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PRODUCTION OF PARTHENOGENETIC PORCINE EMBRYOS

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INTRODUCTION

Although it has been a decade since the birth of the first animal produced from an adult somatic cell, the efficiency of nuclear transfer is still extremely low which hampers the large-scale production of genetically identical animals. According to a recent report, only approximately 1 to 4 % of the embryos reconstructed by nuclear transfer are able to develop to term. In order to enhance productivity, all consecutive steps of the technology need to be improved. Activation of the developmental program of the reconstructed oocyte and providing appropriate culture conditions for the developing embryo are critical for the generation of viable embryos that are capable of full-term development after transfer into surrogate females.

In vertebrate oocytes, meiosis consists of two successive cell divisions that are under the control of the M-phase promoting factor (MPF). MPF was first identified as an activity in the cytoplasm of mature *Rana pipiens* oocytes that was able to initiate maturation after injection into immature oocytes. Later it was shown to be a heterodimer of two subunits: a catalytic subunit, cyclin-dependent kinase 1 (cdk1), and a regulatory subunit, cyclin B. Cdk1 is homologous to the cell division control cdc2 gene product identified in the fission yeast, while cyclin B was demonstrated to belong to a family of proteins involved in the progression of cells through the cell cycle. In immature oocytes, cdc2 binds to cyclin B; at this point MPF is catalytically inactive due to inhibitory phosphorylation of cdc2 on threonine 14, tyrosine 15 and threonine 161 residues. The kinases capable of phosphorylating cdc2 have been identified in frog oocytes (CAK and Myt1) and in yeast (Wee1). MPF gains kinase activity at germinal vesicle break down when the dual-specificity phosphatase cdc25 dephosphorylates cdc2 on threonine 14 and tyrosine 15. Active MPF will then drive cell cycle progression into metaphase I. Exit from MI (like that from MII) is controlled by the anaphase promoting complex (APC), a multi-subunit E3 ligase that ubiquitinates cyclin B, marking it for degradation by the 26S proteasome. The degradation of cyclin B leads to a partial inactivation of MPF which will then facilitate exit from meiosis I and extrusion of the first polar body. A subsequent increase in cyclin B synthesis will generate active MPF again that will drive the cell cycle into meiosis II. Sustained MPF activity then keeps the chromatin in a condensed state and stabilizes the meiotic spindle.

The other signal transduction pathway crucial for the regulation of meiosis is the mitogen-activated protein kinase (MAPK) pathway. MAPK is a serine/threonine protein kinase that, together with its downstream substrates, is believed to stabilize MPF and facilitate the MPF-driven process of progression into meiosis. Shortly after the signal for maturation is initiated, translation of maternal c-mos mRNA begins. c-mos is a proto-oncogene whose product is the MOS protein, a 39-kD germ cell-specific serine-threonine kinase. MOS functions as a MAPK kinase kinase (MEKK) and activates MAPK kinase (MEK1) that in turn activates MAPK through phosphorylation. Active MAPK phosphorylates and thus activates the serine/threonine kinase $p90^{Rsk}$ which then inhibits the inhibitory kinase Myt1, thus facilitating cdc25-mediated MPF activation. Thus the MOS/MEK1/MAPK/$p90^{Rsk}$ cascade directly aids in the activation and stabilization of MPF during G2/M transition at the time of maturation. In addition, the cascade is believed to activate spindle assembly checkpoint proteins which inhibit APC, thus slowing down the rate of cyclin B degradation and preventing metaphase/anaphase transition in the mature oocyte.

Under physiological conditions it is the fertilizing sperm that triggers the resumption of meiosis and entry into interphase by inducing an oscillation in the oocyte's intracellular free calcium concentration. The primary role of the fertilization calcium signal is to downregulate cdk1 and cyclin B; the concomitant decrease in MPF activity then leads to dephosphorylation and subsequent degradation of MOS. Increasing the intracellular free calcium concentration by artificial means can also stimulate meiotic resumption. As opposed to fertilization, most parthenogenetic activation methods are able to generate only a single calcium transient in the oocyte. In mouse oocytes a single calcium transient was insufficient and led to the re-activation of MPF and MAPK. For this reason, parthenogenetic activation methods are clearly needed that can induce all the fertilization-associated changes and stimulate embryonic development with high efficiency. The reduced activities of these crucial cell cycle-related protein kinases are used as an indicator for the success and efficacy of oocyte activation protocols.

For effective *in vitro* embryo production it is also essential to ensure proper culture conditions for the developing embryo. NCSU-23 has been widely used and is one of the most successful media for culture of porcine embryos, but according to recent reports, PZM-3 was superior to NCSU-23 medium in supporting development

of embryos to the blastocyst stage. Another important aspect of embryo culture is the gas composition of the culture environment. In conventional incubators approximately 20% O_2 is used for the developing embryo. However, it has been reported that the O_2 tension in the reproductive tracts of rhesus monkeys, hamsters, and rabbits was between 1.5 and 8.7%. There are indications that a reduced O_2 concentration may be beneficial for development of porcine embryos, however the reports are controversial.

