Development of pronuclei in pig oocytes activated by a single electric pulse

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Summary. Pig oocytes were matured *in vitro* in a modified M-199 medium for 44 h, subjected to electrical stimulation and scored for activation 6 h later. Sham pulsed oocytes, exposed to electroporation medium and an a.c. field, did not develop the female pronucleus any more frequently than occurs spontaneously (8.3% within 50 h of culture). However, a single d.c. pulse proved extremely efficient in activating pig oocytes. Pulses of 0.75–1.65 kV cm⁻¹ lasting 30 or 100 µs activated at least 90% of matured oocytes. The developmental pathway taken by the activated oocytes depended on the parameters of the pulse. The lowest effective stimulation (0.45 and 0.60 kV cm⁻¹ for 30 µs) frequently produced oocytes that remained in pre-pronuclear stages of activation (29.4 and 42.3%, respectively). Extrusion of the second polar body and creation of one pronucleus was the most frequent type of activation (in up to 88.2% among the activated oocytes). The strongest stimulations used (1.05–1.65 kV cm⁻¹ for 100 µs) often yielded oocytes that failed to extrude the second polar body and formed two or more pronuclei (up to 56.3%). Under optimal stimulation (0.75 kV cm⁻¹), the activated oocytes proceed synchronously to interphase of the first mitotic division. Anaphase II is reached within 30 min and telophase II at 1 h after application of the pulse. The second polar body is extruded about 2 h after activation. Well-defined swelling pronuclei were found in oocytes 5–6 h after activation. The relationship between the stage of oocyte maturation and susceptibility to activation was investigated. The period of culture in which the oocytes develop the activation competence (32–36 h of culture) overlapped with the period in which the oocytes complete meiosis (28–38 h). This suggests that ageing in meiotic arrest is not essential for pig oocytes to become activated by electric pulses. Activation of pig oocytes was accompanied by release of cortical granules. In sections of control (metaphase II) oocytes, an average of 7.3 intact cortical granules per 10 µm of overlying cytoplasmic membrane was found. This number dropped to 1.5 in 10 µm within 30 min after the pulse.

Keywords: pig; oocyte; *in vitro* maturation; pronucleus; cortical granules

Introduction

In most mammalian species, matured oocytes are arrested at the second meiotic metaphase and no further development occurs until the oocyte is activated. In natural circumstances, the activation stimulus is provided by a penetrating spermatozoon, but many artificial stimuli can also bring about activation (for review see Kaufman, 1983). The most detailed information on parthenogenetic activation has been obtained in the mouse system. In other species, the cloning of embryos

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by nuclear transplantation, which requires experimental control of activation, has recently become an incentive for more research on parthenogenesis.

In terms of embryo cloning technology, electrically induced activation deserves particular attention. The ability of electric pulses to activate mammalian oocytes was first documented in mice (Tarkowski *et al.*, 1970) and hamsters (Kaufman *et al.*, 1975) *in vivo*. More recent studies, using a simplified *in vitro* technique, have shown that rabbit (Gulyas, 1976; Onodera & Tsunoda, 1989), cattle (Ware *et al.*, 1989) and pig (Prather *et al.*, 1989) oocytes can be similarly activated. However, the activation rates under the conditions being used for cloning appear to have been less than optimal in several studies (Stice & Robl, 1988; Bondioli *et al.*, 1990; Marcus, 1990), suggesting that further studies of electrical activation of oocytes in various species are warranted.

This study aimed to assess the response of pig oocytes, matured *in vitro*, to a single electric pulse. Activation rates were evaluated following various types of pulse and with oocytes of various maturational ages. The cortical reaction was investigated as one of the earliest morphological signs of activation. Finally, the progression of events following activation (completion of meiosis and pronucleus formation) was assessed in relation to its practical relevance for nuclear transplantation.

