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Actin filament distribution in blocked and developing pig embryos

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Summary

Actin filaments play an important role in cell division. The present study was designed to examine the relationship between actin filament distribution and pig embryo development. When *in vivo* matured and fertilised pig oocytes were cultured in TCM 199 or NCSU 23, in various proportions, 45–65% of inseminated oocytes developed to the 2- to 4-cell stages but blastocyst development was observed only in NCSU 23 (34%) or NCSU 23 containing 10% TCM 199 (7%). Supplementation of NCSU 23 medium with 20% or more TCM 199 resulted in no blastocyst formation. Examination of actin filaments indicated that microfilaments were distributed in the cortex, at the junction of blastomeres and in the perinuclear area in the embryos cultured in NCSU 23, but perinuclear actin filaments were not observed in embryos cultured in TCM 199. When 2- to 4-cell stage embryos obtained from TCM 199 were transferred to NCSU 23 medium at 36 h after *in vivo* fertilisation, 57% of the cleaved embryos developed to blastocysts, which was no different from the proportion obtained after culture in NCSU 23 alone (56%). In addition, when 2- to 4-cell stage embryos obtained from TCM 199 were transferred to NCSU 23, most embryos showed perinuclear actin filaments within 6 h. The results indicate that the composition of the culture medium plays an important role in the polymerisation of actin filaments, which in turn influences embryo development. It is possible that pig embryo development was blocked by some components in TCM 199 which prevented actin filament polymerisation.

Keywords: Embryo development, Microfilaments, Pig

Introduction

Actin is a major component of the cytoskeleton and it plays important role(s) in some events in oocyte maturation, fertilisation and embryo development. Recently, it was found that intracellular actin filament distribution was directly related to the 2-cell block in the hamster (Barnett *et al*., 1997) and rat (Matsumoto *et al*., 1998) embryos. Although the mechanism(s) by which actin filaments modulate embryo development is poorly understood, it has been found that microfilaments are required for normal mitochondria distribution (Barnett *et al*., 1997), transfer of mitochondria to daughter cells during cell division (Boldogh *et al*., 1998), distribution and morphology of Golgi complexes (Valderrama *et al*., 1998) and the distribution of some mRNAs (Bassell *et al*., 1994).

Pig embryos are usually blocked at the 4-cell stage (Davis & Day, 1978; Archibong *et al*., 1989). Recently, improvements in culture media have made it possible for pig embryos to overcome the 4-cell block (Beckmann & Day, 1993; Petters *et al*., 1990; Petters & Wells, 1993). Based on these improvements, we have established an *in vitro* system to produce pig blastocysts by *in vitro* maturation (IVM), *in vitro* fertilisation (IVF) and *in vitro* development (Wang *et al*., 1997; Abeydeera & Day, 1997; Abeydeera *et al*., 1998*a, b*). We also found that NCSU 23 medium, which was first reported for culture of *in vivo* produced pig embryos (Petters & Wells, 1993), supported development of 50–60% of cleaved IVM/IVF embryos to the blastocyst stage (Wang *et al*., 1999). However, some embryos were blocked at different cleavage stages but mainly before or at the 4-cell stage. It was found that actin filament distribution may participate in the development of pig

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embryos and *in vitro* culture conditions may not be the optimal for the dynamics of microfilaments (Wang *et al*., 1999, 2000).

In the present study we compared the effects of two culture media – NCSU 23, which supports pig embryo development, and TCM 199, which supports oocyte maturation (Wang *et al*., 1991, 1997; Wang & Niwa, 1995; Abeydeera *et al*., 1998*b*) – on the development of pig embryos produced by IVM/IVF with special reference to actin filament distribution. Our results indicate that TCM 199 did not allow pig embryos to overcome the 4-cell block, possibly through prevention of polymerisation of actin filaments by some components in the medium. The results also provide evidence that microfilaments are critical for normal pig embryo development.

