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Optimisation of porcine oocyte activation following nuclear transfer

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Summary

Experiments were conducted to examine the effects of (a) different activation methods, (b) incubation time in calcium-free medium and (c) bisbenzimide staining on the activation and subsequent development of pig oocytes. Oocytes were matured *in vitro* and activated by one of the following methods: combined thimerosal/dithiothreitol (DTT) treatment, calcium ionophore A23187 treatment followed by incubation in the presence of 6-dimethylaminopurine (6-DMAP), electroporation, and electroporation followed by incubation with cytochalasin B. There were no significant differences in the activation rate (ranging from 70.0% to 88.3%) and the percentage of cleaved embryos after activation (ranging between 48.8% and 58.8%) among the four treatment groups ($p < 0.05$). The rate of development to the blastocyst stage in oocytes activated by thimerosal/DTT (10.0%) or electroporation followed by cytochalasin B treatment (12.3%) was significantly higher ($p < 0.05$) than in the group activated with A23187/6-DMAP (2.5%). Both the activation rate and the rate of blastocyst formation in oocytes that were incubated in Ca^{2+}-free medium for 8 h before thimerosal/DTT activation were significantly lower ($p < 0.05$) than in those incubated for 0, 1 or 4 h. Intracellular Ca^{2+} measurements revealed that the Ca^{2+} homeostasis in these oocytes were severely altered. Staining of oocytes with 5 μg/ml bisbenzimide for 2 h decreased the quality of blastocysts and increased the rate of degenerated embryos at day 6. Two activation protocols (thimerosal/DTT and electroproation) were used for activation after nuclear transfer; the rate of nuclear formation did not differ in the oocytes activated by the two different methods.

Keywords: Activation, Nuclear transfer, Oocyte, Porcine

Introduction

According to recent reports (Tao *et al.*, 1991*a*, *b*), development of porcine nuclear transfer embryos derived from differentiated cells is less efficient compared with that of other species such as sheep (Wells *et al.*, 1997; Wilmut *et al.*, 1997), cattle (Cibelli *et al.*, 1998*a*, *b*; Kato *et al.*, 1998) and mouse (Wakayama *et al.*, 1998). One reason for this low effectiveness is the lack of efficient oocyte activation methods. Many procedures that work well in other species are often ineffective for the activation of porcine oocytes (for review see Prather, 1997*a*). Although many methods of oocyte activation have been described for the pig (Prather *et al.*, 1991, 1997*a*, *b*; Kim *et al.*, 1998; Wang *et al.*, 1998*a*, *b*), limited information is available for directly comparing the activation efficiency of various agents.

Donor nuclei can be fused with the recipient cytoplasm of matured oocytes by means of an electrical pulse, and simultaneous activation can be prevented by using a calcium-free pulse medium (Sun *et al.*, 1992). Exposure of reconstructed embryos to calcium-free medium for a period of time after fusion is thought to be necessary to prevent activation, which might promote complete exchange of nuclear and cytoplasmic proteins and subsequent reprogramming of the transplanted nuclei (Terlouw, 1993). However, the effect of time in calcium-free medium on activation of oocytes is not known.

Another technique that is frequently used during nuclear transfer is the staining of DNA with bisbenzimide (Hoechst 33342). Following staining the cells are exposed to ultraviolet (UV) light which allows

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visualisation of the MII chromosomes (Ebert *et al.*, 1985) and can increase enucleation rates to 100% (Tao *et al.*, 1999*a*, *b*). This method of visualisation has been shown to be without detrimental effects in cattle (Westhusin *et al.*, 1992) and mice (Tsunoda *et al.*, 1998). However, the effect of bisbenzimide and UV exposure on activation and development of pig oocytes has not been reported.

In this study experiments were conducted to examine the effectiveness of the following activation protocols: combined thimerosal dithiothreitol (DTT) activation, activation by the calcium ionophore A23187 followed by incubation in 6-dimethylaminopurine (6-DMAP), electrical stimulation, and electrical stimulation followed by cytochalasin B incubation. We also wanted to investigate the effect of different incubation time in calcium-free medium on activation of pig oocytes; and to identify the effect of bisbenzimide staining on development of oocytes following parthenogenetic activation. Finally, nuclear transfer embryos produced by using differentiated cells and their developmental potential following various activation protocols was investigated.