Materials and Methods

Oocyte collection and culture

Oocytes were collected from ovaries of slaughtered gilts. Follicles, 3–5 mm in diameter, were aspirated through a 20 G needle. The aspirated follicular fluid was pooled in glass test-tubes, stored in a vacuum flask at 37°C and transferred to the laboratory within 2 h. Oocytes were isolated from the fluid and those surrounded by a compact cumulus were selected for experiments. About 15 oocyte–cumulus complexes were cultured on a watch glass in 0.2 ml medium covered with paraffin oil at 38.5°C under an atmosphere of 5% CO₂ in air. The culture medium was modified M-199 (Pavlok *et al.*, 1988) supplemented with 10% bovine serum and 5 iu pregnant mares' serum gonadotrophin ml⁻¹ (PMSG) (Folligon; Intervet Netherlands). Unless otherwise indicated, the oocytes were cultured for 44 h. At the end of the culture period, the oocytes with an expanded cumulus were vortexed for 2 min in the culture medium with 100 iu hyaluronidase ml⁻¹. Cumulus-free oocytes were rinsed several times in the culture medium and evaluated under a stereomicroscope. The oocytes with a discernible first polar body were chosen for activation.

Oocyte activation

Oocytes were activated in a chamber with parallel wire electrodes separated by a distance of 350 µm. The chamber was hooked up to an electrofusion system (CFA 400; Krüss, Hamburg, Germany). The electroporation medium described by Willadsen (1986) of pH 7.0 and osmolarity 297 mosmol was used to fill the chamber and to equilibrate the oocytes (for 10 s) before placing them between electrodes. The pulse generator was programmed to deliver various combinations of the following treatments: (i) an a.c. field of 6 V and 600 kHz for 5 s; (ii) a single d.c. pulse; (iii) reduction of the a.c. to zero over 20 s. The oocytes were removed from the chamber after electrical stimulation, rinsed in the M-199 medium (without PMSG) and cultured in the same medium for the times indicated below. After culture, the oocytes were mounted on slides, fixed in acetic ethanol for 48 h, stained with 1% orcein, and assessed by light microscopy.

Processing oocytes for electron microscopy

The oocytes were fixed in 2.5% glutaraldehyde and 0.75% paraformaldehyde in 0.075 mol cacodylate buffer l⁻¹, pH 7.4, for 60 min. The oocytes were washed in the buffer and postfixed for 60 min in 2% OsO₄, dehydrated in ethanol and embedded in Epon (Crozet *et al.*, 1986). The oocytes were prepared as thin sections contrasted with uranyl acetate for 15 min and with lead citrate for 6 min. The grids were viewed in a Jeol JEM 1200 electron microscope at 80 kV.

Five experiments were carried out to study the response of pig oocytes to various electric fields.

Experiment 1. The activation potential of factors other than the d.c. pulse was evaluated. Three control groups of oocytes were prepared and their activation rates were assessed after extended culture (44 plus 6 h), exposure to electroporation medium (for 30 s) and to a.c. field (electrical treatments (i) and (iii)). The oocytes were fixed 6 h after the treatment and scored for the presence of pronuclei.

Experiment 2. The effect of d.c. pulses on activation of pig oocytes was investigated. The matured oocytes were stimulated in the chamber by the treatments (i)–(iii). The activating d.c. pulse was of $0.3\text{--}1.65\text{ kV cm}^{-1}$, lasting either 30 or 100 μs . The rate and type of activation were scored at 6 h after application of the pulse.

Experiment 3. The progression of events following activation, i.e. completion of the second meiotic division and formation of the female pronucleus, was assessed. The oocytes were activated by the d.c. pulse of 0.75 kV cm^{-1} lasting 100 μs and then cultured for 0.5–6.0 h. The classification of the developing female pronucleus (PN) was based on that used in cattle (Xu & Greve, 1988). Briefly, the following developmental stages were assessed: anaphase II; telophase II; PN-2, second meiotic division is completed, oocyte chromatin remains condensed; PN-3, chromosome decondensation occurs, nuclear envelope starts to form; PN-4, pronucleus is surrounded by a complete envelope, chromosome decondensation proceeds rapidly; PN-5, swelling pronucleus, chromosomes fully decondensed; PN-6, pronucleus reaches maximum size.

Experiment 4. The necessity of the presence of ageing oocytes in meiotic arrest for the development of activation competence was investigated. Oocytes were cultured for 28–42 h and divided into two groups. Oocytes in one group were fixed to assess the maturation rates. Oocytes in the other group were activated by the d.c. pulse of 0.75 kV cm^{-1} lasting 100 μs . The presence of female pronuclei was assessed after 6 h of culture.

