Penetration *in vitro* of bovine oocytes during maturation by frozen–thawed spermatozoa

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Summary. Bovine immature oocytes cultured for various times in TC-199 medium were inseminated with frozen–thawed spermatozoa in Medium BO with caffeine (5 mM) and heparin (10 µg/ml). Very high penetration rates (95–100%) were obtained in all oocytes which had been cultured for 0–20 h. When oocytes cultured for 0 and 4 h were inseminated, 100% of them were penetrated and had a decondensing sperm head and most of the oocytes remained at the stage of condensed germinal vesicle (GV) to telophase-I 20–22 h after insemination. The formation of male and female pronuclei was first observed in oocytes inseminated 8 h after culture. The proportions of polyspermy and average number of spermatozoa in penetrated oocytes gradually decreased as oocyte maturation proceeded. Penetration of at least one spermatozoon with a decondensing head into oocytes at the GV stage (without culture) was almost completed up to 8 h after insemination and at that time most of the penetrated oocytes were still at the stage of GV or condensed GV. These results indicate that maturation of bovine oocytes is not required for sperm penetration into the vitellus or for sperm nuclear decondensation under the *in-vitro* conditions used.

Keywords: *in-vitro* fertilization; immature oocytes; maturation; bovine; sperm nuclear decondensation

Introduction

It has been reported for the mouse (Iwamatsu & Chang, 1972), hamster (Barros & Munoz, 1974), rabbit (Overstreet & Bedford, 1974), rat (Niwa & Chang, 1975) and human (Overstreet & Hembree, 1976; Overstreet *et al.*, 1980) that the zonae pellucidae of immature oocytes at the germinal vesicle stage are penetrable by spermatozoa. However, decondensing sperm heads are seldom observed within the cytoplasm under a phase-contrast microscope. From this it has been concluded that spermatozoa in the perivitelline space do not fuse with the plasma membrane of immature oocytes and are not incorporated into the vitellus. Electron microscopic observations, however, have revealed that, although hamster (Usui & Yanagimachi, 1976; Moore & Bedford, 1978) and rabbit (Berrios & Bedford, 1979) spermatozoa are able to fuse with the plasma membrane of immature oocytes, decondensation of the sperm nucleus does not occur. The nature of factor(s) for preventing sperm nuclear dispersion in immature oocytes is unknown and may not be necessarily constant among different mammals. In the dog, unlike all other mammalian species so far investigated, spermatozoa can penetrate the vitellus and sperm nuclear decondensation follows *in vitro* (Mahi & Yanagimachi, 1976). This seems to be reasonable, because oocytes are ovulated at the germinal vesicle stage in the dog (Van der Stricht, 1923).

The present light microscopic study was designed to determine the possibility of penetration *in vitro* of bovine follicular oocytes at various stages during maturation. It was also investigated whether sperm heads can be decondensed in the cytoplasm of the oocytes at the germinal vesicle stage.

Materials and Methods

The basic medium used for the treatment of spermatozoa and the fertilization of oocytes was essentially the same as that used by Brackett & Oliphant (1975) for the fertilization of rabbit eggs *in vitro*. The medium, designated Medium BO, comprised 112.0 mM-NaCl, 4.02 mM-KCl, 2.25 mM-CaCl₂, 0.83 mM-NaH₂PO₄, 0.52 mM-MgCl₂, 37.0 mM-NaHCO₃, 13.9 mM-glucose, 1.25 mM-sodium pyruvate and 31 µg potassium penicillin G/ml.

