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In Vitro Growth and Maturation of Mammalian Oocytes

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포유동물 난자의 성장과 성숙

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요 약

미성숙난자 성장과 핵성숙을 성공적으로 유도할 수 있는 체외 배양체계의 개발은 난자발생과정 등 기초 생리학 연구에 도움을 줄 뿐만 아니라, 산업적으로 필요한 난자를 공급해 줄 수 있다는데 그 의의가 있다. 최근 보고에 따르면 성장 중에 있는 생쥐 난포란은 체외배양과 체외수정과정을 거쳐 산자 생산까지 가능하지만 돼지 난포란 성숙을 위해서는 좀더 고려해야 할 사항들이 있다. 즉, 난성숙 배양액에 낮은 농도의 FSH 첨가는 난자와 과립세포의 생존성을 유지시키고, Hypoxanthine의 첨가는 난성숙율의 향상에 도움을 준다고 한다. 따라서 금후 생쥐난자에서 얻어진 결과들을 기초로 하여 돼지 난포란의 성장과 성숙에 적합한 배양체계의 개발연구에서 괄목할만한 진전이 있다면 대가축들의 난포란 성숙기술을 개발하는 데 큰 도움을 줄 것이라고 본다.

I. INTRODUCTION

Mammalian oocytes enter the meiotic cell cycle in fetal life, and by the time of birth they have arrested the cycle at prophase in meiosis I. The number of primordial follicles containing a small non-growing oocyte is estimated at several hundred thousands per animal, for example 420, 000 in a pig (Gosden and Telfer, 1987). A very small population of these oocytes start to grow and reach the final size. In turn, a small number of the fully grown oocytes resume meiosis, reach metaphase II, and are ovulated and fertilized. The remaining large number of the oocytes do not grow or degenerated during their growth phase.

Eppig (1977) reported that mouse oocytes can grow to the final size in culture when oocytes maintained junctional communication with surrounding granulosa cells. In 1989, Eppig and his colleague showed successful production of living young derived from oocytes that have been grown and matured *in vitro* (Eppig and Schroeder, 1989). This demonstrates that mouse oocytes in mid-growth phase can acquire full developmental capacity *in vitro*. Now *in vitro* culture systems of growing oocytes are expected to provide a new source of a large population of oocytes for livestock production, for the human *in*

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vitro fertilization program and for preservation of rare animals. Moreover, the systems could assist in basic physiological studies of oocyte growth and follicular development.

Ⅱ. *IN VITRO* GROWTH AND MATURATION OF GROWING OOCYTES

It has been thought that oocytes in the primordial follicles start growing after they are committed to grow in the ovaries, although the commitment signal has been unknown. The remaining large number of small oocytes are quiescent and never enter the growth phase. In such context, growing oocytes isolated from the ovary can continue to grow and reach the final size *in vitro*, when we provide a suitable condition for the growing oocytes or the follicles containing them.

In mice, several culture systems have been developed, which allow to grow the growing oocytes. Follicle culture systems (Spears et al., 1994) and oocyte-granulosa cell complex (OGC) culture systems (Eppig and Schroeder, 1989; Hirao et al., 1990) have been reported. The latter systems need some modifications of attachment to prevent migration away of the surrounding granulosa cells from the oocytes during 10 days of culture. Supplementation of meiotic arresting substance, such as IBMX (isobutyl methylxanthine) or naturally occurring hypoxanthine, is also required for culture to prevent the spontaneous meiotic resumption of the grown oocytes. In any culture systems, growing mouse oocytes can grow to the final size, mature to metaphase Ⅱ, and be fertilized. An essential factor for the growth of mouse oocytes *in vitro* is the maintenance of metabolic coupling between oocyte and its companion granulosa cells. The cells transfer small molecules such as energy substances, nucleotides and amino acids into the oocytes through the gap junction during culture period.

