Optimization of *In Vitro* Culture System of Mouse Preantral Follicles

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생쥐 Preantral Follicles의 체외 배양 시스템에 관한 연구

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Objective: This study was to establish *in vitro* culture system of mouse preantral follicles and to obtain higher *in vitro* development rates and production of live young.

Materials and Methods: Preantral follicles were obtained from 12-day-old FI mouse (C57BL×CBA) by enzymatical methods. Oocyte-granulosa cell complexes (OGCs) of preantral follicles were loaded on Transwell-COL insert and cultured in α MEM supplemented with 5% FBS, 100 mIU/ml FSH and 100 mIU/ml hMG for IVG for 10 days or 12 days. IVM was performed in αMEM supplemented 1.5 IU/ml hCG for 18 hrs and IVF was carried out in M16 medium for 4 hrs, 7 hrs and 9 hrs, respectively. After washing, embryos were cultured in modified M16 medium supplemented 10% FBS with or without cumulus cell for 4 days. Morphologically normal 22 morula and blastocysts were transferred into the uterus of 2 pseudopregnant female ICR recipients.

Results: 1) When the effect of the OGCs size on the nuclear/cytoplasmic maturation of mouse preantral follicle was evaluated, the results of 120-150 ㎛

preantral follicle (MII: 33.0%, ≥2-cell: 36.7%, ≥morula: 20.9%) were significantly higher than those of 70-110 ㎛ preantral follicle (MII: 12.2%, ≥2-cell: 10.2%, ≥ morula: 4.8%) ($p<0.001$). 2) When the effect of period of the IVG days on the nuclear/cytoplasmic maturation of mouse preantral follicle was investigated, the rate of ≥2-cell was higher in 10 days (38.2%) than in 12 days (20.0%) ($p<0.01$). 3) When the effect of period of IVF time on the cytoplasmic maturation of mouse preantral follicle was checked, 9 hrs (≥2-cell: 31.5%, ≥morula: 14.3%) indicated significantly higher cytoplasmic maturation rate than 4 hrs (≥2-cell: 17.5%, ≥ morula: 4.8%) and 7 hrs (≥2-cell: 20.4%, ≥morula: 6.1%) ($p<0.01$). 4) When the effect of co-culture environment on the cytoplasmic maturation was tested, there was no difference between co-cultured preantral follicle (≥morula: 17.4%) and preantral follicle cultured in M16 (≥morula: 17.4%). 5) When experiment was performed as optimal results of size (120-150 ㎛), *in vitro* growth (10 days), insemination period (9 hrs), culture environment (medium only), the rates of MII, ≥ 2-cell and ≥morula were 30.2%, 39.3%, 22.5%, respectively. When 22 morula and blastocysts were transferred to uterus of 2 pseudopregnant recipients, 1 recipient was pregnant and then born 1 live young.

Conclusion: This result demonstrates that *in vitro* culture system of preantral follicles can be used efficiently as another method to supply mouse oocyte.

Key Words: Mouse preantral follicle, *In vitro* growth, *In vitro* fertilization, Co-culture

There are a pool of preantral follicles in the ovaries of mammals. They are valuable for the conservation of rare and endangered species and human. The follicle provides the micro-environment for oocyte growth and maturation. There are three basic types of follicle: (1) primordial follicles (2) preantral follicles and (3) antral follicles.[1] Preantral follicles are surrounded by 1-3 granulosa cell layers and 85-150 ㎛ in size. The oocytes within preantral follicles are arrested in prophase of meiosis I, undergo a great increase in mass and accumulate resources essential for maturation, fertilization and preimplantation embryo development.[2,3,4] Previous studies for culture of preantral follicles have focused primarily on the development and function of follicular somatic cells rather than on oocyte development.[5] When preantral follicles are cultured in the presence of follicle

stimulating hormone (FSH), antrum development appears to mimic closely that process in vivo.[2] However, systems for studies on follicle development are not necessarily optimal for studies on oocyte development. Recently, some researchers have focused on the development of the oocytes in preantral follicles and the acquisition of the oocyte competence to undergo not only maturation but also fertilization and embryonic development.[2,6] Eppig et al. (1989) have developed OGCs on the surface of a collagen matrix, and they obtained live young by embryo transfer.[6]

