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Effects of the Association of Cumulus Cells on Bovine Oocytes on *In Vitro* Maturation and Subsequent Development after IVF

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소 난자의 체외성숙에 있어서 난구세포의 부착이 수정 후의 배발생에 미치는 영향

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요 약

실험1, 난구-난자 복합체(CIO)와 나화난자(DO)의 성숙배양 개시후 3~24시간 동안 각각의 난자에 핵성숙 진행상태를 Hoechst 33342로 염색하여 관찰하였다. GV기는 성숙배양 개시후 3시간에, GVBD기는 6시간에, MⅠ기는 13시간에, AnaⅠ-TelⅠ기는 16시간에, MⅡ기는 24시간에 각각 관찰되었으며, CIO와 DO에 있어 각각의 핵성숙 진행 비율의 차이는 인정되지 않았다. 실험 2, 실험 1에서 결정된 각각의 핵성숙 시간에 CIO로부터 난구세포를 제거하는 것이 난자의 24시간 성숙배양 후 수정능과 그 후의 배발생에 미치는 영향을 검토하였다. 각각의 시간에 난자로부터 난구세포를 제거하여도 MⅡ의 비율과 수정율에는 영향을 미치지 않았다. 성숙배양 개시후 0, 3, 6시간에 난구세포를 제거한 난자의 분할율은 성숙배양 개시후 13, 16, 24시간에 제거한 난자에 비하여 유의하게 낮았다($p<0.01$). 또한 성숙배양 개시후 0, 3, 6, 13시간에 난구세포를 제거한 난자의 배반포배 발생율은 16, 24시간에 난구세포를 제거한 난자에 비하여 유의하게 낮았다($p<0.01$). 이상의 결과는, 체외 소난자의 핵성숙 진행시기는 부착된 난구세포에 의존하지 않으며, 난자와 난구세포의 결합상태를 성숙배양 개시후 13~16시간(MⅠ)까지 즉 MⅠ기에 도달할 때까지 유지시키는 것은 난자의 수정후 배발생에 있어 필수적인 것임을 시사하였다.

(Key words : cumulus cell, maturation, chromosome status, blastocyst)

INTRODUCTION

The maintenance of junctional communication(gap junctions) between cumulus cells and oocytes during maturation has been reported to be important for further development after fertilization(Shioya et al., 1988; Fukui, 1989; Bottcher et al., 1990). In cattle, ultrastructural studies demonstrated that gap junction is decreased during germinal vesicle breakdown up to 12 h of culture *in vitro*, and that gap junctions are no longer found at 18 h, when the oocyte has reached metaphase Ⅰ(Hyttel *et al.*, 1986). Numerous reports on oocyte maturation *in vitro* have suggested that the oocyte appears

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to acquire developmental competency only when the cumulus cells are directly contacting the oocyte(Leibfried-Rutledge *et al.*, 1989). The exact role of the gap junctions on meiotic resumption and oocyte maturation, however, is not clearly understood(Wert and Larsen, 1989). On the other hand, the time course relationship between the existence of gap junctions and oocyte maturation in bovine has not yet been examined. The purpose of this study was to investigate the relationship of the cumulus cells on nuclear progression and the effect of removal of cumulus cells from cumulus intact oocytes on maturation, fertilization, and subsequent development of bovine oocytes *in vitro*.

