Morphology and subsequent development in culture of bovine oocytes matured *in vitro* under various conditions of fertilization

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The objective of these experiments was to evaluate factors affecting *in vitro* fertilization of bovine oocytes matured *in vitro*, and their subsequent development to blastocysts. In Expts 1 and 2, sperm concentration, spermatozoa and oocyte incubation time, motility enhancers and semen source were manipulated. Fluorescent microscopy of microtubules and chromatin was used to observe sperm penetration rate, sperm aster formation and chromatin decondensation. Oocyte penetration rates were affected by sperm concentration but not by spermatozoa and oocyte incubation time. The effect of sperm concentration was due primarily to changes in polyspermy and not monospermy. Motility enhancers had no effect on any parameter measured. In Expt 3, oocytes were matured for 17, 22, 28 and 34 h before fertilization and evaluated for fertilization rates, morphology of cortical granules and exocytosis and blastocyst development. A domain free of cortical granules that was associated with the metaphase chromatin was not observed in mature bovine oocytes. As oocytes matured from 17 to 34 h, the distribution of cortical granules progressed from clustered to diffuse. Although monospermic fertilization rates were similar and cortical granule exocytosis occurred in all groups, polyspermy increased with maturation time. Development to blastocysts increased from 17 to 22 h of maturation but decreased thereafter with increasing maturation time. These results suggest that polyspermy can be reduced by adjusting sperm concentration and spermatozoa and oocyte incubation time with little effect on monospermic fertilization. Increased polyspermy with increased maturation time was not due to a lack of cortical granule exocytosis.

Introduction

Recent experimentation in maturation, fertilization and culture of bovine oocytes *in vitro* has led to marked improvements in the production of embryos *in vitro*. These advances have allowed progress in the study of early fertilization events so that the efficiency of embryo production *in vitro* can be improved. Further manipulation of the bovine genome by cloning and gene transfer requires *in vitro* systems to produce large numbers of transferable, high-quality, diploid (with maternal and paternal chromosomes) embryos. The optimum system would give high penetration rates with minimal polyspermy or other abnormal fertilizations. However, abnormal ultrastructural features of *in vitro* matured (IVM) and *in vitro* fertilized (IVF) oocytes have been described by Hyttel *et al.* (1986, 1988, 1989), including delayed dispersal of cortical granules, incomplete cortical granule release and distribution, polyspermy and delayed pronuclear development. Xu and

Greve (1988) reported four types of abnormalities of IVF and IVM oocytes: polyspermy, polygyny, asynchronous pronuclear development and preactivation of cytokinesis. Although some of the developmental abnormalities have been corrected, polyspermy remains the most prevalent abnormality, as described by Saeki *et al.* (1991), Chian *et al.* (1992) and Pavlok *et al.* (1992). Previous reports have suggested that polyspermically fertilized bovine oocytes can develop to the morula and blastocyst stage, possibly in some cases as androgenotes (Iwasaki *et al.*, 1989; Pinto-Correia *et al.*, 1992; Long *et al.*, 1993).

The objective of these experiments was to investigate factors affecting the penetration of spermatozoa into IVM bovine oocytes, and their further development to transferable embryos. Factors evaluated were sperm concentration, motility enhancers, duration of sperm incubation, source of spermatozoa and oocyte maturation time. Fluorescent microscopy of chromatin, microtubules and cortical granules was used to examine the interaction between spermatozoa and oocytes before and during the early stages after fertilization. The morphology of spermatozoa and oocytes after fertilization and

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Revised manuscript received 18 April 1994.

possible reasons for polyspermy, as well as the relationship between fertilization rates and blastocyst development using IVM bovine oocytes, are presented.

Materials and Methods

In vitro maturation

In Expts 1 and 2, oocytes were obtained by aspirating follicles from ovaries obtained from an abattoir. Ovaries were placed in insulated containers with warm packs for transport to the laboratory. The ovaries arrived approximately 4 h after collection at an average temperature of 30°C, and were washed in warm 0.9% (w/v) saline solution. Follicles 1–10 mm in diameter were aspirated using an 18-gauge needle attached to a collection tube, with a pressure release valve in line to a vacuum pump set to 100 mm Hg. The proportion of follicles < 2 mm in diameter was small and all recovered oocytes were selected for an intact cumulus and evenly pigmented cytoplasm, thus reducing the negative effects of using small follicles (Pavlok *et al.*, 1992).