The follicle size is very important in the *in vitro* culture for development of oocytes from preantral follicles. Oocytes from follicles with a mean diameter ≥16 mm had significantly higher fertilization rates than ones from follicles with a mean diameter ≤14 mm. Oocytes from small antral follicles that complete nuclear maturation are rarely competent to develop to the blastocysts.[2,7,8] In the mouse, Wu et al. (2000) demonstrated that it took small preantral follicles 2 days longer to reach antral follicle than standard follicles reached.[4] Eppig (1992, 1996) demonstrated that the period taking oocytes grown *in vitro* recovered from preantral follicles to reach 50% germinal vesicle breakdown (GVB) was 2 hrs longer than that of oocytes isolated from 18- and 22-day-old mice.[3,10] Zhang et al. (1995) suggested that cumulus cell co-culture started at various stages had no effect on fertilization and cleavage development but significantly improved rates of embryo development to morula or blastocyst stage.[9] During the maturation of oocytes, they undergo nuclear and cytoplasmic processes. Nuclear maturation is a term that refers to the resumption of meiosis and progression to metaphase II (MII), cytoplasmic maturation is other maturation events that prepare the oocyte for fertilization and preimplantation development.[3] Therefore, this study was to test whether follicle size, prolong of IVG days and IVF time and co-culture environment affect to the rates of oocyte maturation and cytoplasmic maturation of mouse preantral follicles.

MATERIALS AND METHODS

1. Animals

12-day-old FI (C57BL×CBA) female mice were used as donors of ovaries. Male mice (FI) that provided sperm for IVF were at least 3 months old.

2. Isolation of preantral follicles

The ovaries were digested in M2 medium containing 1 mg/ml collagenase (Type 1A: Sigma) and 0.2 mg/ml DNase I (Sigma) for 20 min at 37℃ and repeatedly drawn in and out of the pipette.

3. *In vitro* culture of preantral follicles (IVG/IVM/IVF/IVD)

Oocyte-granulosa cell complexes (OGCs) from mouse preantral follicles were collected according to the size. OGCs were washed three times with M2 and culture medium. Culture medium was αMEM (Gibco) supplemented with 0.06 g/l penicillin G, 0.05 g/l streptomycin sulfate, 5% FBS[2,10], 100 mIU/ml FSH and 100 mIU/ml hMG. The 300-400 OGCs were grown *in vitro* (IVG) on Costar Transwell-COL membrane insert (3.0㎛ pore size, 24.5 mm diameter) in Costar six-well cluster dish (Fig. 1A-B). Each dish contained 4 ml of medium and each insert contained 300 ㎕ medium. OGCs were cultured for 10 days (standard, Fig. 1C) or 12 days at 37℃ in 5% CO_2. Half of the culture medium was replaced with fresh medium every other day. After 10 days or 12 days of IVG, OGCs were recovered, washed three times with fresh medium and allowed to mature *in vitro* (IVM) for 18 hrs (Fig. 1D) in α MEM supplemented with 5% FBS, 100 mIU/ml FSH, 100 mIU/ml hMG and 1.5 IU/ml hCG. And then 20-30 OGCs were transferred in 50 ㎕ drop of M16 medium containing 0.4% BSA and fertilized *in vitro* (IVF) with $2x10^6$ cells/ml of FI sperm for 4 hrs (standard), 7 hrs and 9 hrs (Fig. 2A). Oocytes were transferred into medium for *in vitro* development (IVD) and then oocytes undergoing GV, GVB, MII and ≥2-cell were counted and oocytes diameter was observed at 24 hrs after fertilization. The rate of ≥2-cell was counted from matured oocytes (GVB and MII). The rate of ≥morula was counted from GVB and MII. The percentages of morula, blastocysts and hatching blastocysts were also counted from ≥morula stage (Fig. 2C-D) at day 4 after IVF. In addition, the 2-cell embryos (Fig. 2B) were divided into two groups, co-cultured group with bovine cumulus cells and group cultured in

M16 medium only. Culture medium for IVD was modified M16 supplemented with 10% FBS, 1% essential amino acids (Sigma) and 0.5% non-essential amino acids (Sigma), and replaced with fresh medium every day.

4. Embryo transfer (ET) and production of live young

Morphologically normal 22 morula and blastocysts produced at day 4 after IVF were transferred into the uterus of 2 pseudopregnant female ICR recipients on 3 days from plug check (D-1).

