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Control of Sperm Penetration *In Vitro* by Cumulus Cells in Porcine Oocytes

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돼지의 체외수정시 난구세포에 의한 정자침입의 조절

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요 약

본 연구는 체외에서 돼지정자의 난자내 침입에 있어서 난구세포의 기능적인 역할을 검토하기 위하여 실시하였다. 체외수정시 정자침입율은 난구세포 부착(61%) 난자가 제거(25%)된 난자에 비해 높았으나($P<0.01$), 다정자침입에는 영향을 미치지 않았다. 한편 체외수정시 hyaluronidase를 0, 0.01, 0.1 및 1.0mg/ml 농도로 첨가된 배양액내에서 난구세포가 부착된 난자내의 정자침입률은 각각 61, 56, 66 및 39%로 이들 세포를 제거한 난자에서의 침입률 34, 35, 30 및 27%에 비해 높았다. 그러나 다정자침입률은 hyaluronidase의 농도에 관계없이 난구세포를 제거한 난자에서 낮았으며, hyaluronidase의 농도가 높아지면서 다정자침입률이 낮아지는 경향을 나타냈다. 또한 hyaluronidase를 첨가한 배양액내에서 수정후 16 및 24시간에서의 정자침입률은 난구세포를 제거한 경우(25 및 31%) 보다 난구세포가 부착된 난자(48 및 62%)에서 높았으며 ($P<0.05$), 다정자침입률은 난구세포 제거시 유의적으로 낮게 나타났다($P<0.05$). 한편, 난자로부터 채취한 난구세포를 여러 농도로 첨가한 후 난구세포 제거난자를 이용하여 체외수정한 결과 hyaluronidase첨가보다는 무첨가시 정자의 침입률과 다정자 침입률이 낮게 나타났다. 본 연구의 결과로부터 난구세포는 정자의 침입에 효과적으로 작용하였으며, hyaluronidase의 첨가와 난구세포수의 조절이 정자의 침입과 다정자침입에 영향을 미치는 것으로 추측되었다.
(Key words: Cumulus cells, Hyaluronidase, *In vitro* penetration, Polyspermy, Porcine)

I. INTRODUCTION

The mammalian oocyte and its surrounding cumulus cells are metabolically coupled through gap junctions which provide the means of entry into the ooplasm for several metabolites (Moor et al., 1980). Cumulus cells directly enhance oocyte maturation (Sirard and Coenen, 1993) and sperm function (Boatman and Robbins, 1991) and avoid polyspermy (Cummins and Yanagimachi, 1982). Chian et al. (1995) suggested that the cumulus cells act as a sperm trap on *in vitro* fertilization in bovine oocytes. In regard to direct enhancement of sperm function, Yanagimachi (1988) has suggested that the presence of the intact cumulus cells around an oocyte is not absolutely necessary for successful *in vitro* fertilization, but its presence may facilitate fertilization. Evidence in the hamster

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(Corselli and Talbot, 1987) and mouse (Ward and Storey, 1984) indicated that spermatozoa are already functionally capacitated in the oviduct, and that a release of acrosomal content is not required for cumulus penetration. However, some investigators supposed that the cumulus cells directly participate in capacitation of spermatozoa (Soupart and Strong, 1974; Bavister, 1982). It has been demonstrated that the cumulus cells exert a specific effect on human sperm motility and acrosome reaction (Tesarik et al., 1990).

Motlik et al. (1986) have shown that when cumulus-enclosed pig oocytes are matured *in vitro*, the intercellular cooperation is lost much earlier than *in vivo*. Although the cumulus cells disappear within perhaps 3 h in the oviduct of cow *in vivo* (Lorton and First, 1979), they always remain with *in vitro* matured oocytes. It has been believed that there are beneficial roles for cumulus cells on *in vitro* fertilization and subsequent early development in the cow (Fukui, 1990), but the functional role of the cumulus cells in porcine oocytes during *in vitro* fertilization is not clear.

The mechanism by which sperm hyaluronidase is released or exposed to act on hyaluronic acid in the cumulus has been debated for many years. It was long supposed that sperm undergo the acrosome reaction outside of the cumulus or during passage through cumulus, thereby releasing their soluble acrosomal contents including hyaluronidase (Siiteri et al., 1988). The present study was conducted to clarify the role of cumulus cells in sperm penetration and polyspermy during *in vitro* fertilization of porcine oocytes, with special reference to the effect of hyaluronidase on the role of cumulus cells *in vitro*.