MATERIALS AND METHODS

1. Oocyte collection and maturation conditions

Ovaries were collected from slaughtered cows at a local abattoir and were transported to the laboratory within 3 h in physiological saline(0.9% NaCl) at 36℃. After washing the ovaries in fresh physiological saline, oocyte-cumulus complexes were aspirated from small antral follicles (1 to 6mm diameter) using an 22-gauge needle attached to a 10ml sterile plastic syringe. Oocyte-cumulus complexes and follicular fluid were expelled into 10ml glass tubes and maintained at 39℃. Only oocytes with an intact cumulus oophorus and homogeneous, evenly granulated cytoplasm(CIO) were selected using a stereomicroscope(× 20 to 40). CIO were washed twice with phosphate buffered saline(PBS) supplemented with bovine serum albumin(BSA, Fraction V; Sigma Chemical Co. St. Louis, MO, USA; A4503) and twice with maturation medium. After washing, 25 to 30 oocytes were placed in 200μl drops of maturation medium, and covered with paraffin oil(Paraffin Liquid, Light, Nacalai Tesque Inc., Tokyo, Japan) in 60×15 mm culture dishes(Nunclon, Nunc, Roskilde, Denmark). A part of CIO were freed from cumulus cells(DO) using a fine pasteur pipette before culture for maturation. CIO were completely freed from cumulus cells using a fine pasteur pipette at 0, 3, 6, 13, 16, and 24 h during maturation and then cultured for 24 h in total after beginning culture. Inner diameter of a fine pasteur pippette used was almost the same size of the diameter of oocyte. Removal of cumulus cells was performed at 39℃ and complete within 5min/25~30 oocytes. The times of removal of cumulus cells were set according to the results obtained by Kruip *et al.*(1983) and Hyttel *et al.*(1986). Maturation medium consisted of TCM-199 with modified Earle's salts(Nissui Pharmaceutical Co., Ltd., Japan) supplemented with 0.5 mM sodium pyruvate(Sigma; P2256), 3mg/ml polyvinylpyrrolidone(PVP: M. W. 40,000; Nacalai Tesque Inc., Japan), 50 units/ml penicillin G(Sigma), 100 IU/ml hCG(Teikoku Hormone Mfg. Co. Ltd., Japan) and 1μg/ml estradiol(E_2, Sigma; E8875). All media were sterilized by means of a 0.3μm membrane filter(Toy Roshi Kaisha, Ltd., Japan) before use. The oocytes were cultured under 5% CO_2 in air with high humidity at 39℃.

2. Staining of oocytes with Hoechst 33342

CIO and DO cultured for 3, 6, 13, 16 or 24 h were centrifuged at 15,000×g for 5min at 4℃, to localize the lipid granules, then stained with 10μg/ml Hoechst 33342(Sigma; H33342) for 15 to 30min at 39℃, 5% CO_2 in air. The oocytes stained were examined under a fluorescent microscope(DX 50, Olympus) and classified according to chromosome status. The maturation stages of oocytes(GV, GVBD, M I, Ana I, Tel I and M II) were determined by fluorescent microscopy after staining with Hoechst 33342(Fig. 1).

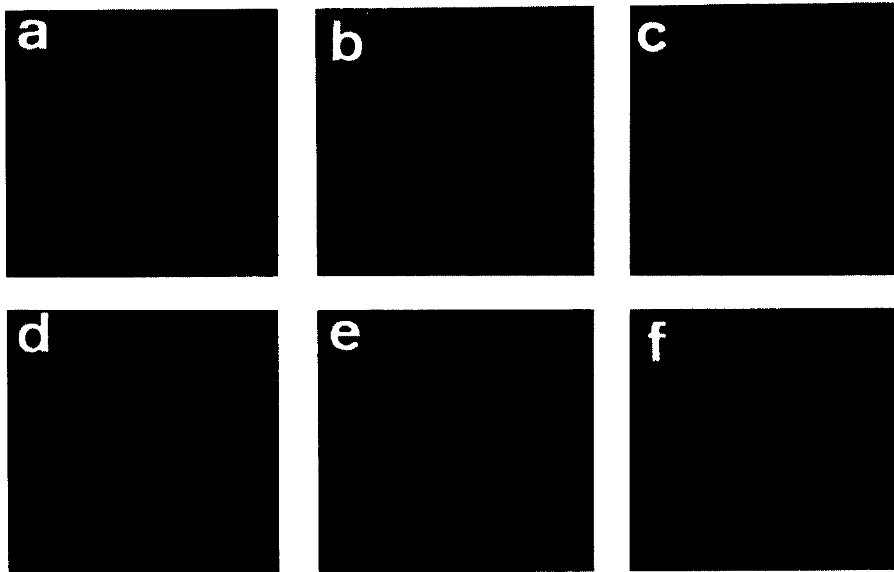

Fig. 1. Bovine oocytes at different stages of maturation stained with Hoechst 33342.
The photographs were taken with a fluorescent microscope(×400). a: germinal vesicle after 3 h in culture, b: metaphase Ⅰ at 13 h, c: anaphase Ⅰ at 13 h(early stage), d: anaphase Ⅰ at 16 h(late stage), e: telophase Ⅰ at 16 h, f: metaphase Ⅱ at 24 h.