5. Measurement of oocyte diameter

OGCs size was measured with an ocular micrometer incorporated in stereomicroscope after collection. At 24 hrs after fertilization, oocyte diameter was measured excluding the zona pellucida with an ocular micrometer attached to an inverted microscope.

6. Experimental design

Experiment I. Effect of the OGCs size on the nuclear/cytoplasmic maturation of preantral follicles

OGCs from preantral follicles were collected according to their size (70-110 ㎛, 120-150 ㎛). 70-110 ㎛ preantral follicles consisted of 1-2 layers of granulosa cells and 120-150 ㎛ preantral follicles consisted of 2-3 layers of granulosa cells.[4,11,12] And then they were taken into IVG for 10 days, IVM for 18 hrs, IVF for 4 hrs and IVD for 4 days, respectively. The rates of GVB, MII, ≥2-cell and ≥ morula were observed.

Experiment II. Effect of the IVG period on the nuclear/cytoplasmic maturation of preantral follicles

OGCs (120-150 ㎛) were grown *in vitro* for 10 days or 12 days at 37℃ in 5%

CO_2 incubator. And they were taken into IVM, IVF for 4 hrs and IVD for 4 days, respectively. GVB, MII, ≥2-cell and ≥morula were recorded.

Experiment III. Effect of insemination period on the cytoplasmic maturation of preantral follicle

OGCs (120-150 ㎛) were grown *in vitro* for 10 days and matured *in vitro*. And then 20-30 complexes were transferred in 50 ㎕ drop of M16 medium and fertilized with $2x10^6$ cells/ml FI sperm for 4 hrs, 7 hrs or 9 hrs. And they were taken into IVD. The rates of ≥2-cell and ≥morula were recorded.

Experiment IV. Effect of co-culture with bovine cumulus cell on the *in vitro* development of preantral follicles

OGCs (120-150 ㎛) were grown *in vitro* for 10 days, matured for 18 hrs and fertilized for 9 hrs, respectively. At 24 hrs after fertilization, the ≥2-cell was observed. The 2-cell embryos were divided into two groups. Co-cultured group was cultured with bovine cumulus cell and M16 group was not. Bovine cumulus monolayer cell was cultured in 50 ㎕ drop of M16 supplemented with 10% FBS, 1% essential amino acids and 0.5% non-essential amino acids. And their rate of ≥ morula was compared.

Experiment V. Production of live young

OGCs (120-150 ㎛) were grown *in vitro* for 10 days, matured *in vitro* and fertilized for 9 hrs, respectively. IVD was performed in M16 supplemented with 10% FBS, 1% essential amino acids and 0.5% non-essential amino acids for 4 days. And morula and blastocyst were transferred into the uterus of pseudopregnant recipients.

7. Statistics

The development rates of GVB, MII, ≥2-cell and ≥morula in each treatment were

compared using Chi-square analysis.

RESULTS

Experiment I. Effect of the OGCs size on the nuclear/cytoplasmic maturation of preantral follicles

To observe nuclear/cytoplasmic maturation of preantral follicles according to the follicle size, preantral follicles were divided into 70-110 ㎛ and 120-150 ㎛. The effect of size on nuclear/cytoplasmic maturation of preantral follicles was summarized in Table 1.

The rate of recovered oocytes from IVG follicles was higher in 120-150 ㎛ (53.9%) than in 70-110 ㎛ (49.5%) ($p<0.05$). A high percentage of oocytes were underwent GVB and MII in 120-150 ㎛ (62.1%) than in 70-110 ㎛ (35.8%) ($p<0.001$). In the nuclear maturation, there were significantly different between 70-110 ㎛ and 120-150 ㎛. The rate of ≥2-cell in 120-150 ㎛ and 70-110 ㎛ was 36.7% and 10.2%, respectively ($p<0.001$). Also, the rate of ≥morula in 120-150 ㎛ and 70-110 ㎛ was 20.9% and 4.8%, respectively ($p<0.001$). Significantly higher percentage in the nuclear/cytoplasmic maturation was 120-150 ㎛ than 70-110 ㎛. Oocytes diameter (㎛) in 70-110 ㎛ and 120-150 ㎛ was 63.3±9 ㎛ and 70.1±7 ㎛, respectively.