OBJECTIVES

The aim of this study was to investigate the effects of different parthenogenetic activation methods and various culture systems on the development of *in vitro*-matured porcine oocytes. We also evaluated the dynamics of the activity of cell cycle-related regulatory proteins such as MPF and MAPK in the oocytes following different activation methods.

MATERIALS AND METHODS

Retrieval and in vitro maturation of oocytes

Ovaries of prepubertal gilts were collected at the local slaughterhouse and transported to the laboratory. Ovarian follicular fluid was aspirated from antral follicles by using a 10 ml disposable syringe and 20-gauge needle and collected in a 50 ml centrifuge tube. After sedimentation, cumulus-oocyte complexes (COC) were recovered under a stereo microscope. Oocytes with several compact layers of cumulus cells were selected for *in vitro* maturation. COC were transferred into 500 µl of TCM-199 medium supplemented with 26 mM sodium bicarbonate, 3.05 mM glucose, 0.91 mM sodium pyruvate, 0.57 mM cysteine, 10 ng/ml epidermal growth factor, 0.5 IU/ml porcine LH, 0.5 IU/ml porcine FSH, 0.1% (w/v) polyvinyl alcohol (PVA), 75 µg/ml penicillin G and 50 µg/ml streptomycin covered with 400 µl of mineral oil in a four-well Nunc dish (Nunc, Roskilde, Denmark) and cultured for 42 to 44 h at 39º C, 5% CO_2 in air.

Activation of oocytes

After maturation, cumulus cells were removed by vortexing of the COC for 8 minutes in Tyrode's lactate (TL)-Hepes medium supplemented with 0.1% (w/v) PVA

and 0.1% (w/v) hyaluronidase. Oocytes with uniform ooplasms and visible first polar bodies were selected and activated. The oocytes were transferred into electroporation medium (300 mM mannitol, 0.1mM $CaCl_2$, 0.1mM $MgSO_4$, 0.5 mM Hepes, 0.01 mg/ml BSA) and washed twice. They were then placed into a chamber consisting of two stainless steel electrodes 0.5 mm apart. An Electro Cell Manipulator (BTX Instrument Division, Harvard Apparatus, Inc., Holliston, MA) was used for electrical stimulation. Two subsequent direct current (DC) pulses of 120 V/mm, 60 µsec each were applied after a brief exposure to an alternating current (AC) of 4 V/mm. After electroporation, the oocytes were randomly allocated into different treatment groups. In the first group, the electroporated oocytes were incubated in the presence of 7.5 µg/ml cytochalasin B for 4 hours. In group 2, the oocytes were incubated in culture medium supplemented with butyrolactone I, a specific blocker of cdc2 and cdk2 kinases (conc. 100 µM) and 7.5 µg/ml cytochalasin B, for 4 hours. In group 3, electroporated oocytes were placed into culture medium containing 10 µg/ml cycloheximide (a protein synthesis inhibitor) and 7.5 µg/ml cytochalasin B, for 5 hours. Following incubation, the oocytes in each group were washed four times in the appropriate culture medium and cultured in 20 µl drops of culture medium for 7 days at 39º C; the additional culture parameters regarding media and gas composition are described below.

Evaluation of pronuclear formation

Six to ten hours after electroporation, the oocytes were mounted on slides under posted coverslips and fixed in ethanol-acetic acid (3:1). Four days later, the oocytes were stained with 1% (w/v) aceto-orcein and the chromatin configuration was examined under a Nikon Eclipse 55i phase-contrast microscope at 400x magnification.

TUNEL assay

At the end of the 7-day culture period, the blastocysts were fixed overnight in 3.7% paraformaldehyde at 4º C, washed twice in phosphate buffered saline (PBS), placed in PBS supplemented with 0.1% Triton X-100 and 0.1% sodium citrate for 2 minutes and transferred to TUNEL labeling medium (Roche Diagnostics GmbH, Mannheim, Germany) for 1 h at 39ºC. After labeling the blastocysts were stained with Hoechst 33342 for 10 minutes. The number of apoptotic nuclei and the number of

total nuclei were counted using a NIKON Eclipse TE2000-U microscope with epifluorescence attachment.