3. *In vitro* fertilization and *in vitro* culture

Frozen-thawed semen(0.5ml×3) was washed twice with m-Tyrode's balanced salt solution supplemented with 10 mM caffeine(sodium benzoate, Sigma; C4144) and 20μg/ml heparin(sodium salt, Sigma; H3125) and centrifuged at 500 ×g for 5min. After decanting the supernatant, pellets containing spermatozoa were resuspended at a density of 5 to 6×10^6 cells/ml in 100μl m-Tyrode's solution with the addition of 5 mM caffeine, 10μg/ml heparin, and 10mg/ml BSA. The sperm suspension was incubated for 1 h in 5% CO_2 in air with high humidity at 39℃.

Oocytes and cumulus cells removed before maturation from CIO were washed 3 times with m-Tyrode's solution containing 10mg/ml BSA. Five to 10 oocytes and cumulus cells were introduced into 100μl of pre-incubated sperm suspension and then cultured for 5 h in 5% CO_2 in air with high humidity at 39℃. After IVF, oocytes were stripped of attached spermatozoa by passing them through a fine-drawn pasteur pipette. The embryos were transferred to the feeder layer of granulosa cells cultured for 30 h in TCM-199 supplemented with 5% calf serum(CS, Gibco, Kim *et al.*, 1996), and then cultured for 8 days. Half the volume of the culture medium was replaced with the equal volume of fresh medium at intervals of 48 h.

To evaluate the maturation and fertilization rates, at the end of the maturation period and at 20 h after insemination, oocytes were fixed with ethanol-acetic acid(3:1) and stained with 1% aceto-orcein. Observations were performed under a phase contrast microscopy(×400). Maturation was assumed by the presence of metaphase Ⅱ chromosomes with the first polar body,

and fertilization was considered complete when more than 2 pronuclei(one female pronucleus and more than one male pronucleus) were evident. Morphological estimation was done at 2 and 8 days after insemination to evaluate the cleavage and the developmental stages, respectively.

4. Statistical analysis

Mean measurements from 5 replicates were taken to determine statistical difference using Fisher's protected least significance difference (PLSD) test following analysis of variance(ANOVA).

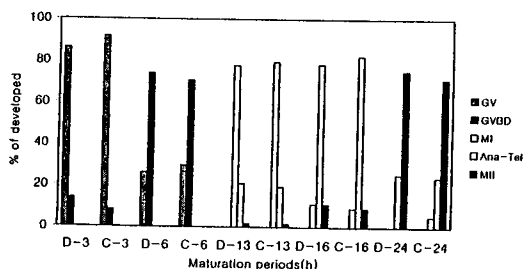

Fig. 2. Changes in nuclear status of bovine cumulus intact(C) or denuded(D) oocytes throughout maturation. GV : germinal vesicle, GVBD : germinal vesicle break down, M I : metaphase I, Ana I-Tel I : anaphase I -telophase I, M II : metaphase II.

RESULTS

The percentages of chromosome configurations of CIO and DO cultured in protein free medium at various times after maturation in culture is shown in Fig. 2. All oocytes remained at the GV stage after 3 h in culture. After 6 h of culture, 65% of oocytes underwent GVBD, and no difference was observed between CIO and DO. The proportion of metaphase I(M I) oocytes increased dramatically at 13 h from the beginning of maturation culture. Oocytes emitting the first polar body appeared after 16 h of culture, although most of the oocytes(80%) remained at anaphase I(Ana I) to telophase I(Tel I). Matured metaphase II(M II) oocytes were observed mainly after 24 h in culture. There was no difference in the proportion of oocytes at each stage between CIO and DO.

There was no difference in the percentages of maturation and fertilization, when oocytes were freed of cumulus cells at 0, 3, 6, 13, 16, and 24 h after culture for maturation(Table 1), on the other hand, the cleavage rates of oocytes freed from cumulus cells at 0, 3, and 6 h after maturation culture was significantly decreased compared to those of oocytes freed at 13, 16 and 24 h(32.3, 50.5 and 51.9% vs 60.8, 59.4, and 68.1%, respectively; $p<0.01$, Fig. 3).

