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Development of Porcine Ova That Were Centrifuged to Permit Visualization of Pronuclei and Nuclei[1]

R. J. WALL,[2,3] V. G. PURSEL,[3]
R. E. HAMMER,[4] and R. L. BRINSTER[4]

U.S. Department of Agriculture[3]
Beltsville, Maryland
and
University of Pennsylvania[4]
Philadelphia, Pennsylvania

ABSTRACT

The obscured pronuclei or nuclei in living one- and two-celled pig ova were revealed after centrifugation for 3 min at 15,000 × *g*. To determine viability of centrifuged ova, one- and two-celled pig ova were collected from superovulated gilts; half of the ova were centrifuged and all ova were transferred into recipient gilts. Prior to transfer all embryos were stained with tetramethylrhodamine isothiocyanate (TRITC) to distinguish the experimental embryos from the recipient's own ova. Centrifuged ova were transferred into one oviduct of recipient gilts and uncentrifuged ova were deposited into the opposite oviduct. Embryos were recovered 4 days after transfer and were stained with lacmoid or Hoechst 33342 to assess their stage of development. Centrifugation had no detectable influence on survival of the recovered embryos to 4 days. Centrifugation is a simple, reliable method for revealing pronuclei and nuclei of one- and two-celled pig ova and apparently does not alter subsequent preimplantation development.

INTRODUCTION

Micromanipulation of nuclei provides a unique means to study early embryonic development and genome function. By removing and/or transferring pronuclei, investigators have attempted to produce homozygous diploids and parthenotes (Surani and Barton, 1983; for reviews see Seidel, 1983 and Markert, 1984). Such manipulations could lead to further understanding of totipotency and cytoplasmic inheritance and eventually may provide a rapid means of producing new "inbred" animal strains. Genomes can also be modified by microinjecting heterologous genes into the pronuclei or nuclei of ova (Palmiter et al., 1983).

All of the aforementioned techniques require visualization of pronuclei or nuclei. However, nuclear structures in living one- and two-celled cattle, sheep, and swine ova are difficult, if not impossible, to visualize by conventional light microscopic techniques. Recently fluorescent stains, Hoechst 33342 (H342; Crister et al., 1983) and 4′,6′-diamidino-2-phenylindole (DAPI; Minhas et al., 1984), have been used to visualize nuclear material in early bovine embryos. In our use, four-celled pig embryos stained with H342 and exposed to ultraviolet (UV) light (100 watts) for 30 s failed to develop when transferred into recipients. Control embryos stained but not exposed to UV light developed normally. It is unclear whether the ultraviolet light or the combination of the stain and UV light was responsible for the embryonic death. Therefore, an alternate method was sought to visualize nuclear structures.

Like cytoplasm of some mammalian ova, cytoplasm of sea urchin eggs is opaque. For more than three decades, sea urchin ova have been centrifuged to isolate cytoplasmic components and allow the examination of nuclei (see Harvey, 1956 for detailed discussion). When sea

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[2] Correspondence and reprint requests: Dr. Robert J. Wall, USDA, Animal Science Institute, Reproduction Laboratory, Bldg. 200, BARC-East, Beltsville, Maryland 20705.
[3] Mention of a trade name, proprietary product, or specific equipment does not constitute a guarantee or warranty by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products that may be suitable.

urchin ova are centrifuged, the cytoplasm becomes stratified into a number of distinct layers including one that contains the nucleus and is devoid of lipid, yolk, and pigment. On the basis of these findings on sea urchin eggs, we undertook experiments to study the appearance of porcine ova following centrifugation and to determine the effect of centrifugation on subsequent development.

MATERIALS AND METHODS

Ovulation Control

Time of ovulation was controlled in mature gilts by the following sequential treatment: 1) altrenogest (R-2267, 17-allyl-hydroxyestra-4,9,11-trien-3-one; Roussel Uclaf, Paris, France) was fed daily at the rate of 15 mg in 1.8 kg of feed/gilt for 5–9 days, beginning on Day 12 to 16 of the estrous cycle; 2) 24 h after the last feeding of altrenogest, each gilt received 1000–2000 IU of pregnant mare's serum gonadotropin (PMSG) by subcutaneous injection; 3) at 78 h after PMSG treatment, each gilt received 500 IU of human chorionic gonadotropin (hCG) by intramuscular injection. On the basis of previous studies (Hunter and Dziuk, 1968; Hunter, 1972), it was assumed the gilts would ovulate 40–44 h after the hCG injection.

