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Control of spermatozoa penetration and polyspermy by cumulus cells in porcine oocytes matured in culture

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Abstract

The functional role of the cumulus cells on sperm penetration and polyspermy during *in vitro* fertilization was examined. The penetration rate was significantly higher($p<0.01$) in oocytes with(61%) than without(25%) cumulus cells. No significant differences, however, was observed in polyspermy. When the hyaluronidase was supplemented to the fertilization medium with different concentrations, penetration rates in oocytes with cumulus cells were higher than 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 1.0 ㎎/㎖(39 vs 27%). On the other hand, the polyspermy rates were lower 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-oocytes culture was prolonged. At 16 and 20hrs after insemination, the penetration rates were significantly higher($p<0.05$) in oocytes with(48 and 62% for 16 and 20hrs) than without(25 and 31% for 16 and 20hrs) cumulus cells in medium with hyaluronidase. However, the polyspermy rates were significantly($p<0.05$) lower in oocytes without(13 and 16%) than with(37 and 48%) cumulus cells at 16 and 20hrs after insemination. In cumulus-free oocytes inseminated in medium with or without hyaluronidase at different concentrations of cumulus cells, the penetration rates were significantly($p<0.05$) higher in medium with than without hyaluronidase at different concentrations of cumulus cells. The proportions of polyspermy were 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 the advantage of culture in medium with cumulus cells and denuded oocytes to inhibit polyspermy with no decrease in the penetration rates during the fertilization *in vitro* in the porcine.

Key words : *In vitro* fertilization, Porcine oocyte, Spermatozoa penetration, Hyaluronidase

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Introduction

The mammalian oocyte and its surrounding cumulus cells are metabolically coupled through gap junctions which provide the unique means of entry into the ooplasm for several metabolites[1]. It is reported that the cumulus cells directly enhance oocytes maturation[2] and spermatozoa function[3]. Changes in intercellular coupling observed during *in vitro* culture of intact sheep follicle were comparable with those occurring *in vivo*[1]. By contrast Motlik et al.[4] have shown that when cumulus-enclosed pig oocytes are matured *in vitro*, the intercellular cooperation is lost much earlier than *in vivo*. In regard to direct enhancement of spermatozoa function, Yanagimachi[5] has suggested that the presence of the intact cumulus cells around an oocytes is not absolutely necessary for successful *in vitro* fertilization, but its presence may facilitate fertilization. Evidence in the hamster[6] and mouse[7,8] indicated that spermatozoa are functionally capacitated already 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[9,10]. It has been demonstrated that the cumulus cells exert a specific effect on human spermatozoa motility and acrosome reaction[11].

Although the cumulus cells are considered to disappear within perhaps 3hrs in the oviduct of cow *in vivo*[12], it always remains with the *in vitro* matured oocytes. It has been believed that there are beneficial roles of the cumulus cells on *in vitro* fertilization and subsequent early development in the cow[13], but the functional role of the cumulus cells in porcine oocytes during the *in vitro* fertilization is unclear. The present study was conducted to clarify the role of cumulus cells on control of penetration and polyspermy during *in vitro* fertilization of porcine oocytes, with special reference to the effect of additional hyaluronidase on the role of cumulus cells *in vitro*.

Materials and Methods

Medium

The basic medium used for the manipulation of oocytes and maturation was tissue culture medium(TCM) 199(with Earle's salts ; Gibco, Grand Island, NY, USA) supplemented with 3.05 mM D-glucose, 0.32 mM Ca-lactate, 2.5 mM Hepes(Sigma Chemical, St-Louis, MO, USA), 10% fetal calf serum, 0.2*m*M Na-pyruvate(Sigma), 50㎍/㎖ gentamycin(Sigma), 1 ㎍/㎖ FSH(Sigma), 5㎍/㎖ LH(Sigma), 1 ㎍/㎖ *oestradiol* 17β(Sigma) and 10%(*v*/*v*) porcine follicular fluid(PFF). This medium was essentially the same as TCM-199 used by Rouillier et al.[14]