The selected cumulus-enclosed oocytes were washed twice in Tyrode–Hepes medium (TL Hepes; Bavister *et al.*, 1983) supplemented with 3 mg BSA ml⁻¹, 1.0% penicillin and 1.0% streptomycin (100 U ml⁻¹ and 100 mg ml⁻¹, respectively; Gibco Laboratories, Grand Island, NY) and transferred to four-well tissue culture plates (Nunc, VWR Scientific, Bridgeport, NJ) containing 250 µl maturation medium per well. Maturation medium consisted of TCM 199 with Earle's salts (Gibco Laboratories) supplemented with 10% (v/v) heat-treated fetal bovine serum (lot number 11112135; Hyclone Labs, Logan, UT), 1 ng oestradiol ml⁻¹ (Sigma Chemical Co., St Louis, MO), 0.5 µg ovine FSH ml⁻¹ [National Institute of Diabetes and Digestive and Kidney Diseases (NIDDKD), Baltimore, MD] and 5.0 µg bovine LH-5 ml⁻¹ (NIDDKD). Fetal bovine serum and the media used in all the experiments were from the same lot, except the maturation medium used in Expt 3. Oocytes were incubated under a humidified atmosphere of 5% CO₂ in air at 38.5°C for 22 h, and routinely > 90% of oocytes that were produced had metaphase II plates.

Oocytes for Expt 3 were acquired from BovaMax Incorporated, Hanford, CA. Oocytes were obtained by harvesting ovaries from an abattoir and aspirating follicles (approximately 3 h after collection), as described above. Cumulus-enclosed oocytes were washed twice in TL Hepes, and approximately 50 oocytes were transferred to each 1.8 ml tissue culture tube containing 500 µl of pre-equilibrated maturation medium covered to capacity with paraffin oil (Dow Corning 500). BovaMax maturation medium consisted of TCM 199 with Earle's salts supplemented with 10% (v/v) heat-treated fetal bovine serum (Hyclone Labs) 0.01 U bovine LH ml⁻¹ (Nobl, Sioux Center, IA), 0.01 U bovine FSH ml⁻¹ (Nobl) and 1.0% (w/v) penicillin and 1.0% (w/v) streptomycin. The tightly sealed tubes were labelled with the starting time of maturation and placed in a battery-powered incubation shipper (Minitube of America, Incorporated, Madison, WI) for transport via overnight express service to our laboratory. The portable incubator temperature was recorded and averaged 38.5°C at the end of the 15–17 h shipment. The tubes

were moved to the maturation incubator until the time of insemination, when the cumulus-enclosed oocytes were washed three times in warm TL Hepes and transferred to fertilization medium. Maturation in this system was effective in producing mature oocytes, since < 5% oocytes had not progressed to metaphase II after 17–22 h in maturation medium followed by 8 h in fertilization medium without spermatozoa.

In vitro fertilization and culture

The fertilization medium consisted of CR1aa (Rosenkrans and First, 1991) supplemented with 6 mg fatty-acid-free BSA ml⁻¹, 5.0 µg sodium heparin ml⁻¹ (Sigma Chemical Co.) to induce sperm capacitation (Parrish *et al.*, 1988) and 20 µmol penicillamine l⁻¹, 10 µmol hypotaurine l⁻¹ and 1 µmol adrenaline l⁻¹ (PHE) to stimulate sperm motility unless otherwise specified. Oocytes were fertilized in groups of 30–50 in four-well tissue culture plates in a final volume of 500 µl fertilization medium incubated under a humidified atmosphere of 5% CO₂ in air at 38.5°C. Semen that had been collected from five sires, pooled, diluted in a milk-based extender and cryopreserved was donated by Eastern AI Cooperative, Incorporated (Ithaca, NY). This semen was used in Expts 1 and 3, and as a control in Expt 2. Oocytes were inseminated using frozen-thawed spermatozoa that had been separated from the extender using a Percoll gradient (45%/90%); the resulting sperm pellet was resuspended in the fertilization medium to desired concentrations. After incubation with spermatozoa, the oocytes were placed in a 15 ml conical tube with 0.5–0.75 ml warm TL Hepes and vortexed using a Genie Vortex 2 (Fisher Scientific, Pittsburgh, PA) on the highest speed for 2 min and 15 s to remove the cumulus cells.