Ovaries were removed from cows at a local slaughterhouse and returned to the laboratory in saline at 30–32°C within 1.5 h. Oocytes were aspirated through a 24-gauge needle into a disposable 1-ml syringe from follicles of 3–5 mm in diameter. The collected oocytes were washed four times with TC-199 medium (with Earle's salts) buffered with 25 mM-N-2-hydroxyethylpiperazine N-2-ethane sulphonic acid (Hepes) and supplemented with 10% (v/v) heated fetal calf serum, 100 µg penicillin G/ml and 100 µg streptomycin/ml. Five to seven oocytes with cumulus cells were carefully transferred into a polystyrene culture dish (35 × 10 mm) containing 100 µl of the same medium covered with warm paraffin oil and cultured at 39°C in an atmosphere of 5% CO₂ in air. After culture for 0, 4, 8, 12, 16 or 20 h, oocytes were washed twice and placed into 50 µl Medium BO supplemented with 20 mg bovine serum albumin/ml (BSA; crystallized and lyophilized, essentially globulin free, No. A-7638; Sigma Chemical Co., St Louis, MO, USA) and 20 µg pig intestinal mucosal heparin/ml (176 USP units/ml; Sigma Chemical Co.), which had been previously covered with warm paraffin oil in a culture dish. The dishes were kept in a CO₂ incubator (5% CO₂ in air at 39°C) for about 30 min until spermatozoa were added for fertilization.

Spermatozoa were treated using the procedures reported previously (Niwa & Ohgoda, 1988; Park *et al.*, 1989). Briefly, two 0.5-ml straws of frozen semen obtained from a Holstein bull (P-123) were thawed in a water bath at 35–37°C. Spermatozoa were washed twice in Medium BO supplemented with 10 mM-caffeine-sodium benzoate (Sigma Chemical Co.) by centrifugation at 833 g for a period of 10 min each. A 50-µl sample of final sperm suspension (5–10 × 10⁶ spermatozoa/ml) was introduced into 50 µl of the medium that included the oocytes. The mixture gave final concentrations of 2.5–5 × 10⁶ spermatozoa/ml, 10 mg BSA/ml, 10 µg heparin/ml and 5 mM-caffeine.

At 20–22 h after insemination, oocytes were mounted, fixed for 48–72 h at room temperature in 25% (v/v) acetic alcohol, stained with 1% (w/v) orcein in 45% (v/v) acetic acid, and examined for evidence of sperm penetration as described previously (Ohgoda *et al.*, 1988). In another experiment, oocytes at the germinal vesicle stage without culture for maturation were incubated with or without spermatozoa in 100 µl Medium BO containing 10 mg BSA/ml, 10 µg heparin/ml and 5 mM-caffeine. Oocytes were examined at various times after incubation for evidence of sperm penetration and oocyte nuclear maturation. Oocytes were considered as penetrated when they had decondensed sperm heads or pronuclei and their corresponding sperm tails in the cytoplasm.

Results

As shown in Table 1, very high penetration rates (95–100%) were obtained equally in all oocytes which had been cultured for 0–20 h for maturation. All the oocytes inseminated 0 and 4 h after culture were penetrated and had a decondensing sperm head and most of the penetrated oocytes were at the stage of condensed germinal vesicle (GV; Fig. 1a, b) to telophase-I. Oocytes penetrated with male and female pronuclei were first observed when they were inseminated 8 h after culture, and their proportions increased as time of insemination was delayed. Extremely high proportions of polyspermy were observed, especially in oocytes inseminated at early stages of maturation, and the proportions decreased as oocyte maturation proceeded. With increased oocyte maturation, there was also a rapid decline in the average number of spermatozoa in penetrated oocytes.

When oocytes at the GV stage were inseminated, a few (7%) were already penetrated 4 h after insemination (Table 2; Fig. 1c–e). Sperm penetration of the oocytes was almost complete up to 8 h after insemination but the number of penetrating spermatozoa increased gradually with the lapse of time after insemination. Although oocytes penetrated and with decondensed sperm nuclei at the GV stage were observed until 20 h after insemination (Fig. 2), GV breakdown was first observed in penetrated oocytes 8 h after insemination. However, very few oocytes matured beyond prometaphase-I, even 20 h after insemination.

To examine whether the suppression of oocyte maturation was due to sperm penetration of the cytoplasm, oocytes at the GV stage were cultured without spermatozoa in Medium BO with caffeine and heparin, conditions which were the same as used for insemination. As shown in Table 3, GV breakdown occurred as usual but maturation of most of the oocytes did not proceed beyond prometaphase-I.

