Development of centrifuged cow zygotes cultured in rabbit oviducts

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Summary. Zygotes from superovulated cows were centrifuged and pronuclei were detected by differential interference-contrast microscopy in 73% of 106 zygotes. Zygotes were then transferred to ligated oviducts of follicular-phase, 1-day pseudo-pregnant or 7-day pseudopregnant rabbits and recovered 5 days later. Their development did not differ from that of uncentrifuged zygotes transferred to the opposite oviduct: 41% of the embryos recovered from rabbit oviducts contained 17–32 nuclei and an additional 5% contained > 32 nuclei. In another experiment, 399 ova from unmated cows were transferred to rabbit oviducts to determine whether centrifugation induced parthenogenetic development. After 7 days, 257 ova were recovered; 16% of the recovered ova had developed parthenogenetically and contained 2–30 nuclei. Neither centrifugation of the ova nor reproductive status of the rabbits influenced the proportion of parthenogenotes found. Parthenogenetic development was also observed in 14 of 71 ova (20%) recovered on Day 7 from uninseminated superovulated cows. In an attempt to increase the probability of detecting treatment differences, centrifuged and control cow zygotes were incubated for 7 (rather than 5) days in opposite oviducts of fourteen 1-day pseudopregnant rabbits. Development was unaffected by centrifugation: 61% of the zygotes recovered had developed beyond the 16-cell stage, with 23, 24 and 15% containing 17–32, 33–64, and > 64 nuclei, respectively. Taking into account the percentage of zygotes in which pronuclei can be seen, the recovery rate from rabbit oviducts, and the proportion of embryos that develop to the morula stage or beyond, 26% of the original group of zygotes would be candidates for transfer into recipient cows.

Keywords: cattle; pronuclei; centrifugation; parthenogenesis; embryo

Introduction

A number of embryo manipulation techniques, such as nuclear transfer and DNA micro-injection, depend upon visualization of nuclei and pronuclei. However, living zygotes of some species, such as cattle, contain optically dense cytoplasm that obscures nuclear structure. To overcome this problem, pronuclei of cattle zygotes have been stained with fluorescent dyes or the zygotes have been centrifuged to displace the optically dense material within the cytoplasm.

After applying the DNA-specific fluorescent dye 4'-6'-diamidino-2-phenylindole (DAPI) to cow zygotes, Minhas *et al.* (1984) were able to view the early stages of fertilization, including pronuclear formation. Subsequently, it was demonstrated that DAPI-treated zygotes can develop into normal calves (Kraemer *et al.*, 1985). Pronuclei have also been revealed in pig and cow zygotes after centrifugation (Wall *et al.*, 1985; Loskutoff *et al.*, 1986). Centrifuged 1- and 2-cell pig embryos continue to develop and, if transferred to a recipient gilt, mature to term, resulting in the birth of normal piglets. Preliminary results suggest that this is also true for cow embryos (Loskutoff *et al.*, 1986).

Several techniques have been used to test the influence of treatments on viability of cattle zygotes. Cow zygotes and cleavage-stage embryos have been evaluated after culture *in vitro* (Tervit *et al.*, 1972; Camous *et al.*, 1984; Heyman *et al.*, 1987) or after transfer into the oviducts of cows (Rowson *et al.*, 1969), sheep or rabbits (Lawson *et al.*, 1972b; Boland, 1984; Sirard *et al.*, 1985; Eyestone *et al.*, 1985; Lambert *et al.*, 1986; Sirard & Lambert, 1986). Boland (1984) reviewed the literature regarding optimal conditions for development of cow embryos in rabbit oviducts. However, direct comparisons of the reproductive status of recipient rabbits are limited (Lawson *et al.*, 1972a).

The present study was conducted to determine the proportion of centrifuged cattle zygotes in which pronuclei could be detected (Exp. 1), to evaluate the potential problem of parthenogenetic development of cow ova in rabbit or cow oviducts (Exp. 2), and to assess the influence of centrifugation on development of cow zygotes for 5 days after their transfer into oviducts of follicular phase, 1-day or 7-day pseudopregnant rabbits (Exp. 3) or for 7 days after their transfer into oviducts of 1-day pseudopregnant rabbits (Exp. 4).

