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Parthenogenetic Development and Cytoskeletal Distribution of Porcine Oocytes Treated by Means of Electric Pulses and Cytochalasin D

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Abstract: Oocyte activation is a key issue in current animal biotechnology. This study was designed to reveal a suitable condition for activation of the pig oocyte. In-vitro matured oocytes were activated by means of double DC pulses (150 V/mm for 60 μsec, 1 sec apart), the same treatment followed by cytochalasin D (CD, 5 μM for 4 hrs), or two sets of the same electricity. Oocytes were examined for their chromosome configurations and the types of pronuclear formation by fluorescence staining 8 hrs post-activation. High rates of pronuclear formation were obtained after each treatment (93–96%). A greater proportion of oocytes having one diploid nucleus or two haploid pronuclei were found by treatment with electric pulses plus CD (84%) than by treatment with one (33%) or two sets of DC pulses (41%). Two sets of DC pulses caused partial loss of cortical microfilaments and a decrease in density of microfilaments and microtubules in the peripheral ooplasm. Oocytes treated with CD had a wavy layer of cortical microfilaments. The incidence of cleavage 48 hrs post-activation was similar for all treatments (69–78%), but the blastocyst formation 168 hrs post-activation was higher after the combined treatment with electric pulses and CD (24%) than the treatment with one (11%) or two sets of electric stimulation (5%). The results suggest that increased development to blastocysts after a combined treatment with double electric pulses and CD may depend on a higher incidence of diploid parthenogenotes, probably due to altered microfilament organization of the ooplasm caused by the CD treatment rather than the DC pulse itself.

Key words: Porcine oocyte, Electric activation, Cytochalasin, Cytoskeleton

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Parthenogenetic activation of mammalian oocytes can be induced by various stimuli, such as exposure to ethanol, calcium ionophore and electric pulse (see review by Kaufman [1]). Recent studies have revealed that multiple pulses of electricity are effective in inducing oocyte activation in pigs [2, 3]. Response of the oocyte to activation stimulation is also associated with oocyte aging in mice and cattle, of which aged oocytes can be more easily activated than young oocytes [1, 4–9]. Nevertheless, our previous study showed that pig oocytes were increasingly fragmented with oocyte aging, when stimulated by double electric pulses [10]. Cytoskeletal organization is involved in the dynamic events during oocyte maturation and activation, such as spindle formation, polar body emission, and pronuclear formation and migration [10–15]. Treatment with cytochalasin, an inhibitor of microfilament polymerization, after parthenogenetic stimulation of the oocytes has enhanced developmental competence in cattle [16, 17], pigs [18], sheep [19] and rabbits [20]. Cytochalasin may function to prevent the release of the second polar body (PBII) after activation of the oocytes, producing diploid parthenogenotes [13], but little information is available on the dynamic changes in the cytoskeleton of the parthenogenotes stimulated by multiple electric pulses and cytochalasin in the pig. The objects of the present study were to examine the cytoskeletal organization and types of pronuclear formation in pig oocytes treated with multiple electric pulses with or without cytochalasin D (CD), and to compare the ability of the parthenogenotes to form blastocysts.

Materials and Methods

Maturation of oocytes

In vitro maturation of pig oocytes was based on the procedures reported previously [10–12]. Briefly, oocytes were aspirated from antral follicles (2–5 mm in diameter) of ovaries collected from slaughtered prepubertal gilts. After being washed with Dulbecco's phosphate buffered saline containing 0.1% polyvinyl alcohol (DPBS-PVA), groups of 10–15 oocytes were transferred to NCSU23 medium supplemented with 10% (v/v) porcine follicular fluid, 10 i.u./ml eCG (Teikoku Hormone Mfg. Co. Ltd., Tokyo, Japan) and 10 i.u./ml hCG (Mochida Pharmaceutical Co. Ltd., Tokyo, Japan). The oocytes were cultured for 24 hrs, then incubated in NCSU23 without hormones for a further 20 hrs in an atmosphere of 5% CO_2 in air at 39°C.