Experiment II. Effect of the IVG period on the nuclear/cytoplasmic maturation of preantral follicles

To evaluate effect of the period of *in vitro* growth on the nuclear/cytoplasmic maturation of preantral follicles, they were divided into group grown *in vitro* for 10 days or 12 days as the IVG period. The result was indicated in Table 2. The rates of recovered oocytes were 53.6% and 43.2% in IVG for 10 days and 12 days, respectively and there was significantly different ($p<0.05$). The proportion of GVB and MII was 76.2% and 74.0% in IVG for 10 days and 12 days, respectively. Therefore, in the nuclear maturation, there was no difference. And the rate of ≥ 2-cell was 38.2% and 20.0% in IVG for 10 days and 12 days, respectively ($p<0.01$).

The rate of ≥morula was 20.6% and 10.0% in IVG for 10 days and 12 days, respectively. However, there was no difference in the rate of ≥morula.

Experiment III. Effect of the insemination period on the cytoplasmic maturation of preantral follicles

To evaluate effect of insemination period on the cytoplasmic maturation of preantral follicles, preantral follicles were divided into IVF for 4 hrs, 7 hrs or 9 hrs according to insemination period. Effect of insemination period was shown in Table 3. At 24 hrs after fertilization, the rate of ≥2-cell was 17.5%, 20.4% and 31.5% in IVF for 4 hrs, 7 hrs and 9 hrs, respectively. The group of 9 hrs was significantly higher in the rate of ≥2-cell than that of 4 hrs and 7 hrs ($p<0.01$). At 96 hrs after fertilization, the rate of ≥morula was 4.8%, 6.1% and 14.3% ($p<0.01$) and the rate of ≥blastocysts was 16.7%, 31.6%, 30.8% in IVF for 4 hrs, 7 hrs and 9 hrs, respectively. Therefore, the group of 9 hrs had significantly higher rate in ≥morula than that of 4 hrs and 7 hrs but there was not different in ≥blastocyst stage among three groups.

Experiment IV. Effect of co-culture with bovine cumulus cell on *in vitro* development of preantral follicles

This experiment was performed to evaluate effect of co-culture with bovine cumulus cell on *in vitro* development of preantral follicles. Preantral follicles were divided into two groups, group co-cultured with bovine cumulus cell in M16 medium and group cultured in M16 medium only. As indicated in Table 4. At 96 hrs after fertilization, the rate of ≥morula was 17.4% and 17.4% in co-cultured group and medium only group, respectively. There was no difference between two groups.

Experiment V. Production of live young

This experiment was performed to evaluate application of collection of 120-150 ㎛ preantral follicles, IVG for 10 days, IVM for 18 hrs, IVF for 9 hrs and then IVD without bovine cumulus cell. As shown in Table 5. The total rate of GVB and MII

was 60.7% and the rate of ≥2-cell was 39.3%. The rate of ≥morula was 22.5%. When morphologically normal 22 morula and blastocysts were transferred into the uterus of 2 pseudopregnant female ICR recipients, 1 recipient was pregnant and then 1 live young was born on day 21 of gestation.

DISCUSSION

This study demonstrates that *in vitro* culture system of preantral follicles can be used efficiently as another method to supply mouse oocyte. In experiment I, the size of mouse preantral follicle was decided by report of Wu et al. (2000): 65-75 ㎛ (class 1 small), 85-110 ㎛ (class 2 small), 120-140 ㎛ (class 3 small), 150-160 ㎛ (standard).[4] The ability of oocytes to complete all stage of development is directly related to oocyte size and consequently the follicle size.[2] Generally, oocytes isolated from 12-day-old mice were 56.00±0.29 ㎛ in diameter and the mean size increased to 68.00±0.23 ㎛ during the 10-day culture period.[6] Eppig et al. (1994) demonstrated that oocytes from small preantral follicles that complete nuclear maturation are rarely competent to develop to the blastocyst stage.[2] In the contrast, oocytes from large preantral follicles are often competent to develop to the blastocyst stage. The increase in oocyte volume is correlated with increased protein content. For example, Wu et al. (2000) demonstrated that preantral follicle of standard size didn't require LH to proceed through antral development but smaller follicles require LH.[4] Also, the time of development from small to large preantral follicle needs the resolution of the specific molecules. Transport of nutritional or regulatory molecules is mediated by gap junction between the oocytes and granulosa cell.[13] In this study, we compared the *in vitro* development capacity of 70-110 ㎛ and 120-150 ㎛ of preantral follicles. Oocyte diameter was 63.3±9 ㎛ in 70-110 ㎛ and 70.0±7 ㎛ in 120-150 ㎛, respectively. Preantral follicles of 70-110 ㎛ has sparse granulosa cells but preantal follicles of 120-150 ㎛ has dense granulosa cells. Preantral follicles of 120-150 ㎛ showed higher nuclear/cytoplasmic maturation than those of 70-110 ㎛. Different size of preantral follicle needs different specific molecules and so culture condition may be improper in 70-110 ㎛.