Materials and methods

Recovery and maturation of oocytes

Porcine oocytes were collected at a local slaughterhouse and transported to the laboratory at 25–29 °C in 0.9% NaCl solution containing 75 μg/ml potassium penicillin G and 50 μg/ml streptomycin sulphate. Follicles 2–5 mm in diameter were aspirated by an 18 G needle and syringe. Cumulus-enclosed oocytes were selected, rinsed three times in the maturation medium and then incubated at 39 °C for 40–42 h with 100% humidity and 5% CO_2 in air (Abeydeera *et al.*, 1998). The maturation medium was a defined medium consisting of tissue culture medium (TCM) 199 (Gibco, Grand Island, NY) supplemented with 0.1 mg/ml cysteine, 0.1% polyvinyl alcohol (PVA), 10 IU/ml eCG, 10 IU/ml hCG and 10 ng/ml EGF. It had previously been covered with paraffin oil and equilibrated in an atmosphere of 5% CO_2 in air at 39 °C.

Activation of oocytes and embryo culture

After maturation, expanded cumulus cells were removed by vigorous pipetting in the presence of 0.3 mg/ml hyaluronidase. Denuded oocytes were stained with 5 μg/ml bisbenzimide in Hepes-buffered Tyrode's (HbT) medium (Bavister *et al.*, 1983) for 30 min, and incubated in HbT supplemented with 3 mg/ml bovine serum albumin (BSA) plus 7.5 μg/ml cytochalasin B for 1 h. This is the same treatment as the oocytes routinely receive before nuclear transfer. They were then assigned to one of the following treatments: (1) Electrical stimulation (ES), consisting of a 120 V/mm DC pulse for 30 μs. Oocytes were held in the electroporation medium (0.3 M mannitol + 5% HbT stock solution) for 5 min prior to the DC pulse and were kept in the same medium for 2 min after the pulse before being transferred to the culture medium. (2) ES followed by incubation with 7.5 μg/ml cytochalasin B for 6 h. (3) Exposure to 200 μM thimerosal for 10 min, followed by incubation in the presence of 8 mM DTT for 30 min. (4) Exposure to 5 μM calcium ionophore A23187 for 5 min, followed by incubation with 2.5 mM 6-dimethylaminopurine (6-DMAP) for 3.5 h. Non-treated oocytes were used as control.

After activation, oocytes were cultured in NCSU-23 medium supplemented with 4 mg/ml BSA and 0.1 mg/ml cysteine at 39 °C in a humidified atmosphere containing 5% CO_2 in air for 6 days (Macháty *et al.*, 1998). The percentage of embryos that developed to the 2-cell stage (cleavage rate) was checked 24 h after activation, while the proportion of embryos that reached the compact morula and blastocyst stage was assessed on day 6 of development.

Intracellular calcium measurements

Intracellular free calcium concentration ($[Ca^{2+}]_i$) was measured using the calcium indicator dye fura-2 (Molecular Probes, Eugene, OR) based on previous reports (Macháty *et al.*, 1997*a*, *b*). Oocytes were incubated in the presence of 2 μM of the acetoxymethyl ester (AM) form of the dye together with 0.02% pluronic F-127 in HbT medium. After the dye was loaded, thimerosal was added to individual oocytes at a final concentration of 200 μM. Fluorescence recordings were performed using a Photoscan-2 photon-counting fluorescence microscope system (Nikon, Tokyo, Japan). The oocytes were alternatively illuminated with 340 and 380 nm wavelength light from a xenon arc lamp. The emitted light was passed through a 510 nm barrier filter and its intensity was measured with a photomultiplier tube after background subtraction. $[Ca^{2+}]_i$ levels are indicated by the fluorescent ratio (R) values of the 340/380 nm excitation wavelengths. Ratios of 1.2 and 6.5 were estimated to correspond to $[Ca^{2+}]_i$ of 65 nM and 602 nM, respectively.