Experiment 5. The release of cortical granules in activated oocytes was investigated. The oocytes were activated by a d.c. pulse of 0.75 kV cm^{-1} lasting 100 μs , cultured for 30 min, then fixed for electron microscopy. Matured unpulsed oocytes were used as controls in this experiment. The number of cortical granules was counted on five sections of each oocyte and related to 10 μm of the overlying cytoplasmic membrane (Cran & Cheng, 1986).

Statistical analysis

The results were compared by χ^2 analysis. The relationship between a pulse strength and occurrence of multiple pronuclei was analysed by correlation coefficient.

Results

Results from Expt 1 show that about 8% of oocytes undergo spontaneous activation during the 50 h culture. Neither equilibration of oocytes in electroporation medium nor their exposure to an a.c. field increased the rate of activation (Table 1). Data from Expt 2 (Table 2) show that a single d.c. pulse is a potent activator of matured pig oocytes. When the duration of the pulse was 100 μs , even the lowest voltage used (0.3 kV cm^{-1}) produced more than 40% activated oocytes. An unequivocal influence of a 30 μs pulse on activation of oocytes was observed when a voltage of 0.45 kV cm^{-1} was used. Increasing the voltage yielded higher proportions of activated oocytes. A pulse of 0.75 kV cm^{-1} , or more, activated at least 90% of oocytes, regardless of the duration of the pulse.

Table 1. The influence of experimental conditions on activation of pig oocytes matured *in vitro*

Treatment	Number of oocytes	Number of oocytes activated (%) ^a
Extended culture	48	4 (8.3)
Exposure to electroporation medium	53	2 (3.8)
Exposure to a.c. field	51	3 (5.9)

^aThe percentages do not differ significantly ($P > 0.05$).

Two major types of parthenogenetic oocyte were produced in this study. In the first, the oocytes extruded the second polar body and created one pronucleus. In the second, the oocytes failed to extrude the second polar body and created two or more pronuclei. The distribution of oocytes

Table 2. The influence of various d.c. pulses on activation of pig oocytes

Pulse duration (μ s)	Pulse strength (kV cm^{-1})	Number of oocytes		Type of activation (%)			
		Pulsed	Activated (%)	1PN	2PN*	pre-PN	d-PN
100	0-30	32	13 (40.6)	11 (84.6)	2 (15.3)		
	0-45	49	38 (77.6) ^{a,b}	31 (81.6)	5 (13.2)	1 (2.6)	1 (2.6)
	0-60	37	24 (64.9) ^a	20 (83.3)	3 (12.5)	1 (4.2)	
	0-75	38	35 (92.1) ^{b,c}	29 (82.9)	5 (14.3)		1 (2.9)
	0-90	65	64 (98.5) ^c	40 (62.5)	19 (29.7)	3 (4.7)	2 (3.1)
	1-05	31	31 (100) ^c	16 (51.6)	15 (48.4)		
	1-20	31	30 (96.8) ^c	12 (40.0)	14 (46.7)	2 (6.7)	2 (6.7)
	1-35	32	32 (100) ^c	13 (40.6)	18 (56.3)		1 (3.1)
	1-50	30	28 (93.3) ^{b,c}	18 (64.3)	10 (35.7)		
	1-65	40	40 (100) ^c	19 (47.5)	21 (52.5)		
	0-30	31	2 (6.5)			2 (100)	
	0-45	32	17 (53.1) ^a	10 (58.8)	2 (11.8)	5 (29.4)	
30	0-60	31	26 (83.9) ^b	13 (50.0)	2 (7.7)	11 (42.3)	
	0-75	37	36 (97.3) ^{b,c}	27 (75.0)	6 (16.7)	2 (5.6)	1 (2.8)
	0-90	36	34 (94.4) ^{b,c}	30 (88.2)	4 (11.8)		
	1-05	37	35 (94.6) ^{b,c}	28 (80.0)	4 (11.4)	1 (2.9)	2 (5.7)
	1-20	34	34 (100) ^c	23 (67.6)	10 (29.4)		1 (2.9)
	1-35	30	30 (100) ^c	16 (53.3)	14 (46.7)		
	1-50	42	38 (90.5) ^{b,c}	26 (68.4)	10 (26.3)		2 (5.3)
	1-65	37	36 (97.3) ^{b,c}	23 (63.9)	11 (30.6)		2 (5.6)

1PN: oocytes with extruded second polar body and one pronucleus; 2PN: oocytes with suppressed extrusion of the second polar body, containing two or more pronuclei in the cytoplasm; pre-PN: oocytes in pre-pronuclear stages of activation, i.e. telophase II and metaphase III (oocytes with extruded second polar body, oocyte chromatin is condensed into discrete unichromated chromosomes); d-PN: oocytes with suppressed extrusion of the second polar body, containing a single diploid pronucleus in the cytoplasm.

