In Vitro Maturation of Round Spermatids Using Porcine Oviduct Epithelial Cell Monolayer Condition Medium

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ABSTRACT

Porcine oviduct epithelial cells (POEC) are widely used in co-culture experiments to improve early embryonic development, *in vitro* fertilization in embryo transfer programs for domestic animals and *in vitro* maturation of immature germ cells. POEC were mechanically isolated and cultured in tissue culture medium 199. Cells grew continuously, and confluent monolayers were formed after 7 days. After forming confluent monolayer of epithelial cells, supernatant was collected as the condition medium for maturing round spermatids *in vitro*. Round spermatids were also separated mechanically and cultured in the POEC condition medium. In this study we observed that 20% of round spermatid cultured were matured into elongating spermatid after 24 h, and about 10% of round spermatid cultured showed complete elongation (elongated spermatid) within 24~48 h of *in vitro* culture. No further development was observed within 50~72 h and transformed cells lost their viability after 72 h. These preliminary findings suggest that the condition medium from POEC may be possible to overcome the round spermatid block by improving the milieu of culture system.

(Key words : Oviduct, Epithelial cell, Monolayer, Round Spermatid, *In vitro* maturation)

INTRODUCTION

Spermatids are the youngest male germ cells with a single set of haploid chromosomes (complete meiosis). Once they have completed meiosis, they undergo a complex cellular differentiation and maturation process known as spermiogenesis. Spermiogenesis starts at puberty and continues throughout the reproductive life of males. During spermiogenesis, round spermatids have approximately 7 μm size, which transform into mature spermatozoa. The use of animal and human cell cultures has become very beneficial for diverse applications in biotechnology and biomedical research. Cell cultures are used as a research technique in fundamental cytology to investigate normal cell metabolism. The epithelial lining of the oviduct that provides microenvironment is a simple columnar epithelium which contains both ciliated and mucus-secreting cells (Verhage *et al.*, 1979; Crow *et al.*, 1994). Tubal epithelium cells from many species, including human, have been grown in monolayer culture, where they lose morphological features associated with the epithelium *in situ* and adopt generally flattened epithelioid morphology with microvilli on their apical cell surface (Thibodeaux *et al.*, 1992; Dickens *et al.*, 1993). The presence of secretory vesicles and the secretions of tubal- specific proteins in bovine or ovine tubal epithelial cell cultures suggest that cells of a secretory phenotype may be retained in culture (Gandolfi *et al.*, 1989; Joshi 1991).

The mammalian oviduct is the natural site where crucial reproductive events, i.e., final gamete maturation, fertilization, and early embryo development, naturally take place (Harper, 1994). Oviduct epithelial cells were assumed to promote early embryonic development at best because of supposed specific glycoprotein secretions (Boice *et al.*, 1990; Roberts *et al.*, 1975) and to improve *in vitro* maturation of oocyte, reduce polyspermy, and increase normal fertilization *in vitro* (Vatzias *et al.*, 1999; Kano *et al.*, 1994).

The low fertilization rate and the high abnormal genetic constitution of the embryos may not result from a deficient meiotic process *in vitro* but rather to the immaturity of the spermatid. The oocyte activating competence was not found at the round spermatid stage in mice (Ogura *et al.*, 1994), but it was demonstrated in hamsters (Ogura *et al.*, 1993) and bovines (Goto *et al.*, 1996). In human, a normal activating competence was demonstrated

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at the roud spermatid stage (Sousa *et al.*, 1996).

We thought that oviduct epithelial cells might provide optimal environment for maturing round spermatids *in vitro*. The aim of the present study was to establish culture system for *in vitro* spermiogenesis using porcine oviduct epithelial cell monolayer condition medium.

MATERIALS AND METHODS

All inorganic and organic chemicals were purchased from Sigma (St. Louis, Mo, USA) unless otherwise stated.

Tissue Acquisition

Oviducts were recovered from the adult sow at a local slaughter house. Once obtained, the oviducts were placed in a sterile receptacle on a filter paper wetted with phosphate-buffer saline at 37℃ to avoid dehydration and damage to the organ and transported to the laboratory within two hours. The oviducts were then cooled gradually during transported to the laboratory by placing the receptacle on ice.

Culture Medium Preparation

The culture medium used for porcine oviduct epithelial cell cultures was tissue culture medium 199 (TCM 199) supplemented with 100 IU /mL penicillin-G and 100 μg /mL streptomycin sulfate. Culture medium used in this experiment was prepared with sterile technique and filtered with 0.20 μm filters (Pall Corporation, USA) and stored at 4℃ until use. The culture medium was strictly maintained at pH 7.4 and equilibrated before use into CO_2 incubator at 37℃ and 5% CO_2 in air.