In experiment II (Table 2) or III (Table 3), the periods of IVG days or IVF time

were prolonged to supplement retardation of growth, maturation and antrum formation of preantral follicles. Generally, 12-day-old mice are cultured for 10-days to be grown *in vitro* to the same total chronological age (22 days). Wu et al. (2000) demonstrated that small preantral follicles took 2 days longer to reach antral follicle than standard follicles reached.[4] However, in this study, prolong of IVG was not significant and rather decreased the rate of ≥2-cell. The result was due to aging of oocyte from large follicles *in vitro*. Eppig (1992, 1996) demonstrated that the period taking oocytes grown *in vitro* recovered from preantral follicles to reach 50% GVB was 2 hrs longer than that of oocytes isolated from 18- and 22-day-old mice.[3,10] The results may be due to deficiency of molecules necessary to derive the resumption of meiosis.[2] Generally, the period of IVF time was 4 hrs, but in experiment III, the period of IVF time was prolonged as well as 3-5 hrs regarding the period that GV reaches GVB. In this study, as insemination time for IVF, 9 hrs had significantly higher cytoplasmic maturation rate than 4 hrs and 7 hrs ($P<0.01$). This result demonstrated that the maturity and fertilization potential of oocytes were obtained during IVF.

Experiment IV was performed to evaluate the effect of co-culture. Eppig (1979) suggested that the junction association between oocytes and granulosa cells must be maintained to promote oocytes growing and development *in vitro* and that the co-culture of oocytes with granulosa cells is not a sufficient condition for oocyte development.[14] Also, in present study, there was not different between M16 and co-cultured medium *in vitro* development to stage of ≥morula of preantral follicle. On the other hand, there are a few data on in vivo development of *in vitro* produced embryos from preantral follicles. Eppig et al. (1989) reported that 137 of 2- to 4-cell stage embryos were transferred to oviducts of 7 pseudopregnant females and bore 7 live young (5.1%).[6,15] However, we transferred blastocysts developed *in vitro* for the first time, when 22 morula and blastocysts produced from *in vitro* culture of preantral follicles were transferred into uterus of 2 pseudopregnant female ICR recipients, 1 recipient was pregnant and then bore 1 live young.

In conclusion, lager size (120-150 ㎛) of preantral follicles, longer period of IVF time and IVG for 10 days improved nuclear/cytoplasmic maturation of preantral follicle. However, preantral follicles were not affected by co-culture.

국문요약

연구목적: 본 연구는 생쥐 preantral follicles의 체외 배양 조건을 확립하고 이를 기초로 높은 체외 발달률 그리고 산자 생산률을 얻고자 하였다.

연구재료 및 방법: Preantral follicles의 oocyte-granulosa cell complexes (OGCs)는 생후 12일된 F1 (C57BL×CBA)으로부터 난소를 적출하여 효소를 이용한 방법을 통해 획득하였다. 회수된 complexes는 10일 또는 12일 동안 체외 성장을 위해 Transwell-COL membrane insert로 옮겨졌고 5% FBS, 100 mIU/ml FSH, 100 mIU/ml hMG가 첨가된 α MEM에서 배양되었다. 체외 성숙을 위해 1.5 IU/ml hCG가 첨가된 αMEM에서 18 hrs 배양을 실시하였다. 그 후 M16에서 수정능력이 획득된 정자와 수정을 하여 4 hrs, 7 hrs, 9 hrs 후에 10% FBS가 첨가된 modified된 M16 배양액에서 4일간 배양하거나 또는 bovine cumulus cell과 co-culture를 실시하였다. 그리고 형태적으로 정상적인 22개의 상실배와 포배를 2마리의 위임신 대리모 (ICR)의 자궁에 이식하여 산자 생산을 유도하였다.