The developmental competency to the blastocyst stage after insemination of oocytes denuded

Table 1. Effects of removal time of cumulus cells from CIO on *in vitro* maturation and fertilization of bovine oocytes

Removal times after culture(hrs)	No. of oocytes examined	No. (%) of oocytes matured	No. of oocytes inseminated	No. (%) of oocytes fertilized
0	71	62(87.3)	57	47(82.4)
3	64	53(82.8)	52	45(86.5)
6	68	61(89.7)	61	50(82.0)
13	51	47(92.2)	47	42(89.4)
16	64	56(87.5)	56	53(94.6)
24	64	53(82.8)	53	50(94.3)

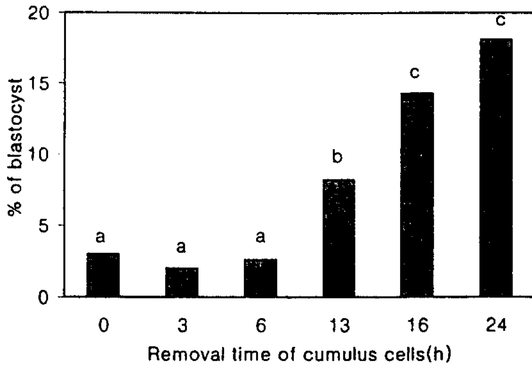

Fig. 3. Effect of the time of removal of cumulus cells from oocytes on *in vitro* development to cleavage (ab, ac: $p<0.01$).

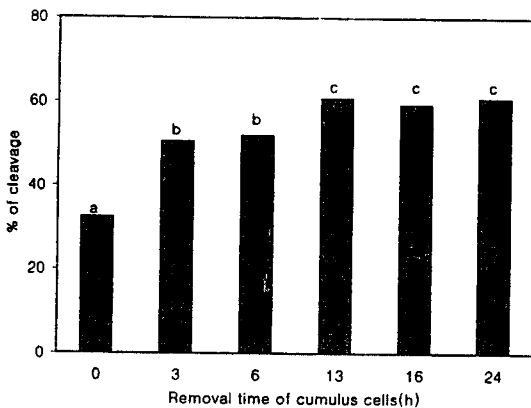

Fig. 4. Effect of the time of removal of cumulus cells from oocytes on *in vitro* development to blastocyst (ab, ac, bc: $p<0.01$).

at 0, 3, or 6 h significantly decreased compared to that of oocytes denuded at 13, 16, or 24 h(3.0, 2.0 and 2.6% vs 8.2, 14.3 and 18.1%, respectively; $p<0.01$, Fig. 4.). Although in the oocytes freed from cumulus cells at 13 h, the percentage of blastocysts increased compared to those of oocytes freed at 0, 3, or 6 h($p<0.01$), but the percentages was significantly lower than for oocytes freed at 16 or 24 h($p<0.01$).

DISCUSSION

The chromosome status of the cumulus intact oocyte and the denuded oocyte were distinguishable by fluorescent microscopy after staining with Hoechst 33342, although the maturation stages of some oocytes whose chromosomes were located within the lipid granules could not be determined(9.5%). The times at which events occurred was confirmed previously in *in vitro* studies(King *et al.*, 1986; Suss *et al.*, 1988; Sirard *et al.*, 1989). During *in vivo* maturation, GVBD occurs between 4 and 8 h after LH peak (Kruip *et al.*, 1983) or between 6 and 9 h in superovulated animals according to Hyttel *et al* (1986). In the present study, the proportion of oocytes reached at each maturation stage was not significantly different between CIO and DO. Finally, more than 80% of oocytes reached M II after 24 h of maturation culture in all treatment(Table 1). Chian and Niwa(1994) has also reported that the presence of cumulus cells during maturation *in vitro* do not significantly influence nuclear maturation of bovine oocytes. This result indicates that the presence of cumulus cells during maturation *in vitro* does not significantly influence nuclear maturation of bovine oocytes. However, it has been reported that a higher percentage of bovine oocytes invested with a compact cumulus cells reached M II than those with loose or no cumulus cell mass(Leibfried and First, 1979; Fukui and Sakuma, 1980). In these experiments, since denuded oocytes at the time of collection from ovaries were used it is possible to consider that these oocytes were already denuded during growing phase.

