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Pronucleus formation, DNA synthesis and metaphase entry in porcine oocytes following intracytoplasmic injection of murine spermatozoa

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Summary

The onset of pronucleus formation and DNA synthesis in porcine oocytes following the injection of porcine or murine sperm was determined in order to obtain insights into species-specific paternal factors that contribute to fertilisation. Similar frequencies of oocytes with female pronuclei were observed after injection with porcine sperm or with murine sperm. In contrast, male pronuclei formed 8–9 h following the injection of porcine sperm, and 6–8 h following the injection of murine sperm. After pronucleus formation maternally derived microtubules were assembled and appeared to move both male and female pronuclei to the oocyte centre. A few porcine oocytes entered metaphase 22 h after the injection of murine sperm, but normal cell division was not observed. The mean time of onset of S-phase in male pronuclei was 9.7 h following porcine sperm injection and 7.4 h following mouse sperm injection. Ultrastructural observation revealed that male pronuclei derived from murine sperm in porcine oocytes are morphologically similar to normal male pronuclei in porcine zygotes. These results suggest that species-specific paternal factors influence the onset of pronucleus formation and DNA synthesis. However, normal nuclear cytoplasmic interactions were observed in porcine S-phase oocytes following murine sperm injection.

Keywords: DNA synthesis, Porcine oocyte, Pronucleus, Sperm injection

Introduction

During fertilisation, morphological and molecular events that affect male and female chromatin are under precise temporal control. In conventional *in vitro* fertilisation of the mouse, meiosis is completed within 2 h of insemination, and male and female pronuclei form between 4–7 h and 5–8 h post-insemination, respectively (Howlett & Bolton, 1985). DNA synthesis is initiated randomly beginning about 11 h post-insemination, and S-phase lasts 6–7 h in both male and female pronuclei (Howlett & Bolton, 1985; Bouniol-Baly *et al*., 1997). The time of onset of replication in the mouse seems to be controlled by maternal factors stored in oocytes, which probably influence the formation of a functional nuclear membrane (Blow & Laskey, 1988). In contrast, in cattle, sperm influences the onset of DNA synthesis during fertilisation (Comizzoli *et al*., 2000). Replication initiates earlier and lasts longer in zygotes fertilised by sperm of high *in vivo* potency, while replication terminates at a time that is independent of the fertility of the bull (Eid *et al*., 1994; Comizzoli *et al*., 2000).

In the pig, sperm penetration is observed 3–5 h after mating and male and female pronuclei form 5–8 h after mating (Hunter, 1972, 1974). The synchronous development of paternal and maternal pronuclei in *in vivo* fertilised zygotes has been demonstrated, but details

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concerning the onset of pronucleus formation and DNA synthesis have not been described (Laurincik *et al.*, 1995). Previously, we reported successful pronucleus formation and apposition in porcine oocytes following injection with either porcine or murine sperm (Kim *et al.*, 1999; Lee *et al.*, 2002). We observed pronucleus formation around 6 h and pronuclear apposition beginning around 9 h following the injection of either porcine or murine sperm. However, little is known about the influence of paternal effects on the onset of pronucleus formation and DNA synthesis following the injection of sperm into porcine oocytes. In the present study we compare these landmarks of fertilisation in porcine oocytes injected with porcine and murine sperm in order to gain insights into species-specific paternal factors that contribute to fertilisation. We additionally describe the ultrastructure of the porcine zygote following murine sperm injection in relation to the chronology of pronuclear S-phase.

Materials and methods

In vitro maturation

Prepubertal porcine ovaries were collected from a local slaughterhouse and transported to the laboratory at 25 °C in Dulbecco's phosphate-buffered saline supplemented with 5.54 mM D-glucose, 0.33 mM sodium pyruvate, 75 μg/ml potassium penicillin G and 50 μg/ml streptomycin sulfate (mDPBS). Cumulus–oocyte complexes (COCs) were aspirated from follicles with an 18 gauge needle into a disposable 10 ml syringe. Fifty porcine COCs were matured in 500 μl of a defined protein-free medium consisting of Tissue Culture medium 199 (TCM199) supplemented with 3.05 mM D-glucose, 0.91 mM sodium pyruvate, 10 ng/ml epidermal growth factor, 0.1% polyvinyl alcohol, 0.57 mM cysteine, 0.5 μg/ml LH and 0.5 μg/ml FSH under paraffin oil at 39 °C for 40–44 h. After maturation, the cumulus cells were removed by vigorous pipetting of the complexes in the presence of 0.3 mg/ml hyaluronidase.