Materials and Methods

Cows, superovulation and collection of ova/zygotes. The 55 parous Holstein dairy and 30 Angus beef cows were superovulated with a follicle-stimulating hormone (FSH-P: Burns-Biotech, Omaha, NE)/prostaglandin F-2 α (Prostin F α : The Upjohn Company, Kalamazoo, MI) regimen as described by Hawk *et al.* (1988). Cows were slaughtered about 40 h after the beginning of oestrus, i.e. ~12 h after the estimated time of ovulation. Reproductive tracts were immediately removed and transported to the laboratory. The oviducts were flushed with 38°C phosphate-buffered saline (PBS) as modified by Tervit *et al.* (1972) and containing 10% heat-inactivated fetal calf serum (GIBCO, Grand Island, NY). Ova were maintained at 38°C in an air atmosphere except as noted below.

Centrifugation of cow zygotes and visualization of pronuclei. Zygotes from 2 or 3 cows were usually pooled and subjected to centrifugation as previously reported (Wall *et al.*, 1985). Briefly, zygotes were transferred to 2-ml microcentrifuge tubes (Sarstedt 72693, Princeton, NJ) containing 38°C Hepes-modified Brinster's medium (Hepes-BMOC, as described by Brinster, 1972, with 25 mM-Hepes substituted for 25 mM-bicarbonate). The tubes were sealed and centrifuged for 3 min at 15 600 g (Eppendorf centrifuge 5412, Fisher Scientific, Richmond, VA).

To visualize pronuclei, centrifuged zygotes were transferred to an oil-covered (360 Medical Fluid, 100 centistokes; Dow-Corning, Midland, MI) microdrop of Hepes-BMOC contained on a siliconized depression slide (Fisher Scientific, 12-560A, Richmond, VA). In Exp. 1, the fluorescent DNA dye, Hoechst 33342 (H342, Sigma, St Louis, MO), was added to the Hepes-BMOC (10 μ l of 2 mg H342/ml H₂O stock solution added to 1 ml Hepes-BMOC) so that observations made with interference-contrast optics could be confirmed by fluorescence (Leitz filter cube 'A', excitation 400 nm, BP 340–380 nm, LP 430 nm). These observations were performed at room temperature. A total of 239 ova and zygotes was examined. Zygotes in which pronuclei were not apparent or were not distinct were classified as not having visible pronuclei. This criterion was chosen because zygotes with poorly defined pronuclei would not be good candidates for microinjection.

Culture of ova/zygotes in rabbit oviducts. Centrifuged and uncentrifuged zygotes were transferred into opposite ligated oviducts of mature New Zealand White rabbits in a manner similar to that described by Boland (1984). Recipient rabbits were in the follicular phase or 1 day or 7 days pseudopregnant. Pseudopregnancy was induced with 150 i.u. hCG (Lypomed, Inc., Melrose Park, IL) administered by intravenous injection. Zygotes were introduced into the fimbriated end of the oviduct with a 5- μ l positive-displacement fixed-volume capillary pipette (Drummond No. 105, Fisher Scientific, Richmond, VA); in some instances, brightly coloured Sephadex-derived density marker beads (Code No. 17-0459-01, DMB-5; Pharmacia Fine Chemicals, Piscataway, NJ) were introduced with each group of zygotes.

To determine whether manipulation of cow ova or their transfer to rabbit oviducts would induce parthenogenetic activation, ova in Exp. 2 were collected from oviducts of unseminated superovulated cows at necropsy about 40 h after the beginning of oestrus. Half of the ova were centrifuged, then centrifuged and uncentrifuged ova were transferred to follicular-phase, 1-day pseudopregnant or 7-day pseudopregnant rabbits. After 7 days, the ova were recovered and evaluated.

Ova were also collected non-surgically from unseminated superovulated cows 7 days after oestrus to ascertain the proportion of unfertilized ova that underwent parthenogenetic development in the cow. The ova were stained and nuclei were counted.