MPF assay

At one hour intervals during the first 6 hours following electroporation, groups of 10 oocytes from each treatment group were removed from culture and assayed. These oocytes were washed in phosphate buffered saline (PBS) supplemented with 0.1% (w/v) PVA, placed into a 0.5 ml centrifuge tube in 4 µl CelLytic™ M detergent solution and kept frozen at -80°C until use. For determination of MPF activity, the cdk1/cdc2 kinase assay kit (Upstate Biotechnology Inc., Lake Placid, NY) was used. The assay is based on phosphorylation of a specific substrate (histone H1) using the transfer of the γ-phosphate of adenosine-5'-[^{32}P] triphosphate ([γ-^{32}P] ATP) by cdk1/cdc2. The assay was carried out according to the manufacturer's directions. Briefly, solutions containing the oocytes were incubated with 10 µl of histone H1 solution (conc. 2 mg/ml) containing 20 mM MOPS, pH 7.2, 25 mM β-glycerol phosphate, 5 mM EGTA, 1 mM sodium orthovanadate, and 1 mM dithiothreitol. In each case the reaction mix was supplemented with 20 µM protein kinase C inhibitor, 2 µM protein kinase A inhibitor and 20 µM Compound R24571, an inhibitor of calcium/calmodulin-dependent protein kinases to reduce interference from other serine/threonine kinases. The reaction was started by the addition of 10 mCi of [γ-^{32}P] ATP (~3000 Ci/mmol, Amersham Biosciences Corp., Piscataway, NJ) in buffer containing 75 mM $MgCl_2$ and 500 µM ATP. After 10 minutes at 30°C, an aliquot of the reaction mixture was removed and blotted onto phosphocellulose paper. The papers were washed thoroughly three times in 0.75% phosphoric acid and once in acetone. They were then placed into 5 ml of scintillation fluid and radioactivity was measured in a scintillation counter. After accounting for nonspecific binding of [γ-^{32}P] ATP and endogenous phosphorylation of proteins in the sample, results were expressed as pmol phosphate incorporated into histone per oocyte per minute (pmol/oocyte/min). The assay was replicated six times.

MAPK assay

MAPK activity in oocytes was determined using the MAP kinase/Erk assay kit (Upstate Biotechnologies, Inc.). This assay uses the Myelin Basic Protein as the substrate for MAPK; the protocol is essentially the same as that described above for

the determination of MPF activity. The data presented show the combined results of a total of six replications.

Experiment 1

In Experiment 1, the effects of the three different activation methods and two culture media (NCSU-23 and PZM-3) were tested in a 3x2 factorial design. The oocytes were randomly allocated into a total of six groups: in the case of each culture medium an electroporation (EP) only, an electroporation + butyrolactone I (EP+BL) and an electroporation + cycloheximide (EP+CHX) group were prepared. As described previously, the initial culture in each group took place in the presence of 7.5 μg/ml cytochalasin B to ensure the formation of diploid embryos. The activated oocytes were incubated for 7 days in an atmosphere of 5% CO_2 in air. The percentage of zygotes (as determined on a subset of embryos having pronuclei), the percentage of cleaved embryos and blastocysts formed were recorded in each group. The experiment was replicated five times.

Experiment 2

In Experiment 2, the effect of O_2 tension on the development of parthenogenetic porcine embryos incubated in different culture media was evaluated in a 2x2 factorial design. The retrieval and in vitro maturation of oocytes were carried out as described above. The most effective activation method was selected based on the results of experiment 1: the oocytes were electroporated and after being allocated into two groups they were incubated in either NCSU-23 or PZM-3 medium supplemented with 100 μM butyrolactone I and 7.5 μg/ml cytochalasin B. After 4 h of incubation the oocytes in each group were subdivided randomly and cultured for 7 days under ~20% O_2 (5% CO_2 in air) or 5% O_2 (5% CO_2, 5% O_2 and 90% N_2). This experiment was replicated five times.

Experiment 3

In Experiment 3, the effect of different oocyte activation methods on the dynamics of MPF and MAPK activity in porcine oocytes were examined. The retrieval, maturation and activation of oocytes were carried out as described above. The oocytes were randomly allocated into three groups: an electroporation (EP) only, an electroporation + butyrolactone I (EP+BL) and an electroporation + cycloheximide

(EP+CHX) group were prepared. For the MPF and MAPK assays, pools of 10 oocytes from each treatment group were collected at 0, 1, 2, 3, 4, 5, and 6 hours after electroporation and used for the experiment.

Statistical analysis

The data were subjected to analysis of variance (ANOVA) using the PROC MIX procedure of the Statistical Analysis System (SAS Institute, Cary, NC, USA). Percentage data were transformed by arc sine transformation before ANOVA analysis. Differences among treatment means were analyzed using the Tukey test; differences with $p<0.05$ was considered significant.

RESULTS

Experiment 1: Effects of activation methods and culture media on development of parthenogenetic porcine embryos

In this experiment the developmental potentials of oocytes activated by three different activation methods and cultured in two different media (NCSU-23 and PZM-3) were compared. Different activation treatments had effected pronuclear formation ($p<0.05$; Table 1). The mean percentage of oocytes having pronuclei after electroporation was 83.9±1.7%; this value was less compared to oocytes activated by EP+BL (92.8±0.8) or EP+CHX (93.0±1.0%). The media, however, had no statistically significant effect on pronuclear formation, the media means for the oocytes forming pronuclei were 89.9±1.4% (NCSU-23) and 89.8±1.6% (PZM-3; $p>0.1$). The frequency of cleaved embryos was similar in most groups; the treatments or media had little effect on this variable (Table 1). However, data analysis within media revealed that in PZM-3 the EP+CHX group had greater ($p<0.05$) development to the two-cell stage than the EP-only group.