Injection of sperm into oocytes

Spermatozoa were centrifuged (400 × *g*, 5 min) and resuspended in TCM-HEPES:10% polyvinylpyrrolidone solution (1:1). A microdrop (5 μl) of this suspension was placed on a slide, and the slide placed in a Nikon Differential Interference Contrast inverted microscope equipped with Narishige micromanipulators. The oocytes were denuded of cumulus cells by repeated pipetting. Oocytes with visible polar bodies and of excellent morphology were used. Oocytes were centrifuged for 10 min in an Eppendorf centrifuge at 12 000 × *g* in a 1.2 ml Eppendorf centrifuge tube. Spermatozoa were injected into the cytoplasm of oocytes using the method of Lee *et al.* (1998). Briefly, the injection needle used was of 6–7 μm inner diameter and 8–9 μm outer diameter. The polar body was at 6 or 12 o'clock and the point of injection at 3 o'clock. An oocyte was penetrated by the injecting micropipette, a small amount of cytoplasm was drawn into the micropipette, and then the cytoplasm together with the spermatozoon and a small amount of medium was expelled into the oocyte. Immediately after ooplasmic injection, the injecting micropipette was quickly withdrawn, and the oocyte released from the holding pipette to reduce the intracytoplasmic pressure exerted on the oocyte. After injection, all oocytes were transferred to NCSU23 medium and cultured at 39 °C under 5% CO_2 in air.

Immunofluorescence microscopy

Microtubules and DNA were detected by indirect immunocytochemical techniques as described by Kim *et al.* (1996). Briefly, the oocytes were permeabilised in modified Buffer M (Simerly & Schatten, 1993; 25% glycerol, 50 mM KCl, 0.5 mM MgCl2, 0.1 mM ethylenediaminetetraacetic acid, 1 mM β-mercaptoethanol, 50 mM imidazole, pH 6.7, 3% Triton X-100 and 25 mM phenylmethylsulfonylfluoride) for 20 min, fixed in methanol at –20 °C for 10 min and stored in phosphate-buffered saline solution (PBS) containing 0.02% sodium azide and 0.1% bovine serum albumin for 2–7 days at 4 °C. Microtubule localisation was performed using anti-α-tubulin (Sigma #T-5168). Fixed oocytes were incubated for 90 min at 39 °C with a 1:100 dilution of anti-α-tubulin in PBS. After several washes with PBS containing 0.5% Triton-X 100 and 0.5% bovine serum albumin (BSA), oocytes were incubated in a blocking solution (0.1 M glycine, 1% goat serum, 0.01% Triton X-100, 1% powdered milk, 0.5% BSA and 0.02% sodium azide) at 39 °C for 1 h. The blocking was followed by incubation with fluorescein isothiocyanate (FITC)-labelled goat anti-mouse antibody (Sigma). DNA was fluorescently detected by exposure to 50 mg/ml propidium iodide (Sigma) for 1 h. Stained oocytes were then mounted under a coverslip with antifade mounting medium (Universal Mount, Fisher Scientific, Huntsville, AL, USA) to slow photobleaching. Slides were examined using laser-scanning confocal microscopy, performed using a Bio-Rad MRC 1024 equipped with a krypton–argon ion laser for the simultaneous excitation of fluorescence for microtubules and propidium iodide for DNA.

Indirect immunofluorescence of DNA synthesis

DNA synthesis was measured by incorporation of 5-bromo-2'-deoxyuridine (BrdU, Sigma). Embryos after

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intracytoplasmic sperm injection were incubated with 100 μM BrdU in NCSU23 containing 10% BSA at 39 °C in 5% CO_2 in air. After labelling, embryos were washed three times in PBS containing 1% BSA (PPB). The zonae pellucidae of the oocytes were removed by a brief exposure to 0.5% protease and then washed in PPB. Zona-free embryos were fixed in methanol at –20 °C for 20 min, washed in PPB, and then permeabilised in 1% (v/v) Triton X-100 in PPB for 15 min and washed three times again in PPB. Embryos were hydrolysed in 4 N HCl for 30 min, washed in PPB, and incubated overnight with monoclonal mouse anti BrdU (Sigma) diluted 1:10 in 1% FBS in PPB at 4 °C in a humidified box. They were then washed in PPB three times and incubated with FITC-conjugated anti-mouse IgG (Sigma) diluted 1:100 in 1% FBS in PPB for 2 h in the dark at room temperature. Afterwards, embryos were washed in PPB three times for 15 min each, and then stained with propidium iodide (200 μg/ml) in PPB for 15 min, washed three times in PPB again, mounted on slides in antifade mounting medium, and viewed with a laser-scanning confocal microscope at a fixed wavelength of 488 nm.