Ⅱ. MATERIALS AND METHODS

1. Collection and maturation of oocytes

Porcine ovaries were collected from a local slaughter-house and kept in saline(NaCl, 0.9% W/V ; Penicillin 100,000 IU/L ; Streptomycin 100mg/L and Amphotericin B 250μg/L ; Sigma Chemical, St-Louis, MO, USA) at 30 to 32℃. Cumulus-oocyte complexes were aspirated from 2 to 6 mm follicles with a 10-ml syringe with an 18-G needle. The collected oocytes were washed three times in Hepes-buffered Tyrode's medium (TLH) and once in maturation medium. Oocytes with compact and complete cumulus cells were introduced to droplets of maturation medium (10 oocytes/50μl droplet), covered with mineral oil and cultured under an atmosphere of 5% CO_2 in air at 39℃ for 42~44 h. The maturation medium consisted of TCM-199 with Earle's salts (Gibco Lab., NY, USA) supplemented with 3.05mM glucose, 0.32mM Ca-lactate, 2.5mM Hepes (Sigma), 10% fetal bovine serum (FBS; Gibco, Life Technologies, Inc. NY 14072 USA), 0.2 mM Na-pyruvate (Sigma), 50μg/ml gentamycin (Sigma), 1μg/ml FSH (from porcine pituitary; Sigma), 5μg/ml LH (from equine pituitary; Sigma), 1μg/ml estradiol 17β (Sigma) and 10% (v/v) porcine follicular fluid (PFF). The PFF was aspirated from follicles(2 to 5mm in diameter) with a syringe and 18-gauge needle, and centrifuged at 3,850×g for 15 min. The supernatant fluid was frozen at −20℃ until used.

2. Spermatozoa preparation and oocyte fertilization

The frozen-thawed spermatozoa were diluted with 2ml of BTS (Beltsville Thawing Solution) and equilibrated in air-tight tubes at 37℃ in a waterbath for 10 minutes. After equilibration, 2ml semen was placed over 2 layers of percoll (65 and 70%) and centrifuged at 2,000×g for 15 minutes. The spermatozoa in the 65% percoll layer were carefully collected, washed in preincub-

ation medium (TCM-199) with Earle's salts (Gibco), supplemented with 3.05 mM glucose, 2.92 mM Ca-lactate (Sigma), 10% FCS, 0.2 mM Na-pyruvate, and 50 μg/ml gentamicin (Sigma) by suspension and centrifugation two times at 250×g for 10 minutes and resuspended in preincubation medium.

The fertilization medium was the same as the preincubation medium, enriched with 2 mM caffeine (Sigma) and adjusted to a pH of 7.2 to 7.4. The final concentration of spermatozoa was adjusted to 1×10^6 cells/ml motile sperm during fertilization *in vitro*.

3. Experimental design

In the first experiment, after oocytes culture for 42~44 h in the maturation medium, the cumulus-oocyte complexes were divided into two groups. A group of the oocytes was completely freed from the cumulus cells by repeated passage through a fine pipette. The oocytes with or without cumulus cells and the denuded were washed three times using fertilization medium, and were placed in 48μl of fertilization medium. The dishes containing the oocytes were kept in an incubator with 5% CO_2 in air and high humidity at 39℃ until spermatozoa were added. A sperm suspension (2μl) was introduced to fertilization droplet that included five oocytes. The mixture had a final concentration of 1×10^6 spermatozoa/ml.

In the second experiment, oocytes were randomly divided into two groups after maturation in culture. One group of the oocytes was completely freed from cumulus cells as described above. The oocytes with or without cumulus cells were washed three times with fertilization medium, and placed with sperm in medium containing different concentrations (0, 0.01, 0.1 and 1.0mg/ml) of hyaluronidase.