Table 1. Penetration *in vitro* of bovine immature oocytes inseminated at various times after culture*

Time of insemination (h after culture)	No. of oocytes inseminated	No. of oocytes penetrated†						No. of polyspermic oocytes (%)‡	Average no. of spermatozoa in penetrated oocytes
		With decondensing sperm head at the stage of:					With male and female pronuclei (%)‡		
		Total (%)	GV	Condensed GV -telophase-I	Metaphase-II	Female pronucleus			
0	78	78 (100)	1	77	0	0	0 (0)	78 (100)	31.9
4	74	74 (100)	1	73	0	0	0 (0)	73 (99)	15.8
8	85	82 (96)	0	60	10	0	12 (15)	67 (82)	5.0
12	85	83 (98)	0	20	8	0	55 (66)	44 (53)	2.4
16	86	82 (95)	0	8	0	5	69 (84)	22 (27)	1.5
20	90	89 (99)	0	0	1	3	85 (96)	18 (20)	1.3

*Experiments were repeated three times at each time of insemination.

†Oocytes were examined 20-22 h after insemination.

‡Percentage of total number of oocytes penetrated.

Fig. 1. Oocytes inseminated at the GV stage and fixed and stained at various times after insemination. **(a, b)** A polyspermic oocyte penetrated 22 h after insemination showing at least 4 decondensing sperm heads, some with penetrating sperm tails (a). (b) This oocyte has not completed maturation because it shows a condensed germinal vesicle (CGV). $\times 609$. **(c–e)** An oocyte penetrated 4 h after insemination. A penetrating spermatozoon (arrow) (c) and germinal vesicle (GV) (d) are clearly visible. $\times 304$. Higher magnification of the penetrating spermatozoon indicated by an arrow in c shows the typical early stage of nuclear decondensation (e). $\times 609$.

Discussion

The results of the present study indicate that bovine ovarian oocytes at the GV stage and those undergoing maturation are equally penetrable by spermatozoa *in vitro*. The findings that oocytes at the GV stage are all penetrated with subsequent decondensation of sperm nuclei 20–22 h after insemination and that decondensation of sperm nuclei can be induced even before GV breakdown are profoundly interesting since, with only the exception of the dog observed *in vitro* (Mahi &

Table 2. Time of penetration *in vitro* of bovine oocytes inseminated at germinal vesicle stage*

Time of examination (h after insemination)	No. of oocytes examined	No. of oocytes penetrated† at the stage of:					No. of polyspermic oocytes (%)‡	Average no. of spermatozoa in penetrated oocytes
		Total (%)	GV	Condensed GV	Prometa-phase-I	Metaphase-telophase-I		
4	55	4 (7)	4	0	0	0	1 (25)	1.5
8	59	56 (95)	36	17	3	0	41 (73)	4.1
12	54	52 (96)	18	14	20	0	50 (96)	9.5
16	54	54 (100)	6	35	11	2	49 (91)	13.9
20	50	50 (100)	7	23	18	2	50 (100)	26.7

*Experiments were repeated twice at each time of examination.

†All oocytes were penetrated with a decondensing sperm head.

‡Percentage of total number of oocytes penetrated.

Table 3. Maturation of bovine oocytes cultured in Medium BO with caffeine and heparin*

Time of examination (h after culture)	No. of trials	No. of oocytes examined	No. of oocytes at the stage of:			
			GV	Condensed GV	Prometa-phase-I	Metaphase-telophase-I
4	2	57	57	0	0	0
8	3	71	39	32	0	0
12	2	59	23	36	0	0
16	3	70	3	40	27	0
20	3	63	0	29	22	12

*For details, see text.

Yanagimachi, 1976), there are no reports to show the same phenomena in other mammalian species. Usui & Yanagimachi (1976) speculated that sperm nuclear decondensing factor is synthesized in the egg cytoplasm, transported to and accumulated in the GV, and then released into the egg cytoplasm at the time of GV breakdown. Although the chemical nature of the factor remains to be determined, the present study indicates that, in immature bovine oocytes, such a factor seems to be active even before the GV material is released into the cytoplasm. Since, in the present study, the medium used for fertilization included caffeine and heparin, it is possible that these chemicals participate in the activation of the factor. This should be clarified in further experiments, but it is very difficult to fertilize bovine oocytes in this system without these chemicals (Niwa *et al.*, 1988).