Transmission electron microscopy

Oocytes were fixed in 2% glutaraldehyde in Dulbecco's phosphate-buffered saline. The specimens were then postfixed in 2% OsO_4 for 1 h, dehydrated in a graded ethanol series, and embedded in epoxy

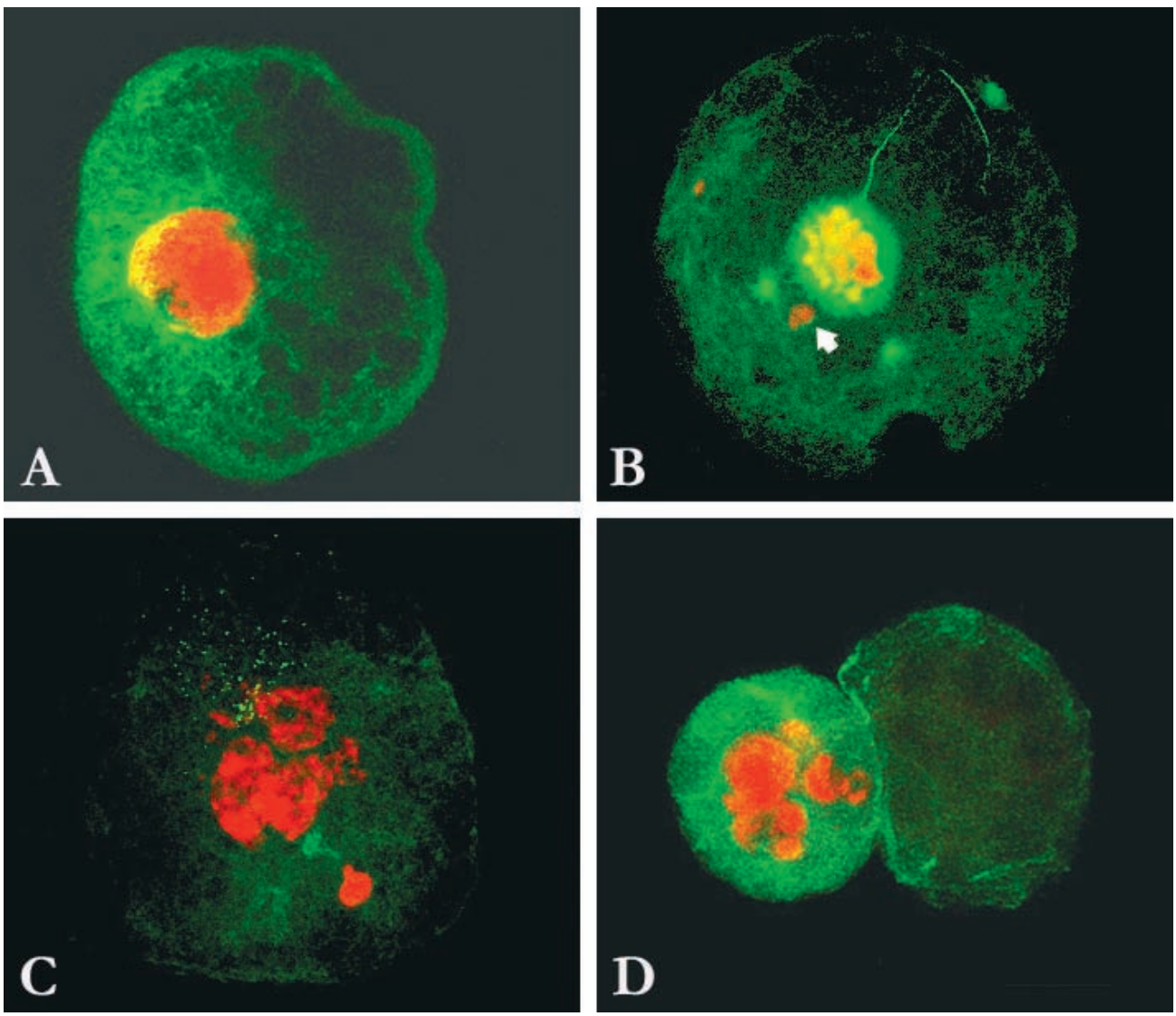

Figure 1 Pronuclear apposition, metaphase entry and abnormal cleavage in porcine oocytes following injection of murine spermatozoa. Red, DNA; green, microtubules. (*A*) Pronuclear apposition. (*B*) Metaphase entry in chromatin. Arrow indicates chromatin debris. (*C*), (*D*) Multiple pronuclear-like structures in a porcine oocyte (*C*) and abnormal cleavage (*D*) at 26 h following murine sperm injection.

