The Effect of Oviductal Epithelial or Granulosa Cells Monolayer Used during Maturation Culture on the In Vitro Maturation and Fertilization of Porcine Oocytes

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Abstract: *We evaluated the effect of two monolayers derived from reproductive cells on in vitro maturation and fertilization of porcine oocytes. Cumulus-oocyte complexes were cultured in medium alone or co-cultured for 48hr on the monolayer of porcine oviductal epithelial cells (pOEC) or granulosa cells (pGC). Assessment of nuclear maturation revealed that oocytes matured in pGC significantly reached metaphase of the second meiotic division compared to those in control (76% vs 55%; p<0.05) and did not differ from the pOEC (69%). Following 18hr of insemination, the proportion of penetrated oocytes in control (82%) was greater (p<0.01) compared with pGC (56%) but did not differ from the pOEC (68%). The proportion of oocytes that underwent polyspermy was lower (p<0.01) in pOEC (38%) and pGC (46%) compared to control (79%). More number of male pronuclei were formed in the oocytes matured on pOEC than pGC (37% vs 19%; p<0.05). There was no difference between pOEC and control (28%). In all groups, irrespective of immature or mature stage, some oocytes at germinal vesicle stage formed a male pronucleus within their cytoplasm. It was suggested that the monolayer of pGC or pOEC was effective for preventing oocytes from polyspermy and that the monolayers from pOEC were superior for promoting male pronuclear formation by increasing monospermy. That immature oocytes may also have an ability to form a male pronucleus in their cytoplasm was indicated.*

Key words: *Porcine oocytes, Meiotic maturation, Fertilization, Co-culture.*

Since the first report of Edwards [5] on the *in vitro* maturation of pig oocytes, the oocytes of this species have been frequently used as a model to study resumption of meiosis in mammals [17, 28]. Although the technique for *in vitro* fertilization in domestic animals has been improved to the extent that the high rate of penetration are not accompanied by major problems of multiple sperm entry, pig oocytes differ from those of most other species in that undergo high incidence of polyspermy when fertilized in *in vitro* [15] and their polyspermy represents the major unsolved problem. It is difficult to distinguish that the high polyspermy is related either to the maturation of the male and female gametes before insemination or to the condition of *in vitro* fertilization. In several studies, it has been suggested that only oocytes cultured in follicles [16], co-cultured with granulosa cells [18, 27], or cultured within the cumulus in medium with LH [26] show good developmental competence after fertilization. There have been some reports that a glycoprotein deposition on the zona pellucida [29] facilitates either more synchronous exocytosis of the cortical granular contents or physiological responses of the substance to cortical granular materia [1, 8, 10], leading to a functional block to polyspermy, and that the presence of a portion of the follicular wall and to a lesser extent, granulosa cells during maturation increases the incidence of oocytes with only one male pronucleus [32]. The recent report by Nagai and Moor [20] suggested that the secretory products from oviduct cells co-cultured with gametes may act to influence the incidence of polyspermy in pig eggs fertilized in *in vitro*. To increase the extent of cytoplasmic maturation which is essential for successful maturation and subsequently for getting normal-fertilized oocytes, it may be expected that the reproductive cells create well condition for co-culture during the *in vitro* maturation. By applying co-culture method, it is

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possible to produce large numbers of viable pig embryos that represent a comparative research resource for the study of early preimplantation development. Therefore, the final purpose of this experiment was to determine the most efficient *in vitro* condition for the production of normally fertilized porcine oocytes.

Materials and Methods

Culture of pOEC: Pig oviducts were obtained from a local slaughterhouse and transported to the laboratroy in mPBS supplemented with 200 units penicillin and 200 μg streptomycin/ml. After dissection, oviducts were briefly immersed in 70% ethanol. Their epithelial cells derived from an ampullar portion of oviduct were gently scraped with a microscopic slide, transferred to a conical tube containing 5 ml of TCM-199 supplemented with 10% FCS and antibiotics and washed twice by centrifugation at 1,000 g for 5 min. Finally, a portion of cells were resuspended in fresh culture medium, seeded in 4-well plates (300 μl suspension per well) and cultured for 4–6 days at 39 °C and 5% CO_2 in humidified air. After culture, medium was renewed with mPBS and some unattached cells were discarded. Monolayers were developed to 70–80% confluence prior to initiation of co-culture with oocytes. Prior (5 hr) to oocytes culture, confluent cells were washed twice with TCM-199 containing 10% FCS and maturation medium, and then used for the co-culture with oocytes.