Materials and methods

IVM of oocytes

In vitro matured oocytes were obtained by culturing follicular oocytes collected from the ovaries of prepubertal gilts. Briefly, oocytes were aspirated from antral follicles (3–6 mm in diameter) of ovaries collected at an abattoir. After washing four times with HEPES-buffered Tyrode's medium containing 0.1% polyvinyl alcohol (HEPES-TL-PVA), oocytes were washed three times with the maturation medium, which was tissue culture medium (TCM) 199 supplemented with 0.57 mM cysteine, 0.1% PVA, 10 IU/ml eCG (Intervet America, Millsboro, DE), 10 IU/ml hCG and 10 ng/ml epidermal growth factor. Groups of 50 oocytes were transferred to 500 µl of maturation medium which had previously been covered with warm paraffin oil (Fisher Scientific, Pittsburgh, PA) in a four-well culture plate and equilibrated overnight in an atmosphere of 5% CO_2 in air. The oocytes were cultured at 39 °C in 5% CO_2 in air for 44 h. After culture, oocytes were freed of cumulus and corona cells by treatment in maturation medium that contained 0.02% hyaluronidase obtained from bovine testis (Sigma Chemical Co., St Louis, MO), followed by repeated passage through a fine pipette.

IVF of oocytes

Cumulus-free oocytes were washed three times with insemination medium and 30–35 oocytes were transferred into a 50 µl droplet of insemination medium covered with paraffin oil which had been equilibrated for 40 h at 39 °C, in 5% CO_2 in air. The dishes were kept in a CO_2 incubator until spermatozoa were added for insemination. The insemination medium was a modified Tris-buffered medium consisting of 110.0 mM NaCl, 0.47 mM KCl, 7.5 mM $CaCl_2$, 0.5 mM sodium pyruvate, 10 mM glucose, 20 mM Tris, 1 mM caffeine and 1 mg/ml bovine serum albumin (BSA), which has been modified in our laboratory (Abeydeera & Day, 1997). For IVF, one 0.1 ml frozen semen pellet, made according to methods reported by Wang *et al.* (1991), was thawed at 39 °C in 10 ml of phosphate-buffered saline (PBS) containing 1 mg/ml BSA and antibiotics. After being washed three times (1900 *g*, 4 min), spermatozoa were resuspended with insemination medium to give a concentration of 5×10^5 cells/ml, and 50 µl of the sperm sample were added to the 50 µl fertilisation drops which contained the oocytes. Six hours after insemination, oocytes were removed from the fertilisation drops and exposed to culture medium.

Culture of embryos

Two culture media were used in the present study: TCM 199 and NCSU 23. Both were supplemented with 4 mg/ml BSA. After washing three times with culture medium, each of the 30–40 inseminated oocytes was cultured in either TCM 199 or NCSU 23. In experiment 1, embryos were cultured in NCSU 23:TCM 199 (v:v) in various proportions (100:0, 90:10, 80:20, 50:50, 20:80, 0:100). Cleavage rate was examined 36 h after IVF and blastocyst formation was examined 6 days after IVF. In experiment 2, inseminated oocytes were cultured either in TCM 199 or in NCSU 23. At 36 h after IVF, cleaved embryos were selected, washed three times and cultured in NCSU 23 for another 4.5 days to examine blastocyst formation. Subgroups of the cleaved embryos were fixed for examination of actin filaments at 0, 2 and 6 h after transfer to NCSU 23.

Assessment of actin filaments

Embryos were fixed in 3.7% paraformaldehyde (Sigma) in PBS for 2 h at room temperature, followed by treatment with 1% Triton X-100 in PBS for 6 h and then washing twice in PBS. The embryos were then cultured in a blocking solution (PBS containing 2 mg/ml BSA and 150 mM glycine) for 30 min. After washing for another hour in PBS, the embryos were stained with 1 µg/ml fluorescein isothiocyanate conjugated phalloidin (Sigma) in PBS-Tween 20 (0.1%) for 1 h. Samples were washed twice in PBS-Tween solution and then stained with 10 µg/ml propidium iodide (PI) in PBS for examination of the nucleus. Finally, these embryos were mounted on slides and examined by lasar confocal microscopy. Confocal microscopy was performed using a BioRad MRC-600 confocal imaging system (Wang *et al*., 1999).

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Statistical analysis

Two (experiment 1) and four (experiment 2) replicate trials were conducted and all percentage data were subjected to arc sine transformation before statistical analysis. Comparisons were conducted by ANOVA. A probability of $p < 0.05$ was considered to be statistically significant.