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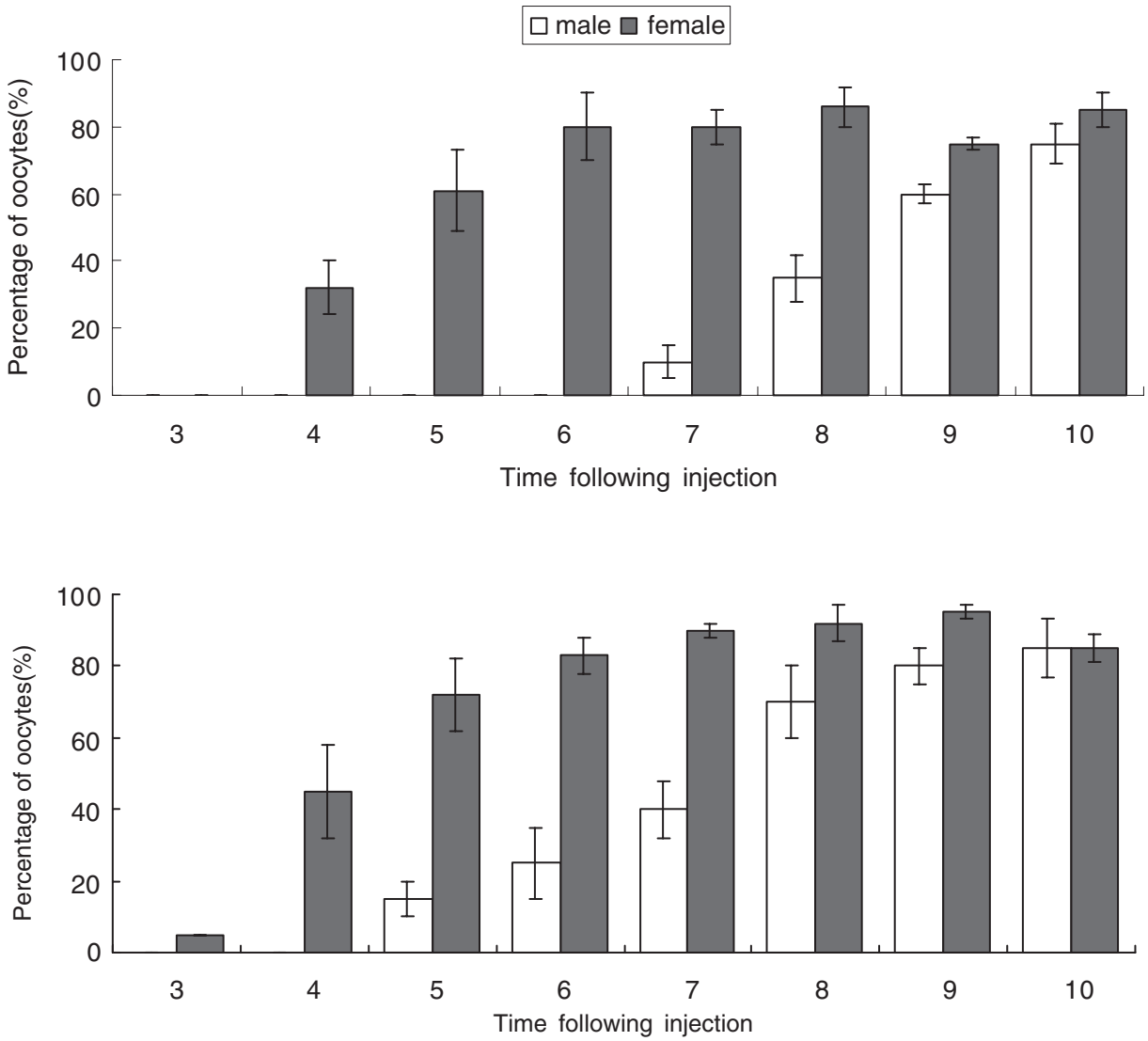