On the other hand, culture systems for growing oocytes of larger species, in which follicles developed to a much larger size than in rodents, have not been established yet. Maintenance of the viability of both oocytes and surrounding granulosa cells is the major problem in such cultures, probably because the follicles are more demanding. We use two culture methods: follicel culture for preantral pig follicles containing oocytes at mid-growth phase (70 ~ 90μm) (Hirao et al., 1994), and OGC culture for oocytes (100μm) from early antral follicles (Miyano et al., unpublished data). These oocytes are, at the time of isolation, incompetent to resume meiosis. When the follicles or OGCs are embedded in collagen gels and cultured, the oocytes are held within the layers of the granulosa cells and grow to nearly final size (120μm). In both systems, an addition of FSH to culture medium is essential for oocyte survival as this maintains the viability of granulosa cells, and induces antrum formation (Fig. 1). Surprisingly, cultured OGCs also make an antrum in collagen gel. When the oocytes grown *in vitro* are liberated from the follicles, some oocytes resume meiosis and mature to metaphase Ⅱ. For the OGCs supplementation of hypoxanthine (2mM), which is a component of pig follicular fluid, promotes the maturational competence of the oocytes (Fig. 2) (Moritake et al., unpublished data). *In vitro* grown and matured oocytes are penetrated by spermatozoa and form a female pronucleus, but decondensation of the sperm head is incomplete (Hirao et al., 1994). All together this demonstrates that pig growing oocytes can grow up to their final size, acquire meiotic competence, and be penetrated by spermatozoa *in vitro*, although there is a very low level or success in fertilization.

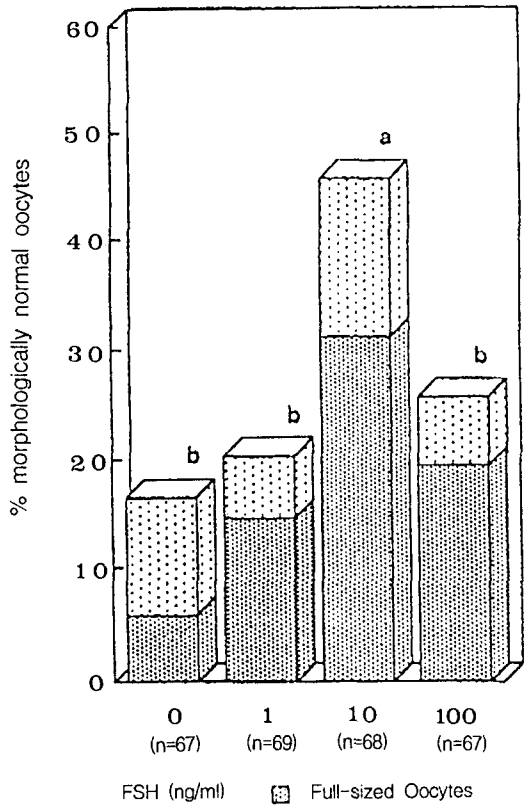

Fig. 1. Effect of FSH on viability of pig oocytes during 8-day culture.

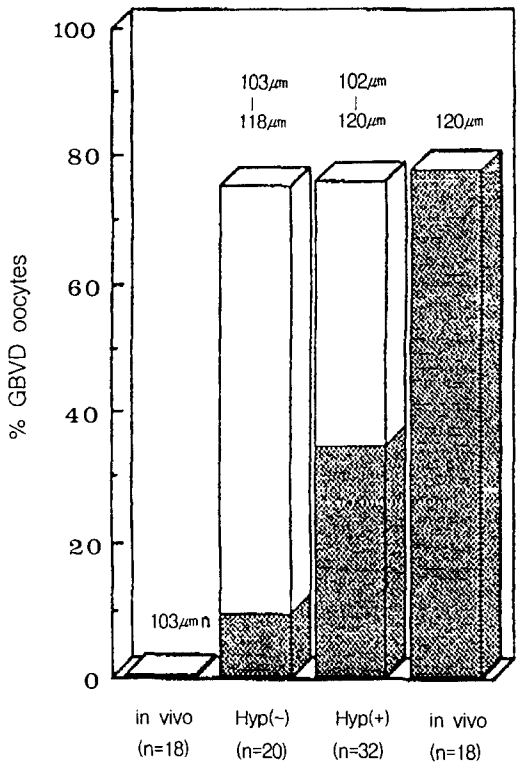

Fig. 2. Effect of hypoxanthine on acquisition of meiotic competence in pig oocytes.