In Exp. 3, centrifuged and uncentrifuged zygotes were randomly assigned to follicular-phase, 1-day or 7-day pseudopregnant rabbits. The embryos were recovered after residing in the ligated rabbit oviducts for 5 days. In Exp. 4, all centrifuged and uncentrifuged zygotes were transferred to 1-day pseudopregnant rabbits and recovered after 7 days.

Recovery of cow embryos from rabbit oviducts improved considerably from Exp. 3 (58%) to Exp. 4 (72%), probably because of a change in the method used to remove the ligature before flushing the oviduct. In Exp. 3, the ligature was removed by sectioning the oviduct on the isthmic side of the ligature. In Exp. 4, the excised oviducts were transilluminated and the brightly coloured beads that were transferred with the zygotes could be seen clustered next to the ligature. The embryos had probably migrated to the same location (Bourdage & Halbert, 1984). Therefore, the oviducts were sectioned on the utero-tubal side of the ligature. Then the ligature was carefully cut but allowed to remain in place, and the end of the oviduct was gently probed to open the lumen under the ligature. Success of the flushing procedure could be immediately assessed by watching the beads appear in the collection dish. Recovery of beads and embryos was highly correlated ($r = 0.94$) in the 54 oviducts in which the comparison was made.

Evaluation of cow embryo development. Cow zygotes and embryos recovered from rabbit oviducts were stained with H342 as previously described (Pursel *et al.*, 1985). When oviducts of 1-day pseudopregnant rabbits were flushed, care was taken to distinguish between rabbit and cow ova (Hafez & Sugie, 1962; Sirard *et al.*, 1985). On rare occasions, differences in thickness of the mucin coat were slight and opacity of the cytoplasm had to be used as the selection criterion.

Statistics. All calculations were performed with the aid of the Statistical Analysis System Version 6 (SAS Institute, Cary, NC). Interference-contrast evaluation of centrifuged zygotes was compared with staining results by χ^2 analysis. In experiments in which development of cow zygotes was studied, nuclear counts were converted to cleavage index [$=\log_2(\text{nuclear count})$] before the data were analysed. Unfertilized ova were excluded from the analysis. For Exps 2 and 3, in which ova were transferred into rabbits of three reproductive states, data were subjected to a split-plot analysis. The influence of reproductive status of recipient rabbits was tested using rabbits nested within reproductive status as the error term; the effect of centrifugation and the interaction between reproductive status and centrifugation were tested using the cross product of rabbits and centrifugation nested within reproductive status as the error term. In Exp. 4, in which cow zygotes were transferred only into 1-day pseudopregnant rabbits, the effect of centrifugation on cow egg development was tested using the residual mean square after correcting for variation attributable to rabbits.

Results

A total of 1660 ova and zygotes was collected from the 85 heifers and cows within 10 min of slaughter. Based on counts of ovulation points, mean $\pm$ s.e.m. egg recovery was $71 \pm 3\%$ and was unaffected by breed.

Experiment 1: visualization of pronuclei

After ova and zygotes were centrifuged, the dense material that normally obscures the pronuclei is concentrated on one side of the ova or zygote (the uppermost side during centrifugation). The pronuclei are usually seen, as in pig zygotes (Wall *et al.*, 1985), near the interface of the dense material and the remainder of the cytoplasm.

Pronuclei were believed to have been observed in 79 of the 239 ova and zygotes examined with interference-contrast optics. Staining with H342 confirmed the presence of pronuclei in 77 out of the 79 zygotes classified as pronuclear. However, 29 of 160 ova (18%) classified as not having apparent pronuclei by interference-contrast contained two nuclear structures within their cytoplasm after H342 staining.

Experiment 2: parthenogenetic development of cow ova in rabbit oviducts and in vivo

Parthenogenetic development was initiated in 41 (16%) of 257 ova recovered from rabbit oviducts (Table 1); the proportion differed ($P < 0.001$) for centrifuged ova (9%) and ova that were not centrifuged (26%). No significant effect of reproductive status of rabbits was detected, although only 1 of six 1-day pseudopregnant rabbits contained parthenogenetically developing cow eggs compared to 5 of 7 follicular-phase and 5 of six 7-day pseudopregnant rabbits that contained parthenogenetic cow ova.