Figure 2 The incidence (mean ± SEM) of oocytes with male pronucleus (open bars) and female pronucleus (filled bars) formation in porcine oocytes following injection of porcine (above) and mouse (below) sperm.

resin. Ultra-thin sections were cut with a diamond knife and post-stained first with 1% uranyl acetate in 30% ethanol and then with Reynolds' lead citrate. A transmission electron microscope (Hitachi 600, Hitachi, Kashiwa, Japan) was used to determine chromatin configuration in porcine oocytes following sperm injection. Negative film was digitised with a scanner (Epson GT-9000) and archived on an erasable magnetic diskette.

Statistical analysis

The effect of post-injection time on the rates of female and male pronuclear formation, and DNA replication, was expressed as a linear regression of the individual percentage and time following sperm injection (Comizzoli *et al.*, 2000). The mean time of these events was defined as the time at which half the zygotes had a male or female pronucleus or were undergoing DNA synthesis. Data were pooled from at least four replicate experiments. Differences in the percentages of oocytes developing to a particular stage were determined by chi-square procedures.

Results

Pronucleus formation following the injection of porcine and murine sperm

Microtubules and chromatin configuration during pronuclear apposition, entry into metaphase, and cell division were monitored at 22 and 26 h following the injection of murine sperm, as shown in Fig. 1.

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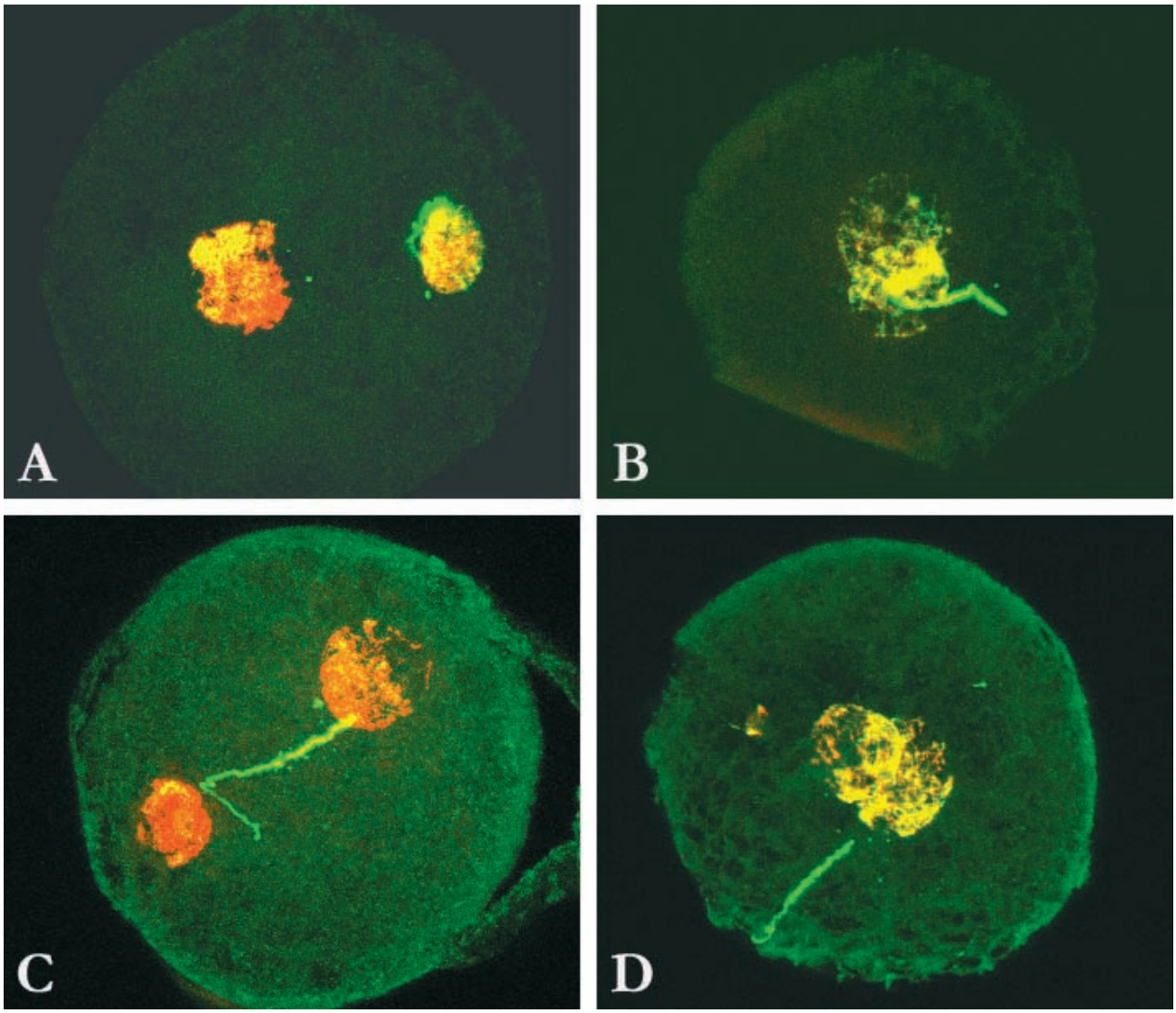

