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Original article

Pronucleus formation in bovine oocytes activated by a single electric pulse

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Summary — Bovine oocytes matured *in vitro* were stimulated by a single pulse of direct current (DC), then cultured for 0.5–6 h and evaluated by light (LM) and transmission electron microscopy (TEM). At the light microscopic level, the beginning of oocyte chromatin decondensation was first observed 3 h post-activation (14%). A well-developed pronucleus with evenly granulated nucleoplasm surrounded by nuclear membrane was found in 12, 61 and 81% oocytes at 4, 5 and 6 h post-activation, respectively. The TEM evaluation revealed that nuclear membrane vesicules were first visible at 0.5 h post-activation and became even more prominent at 1 h. Based on these observations, it is concluded that a nuclear membrane starts to form immediately after oocyte activation, while a well-developed pronucleus appears at 4–6 h.

oocyte / activation / nuclear envelope / pronucleus / bovine species

Résumé — Formation du pronoyau dans les ovocytes bovins activés par un choc électrique unique. *Des ovocytes bovins maturés* in vitro *ont été stimulés par un seul choc électrique (courant continu), cultivés de 0,5 à 6 h et observés en microscopie optique et électronique à transmission (MET). Au microscope photonique, le début de la décondensation de la chromatine est observable 3 h après l'activation (14%). Un pronoyau bien développé avec un nucléoplasme régulièrement granulaire, entouré d'une membrane, est retrouvé dans 12, 61 et 81% des ovocytes, 4, 5 et 6 h après activation. La MET révèle que les vésicules formant l'enveloppe nucléaire apparaissent une demi-heure après activation et deviennent plus évidentes après 1 h. D'après ces observations, on peut conclure qu'une enveloppe nucléaire commence à se former immédiatement après l'activation de l'ovocyte tandis qu'un pronoyau bien formé n'apparaît qu'après 4 à 6 h.*

ovocyte / activation / enveloppe nucléaire / pronoyau / espèce bovine

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INTRODUCTION

In most mammalian species, the ovulated eggs are arrested at the metaphase of the second meiotic division until sperm-egg fusion. The sperm activation stimulus can be simulated artificially, *ie* parthenogenetically, *via,* for example by Ca^{2+} ionophore A23187 (Ware *et al*, 1989), protein synthesis inhibitors (First *et al*, 1992) or ethanol (Nagai, 1987) and also by an electric pulse. It has been demonstrated that electrically activated oocytes exhibit similar characteristics to penetrated eggs: establishment of zona pellucida block prevents polyspermic penetration (Gwatkin *et al*, 1973; Běhalová *et al*, 1992), completion of meiosis, chromatin decondensation and pronuclear formation (Landa and Hájková, 1989; Procházka *et al*, 1992), cleavage (Tarkowski *et al*, 1970; Kaufman *et al*, 1975; Prather *et al*, 1991; Procházka *et al*, 1993) and in particular cases limited post-implantation development (Ozil, 1990). Although the kinetics of pronucleus formation in activated bovine oocytes has been monitored (Landa and Hájková, 1989; Powell and Barnes, 1992), to our knowledge, no reports exist on nuclear envelope development prior to chromatin decondensation. Reaching a better understanding of pronucleus formation will be useful, especially in nuclear transfer experiments.

The present study was therefore conducted to determine pronucleus formation in electrically activated, *in vitro* matured bovine oocytes. The light microscopic (LM) observations were accompanied by transmission electron microscopy (TEM) to evaluate the immediate ultrastructural changes in relation to nuclear membrane development.