^{a-c}Numbers with different superscripts within either pulse duration differ significantly ($P < 0.05$).

*Frequency of 2PN increased with higher voltage ($r = 0.86$ for 100 μ s pulse and 0.81 for 30 μ s pulse).

between the two groups depended on the strength of electrical stimulation during activation; the higher voltages produced greater numbers of oocytes with suppressed extrusion of the second polar body. This relationship was better expressed when the longer pulse (100 μ s) was used (Table 2). The pulses of low intensity (0-45 and 0-60 kV cm^{-1} for 30 μ s) produced some oocytes that initiated, or even completed, the second meiotic division but did not form a nuclear envelope (Table 2).

In Expt 3, the time sequence of the second meiotic division and pronucleus formation was investigated under the optimal activation conditions. Chronology of these events is shown in Table 3. Completion of the second meiotic division occurs synchronously in most of the oocytes. They progress from the second metaphase (Fig. 1a) to the second anaphase (Fig. 1b) within 30 min and reach telophase (Fig. 1c) about 1 h after activation. Two hours after activation, the second polar body is extruded and the oocyte chromatin is still assembled to a homogeneous mass (PN-2). During the third hour of activation, the decondensation of the oocyte chromatin is initiated and the nuclear envelope starts to form (PN-3). At 4 h after activation, the formation of the nuclear membrane is completed and decondensation of chromosomes proceeds rapidly (PN-4). About one-third of the oocytes contained a well-developed and swelling pronucleus (PN-5) at this time. The number of such oocytes rose to 43 of 87 (50%) and 69 of 92 (75%) at 5 and 6 h after activation, respectively. An accelerated progression of activation was observed in oocytes that failed to extrude the second polar body and formed one diploid pronucleus. In these oocytes, a swollen pronucleus (PN-6) could be found 4–6 h after application of the pulse (Table 3).

The data from Expt 4 (Table 4) showed that activation competence develops within the period in which the oocytes complete meiotic maturation. The oocytes reach metaphase II gradually in the period between 28 and 38 h of culture; activation competence was achieved by an overwhelming

Table 3. Pronuclear formation in pig oocytes matured *in vitro* and activated by a single electric pulse*

Duration of culture (h)	Number of oocytes		Developmental stage of activation						
	Pulsed	Activated	AII	TII	PN-2	PN-3	PN-4	PN-5	PN-6
0.5	66	60	60						
1	70	66	12	54					
2	68	66		7	52	5	2		
3	65	62		2	11	35	12	2	
4	100	97	3	3	6	9	46	28	2
5	93	87		2	2	3	34	43	3
6	95	92			7		12	69	4

AII: anaphase II; TII: telophase II; PN-2: second meiotic division is completed, oocyte chromatin remains condensed; PN-3: chromosome decondensation occurs, nuclear envelope starts to form; PN-4: pronucleus is surrounded by a complete envelope, chromosome decondensation proceeds rapidly; PN-5: swelling pronucleus, chromosomes fully decondensed; PN-6 pronucleus reaches maximum size.

*A pulse of 0.75 kV cm^{-1} lasting $100 \mu\text{s}$ was applied to stimulate activation.

Fig. 1. Progression of pig oocyte activation is shown. (a) An oocyte in metaphase II after 42 h of culture *in vitro*. (b) An oocyte in anaphase II 30 min after application of electric pulse. (c) An oocyte in telophase II 1 h after application of electric pulse. Bars = $10 \mu\text{m}$.

majority of oocytes from 32 to 36 h of culture. Pig oocytes therefore acquire the ability to be activated during the final stages of meiotic maturation and ageing in metaphase II arrest is not essential for this process.