Example 1: OGC culture for *in vitro* growth of mouse oocytes and subsequent oocyte-cumulus cell complex (OCC) culture for maturation (Eppig and Schroeder, 1989)

Ovaries obtained from 12-days old F_1 (C57BL/6J×SJL/J) mice were dissociated with collagenase. This produced OGCs without theca cells of basal lamina. The largest follicles are preantral follicles consisting of about 2 layers of granulosa cells around the oocytes. OGCs were cultured on membrane inserts (3.0㎛ pore size) in 6-well cluster dishes with 5ml medium per well. These membranes were treated with collagen solution. This treatment allowed attachment and maintenance of OGCs with only minimal migration of the granulosa cells from the oocytes. Eagle's minimum essential medium (MEM) supplemented with 5% fetal bovine serum, 0.23mM pyruvic acid, 0.01% phenol red, 50㎍/ml streptomycin sulfate, and 75㎍/ml penicillin G potassium salt was used as a basic medium. For oocyte growth culture, the medium was also supplemented with 0.05mM IBMX, and OGCs were cultured for 10 days under an atmosphere of 5% O_2, 5% CO_2 and 90% N_2 at 37℃. Cultures were fed every other day by exchanging approximately 1ml fresh medium for the same volume of used medium.

After 10 days, oocytes that had become dislodged from the membrane were removed and dis-

carded. Those that remained attached to the membrance as an OGC were removed form washed in the basic medium with 1㎍/ml FSH, and OGCs (oocytes completely enclosed by cumulus cells) were liberated. After OGCs were washed, they were transferred to membrane inserts (3.0 ㎛ pore size, not tissue culture-treated) in 6-well cluster dishes with 4ml medium with FSH per well and cultured for oocyte maturation for 16 hr under 5% O_2, 5% CO_2 and 90% N_2 at 37℃. The ova were then collected and washed in Whitten's medium and inseminated. After insemination, the eggs were washed and cultured in 1ml of Whitten's medium in silicone-rubber stoppered glass tubes with 5% O_2, 5% CO_2 and 90% N_2 atmosphere in a 37℃-water bath. Two- to 4-cell stage embryos were transferred to oviducts of pseudopregnant females.

Example 2: OGC culture for *in vitro* growth and maturation of mouse oocytes (Hirao et al., 1990)

Ovaries obtained from 10-day-old mice (ddY strain) were washed with MEM and immersed into MEM supplemented with 2mg/ml collagenase and 50㎍/ml sodium pyruvate for 30min at 37℃. The ovaries were then rinsed in a culture medium of MEM supplemented with 10% fetal bovine serum, 50㎍/ml sodium pyruvate and 2mM bhypoxanthine. Three ovaries were placed in each petri dish (35×10mm) containing 2.5ml of the medium and were pipetted for 2min to liberate the OGCs. A suspension of OGCs was transferred to a dish coated with 1% agar solution to prevent the attachment of OGCs to the plastic surface and the loss of their structural integrity. The OGCs were cultured for 10 days at 37℃ in 5% CO_2 and 95% air. During the culture period OGCs aggregated to form clumps. Oocytes in the clumps grew, while denuded oocytes degenerated by the end of the culture period. Half the medium was changed every 4 days.

After 10 days of growth culture, the OGCs were rinsed in the medium without hypoxanthine and transferred into drops (10㎕) of medium overlain with paraffin oil in a petri dish (60× 15mm). They were cultured for 20hr at 37℃ in 5% CO_2 and 95% air. Following this maturation period, oocytes were examined under the microscope for germinal vesicle breakdown and extrusion of a first polar body. Oocytes without germinal vesicles were recovered and rinsed in a modified Krebs-Ringer bicarbonate solution (mKRB) (Toyoda and Chang, 1974). The prepared oocytes were transferred into 400㎕ of mKRB and inseminated with spermatozoa collected from the cauda epididymis of mature male mouse.

Example 3: Follicle culture for *in vitro* growth of pig oocytes and subsequent OCC culture for maturation (Hirao et al., 1994)

Ovaries were collected from prepubertal gilts at a local abattoir and transported to the laboratory within 1 hr, while preventing any abrupt temperature changes. The ovaries were washed with PBS and Hepes-buffered MEM and cut into small pieces. After the pieces were incubated in Hepes-buffered MEM containing 0.1% collagenase for 1 hr at 39℃, preantral follicles were removed from the connective tissues and freed from adherent thecal cells using fine forceps and a needle. Follicles with diameter of 0.2~0.3mm were collected and washed three times with Hepes-buffered MEM containing 2% fetal calf serum.