MATERIALS AND METHODS

Oocyte recovery and culture

Bovine ovaries, collected at a slaughterhouse, were kept in phosphate-buffered saline (PBS) at 33°C and transported to the laboratory within 1–3 h slaughter. Oocytes were obtained by aspiration from antral follicles (4–8 mm in diameter) and only the cumulus–oocyte complexes (COC) with tightly compacted multi-layers of cumulus cells were selected for culture (Xu *et al*, 1986). About 20 COC were placed in 0.5 ml medium under paraffin oil and cultured at 39°C in an atmosphere of 5% CO_2, 10% O_2 and 85% N_2 for 28 h. The culture medium was composed of modified M-199 (Pavlok *et al*, 1992) supplemented with 20% heat-inactivated estrous cow serum (ECS) and 20 IU per ml PMSG/HCG (Suigonan, Intervet, 2720 Skovlunde, Denmark). Following the maturation period, COC were stripped of their cumulus cells by vortexing in 0.1% hyaluronidase for 2 min and washed several times in TCM-199 supplemented with 10% ECS. The denuded oocytes were evaluated under a stereomicroscope and only those with spherical shape, a visible first polar body and evenly granulated ooplasm were selected for activation.

Oocyte activation

Due to the necessary time required for denudation and selection, all oocytes were pulsed at ≈ 30 h after the onset of culture. Prior to pulse treatment oocytes were equilibrated for 2 min in electroporation medium (Willadsen, 1986) and then placed in a fusion chamber consisting of 2 parallel wire electrodes 0.5 μm apart. The chamber was connected to a Krüss CFA 400 electrofusion apparatus and overlaid with electroporation medium. Activation was electrically induced using a field of alternating current (AC) of 6 V and 600 kHz for oocytes alignment and a single 1, 1.125 or 1.25 kV/cm DC pulse of 30 μs duration. After stimulation the oocytes were washed

through M-199 + 10% ECS and cultured in the same medium under the previously described culture conditions for 0, 0.5 and 1 h (TEM) or for 0.5, 1, 2, 3, 4, 5, 6 and 24 h (LM). Control unpulsed oocytes were cultured for an additional 1 h (TEM) or 24 h (LM).

Oocyte examination

At the end of each culture period, oocytes were processed for LM or TEM (see later). Oocytes for LM were mounted on slides, fixed in acetic ethanol (1:3) for 24 h, stained with 1% acetic orcein and differentiated in 25% acetic acid. Oocytes which had progressed to anaphase II; pronucleus (PN) formation or cleavage were considered activated. For the description of karyokinesis within 6 h post-activation, the modified criteria of Xu and Greve (1988) were adopted. Briefly, PN1 was substituted by anaphase II (AII) and telophase II (TII), oocytes with the second polar body abstricted were classified as PN2 and those with initial chromosome decondensation and nuclear envelope formation as PN3. Oocytes showing complete chromosome decondensation and pronucleus surrounded by nuclear membrane were classified as category PN4 . Oocytes which possessed 2 polar bodies and chromosomes arranged in a metaphase plate were termed as being in the third meiotic metaphase (MIII) (Kubiak, 1989).

Oocytes for TEM were fixed at 0 h (n = 3), 0.5 h (n = 4) and 1 h (n = 5) after stimulation and control oocytes (n = 3) after 1 h of additional culture and processed according to Hyttel and Madsen (1987). Briefly, specimens were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer for 1 h at 4°C, washed twice in 0.1 M phosphate buffer, post-fixed in 1% OsO_4 in 0.1 M phosphate buffer, washed twice in distilled water, uranyl block-stained, dehydrated and embedded in Epon. Subsequently, semi-thin sections were prepared and stained with 1% basic toluidine blue for LM evaluation. Selected sections were reembedded; ultrathin sections were contrasted for TEM.

Statistics

The data were compared by χ^2 analysis at p = 0.05.

RESULTS

Experiment 1

The extent of activation caused by handling and denuding of *in vitro* matured oocytes was examined in experiment 1.

At the end of maturation period, 2 groups of oocytes, *ie* COC *versus* denuded, were cultured for an additional 24 h and after fixation scored for activation. As seen from table I, activation rate in denuded oocytes (13%) was not significantly higher than the COC group (3%) (p > 0.05). Cytologically, the majority of activated eggs (7/11) exhibited formation of a third metaphase.