All gilts were checked for estrus once or twice daily using a mature boar. Donor gilts were bred by a fertile boar or artificially inseminated with fresh semen twice during estrus.

Embryo Recovery

At 18–27 h after the expected time of ovulation, gilts were anesthetized by intravenous injection of ketamine followed by closed circuit administration of halothane. The reproductive tract was exposed by midventral laparotomy. Ova were recovered by flushing 20 ml of modified Brinster's medium (BMOC; Brinster, 1972) from the uterotubal junction through the cannulated infundibular end of each oviduct. Ova were collected in sterile polystyrene culture dishes and maintained in a 5% CO_2/95% air atmosphere except during centrifugation.

Centrifugation and Staining of Ova

Half of the ova from each donor gilt were transferred into 2-ml microcentrifuge tubes (Sarstedt 72.693) containing BMOC. The tubes were sealed and centrifuged for 3 min at approximately 15,600 × *g* (Eppendorf Centrifuge 5412).

The experimental ova (centrifuged and uncentrifuged) were stained with tetramethylrhodamine isothiocyanate (TRITC), a fluorescent dye, to distinguish them from the recipients' ova at recovery. The TRITC staining procedure has been shown to have no effect on embryonic development (Pursel et al., 1984). Centrifuged and uncentrifuged ova were transferred to separate microdrops, under paraffin oil, containing 0.4 mg TRITC/ml BMOC (Sigma T-2018 or T-2639) and incubated at 38°C in an atmosphere of 5% CO_2/95% air for 5 min. Stained ova were transferred through 5 microdrops of BMOC to remove excess TRITC and maintained in BMOC until they were deposited into the oviducts of recipient gilts.

Embryo Transfer

Ova were transferred to the oviducts of recipient gilts by aspirating them into sterile polyethylene tubing, inserting the tubing through the infundibulum, and then expelling the ova into the lower ampulla. Centrifuged ova were transferred into one oviduct and uncentrifuged ova into the other oviduct of recipient gilts who were at the same postestrus stage as were ovum donors. Some of the gilts used as recipients had served as ovum donors. The other recipients were synchronized with altrenogest but were not superovulated (PMSG was not administered). Four days after transfer, recipients were killed and their uteri were flushed with saline.

Embryo Evaluation

Recovered embryos were arranged on a microscope slide and held in place by the procedure described by Dziuk (1971). Embryos were then examined with epi-illumination fluorescence (Leitz Laborlux 12, fitted with fluorescent module N2 containing BP530-560 excitation filter and LP580 barrier filter) to distinguish the experimental embryos (TRITC positive) from the recipients' own ova (unstained). Embryos from 4 of the recipients were fixed with acetic ethanol and stained with lacmoid (Chang, 1952), and embryos from the remaining 6 recipients were stained with H342. A stock solution of 1 mg Hoechst 33342/ml H_2O was diluted 100 times in BMOC or 2.3% sodium citrate (final H342 concentration 10 μg/ml). Embryos were exposed to the diluted stain for 15 min at 38°C before nuclei were counted (Leitz Fluorescent filter module A; excitation filter BP340-380, barrier filter LP430; see Pursel et al., 1984 for details).

Statistics

Percentages of recovered embryos at each of eight developmental stages were calculated for each recipient and subjected to statistical analysis using the General Linear Model Procedure (Statistical Analysis System, SAS Institute, Cary, NC). Recipients, embryo treatments, and stages of embryo development at the time of recovery were specified as main effects. The number of embryos recovered from each recipient was used as a weighting factor to test the main effects and interaction between treatment and stage of development.

RESULTS

Before centrifugation, pronuclei or nuclei cannot be seen with bright-field or interference contrast optics in pig ova (Fig. 1). However, these structures in one- and two-celled pig ova were readily visualized after centrifugation (Fig. 2). To date, a total of 715 one-celled and 359 two-celled pig ova have been centrifuged. Pronuclei or nuclei were observed in 914 (85%) of the ova. Most ova in which nuclear structures were not observed were one-celled ova. Under the conditions we used, embryonal cytoplasm segregates into three regions of varying opacity