Follicles containing oocytes with diameters of 70~79.5 and 80~89.5㎛ were cultured in collagen gel for 16 and 12 days, respectively. The culture medium used was Waymouth MB752/1 supplemented with 50㎍/ml sodium pyruvate, 100 ㎍/ml penicillin G potassium, 50㎍/ml strep-

tomycin sulfate, 5% FCS, 2m Sigma U/ml FSH, 1μg/ml oestradiol. After being washed in the culture medium, five follicles in a small volume of the culture medium were placed in the center of plastic dish (35×10mm) and overlaid with 0.4ml of the collagen gel solution. Collagen gel solution was prepared by mixing 0.3% acid-soluble collagen solutio, 10 times concentrated Waymouth MB752/1 medium without bicarbonate and 0.05M NaOH containing 22mg/ml $NaHCO_3$ and 47.7mg/ml Hepes, at concentration 8:1:1 (v:v:v). When the collagen gels had set after incubation for 20min at 39℃, 3ml of the culture medium was poured into the dishes. The follicles were cultured at 39℃ under the atmosphere of 5% CO_2 and 95% air. One-half of the medium was changed every 4 days.

After growth culture, the collagen gel matrices were treated with 0.1% collagenase solution and the follicles were recovered. The oocytes enclosed by one or two layers of cumulus cells were collected from the follicles, washed with mKRB and transferred into microdrops (10μl) of the solution covered with paraffin oil in a plastic dish. They were cultured for 48 hr at 39℃ in 5% CO_2 and 95% air. After culture, oocytes were transferred into microdrops (100μm) of the fertilization medium, BO solution (Brackett and Oliphant, 1975) containing 10mg/ml BSA and 2mM caffeine, and covered with paraffin oil. The oocytes were inseminated by epididymal boar spermatozoa, which had been frozen-thawed, washed and preincubated.

Example 4: Follicle culture for *in vitro* growth of mouse oocytes and subsequent OCC culture for maturation (Spears et al., 1994)

Ovaries from 32-dyas-old F_1 (C57BL/6×CBA/Ca) mice were aseptically removed and placed in watchglasses of Leibovitz L medium (Gibco-BRL, UK) supplemented with 2mM glutamine and 3mg/ml BSA. Individual preantral (primary) follicles, 190 ± 10μm in diameter were microdissected from ovaries using fine, sharp needles. Each follicle was removed from the ovary with a clump of thecal-stromal tissue attached, since naked follicles fail to develop ful ly in this system. Follicles showing sighs of atresia (dark or shrunken) or with mis-shapen oocytes were discarded.

Follicles were cultured individually in microtitre plates in 20μl droplets of MEM supplemented with 10μg/ml human transferrin, 1IU/ml hFSH and 5% mouse serum under 50μl mineral oil in 5% CO_2 in air at 37℃. Follicles were transferred to a new well with fresh medium every 12hr. After 5~6 days of culture, follicles were ruptured using needles to collect OCCs. The OCCs were cultured for a further 14~16hr in a droplet of M16 medium containing 5% FCS, 1 IU/ml hFSH and 10ng/ml epidermal growth factor (EGF).

In vitro matured oocytes were inseminated and examined the following day, and healthy 2-cell embryos were cultured in 5% O_2, 5% CO_2 and 90% N_2 at 37℃. Blastocysts obtained after 4-days culture were transferred into host pseudopregnant females.

Ⅲ. *IN VITRO* GROWTH OF SMALL OOCYTES IN PRIMORDIAL FOLLICLES

Small oocytes in primordial follicles appear quiescent in the ovaries. However, small mouse oocytes (15~20μm) in primordial follicles in the ovaries at birth start to grow spontaneously in culture. Oocytes in organ-cultured ovaries (Miyano et al., 1988), and denuded oocytes cultured with the ovarian somatic cells (Miyano et al., 1994) start growing. Small oocytes in 4-day-old mice grow up to the final size in cultured ovar-

ies, acquire the meiotc competence, and mature to metaphase II (Hirao et al., 1993). These suggest the possibility that small oocytes in primordial follicles are available as a large source of oocytes for *in vitro* fertilization, although it has not been known whether they have already been committed to grow in the ovaries.