The majority (29) of the activated ova contained between 2 and 4 nuclei: 10 parthenogenotes contained between 5 and 16 nuclei and two contained 30 nuclei. Most parthenogenotes were morphologically abnormal. Although pycnotic nuclei were observed, the majority of the stained

Table 1. Development of ova collected from uninseminated cows, centrifuged, and cultured for 7 days in opposite oviducts of rabbits of three different reproductive states

Treatment of ova	Reproductive state of recipient doe	No. of does	Ova recovered from rabbits			No. of oviducts with cleaved ova
			Total no.	No. at metaphase	No. cleaved (%)	
Centrifuged	Follicular	7	65	55	10 (15)	4
	1-day pseudopregnant	6	30	30	0	0
	7-day pseudopregnant	6	50	47	3 (6)	3
Uncentrifuged	Follicular	7	45	32	13 (29)	4
	1-day pseudopregnant	6	44	35	9 (20)	1
	7-day pseudopregnant	6	23	17	6 (26)	3

nuclei appeared normal. The nuclei of the two most advanced parthenogenotes were strikingly normal in appearance.

The proportion of ova that developed parthenogenetically differed significantly among rabbits ($P = 0.005$): 8 rabbits had no parthenogenetically developing ova and 11 rabbits contained parthenogenetically developed ova; 3 of these rabbits contained only centrifuged parthenogenotes, 4 contained only control parthenogenotes, and 4 contained centrifuged and control parthenogenotes.

Parthenogenetically activated ova were recovered from 5 of the 12 uninseminated cows that were flushed non-surgically. The mean number of parthenogenotes from the 5 cows was 2.8, range 1–5 per cow. Of the 71 ova recovered, 14 (20%) had cleaved; 13 ova contained 2 brightly staining nuclei and one other ovum had 3 nuclei. In the remainder of the ova, the DNA appeared as a diffuse cloud typical of degenerating unfertilized ova or as a single small spot of DNA characteristic of metaphase ova.

Experiment 3: culture of cow zygotes in rabbit oviducts for 5 days

After 5 days, 221 ova and embryos were recovered. Recovery rate of embryos ($58 \pm 8\%$, mean $\pm$ s.e.m.) was not influenced by centrifugation ($P = 0.831$) or reproductive state of the rabbits ($P = 0.708$).

There was a significant interaction between centrifugation and reproductive status of rabbits ($P = 0.045$) on development of the zygotes. Development of cow zygotes, as measured by cleavage index (Table 2), was less advanced for centrifuged zygotes recovered from follicular-phase rabbits and uncentrifuged zygotes collected from 7-day pseudopregnant rabbits than for zygotes recovered from rabbits of other groups. Most of the embryos were at the 5- to 16-cell ($40 \pm 6\%$) or 17- to 32-cell ($41 \pm 6\%$) stage with only $5 \pm 2\%$ of the embryos developed to the 33- to 64-cell stage. Embryo development appeared to be slightly more advanced in 1-day pseudopregnant rabbits than in the others.

Experiment 4: culture of cow zygotes in rabbit oviducts for 7 days

To determine whether longer culture in rabbit oviducts would increase the sensitivity of the viability test and thus increase the chance of detecting effects of centrifugation, 123 centrifuged and 123 uncentrifuged ova and zygotes were transferred to the opposite oviducts of fourteen 1-day pseudopregnant rabbits. At 7 days after transfer, $72 \pm 6\%$ of the ova/embryos were recovered.