Nuclear transfer

Nuclear transfer was performed according to the procedures reported previously (Tao *et al.*, 1991*a*). Briefly, the donor cells used for nuclear transfer were fetal fibroblast cells between passages 2 and 8 of culture, which were synchronised in presumptive G_0 stage by serum deprivation. Mature oocytes were stained with

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5 μg/ml bisbenzimide in HbT medium supplemented with 3 mg/ml BSA for at least 30 min and enucleated in HbT supplemented with 3 mg/ml BSA and 7.5 μg/ml cytochalasin B. Enucleation was accomplished by removing the first polar body and the metaphase II plate together with a small amount of surrounding cytoplasm using a glass pipette (outer diameter 25–30 μm). Confirmation of successful enucleation was achieved by visualising the karyoplast, while still inside the pipette, under UV light. Following enucleation one fetal fibroblast cell was transferred into each enucleated oocyte. The cells were injected into the perivitelline space through the same slit in the zona pellucida as made during enucleation. After micromanipulation, embryos were placed in NCSU-23 medium supplemented with 4 mg/ml BSA and 0.1 mg/ml cysteine for 30–60 min. They were then placed between 200 μm diameter platinum electrodes in a solution of calcium-free 0.3 M mannitol for 2 min. Cell fusion was induced with a DC pulse of 160 V/cm lasting for 40 μs. In the case of oocytes where fusion did not occur, a second DC pulse was applied 30 min later. After the electrical pulse, oocytes were left in 0.3 M mannitol solution for 30 min. They were then activated by electrical stimulation in calcium-containing medium or by the combined thimerosal/DTT treatment.

Assessment of nuclear status

To verify the success of oocyte activation, the rate of pronuclear formation was determined. For this purpose some oocytes 12 h after activation were mounted on slides under posted coverslips and fixed in methanol:acetic acid (3:1) for 24 h. In embryos reconstructed by nuclear transfer, the degree of condensation of the donor cell chromosomes (1 h after fusion) and the rate of nuclear formation (12 h after activation) were assessed. These embryos were also fixed in the same solution. Fixation was followed by staining with 1% (w/v) aceto-orcein and evaluation using Hoffman modulation contrast optics at a magnification of ×200.

Experiments

Experiment 1

The effect of the different activation methods on oocyte activation was investigated. The oocytes were activated by one of the methods mentioned above, and the rates of pronuclear formation, cleavage at 24 h post-activation, and the development to the compact morula/blastocyst stage at 6 days post-activation were recorded in each group. Non-treated oocytes were used as negative controls.

Experiment 2

The effect of incubation time in Ca^{2+}-free medium on oocyte activation was examined. Prior to activation, oocytes were exposed to Ca^{2+}-free HbT medium for 0, 1, 4 or 8 h. Activation was accomplished by the combined thimerosal/DTT method. Results from each group were compared as described in experiment 1. To further investigate the effect of Ca^{2+}-free medium on oocyte activation, some of these oocytes were used for $[Ca^{2+}]_i$ measurements. They were incubated in Ca^{2+}-free HbT medium for 0, 1, 4 or 8 h, and the changes in $[Ca^{2+}]_i$ were monitored after thimerosal treatment.

Experiment 3

In this experiment, the effect of bisbenzimide staining on oocyte activation was studied. Oocytes were exposed to 5 μg/ml bisbenzimide in HbT medium for 0, 0.5, 1 or 2 h. Activation was accomplished by exposure to thimerosal/DTT. In these groups, the rate of cleavage (24 h post-activation), the rate of embryo development to the compact morula or blastocyst stage (after 6 days of culture), and the quality of blastocysts were compared. Blastocysts were classified as follows into three categories according to Nagashima *et al.* (1989); category I (excellent), typical blastocysts with very few (< 5%) degenerated cells and a clearly visible inner cell mass (ICM); category II (fair), blastocysts of a smaller diameter than in group I with a large number (5–10%) of degenerated cells and ICM not clearly visible; category III, small blastocysts without a visible ICM and a large number (> 10%) of degenerated cells or some large non-integrated blastomeres. All blastocysts were stained with bisbenzimide (5 μg/ml) and the number of nuclei was determined (Pursel *et al*., 1985).