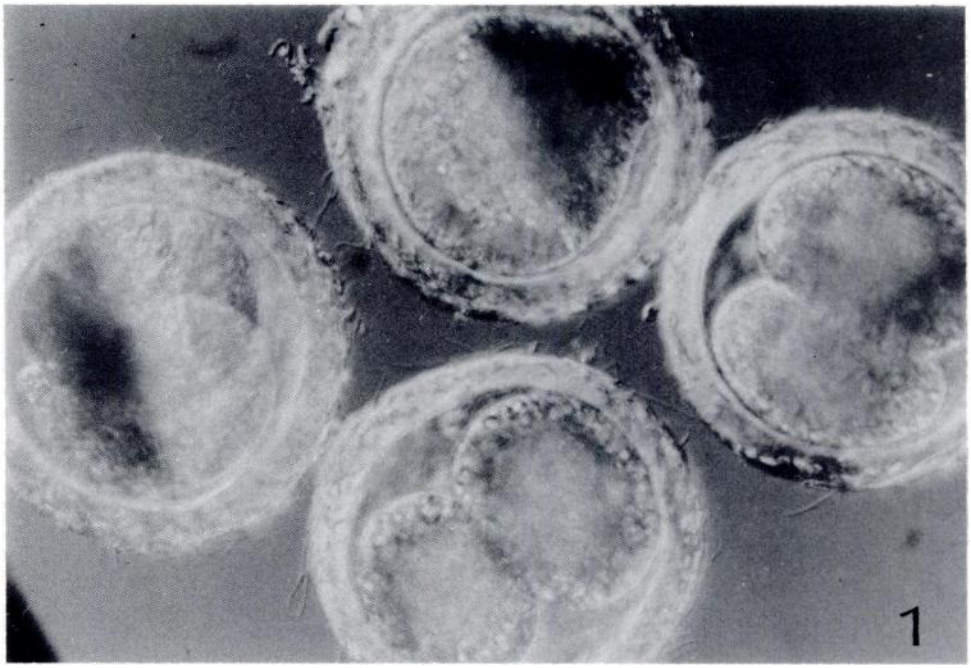

FIG. 1. Centrifuged and uncentrifuged pig ova photographed with interference contrast optics (X200). Note the stratified appearance of the centrifuged zygote (*top*) and two-celled embryo (*left*).

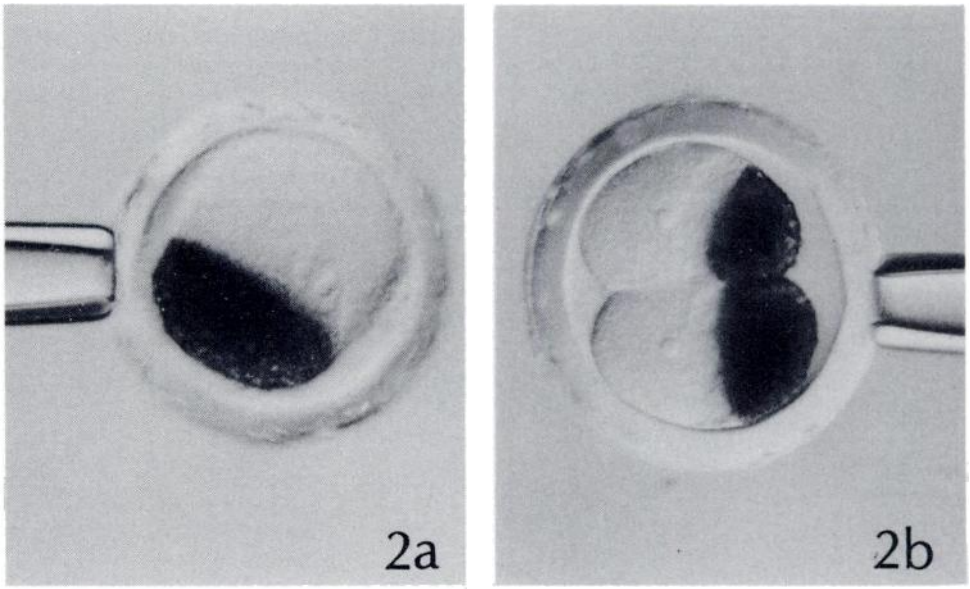

FIG. 2*a*: Centrifuged pig zygote attached to a holding pipet. Three nucleoli can be seen in the equatorial region of the zygote. The nucleus appears as a smooth, nongranular region immediately surrounding the nucleoli. Interference contrast optics (X200). *b*: Centrifuged two-celled pig embryo attached to a holding pipet. A nucleolus can be seen in each blastomere. The smooth, nongranular region surrounding the nucleolus of the bottom blastomere outlines the boundary of its nucleus. Interference contrast optics (X200).