결과: 1) OGCs 크기가 mouse preantral follicle의 핵 및 세포질 성숙에 미치는 영향을 조사하였을 때 120-150 ㎛의 preantral follicle (MII: 33.0%, 난할률: 36.7%, 상실배 이상: 20.9%)은 핵 및 세포질 성숙에 있어서 70-110 ㎛ (MII: 12.2%, 난할률: 10.2%, 상실배 이상: 4.8%) 보다 더 높았다 ($p<0.001$). 2) 체외 성장 기간의 연장이 mouse preantral follicle의 핵 및 세포질 성숙에 미치는 영향을 조사하였을 때 10일 (난할률: 38.2%)은 12일 (난할률: 20.0%) 보다 난할률에서만 더 높았다 ($p<0.01$). 3) 체외 수정 시간의 연장이 mouse preantral follicle의 세포질 성숙에 미치는 영향을 조사하였을 때 9 hrs (난할률: 31.5%, 상실배 이상: 14.3%)은 4 hrs (난할률: 17.5%, 상실배 이상: 4.8%), 7 hrs (난할률: 20.4%, 상실배 이상: 6.1%) 보다 세포질 성숙에 있어서 유의하게 높은 발달률을 나타냈다 ($p<0.01$). 4) 공배양이 mouse preantral follicle의 세포질 성숙에 미치는 영향을 조사하였을 때 공배양 (상실배 이상: 17.4%)을 실시했을 때와 M16 (상실배 이상: 17.4%)에서 배양되었을 때는 차이가 없었다. 5) preantral follicle의 크기 (120-150 ㎛), 체외 성장 기간 (10일), 체외 수정 기간 (9시간), 배양 환경 (단지 medium만 존재)의 적절한 결과들을 종합하여 수행하였을 때 MII 성숙률, 난할률, 상실배 이상의 발달률은 30.2%, 39.3%, 22.5%이었고 총 22개의 상실배 및 포배를 2마리의 대리모에 이식했을 때 1마리가 임신했고 1마리의 산자를 생산했다.

결론: 따라서, 본 실험은 preantral follicle을 이용한 체외배양 시스템이 생쥐 oocyte를 공급하는 또 다른 방법으로 효과적으로 이용될 수 있다는 것을 시사한다.

REFERENCES

1. Torrance C, Telfer E, Gosden RG. Quantitative study of the development of isolated mouse pre-antral follicles in collagen gel culture. J Reprod Fertil 1989; 87: 367-74.
2. Eppig JJ, O'Brien MJ, Wigglesworth K. Mammalian oocyte growth and development *in vitro*. Mol Rep Dev 1996; 44: 260-73.
3. Eppig JJ and O'Brien MJ. Development *in vitro* of mouse oocytes from primordial follicles. Biol Reprod 1996; 54: 197-207.
4. Wu J, Nayudu PL, Kiesel PS, Michelmann HW. Luteinizing hormone has a stage-limited effect on preantral follicle development *in vitro*. Biol Reprod 2000; 63: 320-7.
5. Telfer EE. *in vitro* models for oocyte development. Theriogenology 1998; 49: 451-60.
6. Eppig JJ and Schroeder AC. Capacity of mouse oocytes from preantral follicles to undergo embryogenesis and development to live young after growth, maturation, and fertilization *in vitro*. Biol Reprod 1989; 41: 268-76.
7. Dubey AK, Wang HA, Duffy P, Penzias AS. The correlation between follicular measurements, oocyte morphology, and fertilization rates in an *in vitro* fertilization program. Fertil Steril 1995; 64: 787-90.
8. Wittmaack FM, Kreger DO, Blasco L, Tureck RW, Mastroianni L, Lessey BA. Effect of follicular size on oocyte retrieval, fertilization, cleavage, and embryo quality in *in vitro* fertilization cycles: a 6-year data collection. Fertil Steril 1994; 62: 1205-9.
9. Zhang L, Jiang S, Wozniak PJ, Yang X, Godke RA. Cumulus cell function during bovine oocyte maturation, fertilization, and embryo development *in vitro*. Mol Rep Dev 1995; 40: 338-44.
10. Eppig JJ, Wigglesworth K, O'Brien MJ. Comparison of embryonic developmental competence of mouse oocytes grown with and without serum. Mol Rep Dev 1992b; 32: 33-40.
11. Mcgee EA, Chun SY, Susan L, He YE, Hsueh AJW. Keratinocyte growth factor promotes the survival, growth, and differentiation of preantral ovarian follicles. Fertil Steril 1999; 71: 732-8.