Experiment 2

Experiment 2 assessed the optimal level of an electrical DC pulse for oocyte activation (table II). The exposure to a single DC pulse of 1, 1.125 and 1.25 kV/cm caused activation of 73–79% oocytes fixed 24 h after stimulation. Although the activation rate was similar (p > 0.05) at all pulses used, the stronger stimulations (1.125 and 1.25 kV/cm) increased the number of degenerated oocytes (9–10% compared to 0% at 1 kV/cm) and negatively affected the extru-

Table I. Effect of handling on spontaneous activation in cumulus oocyte complexes (COC) and denuded bovine oocytes (total of 5 replicates per group).

Type of oocyte	*No of oocytes*		*Type of activation*		
	Total	*Activated (%)*	*MIII*	*PN*	*2 cells*
COC	69	2 (3)		1	1
Denuded	72	9 (13)	7	2	

Table II. The activation of bovine oocytes at different parameters of DC pulse (total of 4–5 replicates per group).

Pulse strength (kV/cm)	*No of oocytes*		
	Pulsed	*Activated (%)*	*Degenerated (%)*
1.00	98	74 (76)	0 [a]
1.125	67	49 (73)	7 (10) [b]
1.25	67	53 (79)	6 (9) [b]
Control	107	10 (9)	0

[a,b] Values with different superscripts are statistically different ($p < 0.05$).

sion of the second polar body (45–51% compared to 26% at 1 kV/cm; data not shown). Based on these results, a single DC pulse of 1 kV/cm was chosen for oocyte activation in the following experiments.

Experiment 3

Experiment 3 described the sequence of pronucleus formation in parthenogenetically stimulated oocytes during the first 6 h post-activation. As shown in table III, AII was reached within 0.5 h (88/88, 100%) and TII within 1 h (90/90 100%) post-activation. The majority of the pulsed oocytes (66/83, 80%) extruded the second polar body (PN2) within 2 h post-stimulation. The initial decondensation of the chromatin (PN3) was first observed at 3 h post-activation (13/96, 14%). The well-developed pronucleus with an evenly granulated nucleoplasm, surrounded by nuclear membrane (PN4) was seen in oocytes at 4 h (11/94, 12%) post-activation and increased in frequency at 5 and 6 h (60/98, 61% and 64/79, 81%, respectively).

Experiment 4

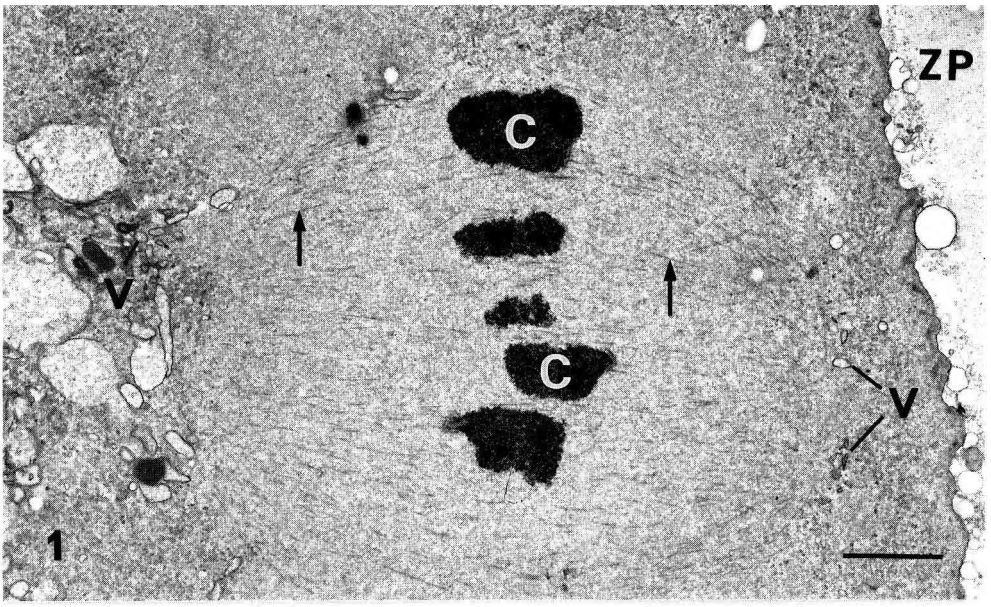
Immediate ultrastructural changes in activated oocytes were evaluated in experiment 4. From the oocytes fixed at 0.5 and 1 h post-stimulation, 7 were activated: 4 in AII and 3 in TII. TEM observation showed that portions of nuclear envelope developing from the smooth endoplasmatic reticulum were visible at 0.5 h post-activation (fig 2) and became even more prominent at 1 h (fig 3b). The nuclear envelope portions were always found at that side of the chromatin, which faced the center of the ovum, leaving space for peripheral attachment of microtubules. All unpulsed oocytes fixed after 1 h were at MII.