Culture of pGC: Cells were recovered during oocytes collection just after follicular aspirations. Oocytes were immediately removed from aspirations, and remaining cells were centrifuged at 250 g for 5 min. Cells were resuspended in culture medium (TCM-199 containing 10% FCS and antibiotics). After further wash, the cellular number and viability was determined by use of a hemacytometer and the method of trypan blue exclusion. A total of 1 × 105 cells/ml was placed in 4-well plate and incubated for 4–6 days before co-culture with oocytes. The subsequent treatments were performed as did in pOEC culture.

Oocytes Collection and Maturation: Oocytes were collected by aspiration of the follicles (2–5 mm in diameter) of pig ovaries. Only those oocytes completely surrounded by compact cumulus cells were used for experiments, washed 3 times with TCM-199 containing 0.25 mM sodium pyruvate, 0.34 ml/100 ml sodium DL-lactate (60% syrup), 23.8 mM hydrogen carbonate, and 0.1 mg/ml dibekacin sulfate. Oocytes were then transferred into the monolayer of pOEC or GC in 4-well plates containing 500 μl of TCM-199 supplemented with 10% FCS (fetal calf serum), 1 mg/ml BSA (bovine serum albumin), 15 IU/ml PMSG (pregnant mare serum gonadotropin) and 15 IU/ml hCG (human chorionic gonadotropin) or into the medium alone (control) and cultured for 48 hr.

In Vitro Fertilization: The cultured oocytes were washed twice in B.O. medium, and groups of 20 oocytes were transferred into 4-well plates containing 400 μl of B.O. medium supplemented with 10 μg/ml heparin, 3 mg/ml BSA and 5 mM caffeine. Motile spermatozoa were obtained by centrifuging a 500 μl of thawed semen for 10 min at 700 g. Spermatozoa were counted by a hemacytometer, diluted with an appropriate volume of fertilization medium and preincubated for 2 hr. The sperm suspension (25 μl) was added to each drop of fertilization medium to obtain a final concentration of 2×10^6 spermatozoa/ml. On 6 hr after insemination, oocytes were freed from cumulus cells in culture medium (TCM-199 supplemented with 10% FCS and 10 μg/ml insulin), washed and cultured for further 12 hr (about 15 zygotes per 100 μl droplet medium) at 39 °C in a humidified atmosphere of 5% CO_2 and 95% air before fixation.

Evaluation of Oocytes: The oocytes were mounted on a slide with a whole-mount procedure and fixed for 48 hr in ethanol : acetic acid (3:1 v/v). The nuclear status of oocytes were examined under a phase-contrast microscope after staining with 1% orcein in 45% acetic acid according to the criteria of Hunter and Polge [9]. Sperm penetration and male pronuclear development were determined as described by Ding *et al.* [4]. The presence of unswollen or swollen sperm heads or male pronuclei in the ooplasm was taken as evidence of sperm penetration. The penetrated oocytes with one or more full size male pronuclei were considered to have undergone normal male pronuclear development.

Data were analyzed by Chi-square analysis to determine the differences between experimental groups.

Results

The effect of co-culture during the *in vitro* maturation of pig oocytes on their fertilization status were evaluated by determining the proportion of oocytes reaching metaphase of the second meiotic division and their subsequent ability to form a male pronucleus. In all groups, the cumulus cells were observed to undergo a dramatic expansion 48 hr after *in vitro* culture. Completion of nuclear maturation was determined by the appearance of chromosomal spreads in metaphase II plate as well as an expulsion of first polar body. As shown in Table

1, the proportion of matured oocytes in pGC group (76%) was significantly higher ($p<0.05$) than in the control (55%). On the other hand, the co-culture on the monolayer of pOEC resulted in the maturation rate of 69%, which was not significantly different from the pGC and control group. The penetration rate was defined as the percentage of oocytes that had at least one sperm in their cytoplasm, regardless of the decondensation stage of sperm nuclei (Table 2). The proportion of oocytes which underwent penetration was significantly greater ($p<0.01$) for oocytes matured in control medium (82%) than in pGC (56%). The penetration rate of oocytes matured in pOEC (68%) was not different compared with pGC and control. On the other hand, the polyspermic penetration rate of oocytes matured in pOEC and pGC was 38% and 46%, respectively, which were significant differences ($p<0.01$) compared with control (79%).