12. Veeck LL, Edward Wortham JW, Witmyer J, Sandow BA, Acosta AA, Garcia JE, Jones GS, Jones HW. Maturation and fertilization of morphologically immature human oocytes in a program of *in vitro* fertilization. Fertil Steril 1983; 39: 594-602.

13. Carroll J, Whittingham DG, Wood MJ. Effect of dibutyryl cyclic adenosine monophosphate on granulosa cell proliferation, oocyte growth and meiotic maturation in isolated mouse primary ovarian follicles cultured in collagen gels. J Reprod Fertil 1991; 92: 197-207.

14. Eppig JJ. A comparison between oocyte growth in coculture with granulosa cells and oocytes with granulosa cell-oocyte junctional contact maintained *in vitro*. J Exp Zool 1979; 209: 345-53.

15. Eppig JJ and O'Brien MJ. Comparison of preimplantation developmental competence after mouse oocyte growth and development *in vitro* and *in vivo*. Theriogenology 1998; 49: 415-22.

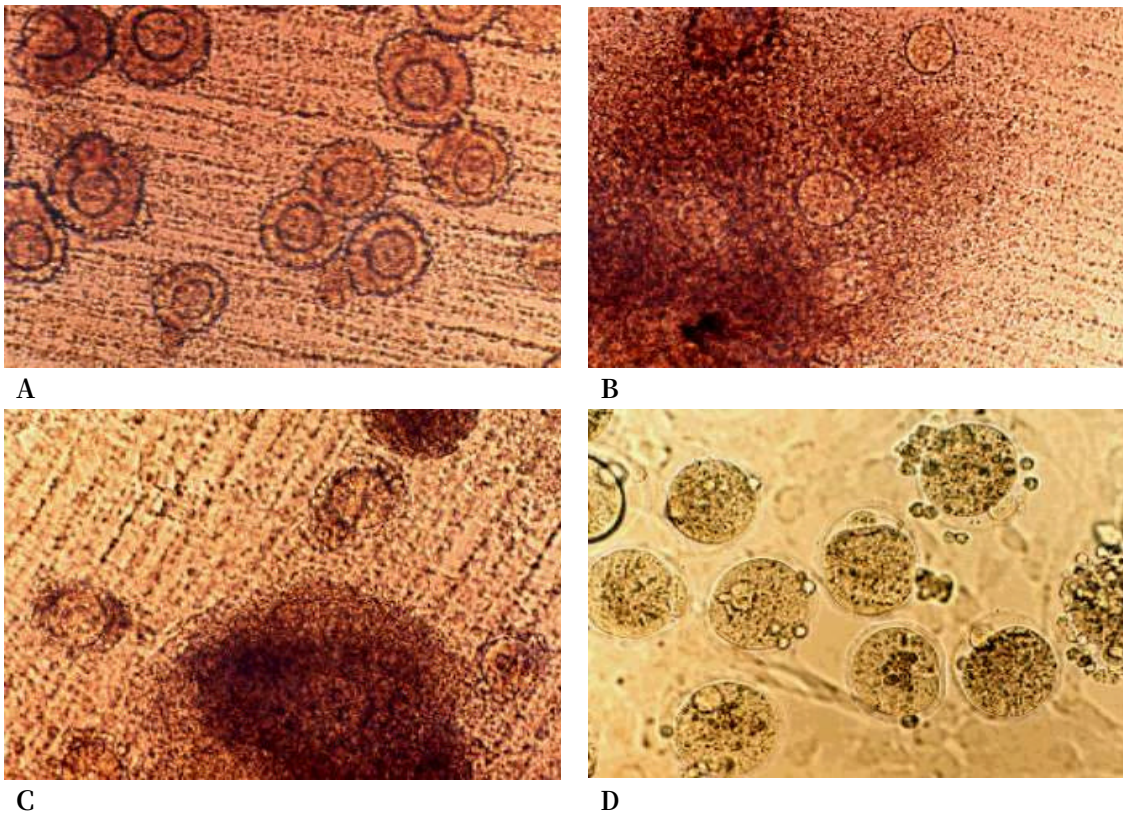

Figure 1. *In vitro* grown preantral follicles. (A) Day 0 of *in vitro* growth. (B) Day 2