DISCUSSION

Parthenogenetic activation of mammalian oocytes was induced by several physical and chemical stimuli. It cannot be excluded that the oocytes may be exposed to one or more of these stimuli during their *in vitro* handling procedures. Graham (1970) and

Fig 1. Detail of an oocyte fixed immediately after electric stimulation with the arrangement of the chromatin (C) in metaphase configuration with microtubules (arrows) attached. Note the location of vesicules (V) at the poles of the spindle. ZP : zona pellucida ; bar = 2 μm.

Fig 2. Detail of an oocyte fixed 30 min after electric stimulation with the chromatin (C) arranged in the anaphase configuration. Note the initial formation of nuclear envelope portions (NE) from the smooth endoplasmic reticulum (SER). Pvs : perivitelline space ; bar = 1 μm.


ZP
C
C
V
V
1

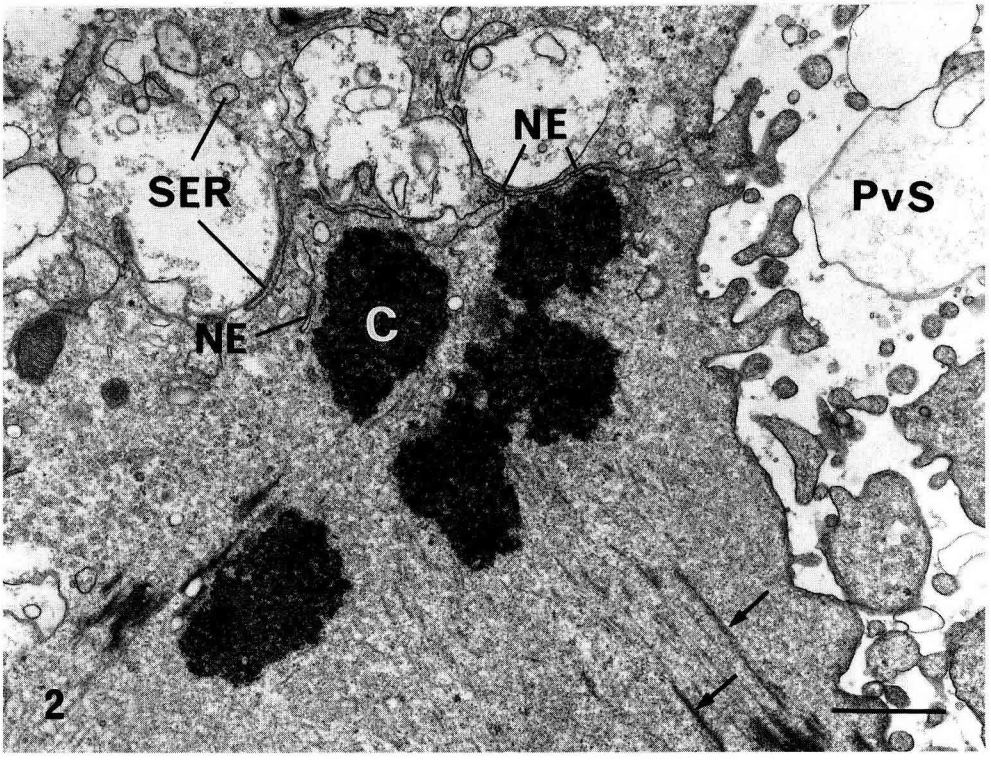

NE
SER
PvS
NE
C
2

Table III. Chronology of pronucleus formation within 6 h post-activation (total of 4–5 replicates per group).

Culture period (h)	*No of oocytes*			*Developmental stage of activation*					
	Pulsed	*Degenerated*	*Activated*	*MIII*	*AII*	*TII*	*PN2*	*PN3*	*PN4*
0.5	99	0	88		40	42	6		
1	102	3	90			37	53		
2	102	4	83			17	66		
3	104	3	96			2	81	13	
4	105	2	94	4		1	45	33	11
5	104	1	98	4		1	14	19	60
6	86	5	79	7			1	7	64
Total	702	18 (3) *	628 (89) *						

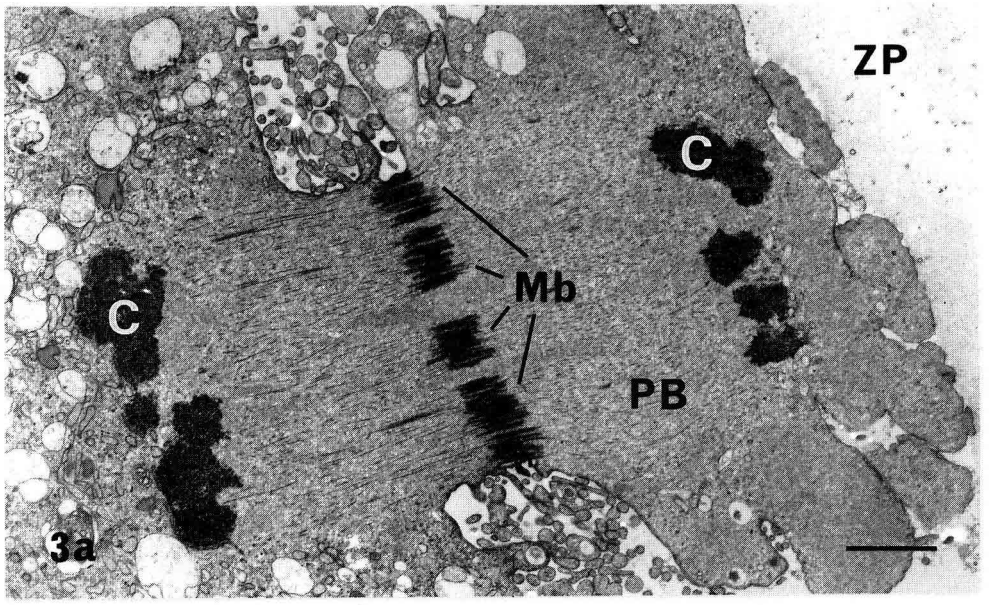
* In % from pulsed oocytes.