Oocyte collection and culture

Porcine ovaries were collected at a local slaughterhouse and kept in saline(NaCl, 0.9% *w*/*v* ; penicillin 100,000 IU/*l* ; streptomycin 100 ㎎/*l* and amphotericin B 250 ㎍/*l* ; Sigma) at 30 to 32℃. Cumulus-oocyte complexes were aspirated from 2 to 6㎜ follicles with a 10 ㎖ syringe of 18G needle. Porcine oocytes were selected on the basis of visual assessment of morphological features for *in vitro* maturation. The collected oocytes were washed three time in Hepes-buffered Tyrode's medium(TLH) and once in maturation medium, 10 oocytes with a compact and complete cumulus cells were introduced to

droplets(50$\mu\ell$) of maturation medium, and covered with mineral oil and cultured under the atmosphere of 5% CO_2 in air at 39℃ for 42-44hrs.

Sperm preparation

Pooled ejaculate from boar were frozen, and the straws were later thawed by immersion in a 37℃ waterbath for about 30 sec. Thawed sperm was diluted with 2 mℓ of BTS(Beltsville Thawing Solution) and equilibrated in air-tight tubes at 37℃ in a water bath for 10min. After equilibration, the 2 mℓ of semen were placed over 2 layers of Percoll(65 and 70%) and centrifuged with 2000 ×*g* for 15min at 20℃. The spermatozoa in the 65% Percoll layer were carefully collected, washed twice in preincubation medium by suspension and centrifugation of 250 ×*g* for 10min and resuspended in preincubation medium.

In vitro fertilization

After the final washing, the motile spermatozoa were adjusted to concentration of 25×10^6 cells/mℓ. The fertilization medium was TCM-199 supplemented with 3 mM glucose, 3 mM Ca-lactate, 0.2 mM Na-pyruvate and 10% FCS. The final concentration of spermatozoa was adjusted to 1×10^6 cells/mℓ for fertilization *in vitro*.

Examination of spermatozo

At the end of spermatozoa-oocyte co-incubation, cumulus cells were removed by repeated passages through a fine pipette. The cumulus-free oocytes were transferred on to the center of glass slide with four vaseline spots, gently compressed with a cover slide, immersed in 25% acetic alcohol for 2-3 days for complete fixations and stained with 1% orcein in 45% acetic acid. Excess stain was removed by infiltrating aceto-glycerol medium(20% glycerol and 20% glacial acetic acid in distilled water) under the phase-contrast microscope at a magnification of ×200 or ×400. At the end of examination, oocytes were considered as penetrated when spermatozoa with a swollen head or pronuclei were found in the vitellus (Fig 1).

In the first experiment, after culture for 42-44hrs in the maturation medium, the cumulus-oocytes 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 were washed three times using fertilization medium, and then the cumulus-intact and the cumulus-free oocytes were introduced respectively to fertilization medium of 48$\mu\ell$. 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. Sperm suspension (2$\mu\ell$) was introduced to the fertilization droplet that included five oocytes. The mixture gave final concentration of 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 in the first experiment. The oocytes with or without cumulus cells were washed three times with fertilization medium, and inseminated 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, they