Preparation of Condition Medium from Porcine Oviduct Epithelial Cells

The oviducts were dissected from the ovarian tissue and washed frequently with Ringer's solution to remove adherent debris, mucus, and red blood cells into a laminar flow sterile hood. Ampulla region of oviducts was used in this study and washed three times with Ringer's solution and PBS containing 100 IU/mL penicillin-G and 100 μg /mL streptomycin sulfate. The ampullary oviducts were cut longitudinally and inner lining of the lumen was opened and cut into pieces of 6~8 mm^2 and suspended in Hank's balanced salt solution (HBSS). Tubules were washed manually two times with HBSS and rinsed into the same solution containing 5 mM EDTA and kept in the sterile hood for 40 min at room temperature. After this treatment, the ciliated epithelium was gently scrapped to dislodge from the remaining cells with wide spatula in fresh HBSS. The sheets of epithelium were dispersed by passage through a 20-gauge needle several times. Cell clumps were washed two times at 800 g for 5 min in the same solution and erythrocyte-lysine buffer (Verheyen *et al.*, 1995). Final pellet was also washed with TCM 199 and finally suspended in the culture medium. Cellular content was controlled and adjusted to 2×10^4 cells /mL and cell suspension of 0.5 mL was cultured in each of 12-well tissue culture dish which was previously filled with 3 mL TCM 199 with 5% or without fetal bovine serum (FBS) and cultured into CO_2 incubator at 37℃ and 5% CO_2 in air. After formation of monolayer supernatant was collected and centrifuged at 5,000 rpm for 30 min at 4 ℃. Supernatant was filtered by 0.20 μm filters and kept at −20 ℃ for future use.

Preparation of Porcine Germ Cells

Testicular tissues were collected in PBS and squeezed with surgical blades. The resultant fluid was diluted again with PBS and washed by centrifuging at 500~600 g, five times for 5 min each. For an excessive number of erythrocytes the pellet was resuspended for 5 min in 2 mL of erythrocyte-lysing buffer (Verheyen *et al.*, 1995) as mention above. After getting the final pellet, it was diluted with POEC condition medium, which was incubated at 37℃ and 5% CO_2 in CO_2 incubator. Five μL culture drops of POEC condition medium were made and kept them in CO_2 incubator before use. Cell suspension of 2 μL was mixed to each of the culture drops. Most of the preliminary works on identifying the different types of spermatogenic cells in an unstained wet preparation was carried out with Nikon inverted microscope equipped with Hoffman optics. Spermatids were identified by previously described criteria (Tesarik and Mendoza, 1996). The cell is characterized by a dense, smooth dark nucleus positioned centrally or inclining towards the cell membrane. In some of these cells, the early acrosomal vesicle or acrosomal cap is clearly visible as a bright white spot or sickle-shape adjacent to the nucleus. Spermiogenic cells were cultured *in vitro* using the standard aseptic technique under 5% CO_2 in air, at 37℃ rather than the usual 39℃ to avoid the adverse effects of the higher temperature on cellular metabolism.

Assessment of Viability

Viability of the germ cells in cellular suspension was determined using the trypan blue exclusion test (Talbot and Chacon, 1981). Trypan blue stains dead cells blue but do not permeate the membrane of living cells, and so they remain unstained (Phillips, 1973). Trypan blue solution (0.5 ml; T-8154; Sigma, Poole, UK) was added to 0.5 mL of cell suspension. The mixture was allowed to stand for 10 min at room temperature and then centrifuged at 500 g for 10 min at 4℃. Supernatants were discarded and the pellets resuspended in 1 mL of enriched Krebs-Ringer bicarbonate medium. A small droplet from each tube was transferred to the culture dish and observed under a microscope. Cells stained blue were considered dead.