In Expt 5, three control and three activated oocytes were examined by electron microscopy. The cortical granules were found in all control (metaphase II) oocytes as dense ball-shaped structures located very close to the inner leaf of the cytoplasmic membrane. Both light and dark forms (Cran & Cheng, 1986) were observed. The granules apparently consisted of a surface membrane and inner contents (Fig. 2a). The mean number of cortical granules was 7.3 per $10 \mu\text{m}$ of the cytoplasmic membrane. By 30 min after activation, the mean number of cortical granules had decreased to 1.52. The surface membrane of cortical granules fused with the cytoplasmic membrane and their contents were evenly dispersed in the perivitelline space (Fig. 2b).

Discussion

The mechanism of oocyte activation in mammals is poorly understood except that it seems unequivocal that calcium ions play a role in this process. Activation is accompanied by release of Ca^{2+}

Table 4. Completion of meiosis and development of activation competence in pig oocytes during culture *in vitro*

Time of culture (h)	Maturation rates*		Activation rates*†	
	Number of oocytes	Number of oocytes in MII (%)	Number of oocytes	Number of oocytes activated (%)
28	44	6 (13.6) ^a	32	1 (3.10) ^a
30	43	9 (20.9) ^{a,b}	36	1 (2.8) ^a
32	47	15 (31.9) ^b	38	3 (7.9) ^{a,b}
34	44	24 (54.5) ^c	38	8 (21.7) ^b
36	44	34 (77.3) ^d	41	37 (90.2) ^c
38	52	47 (90.9) ^{d,e}	48	45 (93.8) ^{c,d}
40	50	46 (92.0) ^e	43	40 (93.0) ^{c,d}
42	46	42 (91.3) ^e	40	40 (100) ^d

III: metaphase II.

*Maturation and activation rates were assessed on the same batch of oocytes divided in two groups following culture *in vitro*. Data are pooled from three replicates performed on different days.

†A pulse of 0.75 kV cm⁻¹ lasting 100 µs was applied to stimulate activation.

^{a-e}Values with different superscripts within a column differ significantly ($P < 0.05$).

from intracellular storage sites (Whittingham & Siracusa, 1978; Miyazaki *et al.*, 1986). However, introducing exogenous Ca²⁺ into the oocyte cytoplasm, for example by microinjection (Fulton & Whittingham, 1978), also results in effective activation.

Application of d.c. pulses across the cytoplasmic membrane creates pores in it (Zimmermann & Vienken, 1982). This transient process is coupled to an increase in conductivity and permeability of the membrane that makes possible an exchange of extra- and intracellular components by diffusion. An oocyte exposed to such conditions may thus not be activated by the electric field *per se*, but through chemical signals elicited by the pulse (Ozil, 1990). These signals could be represented by an ionic influx into the oocyte. This assumption is supported by the finding that the electroporation medium has to contain a certain concentration of ions for activation to occur (Onodera & Tsunoda, 1989; Ozil, 1990).

The present data confirm that an electroporation medium containing Ca²⁺ and Mg²⁺ provides suitable conditions for electrical activation of pig oocytes. The pulse strength resulting in activation of nearly all the treated oocytes was surprisingly low in our experiments (0.75 kV cm⁻¹ for 30 or 100 µs). Similar effects were observed in mouse and rabbit oocytes when a pulse of 1.5 kV cm⁻¹ lasting for 100–200 µs was used (Onodera & Tsunoda, 1989). Collas & Robl (1989) found that three pulses of 3.6 kV cm⁻¹ lasting 60 µs were required to activate rabbit oocytes. In the recent study with pig oocytes, pulses of 0.7 and 0.8 kV cm⁻¹ activated only 34 and 43% of oocytes, respectively (Prather *et al.*, 1991). These differences could be due to many factors such as species specificity, *in vitro* or *in vivo* origins of the treated oocytes, types of fusion chamber and pulse generator and handling of the oocytes before and after the pulse. The important factor may also be the composition of the electroporation medium, especially the balance of ions in the nonelectrolyte solution.

Even the strongest pulses used in this study did not cause any visible degenerative changes in the oocytes. However, the oocytes activated by the relatively strong pulses frequently failed to extrude the second polar body and created two or more pronuclei. This suggests that strong electrical stimulation interferes with the function of the meiotic spindle. This observation is in accord with the data obtained in rabbits in which stronger electrical stimulation produced greater proportions of oocytes with micronuclei (Ozil, 1990).