Table 2. Development of centrifuged and uncentrifuged cow ova and zygotes cultured for 5 days in opposite oviducts of rabbits of three reproductive states (7 recipients in each state)

Egg treatment	Reproductive state of recipient doe	Eggs recovered from rabbits		% Cleaved embryos with:					Cleavage index†
		Total no.	No. cleaved (%)	2-4 nuclei	5-16 nuclei	17-32 nuclei	33-64 nuclei		
Centrifuged	Follicular	32	28 (88)	21	25	54	0	3.48 ± 0.20*	
	1-day pseudopregnant	33	21 (64)	10	38	48	5	3.96 ± 0.23	
	7-day pseudopregnant	47	30 (64)	7	40	43	10	4.02 ± 0.19	
Not centrifuged	Follicular	27	27 (100)	7	41	48	4	3.80 ± 0.20	
	1-day pseudopregnant	42	25 (60)	4	40	52	4	4.11 ± 0.21	
	7-day pseudopregnant	40	31 (78)	16	68	13	3	3.04 ± 0.19	

†Cleavage index = $\log_2$ (nuclei), mean ± s.e.m.*A significant interaction was detected between zygote treatment and reproductive state of doe ($P = 0.045$).**Table 3.** Development of centrifuged and uncentrifuged cow ova and zygotes cultured for 7 days in opposite oviducts of 1-day pseudopregnant rabbits (N = 14)

Egg treatment	Eggs recovered from rabbits		% Cleaved embryos with:					Cleavage index†
	Total no.	No. cleaved (%)	2-4 nuclei	5-16 nuclei	17-32 nuclei	33-64 nuclei	> 64 nuclei	
Centrifuged	84	40 (48)	15	20	23	33	10	4.22 ± 0.23
Not centrifuged	71	40 (56)	10	33	23	15	20	4.20 ± 0.23

†Cleavage index = $\log_2$ (nuclei).

Centrifugation did not significantly ($P = 0.256$) influence development of cow zygotes (Table 3). However, allowing the embryos to reside in rabbit oviducts for an additional 2 days increased the proportion of embryos with 33 or more nuclei (compare percentages in Tables 2 and 3): 12 (15%) of the recovered embryos contained more than 64 nuclei and most were in the expanded blastocyst stage of development.

Discussion

Pronuclei were observed in 73% of centrifuged cattle zygotes (pronuclei detected in 77 and missed in 29 zygotes). In sheep, whose eggs do not require centrifugation for pronuclei to be seen (Nancarrow *et al.*, 1984), pronuclei have been detected in 80% of zygotes (Hammer *et al.*, 1985). Although data of this type are not available for pig zygotes, pronuclei are apparently missed in about 25% of centrifuged zygotes in that species (V. G. Pursel, personal communication). It is not known why a proportion of the pronuclei cannot be detected with differential interference-contrast optics in these zygotes. In centrifuged cow and pig zygotes, pronuclei may migrate with or be covered by the

migration of optically dense material in the cytoplasm. The latter explanation is supported by an electron microscope study of ultracentrifuged mouse zygotes that found orientation of the eggs, during centrifugation, to be random (Mulnard & Puissant, 1984). It is also possible that, in recently fertilized zygotes, in which pronuclei are located near the vitelline membrane, optical contrast is reduced and some of the pronuclei may be overlooked. Although a proportion of observed pronuclei are peripheral, the vast majority are centrally located. Ova that are approaching syngamy, when nuclear envelopes break down, may account for a small proportion of the zygotes in which pronuclei are not observed. However, when stained, the pronuclear material is usually found in distinctly separate locations, not together, as would be the case at the time of syngamy.

Cow zygotes tolerated centrifugation with no detectable loss in viability. However, a higher proportion of centrifuged zygotes that were cultured in rabbit oviducts for 7 days contained 33–64 nuclei and a lower proportion contained more than 64 nuclei when compared to uncentrifuged zygotes (Table 3). The same relationship was observed when viability of centrifuged pig zygotes was tested (Wall *et al.*, 1985). Centrifugation resulted in fewer sheep blastocysts (Nancarrow *et al.*, 1984) and fewer mice born (Mulnard & Puissant, 1984; Nakamura *et al.*, 1986).