RESULT

Culture of Porcine Oviduct Epithelial Cells

Mechanically scrapping of oviduct epithelium caused perfect disassociation of ciliated epithelial cells. Cells were examined by phase-contrast microscopy from the time of seeding to formation of monolayer. Two distinct cell types were observed in these cultures of oviduct epithelium: ciliated and secretory cells. Ciliated cells were generally polygonal in shape, flat and with well-defined contours at 24 h of seeding (Fig. 1a). After 24 h in culture medium, oviduct epithelial cells (OEC) attached to the bottom of culture dish and separated from the cell plague (Fig. 1b). Soon after the epithelial cells attached firmly to the culture wells, epithelial cells began to migrate peripherally out of the cell plaque to form plates on the bottom of the culture wells and begun to divide. Cells gradually adopted a flattened configuration while arranging themselves into a colony-like growth pattern had seen at third day of culture. Epithelial cell confluency in the wells occurred within 4~5 days. The primary OEC cultures lost ciliary activity quickly within 5 days of culture. By day 7, the oviduct epithelial cells formed a confluent monolayer. About 80% of the cell colonies formed a typical epithelial cell appearance defined by tightly dense polygonal cells (Fig. 1c). The resulting monolayer was free of cilia. As time progressed, epithelial cells located at the periphery of the aggregates became flattened and continued their migratory activity as they proliferated. The presence of FBS was found to be beneficial for maintaining the differentiated state of oviduct epithelial cell growth.

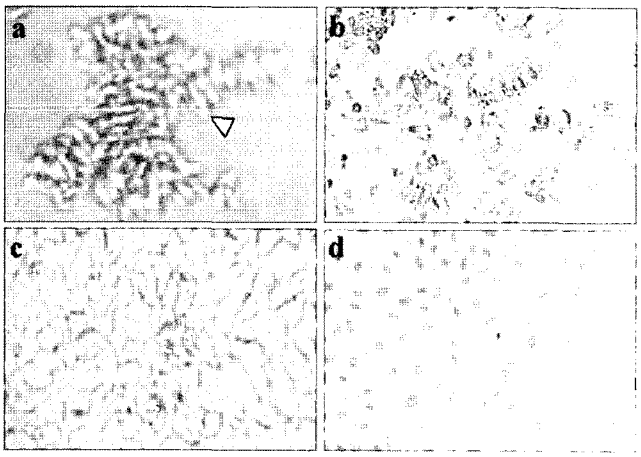

Fig. 1. Growth feature *in vitro* of porcine oviduct epithelial cells isolated by scrapping (× 375). a: Day 1 of oviduct epithelial cells with actively beating cilia (arrow head), b: Epithelial cells migrating out of the cell clusters at day 2 of seeding, c: Oviduct epithelial cells formed a confluent monolayer after 7 d of plating, d: H & E staining of monolayer clearly showed epithelial gathering of polygonal cells that contained large round cell nuclei.

Morphology and Viability of the *In Vitro* Cultured Round Spermatid

The *in vitro* maturation of round spermatids was examined. Round spermatids cultured *in vitro* were also examined for their viability. After 24 h of culture in the condition medium without any hormone source it was observed that the nucleus of the round spermatid started to bulge to the periphery and then the acrosomal cap was appeared at opposite of flagellum. After 48 h of inoculation late elongating spermatids had shortened nuclei that had migrated to the opposite to the flagella. There was no remarkable change found from 50~72 h and cells lost their viability after 72 h. However, Round spermatids cultured in tissue culture medium alone or tissue culture medium supplemented with 20% FBS lost their viability and broke down after 24 h of culture.

DISCUSSION

Mammalian spermatogenesis is a highly synchronous process by which mitotic spermatogonia, meiotic spermatocytes, and haploid spermatids develop in close association with somatic Sertoli cells. The complete recapitulation of spermatogenesis *in vitro* remains an elusive goal in reproductive biology. While germ cell viability and functionality can be maintained for extended periods in culture (Kierszenbaum, 1994), only limited differentiation of spermatogenic cells has been achieved *in vitro* (Tesarik *et al.*, 2000).

The present study showed that condition medium from oviduct epithelial cell monolayers can support maturation of porcine round spermatids. It is generally assumed that the condition medium from OEC monolayers improves cell quality by removing toxic compounds, by supplying small molecular weight metabolites or by providing growth factors (Aslam and Fishel, 1998). Similar advances have not been achieved using isolated and purified epithelium preparations. Four methods to isolate porcine oviduct epithelial cells were compared in the present study: mechanical: squeezing and scraping and enzymatic: collagenase and trypsin treatment (results not shown except scraping). All methods were able to isolate epithelial cells. Mechanical scrapping was the most reliable method. It was easily performed, inexpensive and possible cellular damage during treatment with enzymes was excluded.