Inseminated oocytes were cultured in 50 µl drops of CR1aa supplemented with 10% heat-treated fetal bovine serum (CR2) under paraffin oil. Inseminated oocytes (*n* = 10–20) were cultured in each drop of CR2, without changing or adding CR2, for a total of 8 days (day 0: day of insemination). These conditions allowed a cumulus cell monolayer to be established from those remaining attached to the zona pellucida after vortexing. Development was calculated on day 8 as the number of expanding or hatching blastocysts, with no or a few extruded or dead cells (grade 1 or 2, respectively), divided by the total number of oocytes cultured.

Immunolocalization of chromatin and microtubules

In Expts 1 and 3, chromatin and microtubules were detected by immunofluorescence of whole mounts of zygotes, as described by Long *et al.* (1993). Zygotes were transferred to a microtubule stabilization buffer (Schatten *et al.*, 1985) containing 3% Triton X-100 (v/v) for 6 h at 38°C, which was repeatedly changed, followed by fixation in 2.0% (v/v) paraformaldehyde in PBS for 30 min at 38°C. After washing in PBS and incubating for 10 min in 0.1 mol glycine l⁻¹, the zygotes were incubated overnight at 4°C with anti- α -tubulin monoclonal antibody (Sigma Chemical Co.) diluted 1:100 in PBS containing 0.1% (v/v) Tween 20 (PBS-T). Zygotes were washed for a minimum of 6 h with several changes of PBS-T

and incubated overnight in fluorescein isothiocyanate-conjugated, affinity-purified goat anti-mouse immunoglobulin G (IgG) (FITC; Sigma Chemical Co.) diluted 1:10 in PBS-T at 4°C. After incubation, zygotes were washed for a minimum of 6 h with repeated changes of PBS-T and mounted on glass slides in glycerol containing 0.5 µg Hoechst 33342 ml⁻¹ (Sigma Chemical Co.). Coverslips were sealed to slides with nail polish and stored in the dark at 4°C 1–7 days before evaluation. Control zygotes were incubated with 1:100 affinity-purified mouse IgG (Sigma Chemical Co.) instead of α -tubulin antibody, and always gave negative results.

A direct method of visualizing the chromatin without staining the microtubules was used in Expt 2, since only the sperm penetration rate and developmental capacity were evaluated. After cumulus removal, zygotes were transferred to 3.7% (v/v) paraformaldehyde containing 10.0% (v/v) Triton X-100 for 20 min at 38°C, followed by extensive washing in PBS containing 0.3% (w/v) BSA. Oocytes were mounted in glycerol with Hoechst as above. Cell counts of blastocysts 8 days after insemination produced in Expt 2 were performed by mounting grade 1 and 2 embryos directly to glycerol with Hoechst on glass slides, and counting nuclei.

Cortical granule localization and staining

The staining procedure was modified from previously published reports using hamster oocytes (Cherr *et al.*, 1988). After vortexing to remove the cumulus cells, oocytes and presumptive zygotes were treated with 0.4% (w/v) pronase E (Sigma Chemical Co.) to remove the zona pellucida. The nude cells were washed three times in TL Hepes and transferred to 3.0% (v/v) paraformaldehyde in PBS for 1 h at room temperature. Fixed cells were washed in blocking solution (PBS containing 1.0% (w/v) BSA, 0.1 mol glycine l⁻¹ and 0.05% (v/v) Triton X-100) for 1 h and 40 min with five changes of solution. Cells were transferred to fluorescein isothiocyanate conjugated with *Lens culinaris* agglutinin (10 µg ml⁻¹; Sigma Chemical Co.) in blocking solution for 30 min in the dark at room temperature. Labelled cells were washed in blocking solution as above, followed by a similar wash in PBS containing 1 mg polyvinylpyrrolidone ml⁻¹, and mounted in glycerol with Hoechst. This procedure was first tested using mature mouse oocytes and resulted in cortical granule patterns as described by Ducibella *et al.* (1988).

Microscopy and photography

All oocytes or zygotes stained for chromatin, microtubules or cortical granules were visualized at $\times 1000$ using a Zeiss Axioskop microscope equipped with epifluorescence. Hoechst and FITC signals were detected using 365/400 and 450/520 nm excitation/barrier filter combinations, respectively. A video camera attached to the microscope was used to capture images that were produced using the Image 1 AT software (Universal Imaging, Media, PA). Images were stored in 44 Mb disk cartridges, and photomicrographs were prepared with a video printer (model UP5000; Sony, Park Ridge, NJ). Blastocyst nuclei were counted at $\times 200$ using a Nikon Diaphot

microscope equipped with epifluorescence and Hoechst filter combinations as above.