Activation of oocytes

At the end of the culture, the oocytes were denuded of cumulus cells in NCSU23 supplemented with 0.1% hyaluronidase, washed, and matured oocytes were selected on the basis of the presence of the first polar body (PBI) under a stereomicroscope and the maturation rate was recorded. They were subjected to one of the following treatments: A) one set of double DC pulses (150 V/mm for 60 μsec) 1 sec apart, B) the same treatment followed by incubation in 5 μM cytochalasin D for 4 hrs, and C) two sets of the same electric treatment with a 10 min interval between them. Before electric stimulation, the oocytes were equilibrated for 5 min in pulse medium: 0.3 M mannitol supplemented with 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$ and 0.05% bovine serum albumin (BSA). The chamber for electric stimulation consisted of a glass Petri dish (60 mm in diameter) with two parallel platinum block electrodes 1 mm apart and half-filled with pulse medium before use. The oocytes were randomly placed in groups of 10–15 for a time between the electrodes. Electric stimulation to induce activation was delivered with an Electro Cell Fusion apparatus (Bex LF101L, Tokyo, Japan). Stimulated oocytes were washed and placed in 100 μl-drops of NCSU23 supplemented with 0.4% BSA under mineral oil in an atmosphere of 5% CO_2 in air at 39°C for 8 hrs (4 hrs after CD treatment in treatment B). The oocytes were then processed for fluorescence staining to assess the pronuclear formation and the distribution of microtubules and microfilaments.

To serve as controls at the pronuclear stage, *in vitro* fertilization (IVF) was done as reported previously [10]. Briefly, spermatozoa collected from three boars were preincubated in modified Tris-buffered medium [21] supplemented with 1 mM caffeine and 0.1% BSA (IVF medium) for 90 min. After maturation incubation, cumulus-oocyte complexes were washed three times and placed in 50-μl droplets of IVF medium. Sperm fraction (50 μl) was introduced to the droplets containing oocytes. The oocytes were then cocultured with spermatozoa at a final concentration of 2×10^5 cells/ml at 39°C in an atmosphere of 5% CO_2 in air for 8 hrs.

Fluorescent observations

Methods for preparing samples for fluorescent observations have been reported previously [11]. Denuded oocytes were fixed in a microtubule stabilization buffer [22] at 37°C for 1 hr, thoroughly washed and blocked overnight at 4°C in the washing medium [11]. The fixed samples were later exposed to anti-β tubulin primary antibodies (1:200; Sigma Chemical Co., MO, USA) at 4°C overnight, washed, and then incubated with fluorescein isothiocyanate (FITC)-conjugated secondary antibody (1:200; Sigma Chemical Co.) at 37°C for 2 hrs. After rinsing, the samples were stained with rhodamine-phalloidin (1:1000; Molecular Probes, Eugene, OR, USA) for microfilaments for 1 hr, washed again, then stained for DNA with Hoechst 33342 (10 μg/ml) in mounting medium containing PBS and glycerol (1:1), and finally mounted on slides.

The samples were viewed under an Olympus microscope (BX-FLA, Olympus, Tokyo, Japan). For FITC a filtered U-MWIB set was utilized, a U-WIB set was used for rhodamine and a U-MWU set for Hoechst. A cooled CCD video system (ImagePoint, Photometrics Ltd., Tucson, AZ, USA) was used to obtain images on a computer, and color adjustment was done with IPLab-Spectrum P software (Signal Analytics Corporation, Vienna, VA, USA).

Assessment of developmental ability of the activated oocytes

At 48 hrs after activation, cleavage of oocytes was evaluated under an inverted microscope and blastocyst formation was examined 168 hrs (day 7) post-activation. The number of cells in blastocysts was determined by counting the number of nuclei after the fluorescence staining described above.

Statistical analysis

Data were assessed by analysis of variance with the help of the BMDP program (BMDP Statistical Software, Inc., Los Angeles, CA, USA). When appropriate, percentage data were arcsined transformed.

Table 1. Pronuclear formation of pig oocytes activated by electric pulses and cytochalasin D

Oocyte activation*	No. of oocytes examined	Percentage of oocytes		Percentage of oocytes at**			
		Activated	With PBII	PPN	PN	PN-Frag	MIII
Control	78	0	0	–	–	–	–
One set	134	94 ± 2	63 ± 3[a]	16	75	8	1
Two sets	113	96 ± 3	54 ± 6[a]	6	85	8	1
One set + CD	141	93 ± 2	5 ± 3[b]	3	83	14	0

*, One set included double electric pulses 1 sec apart; two sets included a 10 min interval between sets; one set + CD included additional treatment with cytochalasin D. **, PPN, pre-pronuclear stage; PN, pronuclear stage; PN-Frag, fragmented eggs with a pronucleus (-ei); MIII, metaphase III. [a, b], Values with different superscripts are significantly different (P<0.05).