After 24~48 h of culture in the condition medium without any hormone source it was observed that the round spermatids started to grow flagella. There was no remarkable change found from 50~72 h and cells lost their viability after 72 h. The present experiments also revealed that the optimized conditions were not met because the condition medium was unable to progress complete spermiogenesis only. FSH plays a determinant role in the sur-

Table 1. Effect of porcine oviduct epithelial cell monolayer condition medium on the *in vitro* maturation of round spermatid

Species	No. of round spermatid	Maturation stage of round spermatid		
		Elongating spermatid	Elongated spermatid	Spermatozoa
Porcine	55	11 (20.0)	6 (10.9)	0 (0.0)
Mouse	20	4 (20.0)	2 (10.0)	0 (0.0)

vival of the seminiferous epithelium and in spermatogonia proliferation (Foresta *et al.*, 1998), but suggests primarily that it plays a role in the conversion of round to elongating spermatids. Testosterone induces spermatogonia and spermatocyte proliferation and acts as a crucial element in the conversion of round to elongated spermatids (McLachlan *et al.*, 1994; O'Donnell *et al.*, 1996).

Gerton and Millette (1984) reported that when mouse spermatogenic cells were cultured, flagella were first detected clearly after 6.5 h of culture. After 24 h 20% of round spermatid were the only spermatogenic cells capable of tail formation. Human spermatogenic cells (Aslam and Fishel, 1998) could be maintained in culture and cells remained viable for a short period of time. During initial stage of culture (4~8 h) 22% of spermatids developed flagella. The results of those studies are not comparable with our results. Our study showed that porcine and mouse homogenous population of spermatids developed flagella after 24 h of culture. We believe that this is the first report of limited *in vitro* differentiation of spermiogenic stage of porcine and mouse germ cells.

In mammals other than the human, studies using long-term cultures of mixed spermatogenic populations in hormone supplemented media have also recently shown that meiosis can be completed *in vitro*. However, maturation then became arrested at the round spermatid step (Hue *et al.*, 1998). Round spermatid co-culture on Vero cell monolayers showed that it is possible to mature these cells up to the elongating/elongated phase. *In vitro* maturing of round spermatids could be one method for the study of the round spermatid block (Cremades *et al.*, 1999).

The results of our study suggest that porcine and mouse round spermatids can be cultured *in vitro*, which may enhance spermatid maturation and develop flagella. Maturation of spermatid by the oviduct epithelial cell condition medium provides an excellent way of understanding oviduct physiology and spermiogenesis.

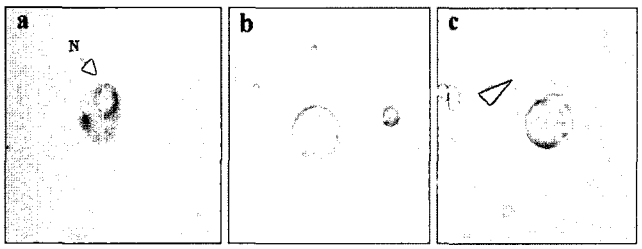

Fig. 2. Transformation of porcine round spermatid in porcine oviduct epithelial cell monolayer condition medium (× 600). a: Round spermatid with well-defined nucleus (N= Nucleus), b: Elongating spermatid where flagellar growth was appeared, c: Elongated spermatid with the prominent flagellum (arrow head).

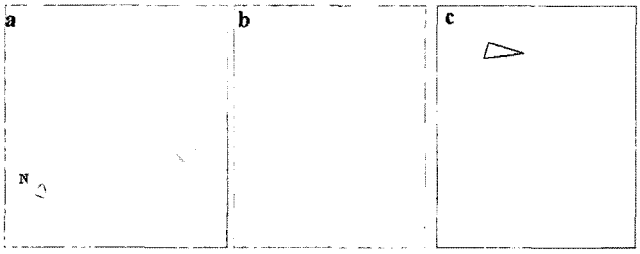

Fig. 3. Transformation of mouse round spermatid in porcine oviduct epithelial cell monolayer condition medium (× 600). a: Round spermatid with well-defined nucleus (N = Nucleus), b: Elongating spermatid where flagellar growth was appeared, c: Elongated spermatid with the prominent flagellum (arrow head).

REFERENCES

1. Aslam I, Fishel S (1998): Short-term *in vitro* culture and cryopreservation of spermatogenetic cells used for human *in vitro* conception. Hum Reprod 13:634-638.
2. Boice ML, Geisert RD, Blair RM, Verhage HG (1990): Identification and characterization of bovine oviduct glycoproteins synthesized at estrus. Biol Reprod 43: 457-465.
3. Cremades N, Bernabeu R, Barros A, Sousa M (1999): *In vitro* maturation of round spermatids using co-culture on Vero cells. Hum Reprod 14:1287-1293.
4. Crow J, Amos NA, Lewin J (1994): Morphology and ultra-structure of Fallopian tube epithelium at different stages of the menstrual cycle and menopause. Hum Reprod 9:2224-2233.
5. Dickens CJ, Southgate J, Leese HJ (1993): Use of primary cultures of rabbit oviduct epithelial cells to study the ionic basis of tubal fluid formation. J Reprod Fertil 98: 603-610.
6. Foresta C, Bettella A, Ferlin A (1998): Evidence for a stimulatory role of follicle-stimulating hormone on the spermatogonial population in adult males. Fertil Steril 69:636-642.

