secondary follicles isolated from immature mice, FSH was critical for follicles survival, growth and antrum formation (Kreeger *et al.,* 2005). Two-layered secondary follicles were FSH responsive, while the multilayered secondary follicles were FSH dependent (Kreeger *et al.,* 2005). Cortvrindt *et al.* (1997) reported that supplementation of 100mUI/ml FSH in *in vitro* culture promoted the survival of follicles and oocyte maturation. While in multilayered secondary follicles (follicles with several layers of granulosa cells surrounding the oocyte), supplementation of 100 mUI/ml FSH produced the maximum rate of growth (Nayudu *et al.,* 1992). During this final stages of antral follicle development, the oocyte become competent enough to resume the meiosis process (Zheng & Dean, 2007) and prior to ovulation, a LH surge causes nuclear maturation, culminating in the completion of the first meiotic division and extrusion of the first polar body, followed by re-arrest at the metaphase of meiosis II (Hutt & Albertini, 2007). Metaphase II of oocytes is mature stage that competence to be fertilized by spermatozoa. However, in this experiment, only 16.7% of oocytes from the antral follicles cultured in DMEM supplemented with FSH that could reach maturation stage (metaphase-II). Therefore further research should be done to increase the number of both the preantral follicle develop to the antral follicles and the number of oocytes reached maturation stage by improvement of the culture system.

CONCLUSION

The supplementation of FSH in the culture medium did not increase the number of preantral follicles, but significantly increased the number of antral follicles. The ovary cutting treatment significantly increased the average number of collected preantral follicles.

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