Experiment 4

In this set of experiments the efficiency of two different activation protocols after nuclear transfer was compared. The two activation methods, thimerosal/DTT and electrical stimulation, were chosen based on preliminary results from experiment 1. Half the reconstructed embryos were fixed to observe the degree of condensation of donor cell chromosomes at 1 h after fusion; the other half was fixed 12 h after activation to determine whether nuclear formation took place.

Statistical analysis

The effects of different methods on activation of pig nuclear transfer embryos were compared using chi-square analysis. Differences were considered to be significant at $p < 0.05$. Differences between the mean values of transient amplitudes and time leading up to the first transient were determined by calculating protected least-significant differences using Multivariate General Linear Hypothesis of Systat (Wilkinson, 1990).

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Results

Experiment 1: Effect of different methods of activation on pig oocytes

Oocytes were subjected to thimerosal/DTT, ES, ES/cytochalasin B, A23187/6-DMAP treatments to compare the activation rates and parthenogenetic development. As shown in Table 1, there were no significant differences in the rate of activation and the rate of development to the 2-cell stage among the four treatments. In all the treatment groups, the rates of both activation (70.0–88.3%) and cleavage (48.8–58.8%) were significantly higher than those in the oocytes (8.3% activation rate; 7.1% cleavage rate). In oocytes treated with ES/cytochalasin B or thimerosal/DTT, the rate of activated oocytes with two pronuclei was significantly higher compared with oocytes treated with A23187/6-DMAP ($p < 0.05$). The rate of development to the blastocyst stage in oocytes treated with thimerosal/DTT (10.0%) or ES/cytochalasin B (12.3%) was significantly higher ($p < 0.05$) than in those treated with A23187/6-DMAP (2.5%). No significant differences in the rate of development to the compact morula stage were found among oocytes treated with A23187/6-DMAP (30.0%), thimerosal/DTT (24.1%) and ES (22.4%). Spontaneous activation of the non-treated control oocytes occurred in 8.3% of the cases; this rate is significantly lower than the activation rate in other groups ($p < 0.05$).

Experiment 2: Effect of incubation time in Ca^{2+}-free medium on pig oocyte activation

The activation rate in oocytes incubated in Ca^{2+}-free medium for 8 h before activation by thimerosal/DTT was significantly lower ($p < 0.05$) than that in oocytes incubated for 0 h, 1 h or 4 h (Table 2). The rate of oocytes with two pronuclei in the control group (0 h) was significantly higher ($p < 0.05$) than that in oocytes incubated in Ca^{2+}-free medium for 1, 4 or 8 h. On the other hand, the rate of oocytes with one pronucleus in the control group was significantly lower ($p < 0.05$) than in oocytes incubated in Ca^{2+}-free medium for 1 or 4 h. Incubation of oocytes in Ca^{2+}-free medium for 8 h before activation negatively affected both activation and subsequent development. These oocytes had a significantly lower rate of pronuclear formation ($p < 0.05$) and none of them developed to the blastocyst stage after activation. There was no significant difference in the cleavage rates and the rates of development to compact morula or blastocyst stages between the 0, 1 and 4 h groups.

The changes in $[Ca^{2+}]_i$ in oocytes treated with 200 μM thimerosal after being incubated in Ca^{2+}-free medium for 0, 1, 4 or 8 h are shown in Fig. 1. Thimerosal induced an increase in $[Ca^{2+}]_i$ in all oocytes but incubation in Ca^{2+}-free medium had a marked effect on the oocytes' response. The transient triggered by thimerosal in the control oocytes had a mean amplitude of $R = 4.2 \pm 0.2$. The amount of Ca^{2+} released from the intracellular stores was significantly smaller ($p < 0.05$) after incubation in Ca^{2+}-free medium; after 8 h incubation the mean amplitude of the Ca^{2+} spike was only $R = 2.7 \pm 0.2$. The time leading up to the first transient was also affected by the Ca^{2+}-free medium: it became increasingly longer with longer incubation times. In control oocytes, the first transient was detected after 568.0 ± 49.2 s, while after 8 incubation in Ca^{2+}-free medium it was 1415.2 ± 49.2 s (Table 3).