Experimental design

Experiment 1. The objective of Expt 1 was to investigate the effects of sperm concentration, spermatozoa and oocyte incubation time and the presence or absence of PHE on sperm penetration rates and subsequent development *in vitro* in a 3 $\times$ 2 $\times$ 2 factorial design. All oocytes were inseminated after 22 h of maturation using the pooled spermatozoa as described above at concentrations of 0.5, 0.25 and 0.125 $\times 10^6$ spermatozoa ml⁻¹ in the presence or absence of PHE. Oocytes were removed from the fertilization medium 8 or 18 h after insemination. Oocytes vortexed 8 h after insemination were cultured in CR2; a proportion of these oocytes ($n = 253$) were removed and fixed 10 h later to evaluate fertilization rates, leaving 347 oocytes in CR2. Oocytes vortexed 18 h after insemination were divided into groups to be fixed immediately ($n = 295$) or cultured in CR2 ($n = 380$). Data were collected over three replicates ($n = 384, 449, 442$ for replicates 1, 2 and 3, respectively) and included degree of sperm penetration, abnormal stages of development and the number of oocytes developing to blastocysts.

Experiment 2. The effect of sire and sperm concentration on the fertilization of IVM bovine oocytes and their subsequent development was evaluated in Expt 2 in a 5 $\times$ 3 factorial design. Semen from four sires of known non-return rates (A: 38%; B: 64%; C: 68%; D: 73%) and the pooled control semen (described above) were compared for fertilization rates and blastocyst development. Spermatozoa from each source were prepared at concentrations of 1.0, 0.5 and 0.25 $\times 10^6$ spermatozoa ml⁻¹ as described, and combined with oocytes that had matured for 22 h. Oocytes and spermatozoa were co-incubated for 8 h in fertilization medium containing PHE and 5 µg sodium heparin ml⁻¹. Cumulus cells were removed by vortexing and a proportion of zygotes from each treatment group was fixed for chromatin evaluation ($n = 890$). The remainder ($n = 1206$) was cultured in drops of CR2 to evaluate development to the blastocyst stage. The experiment was replicated six times ($n = 291$ –510 oocytes per replicate).

Experiment 3. The effect of oocyte age on the block to polyspermy and on nuclear and cytoskeletal morphology during the first cell cycle and subsequent development to blastocysts were evaluated in nine replicates of Expt 3. Oocytes were maintained in maturation medium for 17, 22, 28 or 34 h and then moved to fertilization medium containing PHE and 0.5 $\times 10^6$ spermatozoa ml⁻¹. This combination of spermatozoa and PHE was used because it had induced high rates of polyspermy in previous experiments and therefore challenged the oocytes block to polyspermy at each period. Presumptive zygotes were vortexed 8 h after insemination and fixed immediately, or after 10 h of culture in CR2, to evaluate sperm penetration, sperm aster formation and pronuclear development ($n = 657$; range: 69–109 per replicate). In addition, 1473 (range: 111–202 per replicate) presumptive zygotes were cultured for 8 days in CR2 to compare developmental capability between treatments.

Table 1. Fertilization rates and blastocyst development of bovine oocytes inseminated with different concentrations of spermatozoa and for different incubation times *in vitro*

Parameter	Fixed			Cultured	
	<i>n</i>	2PN (%) ^c	PPN (%) ^d	<i>n</i>	Blastocysts (%) ^f
Sperm concentration ($\times 10^6$ ml ⁻¹)					
0.5	188	49.4 ^a	36.4 ^a	262	28.4 ^a
0.25	183	55.9 ^a	15.9 ^b	211	22.9 ^{ab}
0.125	177	50.5 ^a	10.2 ^b	254	15.3 ^b
		(4.72)	(4.36)		(4.35)
Sperm incubation time (h)					
18	295	48.3 ^a	25.9 ^a	380	23.4 ^a
8	253	55.5 ^a	15.7 ^b	347	21.1 ^a
		(3.85)	(3.56)		(3.55)

^{a,b}Values with different superscripts within a column are significantly different ($P < 0.05$, analysed by SAS least squares means; SEM is given in parentheses).

^cPercentage of fixed oocytes penetrated by only one spermatozoon.

^dPercentage of fixed oocytes penetrated by two or more spermatozoa.

^ePercentage of fixed oocytes penetrated by at least one spermatozoon.

^fPercentage of inseminated oocytes developing to grade 1 and 2 blastocysts on day 8.