After 18 hr of insemination, the percentage of oocytes capable of sustaining the formation of a male pronucleus was significantly higher ($p<0.05$) for the oocytes matured in pOEC (37%) than pGC (19%). Final analysis was focused on the proportion of oocytes that formed the male pronuclei at various stages of maturation (GV-GVBD, MI, AI-TI and MII) by 18 hr of insemination (Table 3). The proportion of oocytes that formed the male pronuclei at GV to GVBD stage was 20% (control), 14% (pGC) and 23% (pOEC). These observations suggest that the porcine oocytes, irrespective of the meiotic stage of maturation, have an ability to form a male pronucleus in their cytoplasm, and that the degree of complete nuclear maturation is not necessary to the formation of male pronuclei.

Table 1. Maturation rate of porcine follicular oocytes cultured *in vitro* by various culture conditions

Culture conditions	No. of oocytes examined	No. of oocytes matured (%)
Control	83	46 (55)[a]
Granulosa cells	42	32 (76)[b]
Oviductal epithelial cells	51	35 (69)[ab]

a vs b; $p<0.05$.

Discussion

It is generally believed that follicular cells surrounding oocyte exert an inhibitory effect on the nuclear maturation of oocyte. However, the recent studies of pig oocyte maturation by either culturing oocytes in medium supplemented with porcine follicular fluid [21] or

Table 2. The rate of fertilization in pig oocytes after *in vitro* maturation with different culture conditions

Treatments	No. of oocytes (%) penetrated	No. of oocytes (%) Polyspermy	M PN*	M & F PN**
Control	71/ 87 (82)[a]	56/71 (79)[a]	20/71 (28)[AB]	3/71 (4)
pGC	74/132 (56)[b]	34/74 (46)[b]	14/74 (19)[A]	2/74 (3)
pOEC	71/104 (68)[ab]	27/71 (38)[b]	26/71 (37)[B]	4/71 (6)

*Male pronucleus, ** Male and Female pronucleus.
a vs b; $p<0.01$, A vs B; $p<0.05$.

Table 3. The proportion of polyspermic penetration and male pronucleus in various maturation stages 18 hr after insemination

Nuclear stages	No. of oocytes (%) Polyspermy Control (56)[A]	pGC (34)[A]	pOEC (27)[A]	Male pronucleus Control (20)[B]	pGC (14)[B]	pOEC (26)[B]
GV-GVBD	27 (48)[a]	10 (29)	14 (52)[a]	4 (20)[ab]	2 (14)	6 (23)
M I	4 (7)[b]	9 (26)	3 (11)[b]	1 (5)[a]	3 (21)	5 (19)
A I-T I	20 (36)[a]	12 (35)	8 (30)[ab]	10 (50)[b]	7 (50)	6 (23)
M II	5 (9)[b]	3 (9)	2 (7)[b]	5 (25)[ab]	2 (14)	9 (35)

A, Total number of polyspermic oocytes.
B, Total number of oocytes that formed a male pronucleus.
a vs b; $p<0.01$.

co-culturing with follicular cells [15] indicate that the somatic follicular cells play an important role in the cytoplasmic maturation of oocytes. The present study clearly demonstrated that the co-culture on pGC led to a significant increase in the percentage of oocytes that completed the meiotic maturation as compared with the control group. The study of metabolic coupling by Motlik *et al.* [19] indicated that follicular tissue actively controls the degree of intercellular cooperation during the development of oocytes. Our data suggest that one of the major effect of such an influence may be due to the ability of granulosa cells either to modulate the interaction or to prolong the persistence of metabolic cooperation between cumulus cells and oocyte during the *in vitro* maturation. It is considered that the low maturation rate in control oocyts might be due to the differences in culture conditions. Two principal factors known to influence the maturation process *in vitro* in many species are protein [2, 20–22] and hormonal supplements. In pig, meiotic maturation of oocytes is induced by culture with media containing PMSG, hCG or PMSG and hCG with or without oestradiol [31]. We have supplemented maturation medium with a high dose (15 iu/ml, respectively) of PMSG and hCG, and also FCS, BSA. Exposure to high concentrations of gonadotropin therefore does not seem to promote nuclear maturation. In addition, it has been reported that fetal calf serum [24] or BSA [32] exerted to inhibition maturation of pig oocytes. Further studies will be needed to determine the relationship between hormonal conditions or protein substances during oocyte maturation.