Table 1 Effect of different activation methods on the activation and subsequent development of *in vitro* matured pig oocytes

Treatment	No. of oocytes stained	No. (%) of activated oocytes			No. of oocytes cultured	No. (%) developed to:		
		Total	With 1 PN	With 2 PN		2-cell	Compact morula	Blastocyst
Thi+DTT	60	50 (83.3)^{a}	16 (26.7)^{a}	34 (56.7)^{a}	170	100 (58.8)^{a}	41 (24.1)^{a,b}	17 (10.0)^{a}
ES	60	53 (88.0)^{a}	29 (48.3)^{a,b}	24 (40)^{a,b}	170	89 (52.4)^{a}	38 (22.4)^{a,c}	16 (9.4)^{a,b}
ES+Cyt. B	60	52 (86.6)^{a}	13 (21.7)^{a}	39 (65.0)^{a}	162	74 (45.7)^{a}	20 (12.3)^{a}	20 (12.3)^{a}
A23187/6-DMAP	60	42 (70.0)^{a}	35 (58.3)^{b}	7 (11.7)^{b}	160	78 (48.8)^{a}	48 (30.0)^{a}	4 (2.5)^{b,c}
Control	60	5 (8.3)^{b}	5 (8.3)^{a}	0 (0.0)^{b}	154	11 (7.1)^{b}	0 (0.0)^{c}	0 (0.0)^{c}

^{a,b,c}Values with different superscript letters within columns are significantly different, $p < 0.05$.
Thi, thimerosal; DTT, dithiothreitol; ES, electrical stimulation; Cyt. B, cytochalasin B; A23187, calcium ionophore; 6-DMAP, 6-dimethylaminopurine; 1 PN, one pronucleus; 2 PN, two pronuclei.

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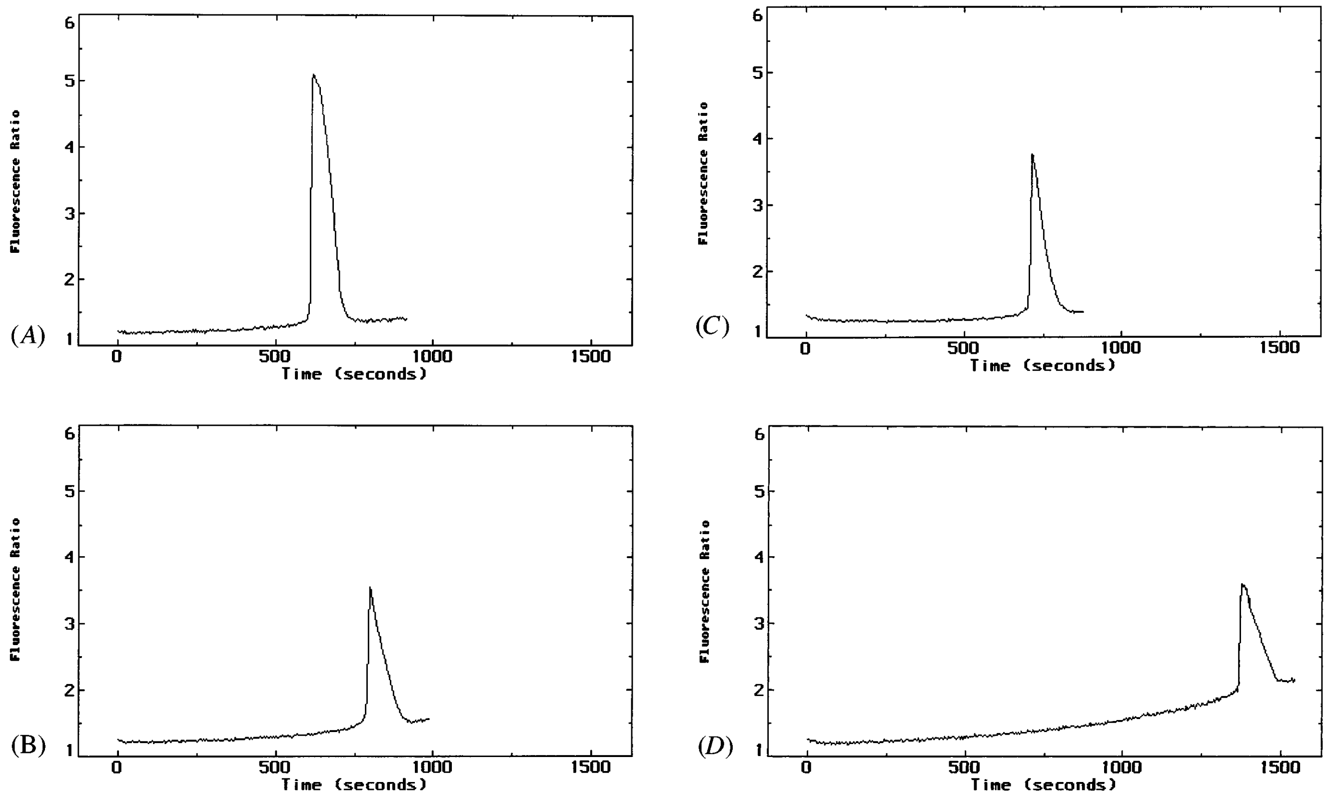