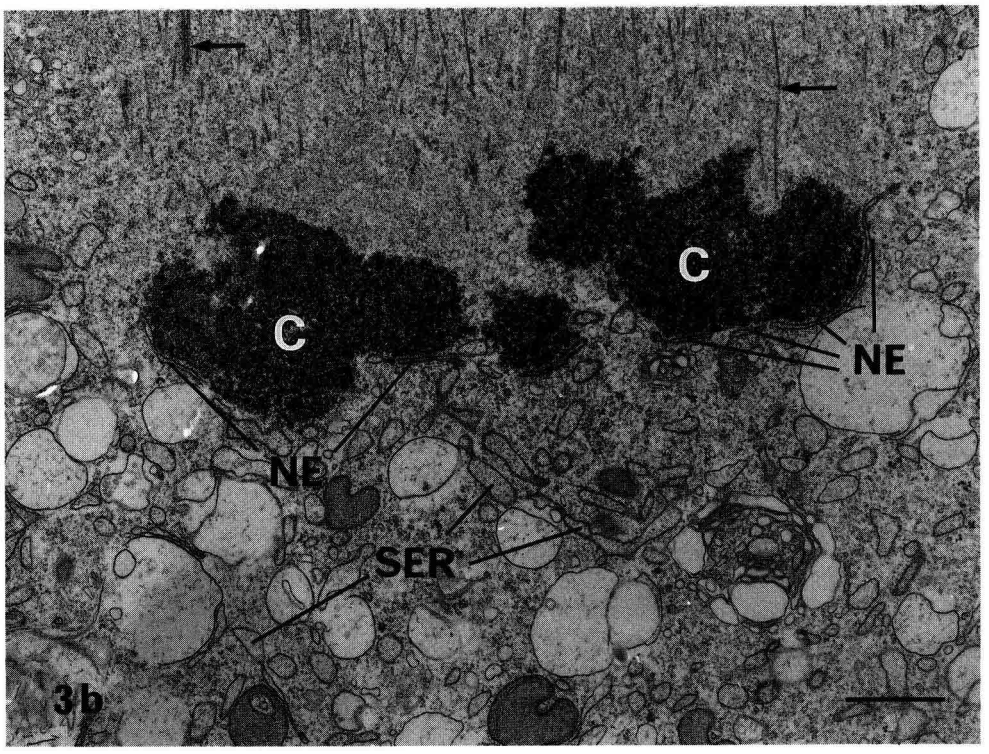
Kaufman (1973) observed parthenogenetic activation of mouse oocytes by exposure to hyaluronidase. On the other hand, hyaluronidase did not appear to activate *in vivo* (King *et al*, 1988) and *in vitro* (Procházka *et al*, 1993) matured bovine oocytes. This is confirmed by the present data where handling and denudation did not result in a significant increase in activation rate compared to the COC group.

Studies of the kinetics of bovine sperm penetration *in vitro* have shown that the first ova with sperm heads in the ooplasm appeared between 3–6 h post-insemination and their number increased until 5–16 h (Xu and Greve, 1988; Park *et al*, 1989; Cox, 1991; Saeki *et al*, 1991). Since the oocytes used for IVF by the above authors were cultured for 24–27 h before insemination, the oocyte spermatic activation occurred between 27–30 h after onset of oocyte maturation culture. It has been demonstrated that the response of bovine oocytes to parthenogenetic stimuli generally increased with their ageing and reached a maximum at 30–32 h (Nagai, 1987; Ware *et al*, 1989; Procházka *et al*, 1993). Moreover, development of zygotes from oocytes matured for 26–28 h led to 45–60% of blastocysts (Běhalová and Avery, unpublished results). It was therefore decided to culture oocytes for 28 h and taking into account the necessary time for denudation and selection, oocytes were pulsed at ≈ 30 h.

Enhanced oocyte activation by numerous pulses has already been established (Kono *et al*, 1989; Collas and Barnes, 1992; Procházka *et al*, 1993). However, under our optimal conditions, a single electric stimulus led to 76–89% of oocytes activation. Compared to the data obtained by

Fig 3. a. Detail of an oocyte fixed 60 min after electric stimulation with the chromatin (C) at the anaphase configuration. Note the prominent midbody (Mb) at the attachment to the developing second polar body (PB); bar: 2 μm. **b**. Detail of the same oocyte as in **a**. Note the chromatin (C) with the attached microtubules (arrows) and the development of large portions of nuclear envelope (NE) from the smooth endoplasmatic reticulum (SER); bar: 1 μm.