When analyzing the frequencies of blastocyst formation, culture media affected development to the blastocyst stage with a greater proportion of embryos reaching the blastocyst stage in PZM-3 medium (59.7±3.6%) as compared to NCSU-23 (39.9±3.1%; $p<0.05$; Table 1). The activation method itself as a main factor was without effect. However, while in NCSU-23 the percentage of blastocyst formation was similar in all three groups, in PZM-3 medium the oocytes that received the

EP+BL or EP+CHX activation treatments had greater development to the blastocyst stage than those activated by electroporation alone. At the end of the 7-day culture period, the frequency of hatching in PZM-3 medium (10.6±1.3%) was also greater than in NCSU-23 (0.2±0.2%; $p<0.001$).

There was also a media effect for average nuclear number of developing blastocysts ($p<0.001$; Table 1). The blastocyst stage embryos that developed in PZM-3 had a greater mean nuclear number (50.2±1.3) than those cultured in NCSU-23 (35.3±1.1). Activation effects were not observed; the number of nuclei per blastocyst did not differ within media. Furthermore, there was no difference in the percentage of apoptotic nuclei per blastocyst between the various treatment groups, the proportion of nuclei showing signs of DNA fragmentation as detected by the TUNEL assay was between 4.7 and 5.3% in all groups.

Experiment 2: Effect of O_2 tension and culture media on development of parthenogenetic porcine oocytes

In Experiment 2, the effects of different O_2 tensions and culture media on the development of pig oocytes activated by the electroporation + butyrolactone I treatment were investigated. The proportions of cleaved embryos were similar in all groups examined; the type of medium or the O_2 concentration did not affect the cleavage significantly. Similarly, no difference was found in the blastocyst formation between embryos cultured under low or high O_2 concentrations (Table 2). The percentage of blastocyst formation was greater in PZM-3 than in NCSU-23 medium (60.4±2.8% and 40.4±4.9%, respectively; $p<0.05$). Furthermore, while no hatched blastocyst was found in NCSU-23, in PZM-3 medium the average frequency of hatching by day 7 of culture was 9.6±1.9% ($p<0.001$). The average nuclear numbers of blastocysts were also greater ($p<0.001$) in PZM-3 medium as opposed to those in NCSU-23 medium (45.5±1.7 and 33.8±1.4%, respectively; Table 2). The TUNEL assay revealed that the media, treatment or media x treatment interaction had no effect on the percentage of apoptotic nuclei in blastocysts; the proportion of nuclei showing signs of programmed cell death was about 5% in all experimental groups.

Table 1. Effect of activation methods and culture media on the development of in vitro-matured porcine oocytes

Media	Treatments	Pronucleus (PN) data		Developmental data					
		N	PN formation N (%±S.E.M.)	N	Cleaved N (%±S.E.M.)	Blastocysts formed N (%±S.E.M.)	Blastocysts hatched N (%±S.E.M.)	No. of nuclei per blastocyst $\bar{x}$ ±S.E.M.	% of apop. nuclei per blastocyst
NCSU-23	EP	110	92 (84.4±2.1)[a]	173	134 (77.1±6.6)[c]	69 (40.8±7.5)[c]	0 (0.0±0.0)[a]	34.2±1.7[a]	5.1±0.5
	EP+BL	117	108 (92.7±1.4)[b]	180	138 (77.0±5.4)[c]	71 (39.6±1.9)[c]	0 (0.0±0.0)[a]	36.8±2.0[a]	4.9±0.5
	EP+CHX	112	104 (92.9±1.8)[b]	173	131 (76.6±6.1)[c]	65 (39.4±6.4)[c]	1 (0.6±0.6)[a]	34.9±2.0[a]	5.3±0.6
PZM-3	EP	116	96 (83.4±3.0)[a]	173	134 (76.0±5.6)[c]	79 (45.6±4.7)[c]	17 (10.2±2.9)[b]	49.9±2.5[b]	5.2±0.5
	EP+BL	110	103 (92.8±1.1)[b]	181	151 (83.3±3.7)[cd]	114 (64.9±5.2)[d]	23 (12.5±1.7)[b]	49.7±2.2[b]	5.2±0.5
	EP+CHX	112	104 (93.1±0.8)[b]	173	154 (89.9±2.9)[d]	117 (68.6±3.5)[d]	17 (9.1±2.4)[b]	50.8±2.1[b]	4.7±0.4

[a, b, c, d] different superscripts in column indicate a difference, [a,b] - $p<0.001$; [c,d] - $p<0.05$

Table 2. Effect of O_2 concentration and culture media on the development of porcine oocytes activated by electroporation followed by butyrolactone I treatment