Cow zygotes have been successfully cultured in rabbit oviducts by numerous investigators (for review, see Boland, 1984). When the embryos are subsequently transferred to cows, pregnancies result (Lawson *et al.*, 1972b; Sirard *et al.*, 1985). In most studies, cow embryos were recovered from rabbits after 3 or 4 days. Culture periods of 5 and 7 days were used in this study to increase the proportion of recovered embryos that would have developed to blastocysts or expanded blastocysts. Because it was anticipated that a high proportion of the embryos recovered from rabbit oviducts would be morulae or blastocysts, nuclei were counted to avoid the problem of distinguishing between morulae and fragmented ova or embryos with few nuclei. To compare the rate of development of cow embryos in these experiments with previously published morphological classifications, the assumption was made that morulae contain 10–20 cells, blastocysts 21–36 cells, and expanded blastocysts more than 36 cells. This classification is an approximation because stained blastocysts were seen with as few as 20 nuclei and morulae with more than 40 nuclei. Nevertheless, the stages of development of embryos recovered after 5 days in rabbit oviducts (collected from cows about 1.7 days after the beginning of oestrus) were similar to stages reported for embryos with a chronological age of 6–7 days collected from cows (Shea, 1981; Lindner & Wright, 1983) and for cow embryos cultured in rabbit oviducts (Boland, 1984). Embryos cultured for 7 days in rabbit oviducts were intermediate in development between embryos recovered from cows 7 and 8 days after oestrus (Shea, 1981; Lindner & Wright, 1983; Boland, 1984). Even though the development of centrifuged cow zygotes appeared to be slowed by about half of a cleavage division as compared to uncentrifuged embryos, development of the centrifuged embryos fell within normal limits when compared to embryos of the same chronological age collected from cows.

Oviducts of follicular-phase (Lawson *et al.*, 1972a; Boland *et al.*, 1976) and pseudopregnant (Hafez & Sugie, 1962; Sirard *et al.*, 1985) rabbits have been used as sites for culturing cow embryos with varied degrees of success. The only direct comparison of reproductive status of rabbits on embryo development was performed with a mixture of 2- and 4-cell sheep embryos (Lawson *et al.*, 1972b). Sheep embryos appeared to develop slightly faster in pseudopregnant hosts, but the results were confounded by the proportion of 2- and 4-cell embryos transferred. In the current study, there was a slight but non-significant advantage in development for cow zygotes transferred to 1-day pseudopregnant rabbits (Table 2). Also, when ova collected from cows that were not inseminated were transferred to rabbits of three reproductive states, parthenogenetic development was least apparent in 1-day pseudopregnant rabbits (Table 1). In experiments designed to study early cleavage events, there may be an advantage in using 1-day pseudopregnant rabbits to limit the confounding effect of parthenogenetic development.

Parthenogenetic development of cow ova matured *in vitro* has ranged between 10 and 16% (Trounson *et al.*, 1977; Xu *et al.*, 1986). In both studies, cow ova were cultured in rabbit oviducts, but in neither study was it determined whether parthenogenetic activation was a consequence of

culture in rabbit oviducts. In the present study, 20% of ova collected from 12 unmated cows 7 days after oestrus showed clear signs of parthenogenetic activation. When ova from unmated cows were transferred to rabbits, the overall rate of parthenogenesis was 16%. These percentages suggest that little if any of the parthenogenetic development observed in previous studies was caused by in-vitro maturation of oocytes. The fact that no parthenogenote recovered directly from cows had more than 3 nuclei compared with as many as 30 nuclei in a few parthenogenotes cultured in rabbit oviducts suggests that rabbit oviducts provided a more hospitable environment for developing parthenogenotes. Unexpectedly, a lower percentage of parthenogenotes were observed in ova that were centrifuged than in the uncentrifuged group. The reason for this difference is unknown. Centrifugation stratifies the cytoplasmic contents of ova and therefore may separate some components that normally interact to cause parthenogenesis. Alternatively, parthenogenetically activated ova may be particularly sensitive to mechanical perturbations and become arrested as a result of centrifugation.

Based on the data presented here, centrifugation makes it possible to see pronuclei in 73% of cattle zygotes. After 7 days of culture in rabbit oviducts, about 70% of the cow eggs can be recovered, and about 50% of those eggs would be candidates for non-surgical embryo transfer.

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