Figure 1 Intracellular Ca^{2+} rises elicited by the addition of thimerosal in pig oocytes. The oocytes were cultured in Ca^{2+}-free medium for 0 h (*A*), 1 h (*B*), 4 h (*C*) or 8 h (*D*) before fluorescent recordings. Each curve represents one oocyte.

Table 2 Effect of culture time in calcium-free medium on pig oocyte activation induced by thimerosal/DTT

Time (h)	No. of oocytes stained	No. (%) of activated oocytes			No. of oocytes cultured	No. (%) developed to:		
		Total	With 1 PN	With 2 PN		2-cell	Compact morula	Blastocyst
0	35	30 (85.7)[(a)]	9 (25.7)[(a)]	21 (60.0)[a]	85	50 (58.8)[a]	22 (25.9)	10 (11.8)[a]
1	35	29 (82.9)[a]	21 (60.0)[b]	8 (22.9)[b]	85	65 (76.5)[a]	18 (21.2)	11 (12.9)[a]
4	35	29 (82.9)[a]	20 (57.1)[b]	9 (25.7)[b]	85	48 (56.5)[a,b]	18 (21.2)	9 (10.9)[a]
8	35	15 (42.9)[b]	7 (20.0)[a]	8 (22.9)[b]	85	23 (27.1)[b]	8 (9.4)	0[b]

[a,b]Values with different superscript letters within columns are significantly different, $p < 0.05$.
1 PN, one pronucleus; 2 PN, two pronuclei.

Experiment 3: Effect of bisbenzimide staining on oocyte activation and development

The effect of staining time with bisbenzimide on parthenogenetic development of pig oocytes is displayed in Table 4. Exposure of oocytes to 5 μg/ml bisbenzimide for 2 h significantly decreased the activation rate and the rate of development to the blastocyst stage, and increased the rate of degenerated embryos at day 6 ($p < 0.05$). Moreover, increasing staining time resulted in lower blastocyst quality as more blastocysts fell into category I in the control and 0.5 h groups than in the other groups where oocytes were stained for 1 and 2 h before activation ($p < 0.05$).

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Table 3 Characteristics of Ca^{2+} responses of oocytes after being incubated in Ca^{2+}-free medium for various periods of time

Incubation time (h)	Peak *R*	Time (in s) until the first transient (mean ± SEM)
0 (control)	4.2 ± 0.2^{a}	568.0 ± 49.2^{a}
1	3.6 ± 0.2^{b}	799.7 ± 49.2^{b}
4	$3.3 \pm 0.2^{b,c}$	843.1 ± 49.2^{b}
8	2.7 ± 0.2^{c}	1415.2 ± 49.2^{c}

^{a,b,c}Values with different superscript letters within columns are significantly different, $p < 0.05$.