Table 2. Types of parthenogenotes derived from pig oocytes activated by electric pulses and cytochalasin D

Oocyte activation*	Percentage of oocytes						
	Not fused with PBI			Fused with PBI			
	1 PN + PBII	1 PN	2 PN	2 PN		≥3 PN	
				+ PBII	– PBII	+ PBII	– PBII
One set	53 ± 5[a]	20 ± 4[b]	13 ± 3[c]	3 ± 2	4 ± 2	4 ± 2	4 ± 2
Two sets	49 ± 6[a]	15 ± 3[b]	26 ± 5[b]	2 ± 1	3 ± 1	1 ± 1	5 ± 1
One set + CD	4 ± 2[b]	32 ± 5[a]	52 ± 4[a]	0	6 ± 2	1 ± 1	6 ± 3

*, See footnote to Table 1. *, PN, pronucleus (-ei); PBI, the first polar body; +/– PBII, with or without the second polar body. [a, b, c], Values with different superscripts within the same column are significantly different (P<0.05).

Differences between the means were determined by means of Duncan's multiple range test. Data were represented as the mean ± s.e.m. and the level of statistical significance was taken as P<0.05.

Results

Pronuclear formation and cytoskeleton distribution

Based on the presence of PBI, the overall maturation rate of the oocytes was 86.9% in this study. As shown in Table 1, spontaneous activation did not occur in the non-treated control group. Most of the parthenogenotes were at the pronuclear stage and some of them (8–14%) were fragmented. One percentage of the activated oocytes showed expulsion of the second polar body and metaphase plate in the ooplasm, which has been reported as metaphase III (MIII) in mice [23]. When all the stages described were considered activated, the overall rate of activation was very high with no significant differences among treatments (93–96%). Types of parthenogenotes are summarized in Table 2. Electric stimulation caused fusion of the PBI into the oocyte with an incidence of 11–15% in each treatment group. There was a high incidence of parthenogenotes with one PBII and one pronucleus in the groups stimulated electrically. When fusion of the PBI occurred, "extra" nuclear-like structures, derived from the chromatin materials incorporated from the PBI, were formed. Treatment with CD after electric stimuli increased the incidence of oocytes with one diploid nucleus or two haploid pronuclei (84%) more than those with electric stimulation alone (33 and 41% for one and two sets of double pulses, respectively).

Fluorescent observation revealed that in the IVF embryos the submembranous layer of microfilaments was observed as a clear ring and female and male pronuclei were included in a microtubule-rich domain supported by microfilament architecture (Figs. 1a–c). The electrically activated oocytes showed decreased density of peripheral microfilaments (Figs. 1d, e) compared to the IVF embryos and some of them displayed a partial loss of cortical actin staining and shrinkage of a microfilament-rich domain. This tendency was more obvious in the oocytes treated with two sets of electric stimulation (Figs. 1f, g) than in those treated with one set of electric pulses. One or more