Results

As shown in Table 1, when inseminated oocytes were cultured in NCSU 23 or NCSU 23 with various proportions of TCM 199, 45–65% of oocytes cleaved 36 h after IVF. However, blastocyst formation was observed only in 100% NCSU 23 medium (34%) and 90% NCSU 23 plus 10% TCM 199 (7%). No blastocyst development was observed in NCSU 23 medium with 20–100% TCM 199. When 2- to 4-cell stage embryos obtained in TCM 199 (36 h after IVF) were transferred to NCSU 23, 57% developed to the blastocyst stage, which was no different from the proportion obtained after culture in NCSU 23 alone (56%; Table 2). When embryos cultured in TCM 199 and NCSU 23 were fixed to examine actin filament distribution, it was found that embryos cultured in NCSU 23 had actin filaments at the cortex, at the junction of blastomeres and in the perinuclear area of most blastomeres (Fig. 1*A, B*). However, embryos cultured in TCM 199 did not have actin filaments in the perinuclear area (Fig. 1*C, D*) or cortex of some blastomeres. When the embryos cultured in TCM 199 were transferred to NCSU 23, perinuclear actin filaments were observed after 2 h of culture in some blastomeres and most embryos had perinuclear actin filaments after culture for 6 h (Table 3).

Table 1 Early development of pig embryos cultured in different media[a]

Medium (v:v)		No. of oocytes cultured	No. (%) of cleaved oocytes	No. (%) of blastocysts
TCM 199 (%)	NCSU 23 (%)			
100	0	80	40 (50)	0[b]
80	20	80	52 (65)	0[b]
50	50	80	48 (60)	0[b]
20	80	80	41 (51)	0[b]
10	90	60	27 (45)	4 (7)[b]
0	100	80	44 (55)	27 (34)[c]

[a]Six hours after *in vitro* fertilisation (IVF), oocytes were removed from insemination medium and placed in culture medium. Cleavage rates were examined 36 h after IVF and blastocyst formation was examined 6 days after IVF.
[b,c] $p < 0.001$.

Table 2 Development of pig embryos culture in two media for 36 h and then transferred to NCSU 23 for continued culture

Medium		No. of oocytes cultured	No. of oocytes at 2- to 4-cell stages (%)	No. (%) of blastocysts/ 2- to 4-cell stages
First 36 h	After 36 h			
NCSU 23	NCSU 23	180	113 (63)	63 (56)
TCM 199	NCSU 23	180	101 (56)	58 (57)

Discussion

Earlier work showed that pig embryos are blocked at the 4-cell stage when exposed to culture medium (Davis & Day, 1978; Archibong *et al*., 1989) and that the block can be overcome by supplementation of the medium with oviductal fluid (Archibong *et al*., 1989) or co-culture with oviductal epithelial cells (White *et al*., 1989). However, when embryos beyond the 4-cell stage collected from oviducts were cultured, most embryos developed to the blastocyst stage (Davis & Day, 1978). Recently, modification in the culture medium has allowed 1-cell stage pig embryos to develop to the blastocyst stage (Petters *et al*., 1990; Beckman & Day, 1993; Petters & Wells, 1993). These improvements have also been extended to successful culture of *in vitro* matured and *in vitro* fertilised zygotes (Abeydeera *et al*., 1998*a*; Wang *et al*., 1997, 1999).

However, the mechanism by which pig embryos overcome the 4-cell block is still unclear. Our previous study indicated that a major difference in actin microfilament distribution was present between *in vitro* produced and *in vivo* produced pig embryos and suggested that such a difference observed under *in vitro* conditions is related to embryo development (Wang *et al*., 1999). In the present study, we found that the culture medium did affect polymerisation of actin filaments and may regulate embryo development under *in vitro* conditions. TCM 199, even at 10% concentration, inhibited microfilament formation in the cytoplasm and blocked embryo development to the blastocyst stage. On the other hand, when embryos from TCM 199 were transferred to NCSU 23 for a few hours, microfilaments appeared in the cytoplasm, and the embryos resumed development. These results indicate that some component(s) in TCM 199 prevents polymerisation and formation of microfilaments in the cytoplasm, which is important for pig embryos to overcome the 4-cell block and to undergo further development.