In large species, no successful culture systems supporting the growth of oocytes in primordial follicles has been developed. Recently, Figueiredo et al. (1994) reported a culture system that supports the viability of bovine primary follicles for 5 days without any information on the oocyte growth in the follicles. When pig primordial follicles are cultured *in vitro*, surrounding granulosa cells soon migrate away from the oocytes, they are denuded and eventually degenerate (Shen et al., unpublished data). Some modifications will be required to prevent the detachment of granulosa cells from the oocyte, because the primordial follicle has no basement membrane outlining the granulosa cells.

Example 5: Organ culture of mouse ovaires (Hirao et al., 1993)

Ovaires were removed from 4-day-old female mice and wahsed 3 times with Hepes-buffered MEM. Five ovaries were placed on each filter support (3mm pore size) in an organ culture dish. Culture medium used was Waymouth MB752/1 supplemented with 50μg/ml sodium pyruvate, 5% FCS, 100μg/ml penicillin and 50μg/ml streptomycin. The ovaris were cultured for 20 days at 37℃ in 5% CO_2 and 95% air. The medium was replaced every 4 days.

After organ culture, the ovaries were incubated in MEM containing 2mg/ml collagenase for 30 min at 37℃. After incubation, they were washed in the fresh culture medium. The oocytes were isolated by pipetting and only oocytes larger than 55μm were used for maturation culture. Each oocyte was transferred to a microdrop of the culture medium (10μl) under paraffin oil and cultured for 24 hr. At the end of the culture period, germinal vesicle breakdown and emission of a first polar body were examined.

Example 6: Culture of bovine follicles (Figueiredo et al., 1994)

Ovaries from adult cows were collected at a local slaughter-house. The ovaries were aseptically removed, stripped of surrounding fat tissue and ligaments, and transferred into 10ml PBS supplemented with 200IU/ml of penicillin and 200μg/ml of streptomycin. Following washing in this solution, each ovary was transferred into 10ml of Hepes-buffered M199 containing the antibiotics and 5% FCS and transported to the laboratory within 1 hr in a thermos flask filled with water at 39℃.

Preantral follicles were isolated from the ovary by applying the mechanical procedure without the use of enzymes. Briefly, the ovaries were cut into small fragments using a tissue chopper. The fragments were put in Hepes-buffered M199 supplemented with 5% FCS, antibiotics and 0.23 mM pyruvate and then suspended 40 times with a pasteur pipette and filtered successively through 500- and 100- μm nylon mesh filters. The follicles with the diameter of 30 to 70 μm were selected using a mouth-operated micropipette. Groups of 3 follicles were palced in 50μl droplets of isolation medium to facilitate the onset of culture.

Isolated preantral follicles were placed in 350 μl of culture medium in groups of 3 follicles per well and cultured for 5 days at 39℃ in 5% CO_2 and 95% air. Culture in groups of 3 follicles per well and their location after attachment at Day 1 of culture allows each follicle to be studied individually during the culture period. A culture medium used was MEM supplemented with

antibiotics, 10% FCS and ITS (6.25 μg/ml Insulin, 6.25 μg/ml transferrin and 6.2 ng/ml selenium). Medium was changed 24 hr after the start of culture, and then every other day.

Ⅳ. SUMMARY

In vitro cultrue systems for the growth of small oocytes and for meiotic maturation are expected to provide a new source of a large population of oocytes as well as assistance in basic physiological studies of oogenesis. Mouse oocytes in mid-growth phase can complete growth and acquire full developmental capacity *in vitro*. On the other hand, growing pig oocytes need some other factors. FSH at a low concentration maintains the viability of both oocytes and granulosa cells, and hypoxanthine promotes the meiotic competence of the oocytes during culture period. Considerable improvement in the culture systems for growth of pig oocytes, suggested from mouse studies, and for oocyte maturation could help to develop this technology in larger species.

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