Experiment 4: Activation of oocytes after nuclear transfer

Enucleated porcine oocytes were fused with fetal fibroblast cells and activated using two different activation protocols (thimerosal/DTT and ES). The rate of chromosome condensation was 45.6% (26/57) in the thimerosal/DTT treated group, while that in the ES oocytes was 42.9% (18/42). There was no significant difference in nuclear formation (36.8% vs 31.0%) between the two groups. The percentage of oocytes with an interphase nucleus 12 h after activation was 36.8% (21/57) in the thimerosal/DTT activated group and 31.0% (13/42) in the group that was activated with an electrical pulse. The difference is not statistically significant.

Discussion

Chemical co-incubation and electroporation are commonly used to induce activation of oocytes following nuclear transfer. In the present study we tested four different methods for their effectiveness to stimulate oocyte activation and subsequent development. We found that the rate of pronuclear formation after activation was similar in the four treatment groups. However, development to the blastocyst stage after treatment with A23187/6-DMAP was significantly lower compared with other treatments. Parthenogenetic activation with calcium ionophore has been extensively studied in many species (Steinhardt *et al.*, 1974; Hagen *et al.*, 1991; Hoshi *et al.*, 1992; Soloy *et al.*, 1997), and the activation results reported vary dramatically. Under our experimental conditions it was the least effective way to trigger embryonic development of porcine oocytes.

We also found different patterns of pronuclear formation following the various activation protocols. Most oocytes had two pronuclei in the ES/cytochalasin B and thimerosal/DTT groups, while only one pronucleus was observed in the A23187/6-DMAP group. Cytochalasin was reported to inhibit extrusion of the second polar body: in the presence of cytochalasin, segregation of the chromosomes occurred but cytokinesis did not take place (Presicce & Yang, 1994*a*, *b*; Liu *et al.*, 1998). This resulted in diploid zygotes with two pronuclei. In our experiment the percentage of electrostimulated oocytes having two pronuclei was also higher following incubation in cytochalasin. The protein kinase inhibitor 6-DMAP, on the other hand, is known to cause the second meiotic spindle to disintegrate and the oocytes to pass directly into interphase (Navara *et al.*, 1994). As a result the oocytes will form one diploid pronucleus. In accordance with this we also found that most oocytes activated by A23187/6-DMAP treatment had only one pronucleus.

It was reported previously that combined thimerosal/DTT treatment also causes activation of pig oocytes (Macháty *et al.*, 1997*a*). In this study most oocytes had two pronuclei following activation with thimerosal/DTT. The rate of pronuclear formation was somewhat higher than reported previously; this may

Table 4 Effect of time of bisbenzimide (Hoechst 33342) staining on development of activated pig oocytes

Time (h)	No. of oocytes	No. (%) cleaved at 24 h	No. (%) developed to blastocyst: Category I	Category II	Category III	Total	Nuclear number in blastocysts (mean ± SEM)	No. (%) degenerate embryos
0	189	77 (40.7)	19 $(63.3)^{a}$	8 (26.7)	3 (10.0)	30 $(15.9)^{a}$	17.8 ± 1.1	48 $(25.4)^{a}$
0.5	185	80 (43.2)	14 $(51.9)^{a,c}$	9 (33.3)	4 (14.8)	27 $(14.6)^{a}$	18.0 ± 1.2	45 $(24.3)^{a}$
1	174	76 (43.7)	9 $(33.3)^{b}$	12 (44.4)	6 (22.2)	27 $(15.5)^{a}$	16.1 ± 1.2	48 $(27.6)^{a}$
2	179	64 (35.8)	4 $(36.4)^{b,c}$	6 (54.5)	1 (9.0)	11 $(6.1)^{b}$	14.9 ± 1.9	66 $(36.9)^{b}$

^{a,b,c}Values with different superscript letters within columns are significantly different, $p < 0.05$.

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be due to the different culture conditions used for oocyte maturation. Recently, a chemically defined system for *in vitro* production of pig embryos has been developed in our laboratory (Prather & Day, 1998), and this was used during the experiments described here. It was demonstrated that cytoplasmic maturation significantly improved by using a defined medium (Abeydeera *et al.*, 1998). Various cytoplasmic factors may affect the response of oocytes to activation stimulators. This observation suggests that oocytes matured in this defined medium may be more competent for activation due to an enhanced cytoplasmic maturation.