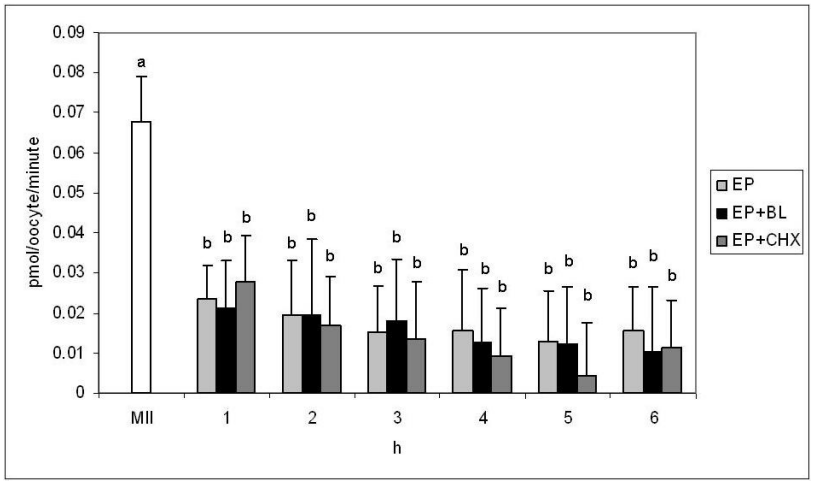
Media	O_2 conc. (%)	N	Cleaved N (%±S.E.M.)	Blastocysts formed N (%±S.E.M.)	Blastocysts hatched N (%±S.E.M.)	No. of nuclei per blastocyst $\bar{x}$ ±S.E.M.	% of apop. nuclei per blastocyst
NCSU-23	20	131	96 (73.2±7.1)	49 (37.5±7.8)[a]	0 (0.0±0.0)[c]	32.2±2.1[c]	5.0±0.9
	5	133	98 (74.0±3.7)	57 (43.2±3.2)[a]	0 (0.0±0.0)[c]	34.9±1.9[c]	4.9±0.5
PZM-3	20	128	105 (82.0±3.7)	76 (59.4±6.6)[b]	13 (10.2±2.1)[d]	44.6±2.4[d]	4.9±0.6
	5	134	107 (79.8±4.0)	82 (61.3±4.9)[b]	13 (9.0±3.4)[d]	46.5±2.4[d]	4.9±0.4

[a, b, c, d] different superscripts in column indicate a difference, [a,b] - $p<0.05$; [c,d] - $p<0.001$

Experiment 3: Changes in MPF and MAPK activities in porcine oocytes activated by different methods

The phosphotransferase activity of cdc2, the catalytic subunit of MPF was determined in activated oocytes by measuring the phosphorylation of its in vitro substrate, histone H1. The original activity of cdc2 in MII oocytes was 0.067 pmol/oocyte/min (Figure 1). One hour after the beginning of activation this activity was significantly lower in every treatment group ($p<0.05$). By this time, the MPF activity in oocytes activated by electroporation only dropped to 34.3% of its original value; additional treatment with cycloheximide or butyrolactone I did not reduce MPF further. The capability to phosphorylate histone H1 remained low in all groups at all time points until 6 hours post electroporation, approximately the time of pronuclear formation.

Figure 1. Dynamic of MPF activity in oocytes activated by different activation methods

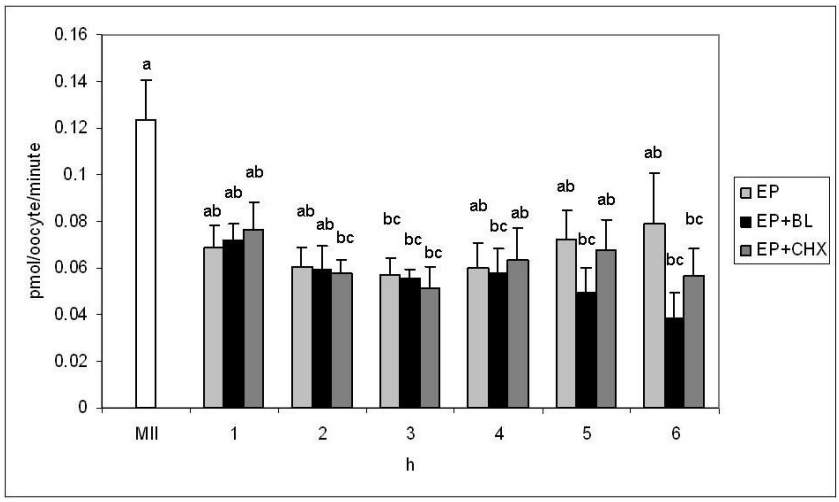


[a,b] different superscripts over the bars between timepoints indicate significant differences ($p<0.05$)

Prior to activation, MAPK activity in mature oocytes was 0.123 pmol/oocyte/min (Figure 2). One hour following electrical stimulation, the activity dropped to 54.4-62.6% of its original value. By 2 hours post electroporation, MAPK activity in the oocytes activated by electroporation plus cycloheximide (0.058 pmol/oocyte/min) was significantly lower ($p<0.05$) compared to that in the non-treated oocytes, and by 3 hours after electroporation it was significantly lower in all

treatment groups ($p<0.05$). After this time, oocytes that were incubated in the presence of butyrolactone I displayed reduced MAPK activity at every time point until 6 hours post activation. However, in oocytes activated by electroporation only, the activity of MAPK started to increase after 3 hours. By 4 hours post electroporation MAPK activity (0.060 pmol/oocyte/min) was statistically no different from activities measured in oocytes prior to activation, and stayed high until 6 hours post electroporation. Some increase in MAPK activity was detected in the cycloheximide-treated oocytes 3 hours after electroporation, but by 6 hours the activity was again significantly lower compared to that measured before activation ($p<0.05$).