In the last three of nine replicates, a portion of unfertilized oocytes from each age group was fixed to determine cortical granule patterns ($n = 35 \pm 5$ per replicate). Cortical granule patterns and sperm penetration rates were determined in 201 inseminated oocytes over the three replicates. After maturation for the appropriate time, oocytes were moved to fertilization medium with or without spermatozoa for 8 h, vortexed and fixed immediately.

Statistical analyses

Data for all experiments were analysed by the General Linear Models (GLM) and Least Squares Means (LSM) procedures (SAS Institute Inc., Cary, NC) to test for significance of main effects and differences between treatments. Arcsin transformations were used when testing significance, although the nontransformed LSM results are presented in tables and text. In Expt 1, PHE, sperm concentration and sperm incubation time were main effects and all possible interactions were tested. The main effects of sire and sperm concentration as well as their interaction with oocytes were tested in Expt 2. In Expt 3 the effect of oocyte age on fertilization rate, cortical granule morphology and exocytosis and blastocyst development was tested. In all experiments a replicate was a percentage calculated from the total number of oocytes in a treatment from each day.

Results

Experiment 1: sperm concentration, sperm incubation time and PHE

Addition of PHE to the fertilization medium did not increase the number of oocytes penetrated by one or more spermatozoa (75.9% versus 69.6%, with and without PHE, respectively),

monospermic (52.7% versus 51.1%, with and without PHE, respectively) and polyspermic (23.1% versus 18.5%, with and without PHE, respectively) penetration rates or blastocyst development (23.8% versus 20.7%, with and without PHE, respectively). There was no interaction between the effects of PHE, sperm concentration or time of sperm removal; therefore, data were subsequently analysed only for the main effects of sperm concentration and the time of sperm removal.

Polyspermic and total penetration rates were greater for 0.5×10^6 spermatozoa ml⁻¹ than for other sperm concentrations ($P < 0.05$). This resulted in an increased development to the blastocyst stage compared with a concentration of 0.125×10^6 spermatozoa ml⁻¹, although monospermic fertilization rates were not different among sperm concentrations ($P > 0.3$) (Table 1).

Incubation of oocytes and spermatozoa for 18 h resulted in a higher rate of polyspermy ($P < 0.05$) but did not affect monospermic fertilization or development to blastocysts (Table 1). There was an increase in the number of zygotes fixed during anaphase or telophase II within the 18 h treatment (7.0% versus 0.0% for 18 and 8 h, respectively; $P < 0.01$). Chromatin anomalies also increased as sperm incubation time increased (8.2% versus 3.8% for 18 and 8 h, respectively; $P < 0.05$). These anomalies included double and split spindles, extrusion of the maternal chromatin and others that have been described by Long *et al.* (1993).

Experiment 2: sperm source

The effect of sire on fertilization rates and blastocyst development is shown in Table 2. Of the three sperm concentrations used per sire, 0.25×10^6 spermatozoa ml⁻¹ yielded the lowest oocyte penetration rate and no significant sire by concentration interactions were observed (data not shown). Therefore, the data for sperm concentration were

Table 2. Fertilization rates and blastocyst development of bovine oocytes inseminated with different semen sources *in vitro*

Semen source	Fixed				Cultured		
	<i>n</i>	2PN (%) ^d	PPN (%) ^e	PENT (%) ^f	<i>n</i>	Blastocysts (%) ^g	Number of cells per embryo
A	139	5.4 ^a (4.02)	0.0 ^a (3.38)	5.4 ^a (8.73)	194	0.5 ^a (3.80)	100.0 ^a
B	190	60.6 ^b (4.02)	11.5 ^b (3.38)	72.1 ^b (8.73)	205	22.9 ^b (3.80)	119.3 ^a
C	190	67.8 ^b (3.67)	23.9 ^c (3.08)	91.7 ^c (7.97)	267	30.2 ^b (3.47)	120.1 ^a
D	185	65.7 ^b (3.67)	17.5 ^{bc} (3.08)	83.2 ^c (7.97)	267	27.3 ^b (3.47)	109.5 ^a
Pooled	186	73.6 ^b (5.19)	17.1 ^{bc} (4.36)	90.7 ^c (11.27)	273	33.8 ^b (4.91)	105.3 ^a

^{a,b,c}Values with different superscripts within a column are significantly different ($P < 0.05$, analysed by SAS least squares means; SEM is given in parentheses).

^dPercentage of fixed oocytes penetrated by only one spermatozoon.

^ePercentage of fixed oocytes penetrated by two or more spermatozoa.

^fPercentage of fixed oocytes penetrated by at least one spermatozoon.