ZP
C
C
Mb
PB
3a


C
C
NE
NE
SER
3b

Kono *et al* (1989), Collas and Barnes (1992) and Procházka *et al* (1993), a single electric pulse was just as effective as multiple stimulations. The principal benefit of the repetitive stimulation was probably caused by the intervals between successive pulses, which allowed further oocyte cytoplasmic maturation (Procházka *et al*, 1993).

The time sequence of pronucleus formation was similar to other reports of parthenogenetically stimulated oocytes (Landa and Hájková, 1989; Powell and Barnes, 1992) and *in vitro* fertilized zygotes (Xu and Greve, 1988). Although the second polar body extrusion was completed within the first 2 h of activation by pulse or gamete fusion, the onset of chromatin decondensation differed according to oocyte age. In ageing eggs (Xu and Greve, 1988; Powell and Barnes, 1992) chromatin decondensation and nuclear membrane formation was noted earlier, at 2 h compared to 3 h post-activation as observed by Landa and Hájková (1989) and the present study. The faster nucleus formation in aged oocytes was probably due to a low level of maturation-promoting factor (MPF) activity known to be responsible for nuclear envelope breakdown, chromosome condensation and spindle formation (Masui and Markert, 1971). This asynchrony became indistinguishable at 6 h post-activation when most of oocytes or zygotes possessed a well-developed pronucleus (Xu and Greve, 1988; Landa and Hájková, 1989; Powell and Barnes, 1992; and the present results).

At the ultrastructural level, the association of the maternal chromosomes with segments of the nuclear envelope was initiated within the first 4 h of ovulation *in vivo* and chromatin decondensation as well as nucleus envelope formation were complete at 5–7 h (Hyttel *et al*, 1988). This time-sequence contrasts with the present results in which nuclear membrane vesicles were already visible by TEM at 0.5 h post-activation and chromatin decondensation by LM occurred at 3 h. This discrepancy may be due to the delay caused by sperm-egg binding and penetration of the zona pellucida under the *in vivo* fertilization situation. In other mammalian species such as in parthenogenetically stimulated mouse, hamster and rabbit oocytes, no onset of pronucleus formation was observed by TEM within the first h post-activation (Gulyas, 1976).

In conclusion, the present study described the time sequence of pronucleus formation in parthenogenetically activated *in vitro* matured bovine oocytes. It was established that the nuclear membrane starts to form immediately after oocyte activation, and that a well-developed pronucleus appears at 4–6 h.

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REFERENCES

Běhalová E, Hyttel P, Smith SD, Greve T, Callesen H (1992) The block against polyspermic fertilization in cattle. *Micron Microsc Acta* 23, 69-70

Collas P, Barnes FL (1992) Parthenogenetic development of bovine oocytes. *Biol Reprod* 46 (suppl 1), 65

Cox JF (1991) Effect of the cumulus on *in vitro* fertilization of *in vitro* matured cow and sheep oocytes. *Theriogenology* 35, 191

First NL, Leibfried-Rutledge ML, Northey DL, Nuttleman PR (1992) Use of *in vitro* matured oocytes 24 h of age in bovine nuclear transfer. *Theriogenology* 37, 211

Graham CF (1970) Parthenogenetic mouse blastocysts. *Nature (Lond)* 266, 165-167

Gulyas BL (1976) Ultrastructural observations on rabbit, hamster and mouse eggs following electrical stimulation *in vitro*. *Am J Anat* 147, 203-218

Gwatkin RBL, Williams DT, Hartmann JF, Kniazuk M (1973) The zona reaction of hamster and mouse eggs: production *in vitro* by a trypsin-like protease from cortical granules. *J Reprod Fertil* 32, 259-265

Hyttel P, Madsen I (1987) Rapid method to prepare mammalian oocytes and embryos for transmission electron microscopy. *Acta Anat* 129, 12-14