In the present study, development of male pronuclei was possible only when oocytes were inseminated at and after 8 h of culture for maturation. GV breakdown of bovine oocytes *in vivo* occurs between 4 and 8 h after the LH peak (Kruip *et al.*, 1983) and this time interval is almost similar to that for oocytes cultured *in vitro* (Sirard *et al.*, 1989). Therefore, the male pronucleus growth factor (Thibault & Gerard, 1973) or the sperm pronucleus development factor (Yanagimachi, 1981) may become only partly active just after GV breakdown. The activation of the factor seems to be intensified gradually as the oocytes develop from GV breakdown to metaphase II. A similar occurrence was observed in oocytes of the mouse (Iwamatsu & Chang, 1972), rat (Niwa & Chang, 1975) and hamster (Usui & Yanagimachi, 1976). On the other hand, most of the oocytes inseminated without culture for maturation were at the stage beyond GV breakdown 20 h later (Table 2) but formation of the male pronucleus was not observed in any of the oocytes penetrated (Tables 1 and 2). This may be due to limitation of the amount of materials supporting sperm pronuclear development present in the cytoplasm since so many spermatozoa were able to penetrate oocytes at the GV stage in the present study. In hamsters, when more than 9 spermatozoa enter one egg, none of the sperm heads develop into pronuclei (Hirao & Yanagimachi, 1979).

Fig. 2. Polyspermic oocytes penetrated at various times after insemination at the stage of the germinal vesicle. **(a, b)** An oocyte penetrated 12 h after insemination showing a germinal vesicle which is undergoing condensation (a) and at least 5 decondensing sperm heads with penetrating sperm tails (b). $\times 609$. **(c, d)** An oocyte penetrated 16 h after insemination. Many spermatozoa are penetrating with various stages of decondensation of sperm heads in different focal planes. This oocyte still has an intact germinal vesicle (c). $\times 304$.

We have reported for cattle that non-preincubated spermatozoa start to penetrate matured oocytes with cumulus and corona cells 3 h after insemination *in vitro* (Park *et al.*, 1989). Since, in the present study, some of the oocytes at the GV stage were already penetrated 4 h after insemination, there seems to be little difference in the time of sperm penetration of mature and immature oocytes. However, the number of spermatozoa penetrating oocytes at the GV stage increased with

time after insemination (Table 2). Since Fulka, Jr *et al.* (1982) have reported that, when zona-free bovine oocytes matured in culture are inseminated *in vitro*, only about 50% of penetrated oocytes are polyspermic, a block to polyspermy at the level of the plasma membrane seems to be strong in bovine oocytes. Therefore, in immature bovine oocytes, there seems to be no block to polyspermy at the level either of the zona pellucida or the plasma membrane. It is known in some species that the contents ejected from the cortical granules into the perivitelline space during fertilization alter the characteristics of the zona pellucida, and thus penetration of excess spermatozoa is prevented (for references, see Wolf, 1981). Ultrastructural studies by Hyttel *et al.* (1988) have revealed that abnormal cortical granule exocytosis does occur during fertilization of in-vitro matured bovine oocytes and allows polyspermy. Although we did not examine in the present study whether exocytosis of the cortical granules does occur during fertilization of oocytes at the GV stage, it has been demonstrated that there is complete failure of cortical granule exocytosis in intact GV oocytes of the hamster (Moore & Bedford, 1978) and rabbit (Berrios & Bedford, 1979).

Apparently, Medium BO with caffeine and heparin is not suitable for maturation of bovine oocytes (Table 3). Therefore, if some other medium which maintains both oocyte maturation and sperm penetration is used, we may be able to observe in more detail the development of the sperm nucleus, after penetration of oocytes at the GV stage, and prior oocyte maturation.

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