^gPercentage of inseminated oocytes developing to grade 1 and 2 blastocysts on day 8.

Table 3. Fertilization rates of blastocyst development of bovine oocytes following different maturation times

Maturation time (h)	Fixed				Cultured	
	<i>n</i>	2PN (%) ^d	PPN (%) ^e	PENT (%) ^f	<i>n</i>	Blastocysts (%) ^g
17	150	45.1 ^a (4.4)	37.0 ^a (3.8)	82.1 ^a (2.6)	366	21.6 ^a (3.1)
22	157	47.6 ^a (4.4)	42.9 ^{ab} (3.8)	90.5 ^b (2.6)	353	38.3 ^b (3.1)
28	192	47.7 ^a (5.0)	51.0 ^b (4.4)	98.7 ^c (2.9)	399	30.1 ^{ab} (3.5)
34	158	39.3 ^a (4.7)	56.2 ^b (4.1)	95.5 ^{bc} (2.8)	355	11.2 ^c (3.3)

^{a,b,c}Values with different superscripts within a column are significantly different ($P < 0.05$, analysed by SAS least squares means; SEM is given in parentheses).

^dPercentage of fixed oocytes penetrated by only one spermatozoon.

^ePercentage of fixed oocytes penetrated by two or more spermatozoa.

^fPercentage of fixed oocytes penetrated by at least one spermatozoon.

^gPercentage of inseminated oocytes developing to grade 1 and 2 blastocysts on day 8.

pooled within sire. Sire A had a lower oocyte penetration rate and led to a lower embryo development rate than did other semen sources tested ($P < 0.001$). Although the semen from sire B tended to give poorer results ($P = 0.07$), no significant differences were observed between individual sires B, C and D and the pooled control semen with respect to monospermic fertilization and development to blastocysts. Sire B displayed lower polyspermy than sire C ($P < 0.01$). Polyspermic fertilization rates were not different among sires C, D and pooled control. The oocyte penetration rate of semen from sire B was lower than that of sires C, D and pooled control ($P < 0.05$), mainly due to decreased polyspermy. In addition, the number of blastomeres per blastocyst on day 8 of culture was not different among sires and pooled control semen.

Experiment 3: oocyte ageing

Increasing oocyte maturation time from 17 to 34 h had no effect on monospermic fertilization of bovine oocytes (Table 3). The number of oocytes penetrated by at least one spermatozoon was lower for oocytes matured for 17 h ($P < 0.05$), due to a decrease in polyspermic fertilization. Blastocyst development was lowest for oocytes inseminated after 34 h of maturation ($P < 0.05$); a maturation time of 17 h resulted in lower development rates than periods of 22 or 28 h ($P < 0.05$), while development tended to be highest in oocytes inseminated after 22 h of maturation ($P < 0.10$).

Evaluation of chromatin decondensation and sperm aster formation was made from zygotes fixed 8 h after insemination.

Fig. 1. Sperm-derived microtubules and chromatin decondensation patterns of bovine oocytes matured *in vitro*. Oocytes were fixed following 8 h of incubation with spermatozoa after they had matured for 25 (a, b), 30 (c, d), 36 (e, f) or 42 (g, h) h. The figure shows (a) a small microtubule aster radiating from the tip of the sperm tail and (b) a slightly decondensed sperm head in an oocyte that had been fertilized and fixed after maturation for 25 h. A large microtubule aster (c, e, g) and a decondensed sperm head (d, f) or pronucleus (h) can be observed in oocytes that had been fertilized and fixed after 30, 36 and 42 h of maturation. Scale bar represents 10 μm .

Figure 1a illustrates the small and irregularly shaped aster extending from the midpiece of the spermatozoon from an oocyte inseminated after 17 h of maturation. The sperm head is still compact having undergone little decondensation (Fig. 1b). This is in contrast to sperm aster development and sperm head decondensation observed in oocytes fertilized at later times. Oocytes inseminated after 22 and 28 h of maturation (Fig. 1c, e) developed large sperm asters, accompanied by moderately decondensing sperm heads (Fig. 1d, f). Insemination after 34 h of maturation resulted in large expanded microtubule asters and greatly decondensed sperm heads (Fig. 1g, h). Microtubule and chromatin decondensation patterns of oocytes matured for 34 h were similar to those observed in oocytes matured for 22 h and fixed 18 h after insemination.