TABLE 1. Number of centrifuged and uncentrifuged pig ova transferred into recipients.[a]

Embryo treatment	1-cell stage	2-cell stage	4-cell stage	Total
Centrifuged	66	48	3	117
Uncentrifuged	57	54	5	116
Total[b]	123	102	8	233

[a]An average of 12 centrifuged and 12 uncentrifuged ova were transferred into each of 10 gilts (range 8–20).

[b]All ova were stained with TRITC before transfer.

(Fig. 2*a*). The most opaque region occupies slightly less than half of the cell's volume and contains granules and droplets of assorted sizes, some of which are pigmented. When centrifuged eggs are allowed to float freely in a Percoll (Pharmacia, Inc., Piscataway, NJ) gradient, the most optically dense region is oriented upward, suggesting that it contains material of relatively low buoyant density. The adjacent region is located approximately at the equator of the ovum and usually appears as a band with poorly defined margins. The equatorial band is composed of translucent droplets ranging in size from 1 to 7 μm in diameter. The remaining region may occupy half or slightly more than half of the egg volume and is usually devoid of large granules or droplets. This region has a translucent rather than transparent appearance. It appears to be composed of a suspension of very fine particles. The pronuclei are often found in the region of the equatorial band, but may also be located in the large translucent region (Fig. 2*b*). Ova may elongate and become slightly tapered (narrowing at the opaque region, Fig. 1) during centrifugation, but generally return to a spherical shape within 5 h.

When ova were suspended in a Percoll gradient and subjected to higher centrifugal forces than used in this experiment, ova were distorted into an asymmetric dumbbell shape, the opaque region forming the smaller of the two lobes. At yet higher *g* forces the ova take on the appearance of a mushroom with the opaque region forming the "stem" of the mushroom. Ova can be successfully centrifuged in preformed Percoll gradients at 15,000 × *g*, but this technique was abandoned because visualization of nuclei was not different from ova processed by the simpler method described in this report.

The numbers of one-, two-, and four-celled experimental ova transferred into recipients are listed in Table 1. Of the 233 ova transferred, 80% (94) of the centrifuged and 73% (85) of the uncentrifuged ova were recovered. The experimental embryos were easily distinguished from the recipients' own ova by their orange color (TRITC) when viewed with fluorescent optics.

No difference was detected in development of centrifuged and uncentrifuged embryos (stage of development by treatment interaction; $p=0.83$). The percentages of centrifuged and

TABLE 2. Stage of development of centrifuged and uncentrifuged pig embryos recovered 4 days after transfer.[a]

Item	Number of embryos		Probability that means differ
	Centrifuged	Uncentrifuged	
Total recovered	94	85	–
Stage of recovered embryos			
Degenerate[b]	5(5)[c]	6(7)	0.84
Metaphase	5(5)	3(4)	0.83
2 nuclei	0(0)	0(0)	–
4 nuclei	1(1)	0(0)	0.90
5–15 nuclei	10(11)	8(9)	0.88
16–32 nuclei	26(28)	14(16)	0.19
33–50 nuclei	8(9)	16(19)	0.22
>50 nuclei	39(41)	38(45)	0.70

[a]Embryos recovered from 10 recipients.

[b]Nuclei were indistinct and number could not be assessed.

[c]Numerals in parentheses are least-squares mean percentages of embryos recovered within treatment groups.

uncentrifuged embryos at various stages of nuclear development are presented in Table 2. The embryos were also arranged in another nuclear grouping (5—32 nuclei and 33—>50 nuclei) and subjected to statistical analysis, but none of the arrangements tested revealed a significant stage of development by treatment interaction.

Fifty-six percent of all embryos recovered contained more than 32 nuclei and were more abundant than embryos with 5—32 nuclei (32%; $P<0.01$) or embryos with fewer than 5 nuclei (5%: $P<0.01$). Six percent of the embryos recovered were classified as degenerate. They contained abnormal nuclei or the number of nuclei could not be determined.