Although the way in which microfilaments affect embryo development is not clear, previous studies

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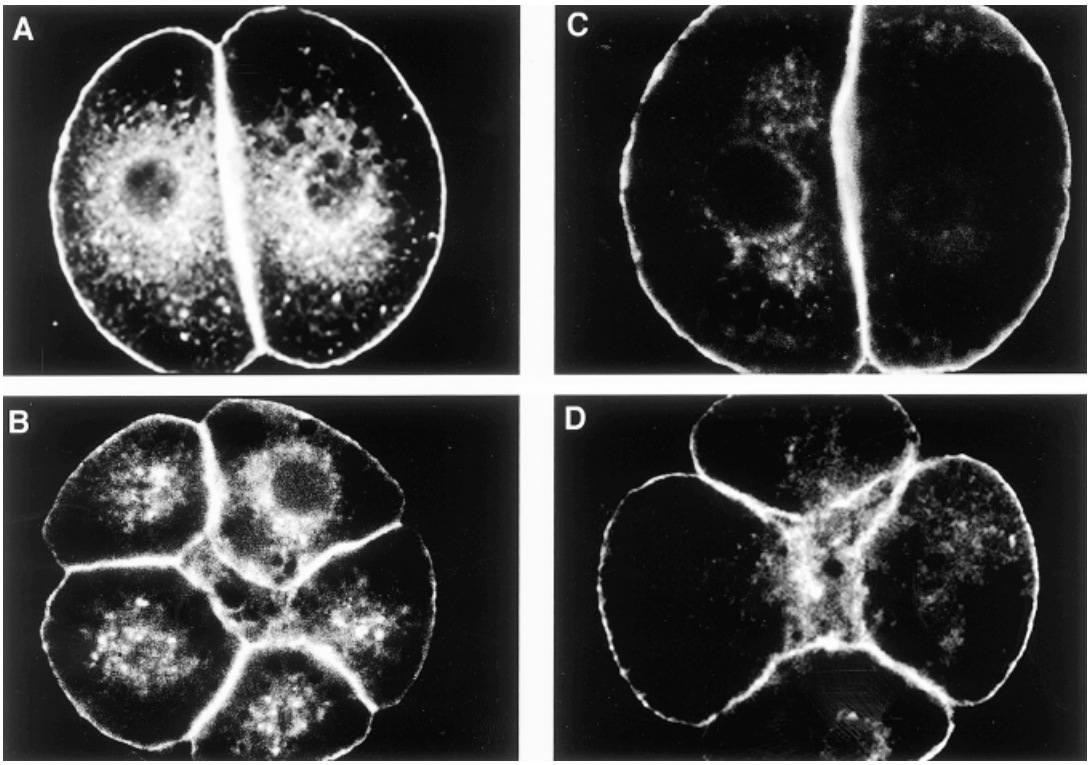


Figure 1 Confocal micrographs of microfilaments in porcine embryos cultured in TCM 199 or NCSU 23. Embryos were stained and examined 36 h after *in vitro* fertilisation. Microfilaments were distributed in the cortex, joints and cytoplasm of blastomeres in embryos cultured in NCSU 23 (*A, B*) but microfilaments were not observed in the cytoplasm of the embryos cultured in TCM 199 (*C, D*).

Table 3 Actin filament distribution in pig embryos cultured in two media

Medium	No. of embryos examined	Actin filaments distributed in the [a]		
		Cortex	Junctions	Perinuclear area
NCSU 23	40	+ (40)[b]	+ (40)	+, ± (28, 12)
Time from TCM 199 to NCSU 23:				
0 h	40	+, ± (34, 6)	+ (38)	– (40)
2 h	38	+ (38)	+ (38)	± (38)
6 h	45	+ (45)	+ (45)	+, ± (30, 15)

[a] +, with microfilaments in all blastomeres; ±, with microfilaments in some blastomeres; –, no microfilaments in any blastomeres.
[b] The number of embryos with different actin filament distribution is shown in parentheses.

conducted either in zygotes, embryos or some somatic cells indicated that microfilaments are necessary for the distribution of mitochondria (Barnett *et al.*, 1997; Matsumoto *et al.*, 1998), Golgi complexes (Valderrama *et al.*, 1998) and some mRNAs (Bassell *et al.*, 1994). They also regulate ion exchange in the cells (Cantiello, 1997). Studies in the hamster (Barnett *et al.*, 1997) and rat (Matsumoto *et al.*, 1998) indicated that phosphate in the medium prevented polymerisation of microfilaments and possibly played a key role in the embryo block. However, in the present study the effect of phosphate could be ruled out since a previous study demonstrated that phosphate does not block pig embryo development in culture (Petters *et al.*, 1990). It has been found that NCSU 23 medium, which contains phosphate, allowed 1-cell pig embryos to develop to the blastocyst stage (Petters & Wells, 1993). However, as in the hamster (Barnett *et al.*, 1997), we found that pig embryos cultured in TCM 199 did not have microfilaments in the perinuclear area. By contrast, most developing embryos in NCSU 23 had microfilaments in all or most blastomeres. Such a positive correlation between microfilament distribution and embryo development indicates that microfilaments play an important role in embryo development. This conclusion was also supported by the findings in our previous two studies. One was that *in vivo* produced pig embryos possess more microfilaments than *in vitro* produced embryos (Wang *et al.*, 1999) and the other was that when cytochalasin D, an inhibitor of polymerisation of microfilaments (Cooper, 1987), was included in the culture medium, all embryos stopped developing and no microfilaments were present in the embryos (Wang *et al.*, 2000).