Cortical granule distribution was analysed in fertilized and unfertilized oocytes within each maturation period and is illustrated in Fig. 2. After incubation for 8 h in fertilization medium without spermatozoa, the cortical granules of oocytes that had matured for 17 h were predominantly clustered (Fig. 2a). As maturation time increased, cortical granules dispersed. In oocytes that had matured for 22 h, cortical granules were often still in small clusters, but after 28 or 34 h of maturation they were mostly dispersed (Fig. 2c, e, g). No domain that was free of cortical granules was ever observed. The chromatin was generally in metaphase II as expected. Cortical granule exocytosis occurred in fertilized oocytes at all

maturation times (Fig. 2b, d, f, h). The completeness of cortical granule exocytosis, however, increased with increasing maturation time, even though the rate of polyspermy increases as the maturation time increases.

Discussion

Maturation in Expts 1 and 2 was performed in different media and different incubation conditions than in Expt 3. Although no direct comparisons were made between these groups, it is noteworthy that oocytes matured during transport to our laboratory fertilized and developed to blastocysts at comparable rates to those matured in our laboratory. These data suggest that viable oocytes can be obtained from oocytes collected at distant locations and transported during the maturation period.

Sperm penetration occurs over an extended period, as oocytes cultured with spermatozoa for 18 h often had condensed sperm heads as well as anaphase or telophase chromatin arrangements at the time of fixing. In addition, sperm heads were observed in oocytes containing pronuclear structures, indicating penetration of previously fertilized oocytes. Decreasing the incubation time with spermatozoa decreases the rate of polyspermy while still resulting in similar monospermic penetration and development to the blastocyst stage. These

Fig. 2. Cortical granule patterns of bovine oocytes matured *in vitro* before and after sperm penetration. Oocytes were fixed following 8 h of incubation in fertilization medium with or without spermatozoa after the oocytes had matured for 25 h (a, b), 30 h (c, d), 36 h (e, f) and 42 h (g, h). The figure shows clustered cortical granules of oocytes that had matured for 25 h (a) before and (b) after fertilization, leaving slight traces of stainable material. (c) Oocytes that had matured for 30 h contained small clusters of cortical granules, but no staining was visible following fertilization (d). (e, g) Oocytes that had matured for 36 h and 42 h displayed well-distributed cortical granule staining but no staining was visible after fertilization (f, h). Scale bar represents 10 μ m.

data support previous reports by Saeki *et al.* (1991) and suggest that decreasing the spermatozoa and egg incubation time reduces undesirable polyspermic or anomalous fertilizations but does not result in significant improvements in monospermic fertilization or development to blastocysts.

Increasing the sperm concentration increased the incidence of polyspermy without affecting the number of monospermic penetrations. However, the rate of development to blastocysts was increased, suggesting that some blastocysts may be polyploid, androgenetic or able to eliminate accessory spermatozoa, as described by Long *et al.* (1993). Alternatively, the presence of numerous accessory spermatozoa during fertilization could produce a stimulatory effect on development *in vivo*, as described by DeJarnette *et al.* (1992) and Nadir *et al.* (1993). Lowering the sperm concentration to decrease the rate of polyspermy did not result in increased monospermic fertilizations and subsequent development to a transferable stage. However, if polyspermic zygotes develop to the blastocyst stage, this would have a deleterious effect on pregnancy rates after transfer to recipients.

The addition of PHE had no effect on any of the observed parameters in Expt 1, which is contrary to effects reported by Susko-Parrish *et al.* (1990). The positive influence of motility enhancers might be observed in fertilization systems, in which penetration rates are low. Over the three experiments described, sperm penetration of control oocytes was greater than 85% and appears to be high enough to eliminate the positive effects of PHE.

The source of semen affected the fertilization rate and subsequent development to blastocysts in these experiments. Although a limited number of sires were used, these results support previous observations by Parrish *et al.* (1986), Ohgoda *et al.* (1988) and others suggesting that the *in vitro* fertilization rate of bovine sires is highly variable.

As previously reported, *in vitro* maturation time of oocytes affects fertilization rates and development to blastocysts *in vitro* (Chian *et al.*, 1992; Dominko and First, 1992; van der Westerlaken *et al.*, 1992), as well as cortical granule distribution (Sathananthan and Trounson, 1982; Cran and Esper, 1990). The aim of the experiments described here was to focus on the mechanisms of fertilization or developmental failure in oocytes that had matured for short or long periods.