Hyttel P, Greve T, Callesen H (1988) Ultrastructure of *in vivo* fertilization in superovulated cattle. *J Reprod Fertil* 82, 1-13

Kaufman MH (1973) Parthenogenesis in the mouse. *Nature (Lond)* 242, 475-476

Kaufman MH, Huberman E, Sachs L (1975) Genetic control of haploid parthenogenetic development in mammalian embryos. *Nature (Lond)* 254, 694-695

King WA, Xu KP, Sirard MA, Greve T, Leclerc P, Lambert RD, Jacques P (1988) Cytogenetic study of parthenogenetically activated bovine oocytes matured *in vivo* and *in vitro*. *Gamete Res* 20, 265-274

Kono T, Iwasaki S, Nakahara T (1989) Parthenogenetic activation by electric stimulus of bovine oocytes matured *in vitro*. *Theriogenology* 32, 569-576

Kubiak JZ (1989) Mouse oocytes gradually develop the capacity for activation during the metaphase II arrest. *Dev Biol* 136, 537-545

Landa V, Hájková M (1989) Parthenogenetic activation of cattle oocytes by a single pulse of direct current. *Folia Biol (Prague)* 35, 307-313

Masui Y, Markert CL (1971) Cytoplasmic control of nuclear behavior during meiotic maturation of frog oocytes. *J Exp Zool* 177, 129-145

Nagai T (1987) Parthenogenetic activation of cattle follicular oocytes *in vitro* with ethanol. *Gamete Res* 16, 243-249

Ozil JP (1990) The parthenogenetic development of rabbit oocytes after repetitive pulsatile electrical stimulation. *Development* 109, 117-127

Park CK, Ohgoda O, Niwa K (1989) Penetration of bovine follicular oocytes by frozen-thawed spermatozoa in the presence of caffeine and heparin. *J Reprod Fertil* 86, 577-582

Pavlok A, Lucas-Hahn A, Niemann H (1992) Fertilization and developmental competence of bovine oocytes derived from different categories of antral follicles. *Mol Reprod Dev* 31, 63-67

Powell R, Barnes FL (1992) The kinetics of oocyte activation and polar body formation in bovine embryo clones. *Mol Reprod Dev* 33, 53-58

Prather RS, Eichen PA, Nicks DK, Peters MS (1991) Artificial activation of porcine oocytes matured *in vitro*. *Mol Reprod Dev* 28, 405-409

Procházka R, Kaňka J, Šutovský P, Fulka J, Motlík J (1992) Development of pronuclei in pig oocytes activated by a single electric pulse. *J Reprod Fertil* 96, 725-734

Procházka R, Durnford R, Fiser PS, Marcus GJ (1993) Parthenogenetic development of activated *in vitro* matured bovine oocytes. *Theriogenology* 39, 1025-1032

Saeki K, Kato H, Hosoi Y, Miyake M, Utsumi K, Iritani A (1991) Early morphological events of *in vitro* fertilized bovine oocytes with frozen-thawed spermatozoa. *Theriogenology* 35, 1051-1058

Xu KP, Greve T, Smith SD, Hyttel P (1986) Chronological changes of bovine follicular oocyte maturation *in vitro*. *Acta Vet Scand* 27, 505-519

Xu KP, Greve T (1988) A detailed analysis of early events during *in vitro* fertilization of bovine follicular oocytes. *J Reprod Fertil* 82, 127-134

Tarkowski AK, Witkowska A, Nowicka J (1970) Experimental parthenogenesis in the mouse. *Nature (Lond)* 226, 162-165

Ware CB, Barnes FL, Maiki-Laurila M, First NL (1989) Age dependence of bovine oocyte activation. *Gamete Res* 22, 265-275

Willadsen SM (1986) Nuclear transplantation in sheep embryos. *Nature (Lond)* 320, 63-65