Figure 3 DNA synthesis in porcine oocytes following porcine (*A*, *B*) and murine sperm injection (*C*, *D*). Red, chromatin; green: BrDU.

Following pronucleus formation, maternally derived microtubules assembled and appeared to move both the male and female pronuclei into the oocyte centre (Fig. 1*A*; Kim *et al*., 1999). Entry into metaphase was observed in a few oocytes 22 h following murine sperm injection (6/95, 6.3%). In all cases, chromatin debris was seen in metaphase oocytes (Fig. 1*B*). However, porcine oocytes injected with murine sperm did not cleave normally when they were fixed at 26 h (Fig. 1*C*, *D*; Kim *et al*., 1999).

The frequencies of female pronucleus formation were similar in oocytes injected with porcine sperm (32 ± 8% and 61 ± 12% at 4 and 5 h, respectively) and murine sperm (45 ±13% and 72±10% at 4 and 5 h, respectively; Fig. 2). The calculated mean time at which 50% of oocytes had a female pronucleus was 4.6 h after porcine sperm injection and 4.8 h after murine sperm injection (Fig. 2). In contrast, male pronuclei formed 8–9 h following porcine sperm injection and 6–8 h following murine sperm injection. The calculated mean time of male pronucleus formation was significantly later following porcine sperm injection (8.7 h) than following murine sperm injection (7.3 h; $p < 0.05$).

DNA synthesis following sperm injection

The synthesis of DNA after sperm injection was evaluated by BrDU incorporation into pronuclei (Fig. 3). DNA synthesis occurred only after complete decondensation of the spermatozoon. Although a fully formed female pronucleus was present in oocytes before sperm decondensation, DNA synthesis was not seen in either pronucleus (Fig. 3*C*, *D*). Oocytes with