The stage of development of ova at the time of transfer influenced the number of nuclei at the time of recovery ($P<0.01$; Table 3). Four days after transfer, recipients that received only two-celled ova had a significantly larger percentage of blastocysts (>50 nuclei, 79%) than did recipients that received only one-celled ova (>50 nuclei, 16%). Recipients into which mixtures of one- and two-celled ova were transferred contained an intermediate proportion of blastocysts (47%) compared with the other two recipient groups. The differential development of one- and two-celled ova was not affected by centrifugation ($p=0.98$).

DISCUSSION

Centrifugation had no detectable influence on development of one- and two-celled pig ova when compared with uncentrifuged controls (Table 2), and the rate of embryo development appeared to be normal. The distribution of embryos recovered by Hancock (1961) 144 h after mating was very similar to the distribution of embryos recovered in this experiment [5—15-cell stage, 7% (Hancock) vs. 10% (this experiment); 16—32-cell stage, 27% vs. 22%; >32-cell stage, 60% vs. 57%].

The development of both centrifuged and uncentrifuged one-celled ova was less advanced than that of two-celled ova at the time of recovery (Table 3). The difference in development of the one- and two-celled ova may reflect the difference in age of the ova at the time of transfer or may indicate that one-celled ova are more sensitive than two-celled ova to handling. The distribution of embryonic stages reported by others between 120 and 144 h after the onset of estrus suggests that our one-celled ova developed at a normal rate, and the higher proportion of blastocysts resulting from transfer of two-celled ova reflected their more advanced stage at the time of transfer. From the combined data (120 embryos) of Hancock (1961) and Perry and Rowlands (1962), one would predict 24% morula and 21% blastocysts to be recovered

TABLE 3. Influence of stage of development at transfer on stage of embryo development at recovery on day 4.

Item	Number of embryos		
	1-celled transfers	2-celled transfers	Mixed transfers
Embryos transferred			
1-celled	78	—	36
2-celled	—	52	25
Embryos recovered	63	48	39
Stage of recovered embryos[a]			
Degenerate[b]	5(8)[c]	2(4)[c]	4(10)[c]
Metaphase	6(10)[c]	0(0)[c]	2(5)[c]
2 nuclei	0(0)[c]	0(0)[c]	0(0)[c]
4 nuclei	0(0)[c]	0(0)[c]	1(2)[c]
5—15 nuclei	17(27)[c]	0(0)[d]	0(0)[d]
16—32 nuclei	13(21)[c]	7(15)[c]	12(31)[c]
33—50 nuclei	12(19)[c]	1(2)[d]	2(5)[c,d]
>50 nuclei	10(16)[c]	38(79)[d]	18(47)[e]

[a]Numerals in parentheses are least-squares mean percentages of embryos recovered within transfer group.

[b]Nuclei were indistinct and number could not be assessed.

[c,d,e]Mean percentages within rows with different superscripts differed significantly ($P<0.05$).