In the third experiment, to examine the timing pattern of sperm penetration to oocytes with or without cumulus cells, oocytes were placed with sperm in fertilization medium with 0.1mg/ml hyaluronidase. Four, 8, 12, 16 and 20 h after sperm addition, spermatozoa and/or cumulus cells were completely removed from the surface of the oocytes by repeating passage through a fine pipette in the fertilization medium, and the oocytes were fixed for examination. Finally, oocytes was completely freed from cumulus cells as described in the first experiment. The cumulus cells obtained by repeated pipetting were washed three times using centrifugation 250×g for 10 min and resuspended in fertilization medium. The cumulus cell-free oocytes were placed with sperm in medium with or without hyaluronidase (0.1mg/ml) and different concentrations(0, 10^2, 10^4 and 10^6 cells/ml) of cumulus cells prepared above. 20~22 h after sperm addition, oocytes were examined for penetration and polyspermy.

4. Evaluation of oocyte fertilization

At the end of each examination, the oocytes were mounted, fixed(acetic acid : ethanol 1:3) for 2-3 days and stained with 1% acetic-orcein in 40% acetic acid : 60% water solution. The proportions of penetration and polyspermy were examined with the light microscope at ×200 and ×400 magnification. Oocytes were considered as penetrated when spermatozoa with a swollen head or pronuclei were found in the vitellus. Oocytes penetrated by only one spermatozoon were judged to be monospermic oocytes.

5. Statistics

Chi-square analysis with the Yates correction was used to test the significance of individual comparisons for rates of penetration and polyspermy.

Ⅲ. RESULTS

As shown in Table 1, when the oocytes with or without cumulus cells were inseminated with frozen-thawed spermatozoa, the proportion of penetrated oocytes was significantly ($P<0.01$) higher in the oocytes with (61%) than without (25%) cumulus cells. However, the polyspermy rate was not significantly difference between cumulus-intact (42%) and cumulus-free (30%) oocytes.

When oocytes with or without cumulus cells were inseminated in medium containing different concentrations of hyaluronidase, the penetration rates were significantly ($P<0.05$) higher in cumulus-intact(61, 56 and 66% for 0, 0.01 and 0.1 mg/ml hyaluronidase) than in oocytes without (34, 35 and 30% for 0, 0.01 and 0.1 mg/ml hyaluronidase) cumulus cells (Table 2). On the other hand, the proportions of polyspermy decreased as concentrations of hyaluronidase increased. However, there were no significant differences in the polyspermy rates between cumulus-intact and cumulus-free oocytes.

Table 3 shows the timing pattern of sperm penetration and polyspermy in cumulus-intact and cumulus-free oocytes in medium with hyaluronidase. The proportions of penetrated oocytes were higher in cumulus-intact than in cumulus-free oocytes at 4, 8, 12, 16 ($P<0.05$) and 20 h ($P<0.05$) after insemination. However, polyspe-

Table 1. Effect of cumulus cells on sperm penetration *in vitro* in porcine oocytes (n=3)

Presence of cumulus cells	No. of oocytes examined	No. of oocytes penetrated			No. of polyspermic oocytes(%)†
		Total (%)	Enlarged sperm head	Male and female pronuclei (%)†	
+	119	73(61)	52	21(29)	31(42)
−	131	33(25)**	21	4(12)	10(30)

† Percentage of total number of oocytes penetrated.

** $P<0.01$.

Table 2. Effect of hyaluronidase concentrations and cumulus cells on penetration *in vitro* in porcine oocytes matured in culture (n=3)

Conc. of hyaluronidase (mg/ml)	Presence of cumulus cells	No. of oocytes examined	No. of oocytes penetrated			No. of polyspermic oocytes(%)†
			Total (%)	Enlarged sperm head	Male and female pronuclei (%)†	
0	+	62	38(61)*	28	10(26)	20(53)
	−	70	24(34)	20	4(17)	10(42)
0.01	+	75	42(56)*	24	18(43)	18(43)
	−	69	24(35)	18	6(25)	8(33)
0.1	+	68	45(66)*	25	20(44)	18(40)
	−	67	20(30)	16	4(20)	6(30)
1.0	+	71	28(39)	22	6(21)	8(29)
	−	73	20(27)	14	6(30)	3(15)

† Percentage of total number of oocytes penetrated.

* $P<0.05$, difference between with and without cumulus cells.