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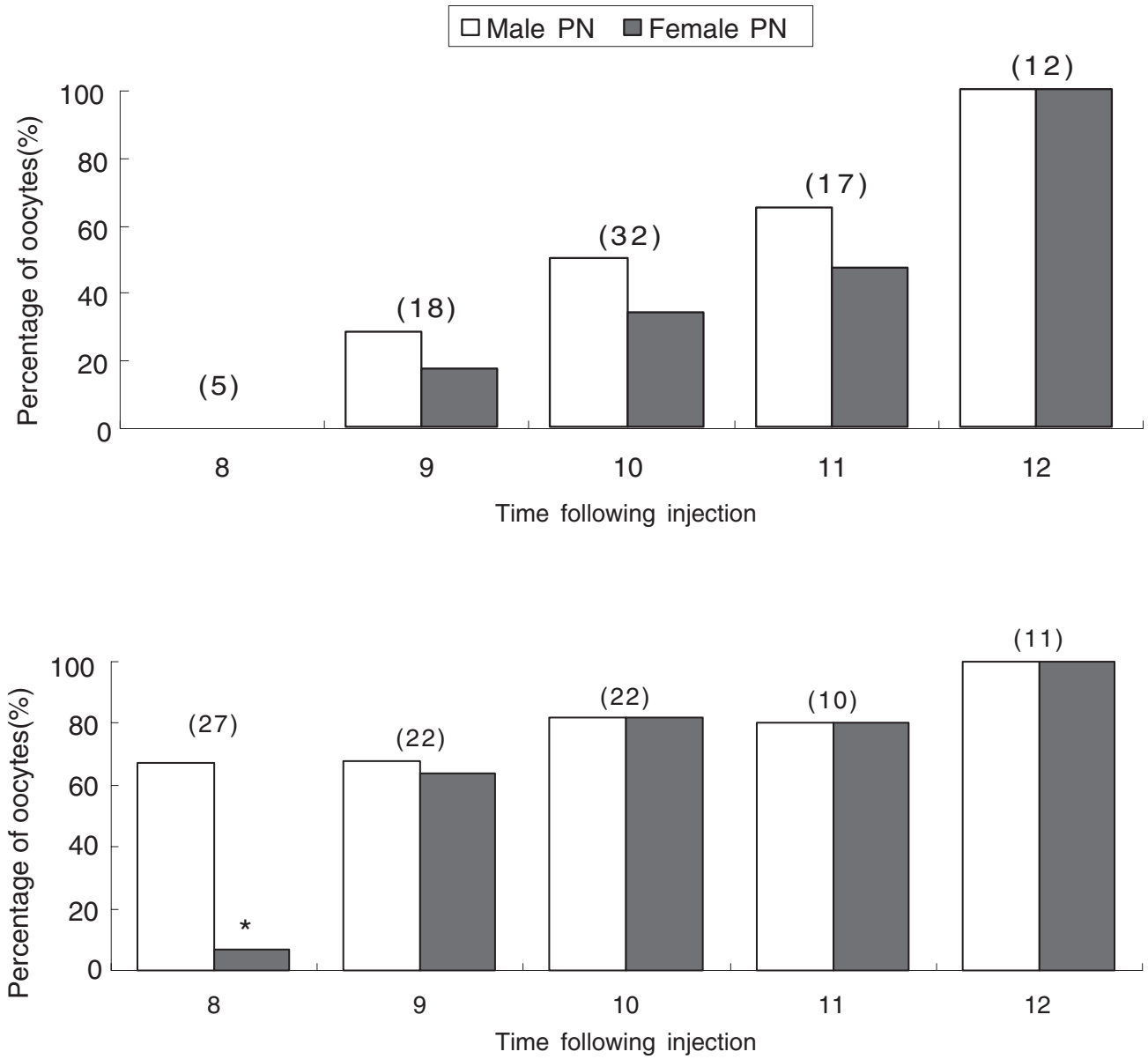

Figure 4 Onset of DNA synthesis in male pronuclei (open bars) and female pronuclei (filled bars) in porcine oocytes injected with porcine (above) and murine sperm (below). The number of zygotes examined in each experimental group is given in parentheses.

complete male and female pronuclei were assayed for DNA synthesis (Fig. 3). As shown in Fig. 4, the mean time of onset of S-phase in the male pronucleus was 9.7 h following porcine sperm injection and 7.4 h following murine sperm injection. The mean time of onset of S-phase in parthenotes was 6.3 h (n = 25). Eight hours after sperm injection the frequency of male pronuclei undergoing DNA synthesis (67%) was higher than that of female pronuclei (7%, Fig. 4; $p < 0.01$). This result suggests that DNA synthesis is delayed in both pronuclei, until male pronucleus formation and entry into S-phase.

Ultrastructure of porcine zygotes produced by murine sperm injection

Between 10 and 13 h after sperm injection, both the paternal and maternal pronuclei are surrounded by an essentially complete nuclear envelope. Nuclear precursor bodies were observed in each pronucleus (Fig. 5*A*, *B*). Most of the chromatin was dispersed, but small portions of condensed chromatin were distributed throughout the nucleoplasm. The status of chromatin was not specifically related to the presence of nuclear precursor bodies. A cluster of large and small granules was attached in the nucleolus precursor body (Fig. 5*a*). After the apposition of male and female chromatin,