Figure 2. Dynamic of MAPK activity in porcine oocytes stimulated by various methods



[a,b] different superscripts over the bars between timepoints indicate significant differences ($p<0.05$)

The efficiency of the different parthenogenetic activation methods to induce meiotic resumption and entry into the first embryonic interphase has also been evaluated. The combined activation methods proved to be more effective in triggering pronuclear formation. By 6 hours after the initial activation stimulus, pronuclear formation was significantly higher in oocytes that were incubated in the presence of butyrolactone I (86.32±3.27%) or cycloheximide (87.56±3.73%) than in those activated by electroporation only (69.39±3.32%; $p<0.05$).

DISCUSSION

Effects of activation methods and culture media on development of parthenogenetic porcine embryos

In the present study, the frequency of pronuclear formation was less in electroporated oocytes compared to that in oocytes activated by the electroporation plus butyrolactone I or electroporation plus cycloheximide methods. This developmental advantage provided by the protein synthesis/protein kinase inhibitors not longer was present at the two cell stage in NCSU-23 medium. However, in PZM-3 medium the proportion of cleaved embryos was greater in the cycloheximide-treated group than it was in the group where activation was induced by electroporation only. Furthermore, in PZM-3 the electroporation plus butyrolactone I or electroporation plus cycloheximide activation methods induced greater development to the blastocyst stage than electroporation alone. The beneficial effects of protein synthesis/protein phosphorylation inhibition were not observed in NCSU-23 beyond the pronuclear stage; this suggests that the NCSU-23 medium has somehow limited the developmental potential of the parthenogenetic embryos. This is in agreement with previous findings that in NCSU-23 medium supplemented with 1% MEM nonessential amino acids, cycloheximide treatment of the electroporated oocytes did not improve development to the blastocyst stage compared with electroporation alone. Although in the present experiments, the two media supported similar pronuclear formation and (with the exception of the electroporation + cycloheximide treatment) similar cleavage frequencies, development to the blastocyst stage and the average cell number of the blastocyst stage embryos improved in PZM-3 medium. When studying development of *in vivo*-derived porcine embryos in different culture media, YOSHIOKA et al., (2002) also reported superior development in PZM-3 medium compared with that in NCSU-23. Furthermore, the number of hatched blastocysts was greater in PZM-3 than in NCSU-23 medium by the end of the 7-day culture in the present study. Proportion of hatched blastocysts produced by *in vitro* fertilization was greater in PZM-3 than in NCSU-23 medium on day 7, although by the ninth day of culture the difference was not apparent. This suggests that the PZM-3 medium facilitates faster development to the hatched blastocyst stage. NCSU-23 has been formulated to meet the metabolic and nutrient needs of pig embryos, while PZM-3 is

a chemically defined medium based on the composition of pig oviductal fluid. The beneficial effects of PZM-3 on porcine embryonic development may be attributed, at least in part, to the amino acid supplementation in the medium. Non-essential amino acids support early cleavage and blastocyst expansion, whereas essential amino acids induced an increase in the cell number of the blastocysts.

Effect of O_2 tension and culture media on development of parthenogenetic porcine oocytes

The O_2 concentration in the reproductive tract is $\leq$ 8.7%. However, for embryo culture a mixture of 5% CO_2 and 95% air is used most frequently that provides an atmosphere of approximately 20% O_2. Greater O_2 concentrations during culture lead to an increase in the formation of reactive oxygen species (ROS) that may have detrimental effects on the cells by inducing DNA damage, lipid peroxidation and oxidative modification of proteins. For embryos produced by *in vitro* fertilization, decreased (5%) O_2 during culture improved blastocyst formation or resulted in greater cell numbers in the blastocyst stage embryos; in the case of *in vivo*-derived embryos it led to improvements in both parameters. However, studies on the culture of parthenogenetic embryos provided inconsistent results. CALLESEN et al. (2000) reported an improvement in pig blastocyst yield when using reduced O_2 tension during culture. However, no difference in blastocyst formation between two O_2 concentrations has been reported with lesser O_2 concentrations supporting development of greater average cell numbers per embryo. Neither blastocyst yields nor the mean cell number per blastocyst was affected by a reduction in the O_2 tension. In the present study, there was no improvement in cleavage or blastocyst formation using lower O_2 tension; the average nuclear numbers per blastocyst were also similar between the two O_2 concentrations. Because of the rigorous selection prior to activation, only good quality oocytes were used for the experiments and these oocytes showed enhanced tolerance against oxygen radicals; lesser quality oocytes with reduced developmental competence may have been more severely affected by the reactive oxygen species. Additional experiments are needed to understand the effect of low O_2 concentration on porcine embryo development under different culture conditions.