The reduced rate of sperm penetration of the oocytes cultured in pGC may be due to the reduction of cell activity following the formation of confluent monolayer. Available evidence suggests that granulosa cell are involved in the synthesis of zona pellucida material and some zona proteins are produced by granulosa cells cultured *in vitro* [14, 19, 24]. Like these proteins, a sperm receptor of zona that interacts with 15 kDa glycoprotein may be synthesized in cumulus cells and then transferred to the zona pellucida [25]. However, the chemical nature of such a factor involved in the sperm penetration remains to be determined in detail.

The presence of pOEC during *in vitro* maturation increased the monospermic penetration and the formation of male pronucleus. Although the role of pOEC for cytoplasmic maturation required to decondense the penetrated spermatozoa and subsequently to form a male pronucleus is still unknown, the effect of pOEC on the male pronuclear formation of pig oocytes may be related to its ability to prolong the functional interaction with cumulus cells during the maturation. Most likely, the result of this study indicates that pOEC efficiently mediated the metabolic process of cumulus cells involved in cytoplasmic maturation, resulting in the male pronucleus formation. In all groups of this study, some immature oocytes could be penetrated by sperm, and then sperm nucleus underwent decondensation and/or transformation to a male pronucleus. Our result agreed with the studies suggesting that sperm nuclear decondensation within the cytoplasm of GV stage oocytes has been observed in dogs [13], cattle [22] and pigs [30]. It is known that the somatic compartment is also required for the transmission of certain amino acid, nucleosides, and phospholipid precursors to the oocyte. Apart from their nutrient role, the follicle cells also generate instructional signals which influence the nucleus for direct synthesis of certain structural proteins [3]. The second important factor in the aquisition of competence by the matured oocytes is hormone which regulates many events within the follicle cells, including steroid biosynthesis [6], protein synthesis [12], and the secretion of glycosaminoglycan and hyaluronic acid [7, 11].

In vivo, sperm head swells 1 hr after penetration, transforms to a male pronucleus 6–8 hr later and first cleavage of pig eggs occurrs 12–14 hr after sperm penetration [10]. In the present study, we observed that the sperm head remained enlarged or transformed to male pronucleus 18 hr after insemination, but none of the eggs cleavaged. It seems that cytoplasmic maturation of the oocytes was not completed until insemination, followed by an arrest of development as suggested by Polge and Dziuk [23]. To obtain the tansferable embryos which are normally fertilized *in vitro*, the interaction or synergistic action of somatic cells with a various chemical components involved in the development of pronuclei as well as hormone levels remains to be further studied.

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卵管上皮細胞又は顆粒膜細胞の単層がブタ卵母細胞の体外成熟および受精率に及ぼす影響

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卵管上皮細胞と顆粒膜細胞を4～6日間培養し，単層細胞シートを前もって形成させた．採取した卵母細胞をwell当り10～15個ずつこれらフィーダ層上に導入し，又は培地のみに入れて48時間成熟培養を行った．各実験区の培養卵母細胞の半分は成熟度判定に用い，残りは凍結射出精液を用いた体外受精に供し位相差顕微鏡下で受精状況を調べた．顆粒膜細胞との共培養によって卵母細胞の成熟率が対照区に比べて有意に高く (76% vs 55%; $p<0.05$)，卵管上皮細胞区 (69%) とは差がなかった．精子侵入率においては，顆粒膜細胞区（56%）に比べて対照区（82%）で有意に ($p<0.01$) 高い価を示し，卵管上皮細胞区 (68%) とは差がなかった．卵管上皮細胞 (38%) あるいは顆粒膜細胞 (46%) と共培養した卵母細胞で単精子侵入率が対照区(79%)より低かった ($p<0.01$)．卵管上皮細胞と共培養により雄性前核形成が顆粒膜細胞区に比べて有意に高い値を示したが (37% vs 19%; $p<0.05$)，対照区 (28%) とは差が認められなかった．本研究において，体外受精後に核が未成熟段階にある一部の卵母細胞の細胞質で精子核の膨潤あるいは雄性前核の形成が見られた．これらの結果から，卵管上皮細胞と顆粒膜細胞の単層は単精子侵入率を高める有効な方法であり，卵管上皮細胞の単層は細胞質の成熟を促進し受精後に雄性前核の発育に適切な条件を与えたことが示唆された．また，卵母細胞のいずれの成熟段階のものでも精子侵入後雄性前核が形成されることが判った．

キーワード：ブタ卵母細胞，成熟分裂，受精，共培養．