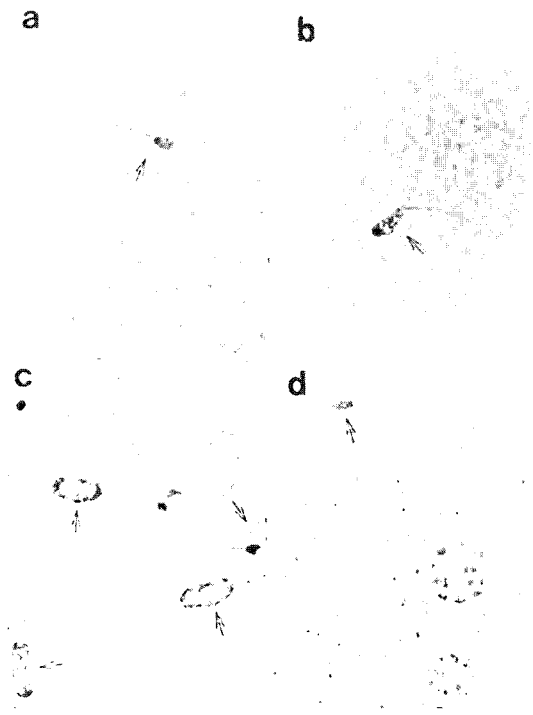

Fig 1. Oocytes penetrated by spermatozoa. (a) An oocyte penetrated by a sperm(arrow) 20-22hrs after insemination. ×400. (b) An oocyte penetrated by a sperm 20-22hrs after insemination showing an enlarged sperm head(arrow) with a penetrating sperm tail. ×400. (c) A polyspermic oocyte penetrated 20-22hrs after insemination showing decondensing sperm heads (arrows), some with penetrating sperm tails. ×400. (d) An oocyte examined 20-22hrs after insemination showing two pronuclei and second polarbody (arrow). ×400.

were inseminated in fertilization medium with 0.1 ㎎/㎖ hyaluronidase. At 4, 8, 12, 16 and 20hrs after insemination, 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 then the oocytes were fixed for examination.

Finally, in the fourth experiment, 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-free oocytes were inseminated in medium with or without hyaluronidase(0.1 ㎎/㎖) and different concentrations(0, 10^2, 10^4 and 10^6 cells/㎖) of cumulus cells.

Results

As shown in Table 1, when the oocytes with or without cumulus cells were inseminated with a 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, there was no difference in polyspermy rate between cumulus-intact(42%) and cumulus-free(30%) oocytes.

When oocytes with or without cumulus cells were inseminated in medium with

Table 1. Effect of cumulus cells on sperm penetration *in vitro* in porcine oocyte

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

*Percentage of total numbers of oocytes penetrated.
[a,b]$p<0.01$

Table 2. Effect of hyaluronidase concentrations and cumulus cells on penetration *in vitro* in porcine oocytes matured in culture

Conc of hyaluronidase (㎎/㎖)	Presence of cumulus cells	No of oocytes examined	No of oocytes penetrated			No of polyspermic oocytes(%)†
			Enlarged sperm head*	Male and female pronuclei(%)†	Total (%)	
0	+	62	28	10(26)	38(61)*	20(53)
	−	70	20	4(17)	24(34)	10(42)
0.01	+	75	24	18(43)	42(56)*	18(43)
	−	69	18	6(25)	24(35)	8(33)
0.1	+	68	25	20(44)	45(66)*	18(40)
	−	67	16	4(20)	20(30)	6(30)
1.0	+	71	22	6(1)	28(39)	8(29)
	−	73	14	6(30)	20(27)	3(15)

† Percentage of total numbers 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(0.1㎎/㎖)

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

† Percentage of total numbers of oocytes penetrated
* $p<0.05$, difference between with and without cumulus cells

different concentrations of hyaluronidase, the penetration rates were significantly($p<0.05$) higher in cumulus-intact than in cumulus-free oocytes at a low concentrations(0, 0.01 and 0.1㎎/㎖) of hyaluronidase(Table 2). On the other hand, the proportions of poly-

spermy were decreased as concentrations of hyaluronidase were 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 20hrs($p<0.05$) after insemination. However, polyspermy were inhibited in cumulus-free than in cumulus-intact oocytes at 4, 8, 12, 16($p<0.05$) and 20 hrs($p<0.05$), respectively.

When cumulus-free oocytes were inseminated in medium with or without hyaluronidase(0.1 ㎎/㎖) and different concentrations of cumulus cells, the penetration rates were significantly($p<0.05$) higher in medium with(33, 44, 66 and 77%) than without(8, 12, 39 and 56%) hyaluronidase at concentrations of 0, 10^2, 10^4 and 10^6 cumulus cells/㎖(Table 4). However, 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/㎖.

Discussion

This study reported two principal findings regarding the roles of cumulus cells on *in vitro* penetration in porcine oocytes matured in culture. First, sperm penetration is under the control of cumulus cells, and these can have at least partial effect on inhibition of polyspermy in cumulus-free oocytes. Second, hyaluronidase is apparently not an important component for the penetration induction process that occurring by cumulus cell's stimulation, but can control spermatozoa penetration and polyspermy partially.