In cattle, the susceptibility of matured oocytes to parthenogenetic stimulation rose essentially after 26 h of culture *in vitro* and reached a maximum at 30–32 h (Nagai, 1987; Ware *et al.*, 1989). As

(a)

(b)

Fig. 2. Cortical reaction in pig oocytes activated by a single electrical pulse. (a) The cortical granules represent a prominent structure located under the cytoplasmic membrane in control matured unpulsed oocytes. Notice the surface membrane of the cortical granules and their dense contents. (b) The cortical reaction was obvious at 30 min after application of the pulse. The cortical granules disappeared from the oocyte and their contents were evenly distributed in the perivitelline space. Bars = 0.5 μ m.

cattle oocytes complete meiosis between 18 and 24 h of culture (Xu *et al.*, 1986; Nagai, 1987; Sirard *et al.*, 1989), it appears that they require several hours of ageing in meiotic arrest to develop a responsiveness to parthenogenetic stimuli. However, Landa & Hájková (1989) observed that cattle oocytes respond to parthenogenetic stimulation even before completion of maturation. Our present data and those published by Hagen *et al.* (1991) demonstrate that ageing in metaphase II is not essential for the development of activation competence in pig oocytes. It has also been shown in mice that cytoplasmic maturity necessary for activation can develop even in oocytes that did not pass through the second metaphase (Kaufman & Howlett, 1986; Clarke *et al.*, 1988). All the data suggest that the ability to respond to the activation stimulus is related more to cytoplasmic maturity than to the events associated with ageing in metaphase II. Biochemical events related to achievement of activation competence may occur in pig oocytes, according to our experiments, between 32 and 36 h of culture.

The release of cortical granules may not be a prerequisite for advanced parthenogenetic development (Kaufman, 1983). For two reasons, we still wondered whether this phenomenon occurs in pig oocytes activated by an electrical pulse. First, considerable interspecies variability has been reported on the occurrence of a cortical reaction after electrical stimulation (Gulyas, 1976). Second, the detection of cortical reaction could serve as a recognition of activation in enucleated oocytes used for cloning. Our data suggest that such an approach would be useful since the cortical reaction takes place in pig oocytes after electrical activation. Moreover, no abnormalities in distribution of cortical granules or dispersion of their contents, as described by Cran & Cheng (1986) and Hyttel *et al.* (1989), were seen in this study.

The chronology of pronucleus formation after fertilization *in vivo* has been studied in pigs (Hunter, 1972). At 3 and 4 h after mating, most oocytes were found in anaphase and telophase, respectively. Oocytes recovered 5 and 6 h after mating were mostly in pronuclear stages. Treatment of oocytes by a single electric pulse permits more precise determination of the time of activation and enabled us to present a detailed view of the development of the female pronucleus in pigs.

The second meiosis was completed within 2 h of activation. The duration of both the second anaphase and telophase does not exceed 1 h. The 2 h that elapsed from the time of activation thus represents the period in which the oocyte cytoplasm undergoes the M-phase–interphase transition. The main factor regulating both mitotic and meiotic M-phase is the maturation-promoting factor (MPF) (for review see Kishimoto, 1988; Motlík & Kubelka, 1990). It has been shown in starfish (Picard *et al.*, 1985; Dorée *et al.*, 1983), amphibians (Gerhart *et al.*, 1984) and mice (Hashimoto & Kishimoto, 1988) that the metaphase–anaphase transition is accompanied, and perhaps triggered, by an abrupt reduction in MPF activity. No detectable amounts of MPF were found in telophase in those studies. On the basis of these results, we postulate that, in pig oocytes, the MPF disappears within the first hour after activation. This conclusion may be taken into consideration when using the matured oocyte cytoplasm as a recipient for foreign nuclei in nuclear transplantations. It has been shown in mice that only nuclei entering an environment of high MPF activity undergo extensive remodelling including the disassembly of the nuclear membrane and chromosome condensation (Szöllösi *et al.*, 1986, 1988). These conditions make a direct contact of the denuded chromatin with the cytoplasm possible; this is believed to be beneficial for successful reprogramming of the introduced nucleus (Collas & Robl, 1991).

We show in this study that pig oocytes matured *in vitro* undergo a range of developmental events following activation by a single electric pulse. Further studies are necessary to assess the degree of parthenogenetic development that can be achieved by those oocytes. In this context, however, repetitive electrical stimulation may be a more relevant approach, as has been reported in rabbits (Ozil, 1990; Collas & Robl, 1990).

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