Artificial activation of oocytes to resume meiosis can be accomplished with the same electrical pulse that is applied to induce fusion of the membranes of the oocyte and the nuclear donor cell. Studies have shown, however, that when the donor cell is in the G_0 stage of its cell cycle, it is advantageous to activate the oocytes after the cell fusion (Wells *et al.*, 1998, 1999). Exposure of reconstructed embryos to Ca^{2+}-free medium for a period before activation is thought to be necessary to prevent activation and thus facilitate nuclear-cytoplasmic exchange of proteins and maintain a high level of maturation promoting factor (MPF) in the cytoplasm. This can result in nuclear envelope breakdown and reconfiguration of the structure of the nucleus such that RNA synthesis is altered. In the present study there was no difference between the activation rate of control oocytes and that of oocytes exposed to Ca^{2+}-free conditions for up to 4 h. A 4 h exposure is thought to be long enough to facilitate the protein exchange necessary for nuclear reprogramming and seems not to have a negative effect on pronuclear formation. However, incubation for 8 h under such conditions prevented development to the blastocyst stage.

Although the rate of activation did not decrease in oocytes that were incubated in Ca^{2+}-free medium for 1–4 h, their Ca^{2+} homeostasis was affected by the culture. While a Ca^{2+} transient was also observed following thimerosal stimulation in these oocytes, the amplitude of the transients was smaller and the calcium transients were delayed compared with the control oocytes. This was most obvious in oocytes that were incubated in Ca^{2+}-free medium for 8 h before activation, where the release of Ca^{2+} from the intracellular stores was delayed by about 10 min. It is known that Ca^{2+} transients are associated with the initiation of activation in the hamster (Miyazaki *et al.*, 1992), mouse (Kline & Kline, 1992), cow (Fissore *et al.*, 1992), rabbit (Fissore & Robl, 1992) and pig (Sun *et al.*, 1992). Treatment with Ca^{2+}-free medium for 1 or 4 h resulted in most of activated oocytes having one pronucleus while the majority of control oocytes possessed two pronuclei. These results indicate that a sufficient increase in $[Ca^{2+}]_i$ is necessary to maintain the correct ploidy of the oocytes after activation. Ca^{2+} signalling has been suggested to release the connection between chromosomes prior to anaphase in somatic cells (Poenie *et al.*, 1986; Ratan *et al.*, 1986), and Ca^{2+} is necessary and sufficient to stimulate the transition from metaphase II to anaphase II (Tombes & Borisy, 1989; Vitullo & Ozil, 1992).

The chromatin in mammalian oocytes and early embryos can be visualised using the fluorescent dye bisbenzimide (Critser & First, 1986; Luttmer & Longo, 1986; Conover & Gwatkin, 1988; Tsunoda *et al.*, 1988). Although bisbenzimide is readily permeable for the cell and provides bright fluorescence to the genetic material upon UV exposure, it can irreversibly modify nucleic acid and prevent normal configurational changes during mitosis and meiosis (Albertini, 1984). This fluorescent dye specifically binds to the adenine and thymine bases of DNA. The present study clearly indicated that exposure of MII oocytes to bisbenzimide for over 1 h reduces the rate of blastocyst formation, and even the blastocysts that formed after the 1 h exposure were of lower quality. This is in contrast to the results reported using bovine oocytes (Westhusin *et al.*, 1992) and should be taken into consideration during visual enucleation.

In the present study, the chromatin staining demonstrated that both electrical stimulation and treatment with thimerosal/DTT resulted in progression of pig nuclear transfer embryos to first interphase. Once the chromosomes of the donor fetal fibroblast nuclei condensed within the cytoplasm of recipient oocytes, a high percentage (80.8%, 21/26) of the reconstructed embryos formed pronuclei; this rate is similar to the rate of pronuclear formation in activated mature pig oocytes. This fact indicates that the activation methods applied in these experiments worked efficiently.

Recently the interest in the field of porcine nuclear transfer has increased tremendously. Although much work has been done to improve the efficiency of the technology, it still has not resulted in pigs produced by nuclear transfer using differentiated cell lines. The information obtained through the present experiments might be useful in that effort.

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