Apoptosis, as a response to suboptimal developmental conditions and stress, has been extensively investigated in embryonic cells. Apoptosis or programmed cell death is a physiological process and occurs in both *in vivo* and *in vitro* embryos. It has been suggested that evaluation of apoptosis in preimplantation embryos should be part of embryo quality assessment. No difference in the incidence of apoptosis in embryos produced using different activation methods, culture media or O_2 tensions was found in the present study. The frequency of apoptotic nuclei in blastocysts was around 5%, somewhat less than what was reported previously (7%). The slightly lesser proportion of apoptotic cells in the present study may be attributable to the exposure of the electroporated oocytes to cytochalasin B, a microfilament polymerization inhibitor. Cytochalasin B prevents the extrusion of the second polar body and induces the formation of diploid embryos. A haploid chromosomal set caused an increased incidence of apoptosis through a decrease in Bcl-xL and an increase in Bak gene expression. In addition, cytochalasin B was suggested to prevent fragmentation of embryos during culture and improve embryonic development to the blastocyst stage in the pig.

In conclusion, these studies demonstrated that electroporation combined with chemical treatments that inhibit protein synthesis or specific protein kinases provides a stimulus that triggers development to the blastocyst stage more effectively than electroporation alone. The PZM-3 medium proved superior to NCSU-23 in supporting development of parthenogenetic porcine embryos up to the blastocyst stage. Defining the optimal activation and culture variables for the production of transferable stage porcine embryos is very important for increasing efficiency of various assisted reproductive technologies including nuclear transfer.

Changes in MPF and MAPK activities in porcine oocytes activated by different methods

It is generally accepted that a major role of the fertilization calcium signal is to induce a decline in cyclin B levels. Mammalian oocytes continue synthesizing proteins while awaiting fertilization. In fact, continuing cyclin synthesis was shown to be required for the maintenance of the meiotic arrest and this is why inhibitors of protein synthesis trigger activation in oocytes of some species, including the mouse. In ascidian oocytes, sperm-induced calcium transients correlate well with episodes of

cyclin destruction; the repetitive nature of the calcium signals is believed to be important to bring cyclin levels down sufficiently for complete MPF inactivation. Nevertheless, the high voltage DC pulses applied in low ionic-strength medium most frequently used for parthenogenetic activation induce only a single calcium transient in the oocyte cytoplasm; no additional calcium spikes follow the initial rise. In the mouse, a single calcium transient induced activation in aged oocytes where the protein synthetic capacity is compromised; it was found to be less effective in freshly ovulated oocytes. Similarly, in bovine oocytes a single electric pulse could induce MPF inactivation in 1 hour, but by 2 hours after stimulation MPF was dramatically reactivated and by 6 hours it was higher than in the original MII oocyte. When three electric pulses were applied, MPF reactivation occurred after 4 hours and six pulses could maintain low MPF activity for at least 8 hours. The importance of a long-lasting activation signal was further demonstrated in experiments where freshly ovulated murine oocytes were stimulated with finely defined and controlled calcium transients. According to these findings, MPF inactivated after 24 electric pulses, while lower numbers of calcium transients led to no inactivation or a rebound in MPF activity. Thus, the duration and frequency of the stimulation determined the onset of cellular responses including the inactivation of MPF and MAPK. In pig oocytes, a single electric pulse of 180 V/mm for 15 μsec was efficient for the inactivation of MPF; but interestingly, inactivation occurred 4 hours after the application of the electrical stimulus. In our experiments, two consecutive DC pulses of 120 V/mm, 60 μsec each initiated release from the metaphase II arrest. Using these parameters MPF activity dropped significantly after only 1 hour and remained low for 6 hours, about the time of pronuclear formation. Since electroporation parameters including voltage, pulse duration and extracellular calcium concentration determine the amplitude and length of the calcium transients generated, these results support the notion that characteristics of the calcium signal affect the function of the MPF inactivation machinery. Our data also indicate that changes in the intracellular calcium levels induced by the electrical pulses were sufficient to downregulate MPF activity; additional inhibition of the cdc2 kinase subunit by butyrolactone I or blocking the synthesis of the regulatory subunit cyclin B by cycloheximide did not stimulate additional changes in the MPF activity.

After fertilization, activation of CaMKII by calcium/calmodulin also induces MAPK inactivation, but unlike the rapid decline in MPF activity, the

MOS/MEK1/MAPK/$p90^{Rsk}$ cascade remains active for 30-45 min following the initial calcium transient. Because the inactivation of MAPK takes place after MPF inactivation, MAPK may not mediate the release from the MII arrest. In mouse oocytes, MAPK was suggested to be involved in the regulation of microtubule nucleation and spindle assembly, and its inactivation is believed to be essential for pronuclear formation after oocyte activation. When high MAPK activity was maintained artificially after fertilization, the oocytes showed a decrease in MPF activity and extruded their second polar bodies but could not undergo pronuclear formation and progressed to MIII. After activation of pig oocytes, $p90^{Rsk}$ was dephosphorylated (inactivated) shortly before pronucleus formation, which coincided with the inactivation of MAPK; this indicates that the cascade plays functional roles in the regulation of nuclear status and microtubule organization. In our experiments, MAPK activity showed unique dynamics following various activation stimuli. In oocytes that were treated with an electrical pulse only, MAPK activity dropped markedly after 1 hr and reached its lowest value 3 hours after electroporation. However, by 4 hours the activity was increasing again and it remained at elevated levels until the time of pronuclear formation. Since high MAPK activity may impair the inhibitory control of Myt1 on cdc2 kinase activity through its substrate $p90^{Rsk}$ and may interfere with pronuclear formation, this is clearly disadvantageous. No rebound in MAPK activity was observed after the initial drop when electroporation was followed by incubation in the presence of butyrolacone I. The activity in these oocytes stayed low until pronuclear formation. Although butyrolactone I primarily inhibits MPF activity by acting on the ATP binding site of cdc2, it was suggested to have non-specific inhibitory effects on other kinases including MAPK. Treatment of porcine oocytes with butyrolactone-I alone induced a gradual inactivation of MAPK (after 3 hours, MAPK was still active in these oocytes), while incubation of oocytes in butyrolactone I after electroporation was effective in supporting development of pig embryos to the blastocyst stage.