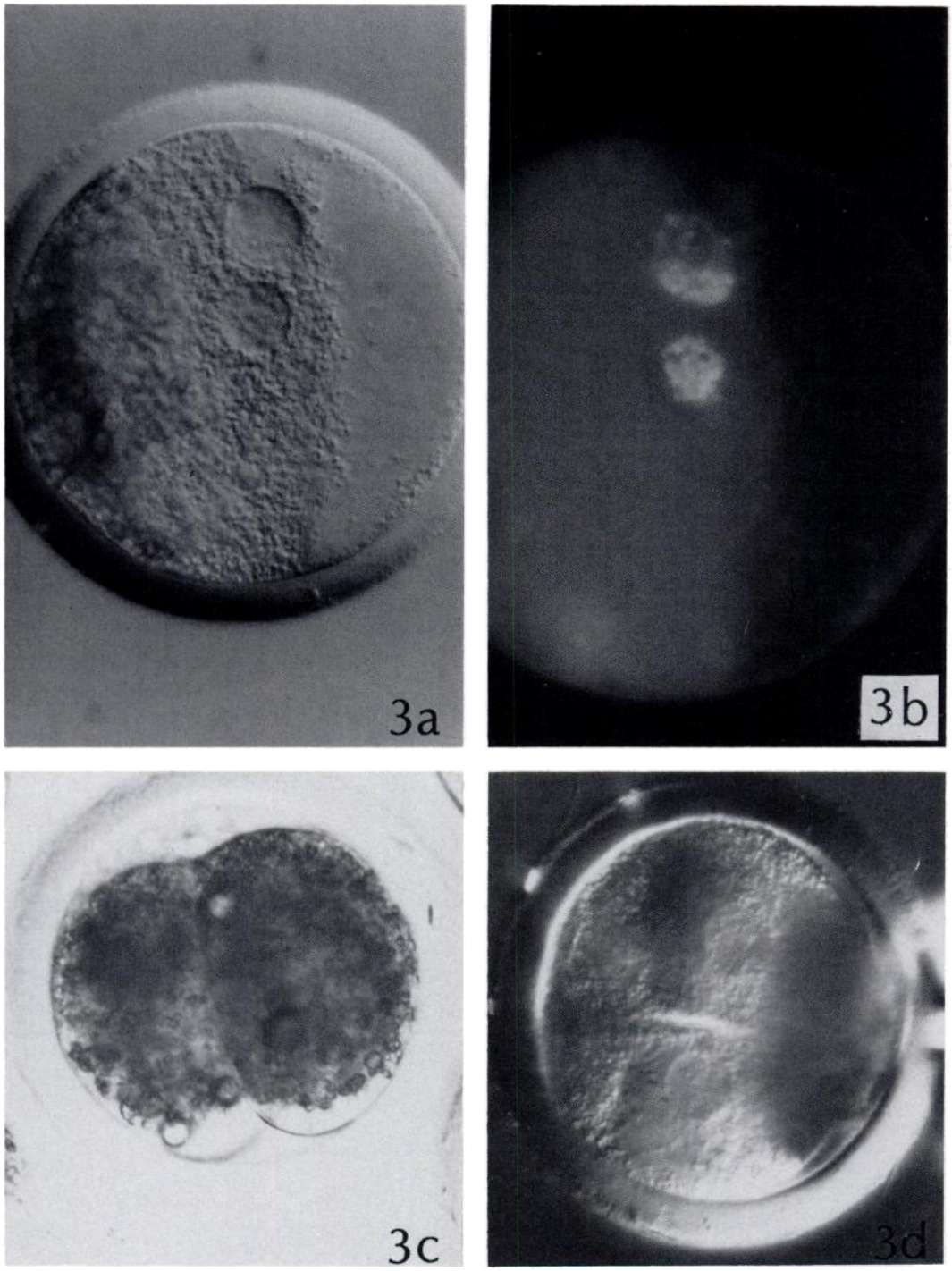

FIG. 3*a*: Centrifuged cattle zygote slightly compressed by a coverslip suspended by petroleum jelly-wax supports. Pronuclei appear as two clear circles. Interference contrast optics (×320). *b*: Same zygote as in *a* after staining for 15 min with H342. Stained pronuclei appear as brightly stained regions, and one of the polar bodies out of the plane of focus at 11 o'clock appears as a faintly stained dot. Epi-illumination fluorescence with Excitation filter BP340–380, Barrier filter LP 430 (×400). *c*: Uncentrifuged two-celled cattle embryo, recovered as a zygote 44 h after administration of $PGF_{2\alpha}$ and incubated in BMOC at 39°C for 20 h in an atmosphere of 5% CO_2/95% air. Interference contrast optics (×320). *d*: Same two-celled cattle embryo as in *c*, after centrifugation. Nuclei appear as clear circular regions in the center of each blastomere. Interference contrast optics (×320).

between 120 and 144 h after the onset of heat. When only 1-celled ova were transferred, in this experiment, 21% morula (16–32 nuclei) and 35% blastocysts (>32 nuclei) were recovered.

As mentioned, nuclear structures were not detectable in 15% of centrifuged pig ova. In almost all cases, these ova were at the one-cell stage. It is not clear why nuclear structures were not observed, although it is possible that some of the ova were not fertilized and others may have been in metaphase or anaphase of the first mitotic division.

In another embryo transfer experiment, centrifuged one- and two-celled ova have developed to normal 30-day-old fetuses. When gilts were allowed to go to term, 11 litters of normal piglets have been born to date (unpublished data).

We have attempted to extend the centrifugation technique to sheep and cattle embryos. We found, as did Nancarrow and coworkers (1984), that centrifugation does not improve the visualization of nuclear structures in sheep ova. However, cattle ova seem to withstand centrifugation, and pronuclei in zygotes and nuclei in two-celled embryos were clearly visible (Fig. 3).

Thus, it is clear that centrifugation is without significant effect on subsequent development of pig zygotes and two-celled embryos into normal-appearing blastocysts.

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