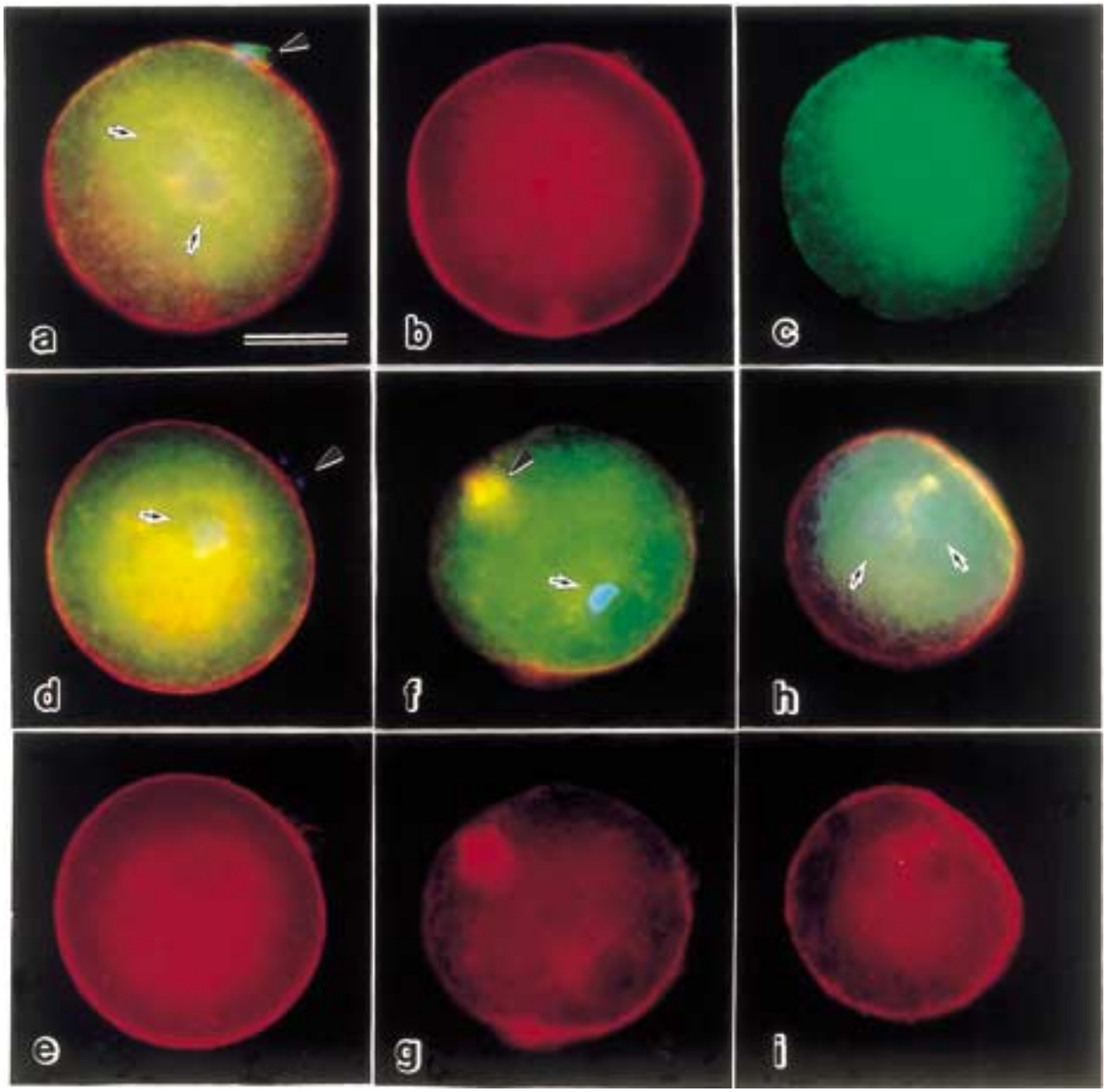

Fig. 1. Porcine activated oocytes triple stained to visualize chromatin (blue), microtubules (green) and microfilaments (red). Yellow shows the overlap of microtubules and microfilaments. The bar represents 50 μm. a-c) A zygote at 8 hrs after insemination. a) Female and male pronuclei (arrows) and the second polar body (arrowhead) can be seen. b) Actin labeling. Microfilaments are mainly located at the cell cortex and around the pronucleus. c) Tubulin labeling. Microtubules show a typical interphase network throughout the ooplasm. d,e) An oocyte activated by double DC pulses having one pronucleus (arrow) and one polar body (out of focus, arrowhead). e) Actin labeling shows a decreased staining intensity, although a similar distribution of microfilaments to that in Fig 1b can be seen. f, g) An oocyte activated by two sets of DC pulses. Note one pronucleus (arrow) and the polar body (out of focus, arrowhead). g) Actin labeling. Microfilaments have decreased in the peripheral ooplasm. Cortical microfilaments are partially lost (on the upper left of the oocyte). h, i) An oocyte treated with cytochalasin D after electric stimulation. Two pronuclei (arrows) can be seen in a microtubule-rich domain. i) Actin labeling. Cortical microfilaments have become wavy. Note a depression in the plasma membrane (on the upper right of the oocyte).