The combined electroporation plus cycloheximide treatment was similarly effective in reducing active MAPK levels. In these oocytes some increase in MAPK activity was detected after the initial drop but by the time of pronuclear formation the activity was less than half of that measured in oocytes prior to activation. Although cycloheximide exerts its beneficial effect on oocyte activation by inhibiting the production of cyclin B, in the presence of this protein synthesis inhibitor MAPK

phosphorylation (activation) also fails to occur. Since reduction of both cdc2/cyclin B activity and MAPK activity are essential for successful parthenogenetic activation in porcine oocytes, the insufficient downregulation of MAPK activity after electro-activation might be the reason for poor pronuclear formation when oocytes were cultured without butyrolactone-I or cycloheximide supplementation. This seems to explain the beneficial effects of cycloheximide on oocyte activation demostrated previously. These results confirm our earlier findings where we demonstrated that the frequency of pronuclear formation, cleavage and blastocyst formation were all significantly higher in oocytes where activation was induced by electroporation followed by protein synthesis or protein kinase inhibition as opposed to those activated by electroporation only.

In conclusion, electroporation by two consecutive electric pulses could efficiently induce MPF inactivation, but MAPK activity rebounded by 4 hours after the electric stimulus. The incomplete inactivation of MAPK resulted in lower frequency of pronuclear formation. However, rapid and long-lasting inactivation of MAPK can be achieved by using protein synthesis or protein kinase inhibitors after the electrical pulse. The combined activation methods were also highly effective in stimulating pronuclear formation and thus may be part of a successful oocyte activating scheme during porcine nuclear transfer.

NEW SCIENTIFIC RESULTS

1. Parthenogenetic activation of porcine oocytes by combined activation methods - using protein synthesis or specific cyclin-dependent kinase inhibitors after an electroporation-induced Ca^{2+} influx - provided a stimulus that triggered better embryonic development than electroporation alone.

2. The PZM-3 medium proved superior to NCSU-23 in supporting development of parthenogenetic porcine embryos up to the blastocyst stage during the culture period.

3. During culture in PZM-3, high O_2 tension (~20%) had no detrimental effect on the development of parthenogenetic porcine embryos indicating that traditional CO_2 incubators (that provide 5% CO_2 in air) can efficiently be used for embryo production.

4a. Efficient and rapid inactivation of MPF can be achieved using electroporation by two subsequent direct current pulses of 120 V/mm, 60 μs.

4b. Electroporation alone causes a decrease in MAPK activity, however, MAPK reactivated following the initial drop in enzyme activity.

4c. After electroporation, using protein synthesis- or cdc2/cdk2 kinase inhibitors can efficiently keep MAPK activity at low levels resulting in better preimplantation embryo development. This explains the beneficial effect of the combined activation methods.

LIST OF PUBLICATIONS IN THE SUBJECT OF THE THESIS

Articles

Nánássy L., Lee K., Jávor A., Macháty Z. (2007): The effect of activation methods and culture conditions on the development of parthenogenetic porcine embryos. Animal Reproduction Science, doi:10.1016/j.anireprosci.2007.01.019

Nánássy L., Lee K., Jávor A., Macháty Z. (2007): Changes in MPF and MAPK activities in porcine oocytes activated by different methods. Theriogenology (*in press*)

Poster abstracts

Nánássy L., Lee K., Jávor A., Macháty Z. (2007): Dynamics of mitogen-activated protein kinase activity in pig oocytes following parthenogenetic activation by different methods. 33rd Annual Conference of the International Embryo Transfer Society; Reproduction, Fertility and Development 19(1):282. p.

Nánássy L., Lee K., Jávor A., Cabot R., Macháty Z. (2006): Optimization of parthenogenetic oocyte activation for porcine nuclear transfer. 39th Annual Meeting of the Society for the Study of Reproduction; Biology of Reproduction, Special Issue 123. p.

Lee K., **Nánássy L.**, Macháty Z. (2006): The distribution of STIM 1 in porcine oocytes and its potential role in capacitative Ca^{2+} entry. 39th Annual Meeting of the Society for the Study of Reproduction; Biology of Reproduction, Special Issue 142. p.

Nánássy L., Lee K., Jávor A., Macháty Z. (2006): Changes in MPF activity in porcine oocytes following activation by various methods. 32nd Annual Conference of the International Embryo Transfer Society; Reproduction, Fertility and Development 18(1,2):265. p.