Our data show no difference in fertilizability at least the ability of pronuclear formation of oocytes denuded before maturation culture. Although, Fukui(1989) reported that oocytes should be surrounded at fertilization by expanded

cumulus cells, other studies have indicated that sperm penetration *in vitro* is improved when oocytes matured *in vitro* are mechanically denuded before insemination(Fukui and Ono, 1989; Lu *et al.*, 1987; Xu *et al.*, 1987).

The cleavage rates significantly decreased when removal of cumulus cells occurred at 0, 3, or 6 h after culturing for 24 h to produce maturation.

The developmental competency to the blastocyst stages after insemination of oocytes denuded at 0, 3, or 6 h(GV to GVBD) after culture for maturation significantly decreased compared to that of oocytes denuded at 16 or 24 h(Ana I to M II stage). On the other hand, in oocytes denuded at 16 h(Ana I to Tel I) after culture for maturation, no difference was observed in the percentage of blastocysts compared to that of oocytes cultured with that of cumulus cells for 24 h(M II). This indicated that the presence of cumulus cells for the first 16 h(Ana I to Tel I) in culture is sufficient for the oocytes to develop normally into blastocysts. In several species only oocytes isolated from preovulatory follicles with cumulus cells expanded, and which have progressed to MI are able to attain full development(Motlik *et al.*, 1975; Niwa *et al.*, 1976). This means that the intimate contact between the oocyte and its cumulus cells up to MI is a prerequisite to further development of normal embryos. The metaphase I stage of *in vitro* cultured bovine oocytes was present at 10.3 to 15.4 h after the beginning of culture(Sirard *et al.*, 1989). Under our experimental conditions, the M I stage was present at 13 h of culture. Chian and Niwa(1994) have shown that the presence of intact cumulus cells for at least 12 h is necessary for normal cytoplasmic maturation of bovine oocytes *in vitro*. Our results suggest that maintenance of coupling between cumulus cells and oocytes within the first 13 to 16 h(M I to Tel I stage) of culture was important for full maturation and further development after fertilization. Mattioli *et al*,(1988) reported that premature breakdown of the cell contact with the oocyte reduces the developmental potency of the oocyte. It has also been reported that gap junctions between oocytes and cumulus cells in bovine are important for full maturation(Skoblina, 1988), and that maturation rates are lower if oocytes are denuded before maturation(Fukui and Sakuma, 1980). Semple *et al*,(1993) reported that bovine oocytes achieve developmental competency within 14 h of commencing *in vitro* maturation.

These results demonstrate that timing of nuclear progression *in vitro* of bovine oocytes is independent of attached cumulus cells and that the presence of directly attached cumulus cells to oocytes for at least 13 to 16 h(M I) after initiation of culture for maturation is necessary for normal cytoplasmic maturation of bovine oocytes *in vitro*, and that the direct communication of cumulus cells with the oocyte may be important to cytoplasmic maturation.

SUMMARY

First, we determined the chromosomal progression of cultured cumulus intact oocytes (CIO) and denuded oocytes(DO) for 3 to 24 h after the start of maturation culture by staining with. The germinal vesicle(GV) was observed at 3 and 6 h and additional chromosomal statuses were observed as follows, germinal vesicle break down(GVBD) at 6 h, metaphase I at 13 h, anaphase I to telophase I at 16 h, and metaphase II at 24 h after initiation of culture. No difference was found in the proportion of chromosomal status between CIO and DO at any time. Second, we investigated the effect of removal of cumulus cells from CIO that defined their

chromosomal status on the maturation, fertilizability, and subsequent development to blastocysts. Removal of cumulus cells at each maturation stage had no effect on the maturation and fertilization rates of oocytes. The cleavage rates of oocytes freed from cumulus cells at 0, 3 or 6 h were significantly lower($p<0.01$) than those of oocytes denuded at 13, 16, or 24 h. In addition, those oocytes with cumulus cells which were removed at 0, 3, 6, or 13 h after beginning of maturation significantly decreased the percentages of blastocyst formation compared to those of oocytes from which cumulus cells were removed at 16 and 24 h($p<0.01$). These results demonstrated that the timing of nuclear progression of bovine oocytes *in vitro* was independent of attached cumulus cells, and suggest that the first 13 to 16 h(metaphase I stage) of the maintenance of coupling of oocyte with cumulus cells during nuclear progression is essential for further development of oocytes after fertilization.

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