7. Gandolfi F, Brevini TAL, Richardson L (1989): Characterization of proteins secreted by sheep oviduct epithelial cells and their function in embryonic development. Development 106:303-312.
8. Gerton GL, Millette CF (1984): Generation of flagella by cultured mouse spermatids. J Cell Biol 98:619-628.
9. Goto K, Kinoshita A, Nakanishi Y, Ogawa K (1996): Blastocyst formation following intracytoplasmic injection of *in vitro* derived spermatids into oocytes. Hum Reprod 11:824-829.
10. Harper MJK, Gamete and zygote transport. In: Knobil E, Neill JD (eds), The Physiology of Reproduction, vol. 1, 2nd ed. New York: Raven Press, Ltd; 1994:123-187.
11. Hue D, Staub C, Perrard Sapori MH (1998): Meiotic differentiation of germinal cells in three-week cultures of whole population from rat seminiferous tubules. Biol Reprod 59:379-387.
12. Joshi MS (1991): Growth and differentiation of the cultured secretory cells of the cow oviduct on reconstituted basement membrane. J Exp Zool 260: 229-238.
13. Kano K, Miyano T, Kato S (1994): Effect of oviduct epithelial cells on fertilization of pig oocytes *in vitro*. Theriogenology 42:1061-8.
14. Kierszenbaum AL (1994): Mammalian spermatogenesis *in vivo* and *in vitro*: a partnership of spermatogenic and somatic cell lineages. Endocr Rev 15:116-134.
15. McLachlan RI, Wreford NG, Meachem SJ (1994): Effects of testosterone on spermatogenic cell populations in the adult rat. Biol Reprod 51:945-955.
16. O'Donnell L, McLachlan RI, Wreford NG (1996): Testosterone withdrawal promotes stage specific detachment of round spermatids from the rat seminiferous epithelium. Biol Reprod 55:895-901.
17. Ogura A, Yanagimachi R, Usui N (1993): Behaviour of hamster and mouse round spermatid nuclei incorporated into mature oocytes by electrofusion. Zygote 1: 1-8.
18. Ogura A, Matsuda J, Yanagimachi R (1994): Birth of normal young after electrofusion of mouse oocytes with round spermatids. Proc Natl Acad Sci USA 91: 7460-7462.
19. Phillips HJ (1973): Evaluation of culture dynamics. Dye exclusion test for cell viability. In Kruse, P.K. and Patterson, M.K. (eds), Tissue Culture Methods and Applications. Academic Press New York NY USA 406-408.
20. Roberts GP, Parker JM, Symonds HW (1975): Proteins in the luminal fluid from the bovine oviduct. J Reprod Fertil 45:301-313.
21. Sousa M, Mendoza C, Barros A, Tesarik J (1996): Calcium responses of human oocytes after intracytoplasmic injection of leukocytes, spermatocytes and round spermatids. Mol Hum Reprod 2:853-857.
22. Tesarik J, Mendoza C (1996): Spermatid injection into human oocytes. I. Laboratory techniques and special features of zygote development. Hum Reprod 11:772-779.
23. Tesarik J, Mendoza C, Anniballo R, Greco E (2000): *In vitro* differentiation of germ cells from frozen testicular biopsy specimens. Hum Reprod 15:1713-1716.
24. Thibodeaux JK, Menezo Y, Roussel JD (1992): Coculture of *in vitro* fertilized bovine embryos with oviductal epithelial cells originating from different stages of the estrous cycle. J Dairy Sci 75:1448-1455.
25. Tilbot P, Chacon RS (1981): A triple-stain technique for evaluating normal acrosome reaction of human sperm. J Exp Zool 215:201-208.
26. Vatzias G, Hagen DR (1999): Effects of porcine follicular fluid and oviduct-conditioned media on maturation and fertilization of porcine oocytes *in vitro*. Biol Reprod 60:42-8.
27. Verhage HG, Bareither ML, Jaffe RC, Akbar M (1979): Cyclic changes in ciliation, secretion and cell height of the oviductal epithelium in women. Am J Anat 156: 505-521.
28. Verheyen G, De Croo I, Tournaye H (1995): Comparison of four mechanical methods to retrieve spermatozoa from testicular tissue. Hum Reprod 10:2956-2959.

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