The beneficial effect of cumulus cells on maturation of oocytes *in vitro* has been

Table 4. Effect of concentrations of cumulus cells and hyaluronidase on penetration and polyspermy in porcine oocytes

Conc of cumulus cells (cells/㎖)	Presence of hyaluronidase	No of oocytes examined	No of oocytes penetrated			No of polyspermic oocytes(%)†
			Enlarged sperm head†	Male and female pronuclei(%)†	Total (%)	
0	+	180	42	18 (30)	60 (33)*	6 (10)
	−	192	16	0 (0)	16 (8)	0 (0)
10^2	+	189	69	15 (18)	84 (44)*	21 (25)
	−	150	8	4 (33)	12 (12)	0 (0)
10^4	+	174	102	12 (11)	114 (66)*	27 (24)
	−	171	66	0 (0)	66 (39)	9 (14)
10^5	+	186	40	24 (17)	144 (77)*	42 (29)**
	−	256	84	3 (3)	87 (56)	9 (10)

† Percentage of total numbers of oocytes penetrated.
*$p<0.01$, **$p<0.05$, differences between with and without hyaluronidase.

reported in rabbit and cow[15], mouse[7] and human[16]. It has also been reported that the roles of the cumulus cells include facilitation of oocytes transport, enhancement of spermatozoa function, and avoidance of polyspermy[17]. In particular, cumulus cells may influence on spermatozoa by correctly orienting sperm to oocytes[18] and by triggering the acrosome reaction[11]. In the present study, the proportions of penetrated oocytes were significantly higher in oocytes with that than without cumulus cells(Table 1). As be showed in some study[19,20], cumulus cells may not essential for fertilization because removal of cumulus cells is not necessarily related to the changes of incidence of fertilization. However, removal of cumulus cells in mouse oocytes caused variability and occasional reduction of *in vitro* fertilization[21]. The present results reinforced the fact that cumulus-intact porcine oocytes were more likely to be penetrable than cumulus-free oocytes.

When cumulus cells are removed, the zona pellucida was hardened[22], and this occurs in GV-stage oocytes prolonged culture period. Furthermore, it has been reported that cumulus cells surrounding the oocytes[22] and serum[29] protect the zona pellucida against hardening. Hardening of zona pellucida in culture was correlated with a decrease of penetration rates[23]. Chian et al.[24] reported that there were no significant differences between oocytes matured with and without cumulus cells, which suggests that hardening of zona does not occur or not prevent sperm penetration during the culture of bovine oocytes even without cumulus cells. They also demonstrated that the hyaluronidase-mediated denudation on oocytes is similar to the mechanical denudation in spermatozoa penetration. Regardless of hyaluronidase presence in medium, this study indicated that the sperm penetration rates in medium with cumulus cells is higher than without cumulus cells. Therefore, it seems that the effect of the hyaluronidase is similar 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[17,24]. Bavister[25] has suggested a function of the cumulus cells that regulate the numbers of spermatozoa reaching the oocyte surface. However, Bedford and Cooper[26] 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[27]. Moreover, it has been suggested that the cumulus cells act as a sperm trap *in vivo*[17] and *in vitro*[28]. In the present study, polyspermy was observed 8hrs 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 20hrs after insemination in fertilization medium including 0.1 ㎎/㎖ 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 not also inhibited in high concentrations of cumulus cells and without hyaluronidase. This results suggests that dispersed cumulus cells may induce more sperm capacitation and acrosome reaction in the surface of the zona

pellucida. It is postulated that the proportion of polyspermic oocytes was directly affected by the cumulus-intact or free on the zona pellucida of oocytes *in vitro*.

In conclusion, while it is clear that cumulus cells can have a positive influence on spermatozoa penetration, its actions on polyspermy control do not appear to function primarily in zona pellucida of cumulus-intact oocytes in medium with or without hyaluronidase. However, the present experimental approaches were used to demonstrate that advantage of culture with high concentration of cumulus cells and cumulus-free oocytes to inhibit polyspermy with no decrease in the penetration rates during the *in vitro* fertilization in porcine.

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