Many factors affect embryo development, such as nutrients, energy substrate, pH and osmolarity. In the

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present study, when 10% TCM 199 was added to 90% NCSU 23 medium the differences (in the above-mentioned factors) compared with 100% NCSU 23 were minimal. However, blastocyst formation was reduced significantly. Undoubtedly, some components of TCM 199 are detrimental to embryo development and the embryos are very sensitive to such a component(s). One of the possibilities was thought to be heavy ions in TCM 199. When we supplemented ethylenediaminetetraacetic acid, a chelator of heavy ions in medium which was beneficial to mouse embryo development (Gardner & Lane, 1996), in 10% TCM 199 plus 90% NCSU 23 medium, we did not find any effect on blastocyst production (data not shown). On the other hand, the inhibitory effect of TCM 199 on embryo development is reversible. When 2- to 4-cell stage embryos cultured in TCM 199 were transferred to NCSU 23 and continued in culture, most embryos developed to blastocysts and the rate was the same as for those embryos cultured in NCSU 23 alone. Examination of microfilament formation indicated that most embryos reformed microfilaments in the cytoplasm after culture for 2–6 h in NCSU 23. These results suggest that some components in TCM 199 reversibly inhibit polymerisation of actin filaments.

Polymerisation of actin from the non-filamentous to filamentous state is a complicated process. Many factors have been reported to be responsible. Recently, it has been found that actin binding protein, such as cofilin (Sun *et al*., 1995; Lappalainea & Drubin, 1997), and phosphorylation of cofilin LIM-kinase (Arter *et al*., 1998), regulate the dynamic behaviour of microfilaments. The compounds in the culture medium may participate in the regulation of these actin binding proteins or associated enzymes. One previous study indicated that the amount of actin protein itself showed little change during embryo development from 2-cell to blastocyst stages (Wang *et al*., 2000). Therefore, it is inappropriate culture conditions that cause actin to be in a non-functional state, i.e. non-filamentous actin.

TCM 199 has been used for culture of oocytes in many animals, including pigs (Wang *et al*., 1991, 1997; Wang & Niwa, 1995; Abeydeera *et al*., 1998*b*). We found that 20–30% of oocytes matured in TCM 199 developed to the blastocyst stage (Wang *et al*., 1997; Abeydeera *et al*., 1999, present data). TCM-199 was also used for culture of embryos to the blastocyst stage in cattle after IVM/IVF (Pinyopummintr & Bavister, 1991). However, in the present study no blastocyst formation was observed in pig embryos when cultured in TCM 199 although most embryos developed to the 2- to 4-cell stages. The sensitivity of oocytes or embryos to some chemicals is clearly different between stages of development and between species. In the present study, the main difference observed between embryos cultured in NCSU 23 and TCM 199 was the distribution of cytoplasmic microfilaments. It is possible that cytoplasmic microfilaments regulate the distribution of other organelles which are important for embryo division. It is also possible that other differences are present between the embryos produced in NCSU 23 and TCM 199. Further examination of these differences may provide a more detailed explanation as to the blocking role of TCM 199 on pig embryo development. Although NCSU 23 medium supports pig embryo development *in vitro*, some embryos are still blocked at various stages and the cell number of blastocysts is small (Wang *et al*., 1999). Thus modifications in culture conditions, especially in the composition of the medium, are still necessary.

In conclusion, our present study indicates that more than 50% of cleaved pig embryos produced by IVM/IVF can develop to the blastocyst stage *in vitro*. However, some embryos are still blocked at various stages and the cell number in blastocysts is low. The formation of microfilaments in the cytoplasm plays an important role(s) in overcoming the 4-cell block. Modifications in the culture conditions by monitoring dynamics of microfilaments may be helpful for further improvement of an *in vitro* system to produce high-quality and viable embryos.

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