Reports by Xu and Greve (1988) indicate that without prior capacitation procedures spermatozoa require approximately 6 h to penetrate bovine oocytes *in vitro*. Although sequential fixations of oocytes following insemination were not performed in the present study, we predict that sperm capacitation and the acrosome reaction would require at least 4–6 h in our system. This leaves 2–4 h for the development of microtubule asters and sperm head decondensation before fixation of oocytes 8 h after insemination. Fertilized oocytes that had matured for 17 h predominately displayed sperm aster and chromatin decondensation patterns that were less developed. This could indicate either a delayed fertilization or a slower progression of events after fertilization. In contrast, oocytes that had matured for longer periods exhibited extensive microtubule asters and decondensed pronuclei following the same fertilization protocol. Similarly, experiments on delayed fertilization in mice by Fraser (1979) and Ishikawa *et al.* (1992) indicate a more rapid development to the pronuclear stage and

accelerated embryonic development in mice undergoing delayed matings.

Further morphological evidence of oocyte variability due to ageing in maturation media comes from observing the cortical granule patterns. The distribution of cortical granules in bovine oocytes varied with the time of maturation, beginning with a clustered pattern which progressively became more uniformly distributed. No domain without cortical granules was observed associated with the metaphase II spindle, which is in contrast to patterns described by Ducibella *et al.* (1988) for mature mouse oocytes and Cherr *et al.* (1988) for hamster oocytes. Electron microscopy of mature mouse (Okada *et al.*, 1986) and pig (Cran and Cheng, 1985) oocytes also indicate a cortical-granule-free domain. The clustered pattern of cortical granules in this study is similar to those reported by Kruip *et al.* (1983) using transmission electron microscopy of maturing bovine oocytes *in vivo*. These observations are also supported by Hyttel *et al.* (1988), who used transmission electron microscopy to observe clusters of cortical granules before fertilization in bovine oocytes matured *in vitro* for 28 h. In our experiments, no bovine oocytes in any age group developed a cortical-granule-free area; this is comparable to cortical granule distribution patterns in domestic cats (Byers *et al.*, 1992). Ooplasmic maturation of bovine oocytes and the ability to initiate sperm aster formation and sperm decondensation may be morphologically linked to progressive dispersal of cortical granules.

Cortical granules appear to be released at all stages of maturation in response to sperm penetration, although not to the same extent in some less mature oocytes. Therefore, a lack of cortical granule exocytosis does not explain the increase in polyspermic fertilization that occurred with increased maturation time. Rather, a decrease in the effectiveness of the cortical granule exudate must occur with increased maturation time.

Incomplete dispersal of exudate upon exocytosis during IVF has been reported in cattle and is associated with polyspermic fertilization of *in vitro* and *in vivo* matured oocytes (Hyttel *et al.*, 1988, 1989). Cran and Cheng (1985) have observed similar patterns of cortical granule exudate following *in vitro* fertilization in pig oocytes. Failure to distribute these granules could lead to a defective formation of a cortical granule envelope, as described in mice by Dandekar and Talbot (1992), and thus to an ineffective block to polyspermy. Alternatively, the zona pellucida could become less responsive to the cortical granule contents as maturation time *in vitro* is increased. Zona hardening during ageing *in vitro* has been described in mice (Gianfortoni and Gulyas, 1985; Downs *et al.*, 1986); although this phenomenon has not been described for bovine oocytes, it is possible that the ageing of the zona alters its response to the cortical granule exudate.

In summary, these experiments have demonstrated an effective fluorescent staining procedure for bovine oocyte cortical granules and its use in determining the cytoplasmic distribution during extended maturation periods. Cortical granules were progressively more distributed as the maturation time increased and were effectively released by sperm penetration, but were ineffective in preventing polyspermic fertilizations. Penetration of aged oocytes by spermatozoa results in the rapid development of a sperm aster and

decondensed pronuclei, in contrast to undeveloped asters and pronuclei in less mature oocytes, suggesting an inability of less matured oocytes to respond to fertilization-induced intracellular signals. Both of these conditions may account for the subsequent lower rate of development of these zygotes to blastocysts. These results indicate that the time of incubation of spermatozoa with oocytes, sire selection and the age of oocytes at fertilization are all important factors in the success of *in vitro* fertilization of bovine oocytes. Manipulation of these parameters can reduce polyspermy, although this is most often accompanied by a decrease in total oocyte penetration rates and has little effect on monospermic fertilization.

The authors thank J. J. Balise for excellent technical assistance and Eastern A. I. Cooperative, Incorporated, Ithaca, NY for providing financial support and the frozen semen used in these experiments.

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