of *in vitro* growth. (C) Day 10 of *in vitro* growth. (D) *In vitro* grown and matured oocyte-cumulus cell complexes. (A)-(D): ×200.

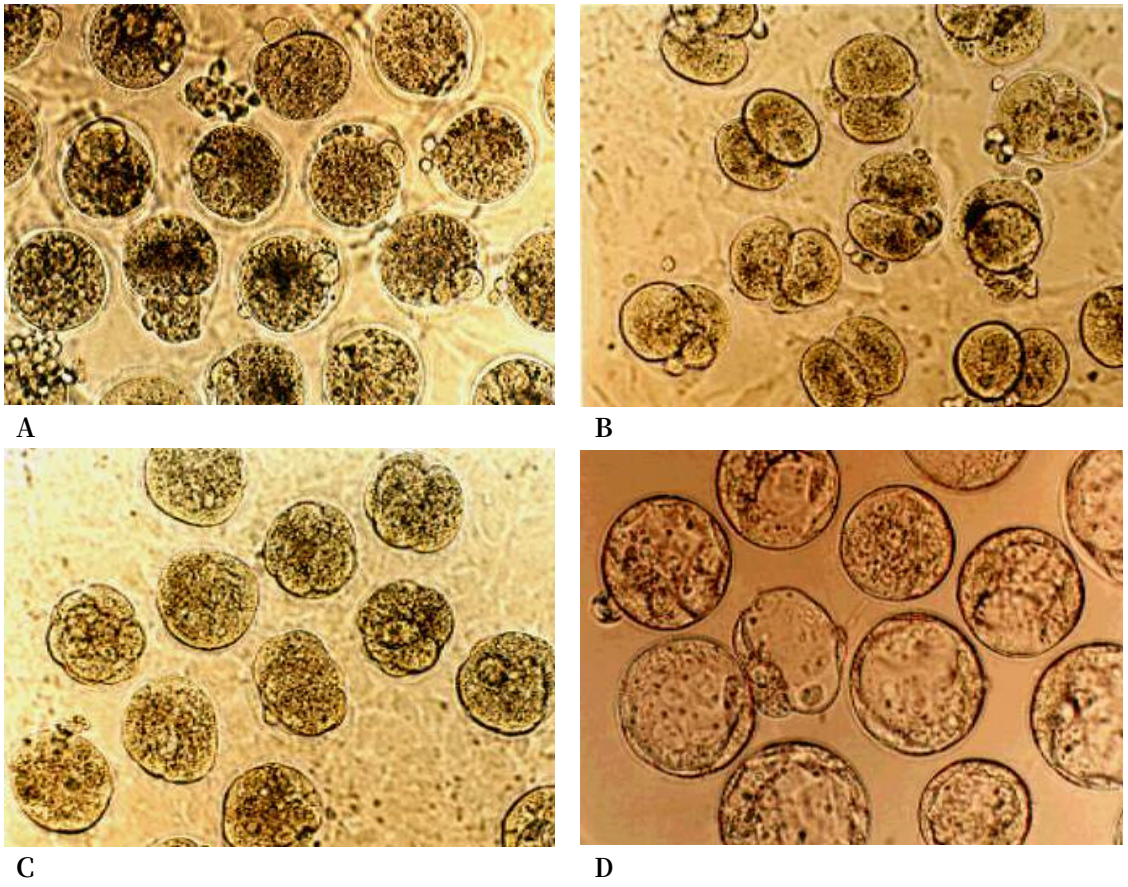

Figure 2. *In vitro* development of oocytes derived from the *in vitro* grown preantral follicles. (A) Fertilized oocytes. (B) 2-cell embryos. (C) Morula. (D) Blastocysts and hatching blastocysts. (A)-(D): ×200.