Table 3. Effect of cumulus cells on sperm penetration *in vitro* at various time of insemination in medium with hyaluronidase (n=3)

Time after insemination (h)	Presence of cumulus cells	No. of oocytes examined	No. of oocytes penetrated			No. of polyspermic oocytes(%)†
			Total (%)	Enlarged sperm head	Male and female pronuclei (%)†	
4	+	107	2(2)	2	0(0)	0(0)
	−	112	1(1)	1	0(0)	0(0)
8	+	109	15(14)	15	0(0)	1(7)
	−	103	5(5)	5	0(0)	0(0)
12	+	120	28(23)	27	1(4)	5(18)
	−	111	10(9)	10	0(0)	1(10)
16	+	124	60(48)*	55	5(8)	22(37)*
	−	116	29(25)	29	1(3)	4(13)
20	+	118	73(62)*	52	21(29)	35(48)*
	−	103	32(31)	28	4(13)	5(16)

† Percentage of total number of oocytes penetrated.

* $P<0.05$, difference between with and without cumulus cells.

Table 4. Effect of concentrations of cumulus cells on penetration and polyspermy in fertilization medium with or without hyaluronidase (n=3)

Conc. of cumulus cells (cells /ml)	Presence of hyaluronidase (0.1mg /ml)	No. of oocytes examined	No. of oocytes penetrated			No. of polyspermic oocytes(%)†
			Total (%)	Enlarged sperm head	Male and female pronuclei (%)†	
0	+	180	60(33)*	42	18(30)	6(10)
	−	192	16(8)	16	0(0)	0(0)
10^2	+	189	84(44)*	69	15(18)	21(25)
	−	150	12(12)	8	4(33)	0(0)
10^4	+	174	114(66)*	102	12(11)	27(24)
	−	171	66(39)	66	0(0)	9(14)
10^6	+	186	144(77)*	40	24(17)	42(29)*
	−	256	87(56)	84	3(3)	9(10)

† Percentage of total number of oocytes penetrated.

* $P<0.05$, differences between with and without hyaluronidase.

rmy was inhibited in cumulus-free oocytes (13 and 16%) compared with cumulus-intact oocytes (37 and 48%) at 16 ($P<0.05$) and 20 h ($P<0.05$) after insemination, respectively.

When denuded oocytes and sperm were cocultured in medium with or without hyaluronidase (0.1 mg /ml) and different concentrations of cumulus cells (Table 4), the penetration rates were significantly higher in medium with(33, 44, 66 and 77%) than without (8, 12, 39 and 56%) hyaluronidase at concentrations of 0, 102, 104 and 106 cumulus cells /ml respectively ($P<0.05$). The proportions of polyspermy were inhibited in medium without(10, 25, 24 and 29%) than with(0, 0, 14 and 10%) hyaluronidase at concentrations of 0 10^2, 10^4 and 10^6 ($P<0.05$) cumulus cells /ml.

Ⅳ. DISCUSSION

This study reports two principal roles for cumulus cells on *in vitro* penetration of porcine oocytes matured in culture. First, sperm penetration is under control by cumulus cells, and this can have at least a partial effect on inhibition of polyspermy in oocytes without cumulus cells. Second, hyaluronidase is apparently not an important component of the penetration induction process that occurs by cumulus cells stimulation, but can control spermatozoa penetration and polyspermy partially.

The beneficial effect of cumulus cells on maturation of oocytes *in vitro* has been reported for rabbit and cow (Robertson and Baker, 1969), mouse (Cross, 1970) and human (Kennedy and Donahue, 1969). It has also been reported that the roles of the cumulus cells include facilitation of oocyte transport, enhancement of spermatozoa function, and avoidance of polyspermy (Bedford and Kim, 1993). In particular, cumulus cells may influence spermatozoa by correctly orienting sperm to oocytes in the hamster (Drobnis et al., 1988) and by triggering the acrosome reaction (Tesarik, 1985). In the present study, the proportions of penetrated oocytes were significantly higher in oocytes with than without cumulus cells. As has been shown (Ball et al., 1983; Mahadevan and Traunson, 1985), cumulus cells may not be necessary for fertilization because removal of cumulus cells is not necessarily ralated to the changes in the incidence of fertilization. However, removal of cumulus cells from mouse oocytes caused variability and occasional reduction of the *in vitro* fertilization (Itagaki and Toyoda, 1992). The present results reinforced the fact that cumulus-intact oocytes were more likely to be penetrable by spermatozoa than cumulus-free oocytes in the pig.