Table 3. Developmental ability of pig oocytes activated by electric pulses and cytochalasin D

Oocyte activation*	No. of oocytes examined	Mean % of oocytes cleaved	Mean % of Blastocysts	Mean number of nuclei in Blastocysts
One set	86	70 ± 2	11 ± 4[ab]	37.8 ± 4.2
Two sets	98	69 ± 6	5 ± 2[b]	23.0 ± 1.8
One set + CD	95	78 ± 7	24 ± 5[a]	32.8 ± 3.1

*, See footnote to Table 1. [a, b], Values with different superscripts are significantly different ($P<0.05$).

pronuclei were always found in a microtubule-rich domain of the ooplasm of parthenogenotes, around which microfilaments were concentrated. The CD treatment induced occasional depression of the plasma membrane of the oocyte, where the microfilament-rich domain became smaller (Figs. 1h, i). Those oocytes also contained a wavy layer of cortical microfilaments.

Developmental ability of the activated oocytes

Table 3 shows *in vitro* development of oocytes after each treatment. Percentages of cleavage were similar for all treatments (69–78%). Nevertheless, the frequency of oocytes which developed to the blastocyst stage was increased by the combined treatment with electric pulses and CD (24%) more than in those with electric stimulation alone (11% and 5% after one and two sets of electric pulses, respectively). The number of nuclei in blastocysts was not significantly different among treatments.

Discussion

The present results show that the optimal parthenogenetic development of porcine oocytes can be obtained by repeated electric pulses plus CD treatment. The results were in accordance with those by Grupen *et al.* [2] and Nagai *et al.* [3]. Lin and Moor [24] also have reported that three pulses at 5 min intervals induced a higher incidence of parthenogenetic development of porcine oocytes than one pulse activation. In the present study, the rates of activation and development after two sets of double electric pulses (150 V/mm for 60 μsec) were lower than those after one set of the same electric pulses. Grupen *et al.* [2] also failed to find more effectiveness in three sets of double pulses than in one or two sets of the same electric pulses. It seems therefore that a moderate number of DC pulses are more effective in inducing parthenogenetic activation in porcine oocytes than a single pulse employed by others [14].

Our data also confirm previous findings showing that cytochalasins inhibit the polar body release and significantly improve blastocyst development [18]. Similar data have been reported in cattle [16, 17], sheep [19] and rabbits [20]. Cha *et al.* [18] have suggested a significant role of ploidy in the development of embryos. As demonstrated by fluorescence staining, shrinkage and peripheral loss of microfilaments after DC pulses and further disorganization of microfilaments by CD treatment may be associated with the retention of diploid chromatin in the ooplasm. Recent studies have revealed that microtubules and microfilaments play important roles in polar body expulsion and pronuclear migration in porcine oocytes [13, 15]. Our previous studies showed that the dynamic change in the cytoskeleton of the ooplasm is involved during pig oocyte maturation [11, 12], and that microtubules and microfilaments in aged oocytes are drastically reduced in density, resulting in increased fragmentation of the parthenogenotes [10]. For this reason, aged oocytes are not suitable for recipient cytoplasts of nuclear transfer programs in the pig. The present study revealed that the density of microfilaments in the ooplasm decreased after multiple electric pulses, suggesting that such altered microfilament architectures might be one of the causes of egg fragmentation regardless of pronuclear formation.

Different types of parthenogenotes, which were not previously described by Kaufman [1], were observed due to electric fusion of PBI into the oocyte as observed previously [10]. In the present study, 11–15% of oocytes were fused with PBI. On the other hand, 52% of the oocytes treated were fused with PBI, when the cleavage plane of PBI was oriented perpendicularly to the line between the two electrodes [10]. Attention has to be paid to avoid incorporation of extra chromatin materials into the oocyte when it is electrically activated so that the development of embryos may progress further.

In conclusion, a combination of a moderate number of DC pulses and CD treatment are effective for the

activation and development of porcine oocytes, but more frequent electric pulses may exert adverse effects on parthenogenetic development of the oocyte, because electric stimulation itself can alter the actin microfilament architecture of the ooplasm. The mechanism by which the diploid chromosomal components contribute to increased development to blastocysts remained unclear.

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