When cumulus cells are removed, the zona pellucida hardens (Katska et al., 1989), and this occurs under a prolonged culture period of germinal vesicle-stage oocytes. Furthermore, it has been reported that cumulus cells surrounding the oocytes (Katska et al., 1989) and serum (Downs et al., 1986) protect the zona pellucida against hardening. Hardening of zona pellucida in culture is correlated with a decrease in penetration rates (Gianfortoni and Gulyas, 1985). Chian et al (1994) reported that there were no significant differences between oocytes matured with and without cumulus cells, which suggests that zona hardening does not occur or does not prevent sperm penetration during the culture of bovine oocytes even without cumulus cells. They also demonstrated that the hyaluronidase-mediated denudation of oocytes is similar to the mechanical denuation of oocytes in sperm penetrations. Regardless of with or without hyaluronidase within medium, this study indicated the sperm penetration in medium with cumulus cells is higher than oocytes without cumulus cells. Therefore, it seems that the effect of the hyaluronidase not different between bovine and porcine oocytes with or without cumulus cells during *in vitro* fertilization.

The notion that the presence of the cumulus cells around the oocytes protects against polyspermy was contradicted by some investigators (Bedford and Kim, 1993; Chian et al., 1995). Bavister (1986) has suggested a function of the cumulus cells that regulate the number of spermatozoa reaching the oocyte surface. However, Bedford and Cooper (1978) proposed that the condition of the oocytes rather than the presence or absence of the cumulus cells is important for normal monospermic fertilization. It has been reported that oocytes do not become polyspermic in the absence of the cumulus cells, and those cumulus-intact oocytes do not protect

against polyspermy (Hunter and Nichol, 1988). Moreover, it has been suggested that the cumulus cells act as a sperm trap *in vivo* (Bedford and Kim, 1993) and *in vitro* (Chian et al., 1995). In the present study, polyspermy was observed 8 h after insemination in cumulus-intact oocytes (Table 3). The polyspermy rates were decreased in oocytes without cumulus cells compared with cumulus-intact oocytes at 16 and 20 h after insemination in fertilization medium including 0.1mg/ml hyaluronidase. In another experiment, when cumulus-free oocytes were cultured with various concentrations of cumulus cells under the medium with or without hyaluronidase during the *in vitro* fertilization, the polyspermy was also inhibited in high concentrations of cumulus cells and without hyaluronidase. In conclusion, these results suggests that dispersed cumulus cells may induce more sperm capacitation and acrosome reaction in the surface of the zona pellucida. Furthermore, polyspermic oocytes was directly affected by the cumulus-intact or free on the zona pellucida of oocytes *in vitro*.

V. SUMMARY

The functional role of cumulus cells on the penetration and polyspermy during *in vitro* fertilization in porcine was examined. The penetration rate was significantly higher ($P<0.01$) in oocytes with (61%) than without (25%) cumulus cells, but significant differences in polyspermy rates were not observed. When hyaluronidase was added to the fertilization medium with different concentrations, penetration rates in oocytes with cumulus cells were higher than in oocytes without cumulus cells at 0 (61% vs 34% ; $P<0.05$), 0.01 (56% vs 35% ; $P<0.05$), 0.1 (66% vs 30% ; $P<0.05$) and 1.0mg/ml (39% vs 27%). The polyspermy rates were lower in oocytes without than with cumulus cells, and had a tendency to decrease with high concentrations of hyaluronidase. In another experiment, the penetration and polyspermy rates had a tendency to increase as time of sperm-oocyte culture was prolonged. At 16 and 20 h after insemination, the penetration rates were significantly higher ($P<0.05$) in oocytes with (48 and 62% for 16 and 20 h) than without (25 and 31% for 16 and 20 h) cumulus cells in medium containing hyaluronidase. Polyspermy rates were significantly ($P<0.05$) lower in oocytes without (13% and 16%) than with (37% and 48%) cumulus cells at 16 and 20 h after insemination. In cumulus-free oocytes inseminated in medium containing different concentrations of cumulus cells, the penetration rates were significantly ($P<0.05$) higher in medium with than without hyaluronidase. The proportion of polyspermy was lower in medium without than with hyaluronidase at 0 (10% vs 0%), 10^2 (25% vs 0%), 10^4 (24% vs 14%) and 10^6 (29% vs 10% ; $P<0.05$) cumulus cells/ml. These results suggest that cumulus cells can have a positive influence on sperm penetration, its action on polyspermy control does appear to function primarily on zona pellucida by co-culture of cumulus cells and oocytes in medium without hyaluronidase.

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