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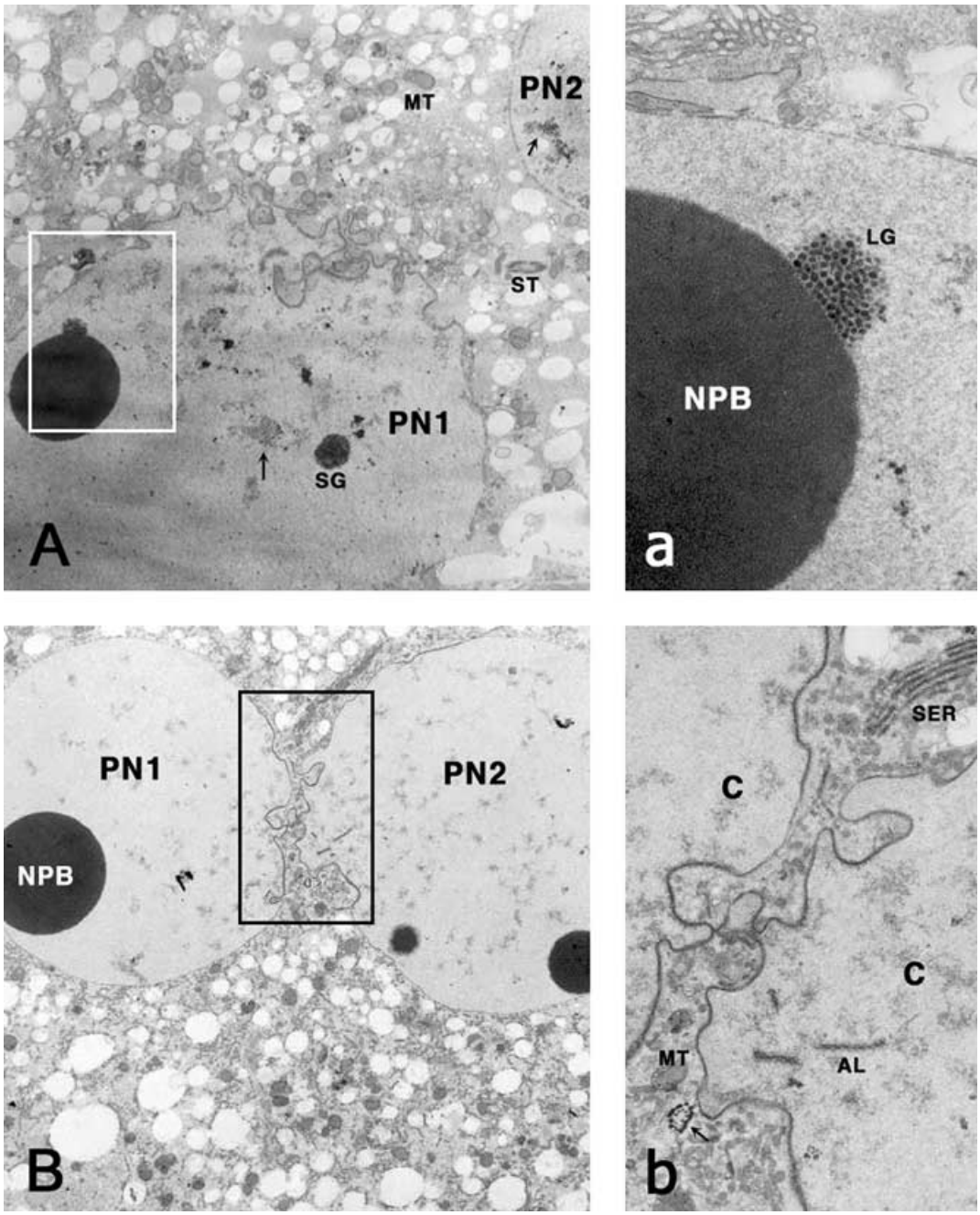

Figure 5 Ultrastructure of two pronuclei in porcine oocytes following murine sperm injection. (*A*) Two pronuclei (PN1, PN2) can be seen. The midpiece of the spermatozoon (ST) was associated with a pronucleus. Mitochondria (MT) were also seen. Soft granules (SG) and chromatin (arrows) are present in the pronucleus. (*a*) The boxed area from (*A*) showing a nuclear precursor body (NPB) that associated with large granules (LG). (*B*) The two pronuclei were separated by a narrow cytoplasmic bridge containing well-developed mitochondria (MT) and smooth endoplasmic reticulum (SER). (*b*) The boxed area from (*B*) showing the midpiece of the sperm (arrow) remaining associated in the pronucleus. Chromatin (C) and intranuclear annulated lamellae (AL) were seen.

chromatin condensation was observed throughout the nucleoplasm (Fig. 5*B*, *b*). Well-developed smooth endoplasmic reticulum, mitochondria and intranuclear annulate lamellae could be seen (Fig. 5*B*, *b*).

At 15–18 h after injection, in some cases the spherical fibrillar bodies were associated with less electron-dense material and contained a single large vacuole, conferring a ring-shaped appearance in sectioned profiles (Fig. 6*A*, *a*). The nuclear envelopes adopted an undulating appearance (Fig. 6*a*). Nucleolus precursor bodies and condensed chromatin in contact with clusters of small and large granules and the nuclear

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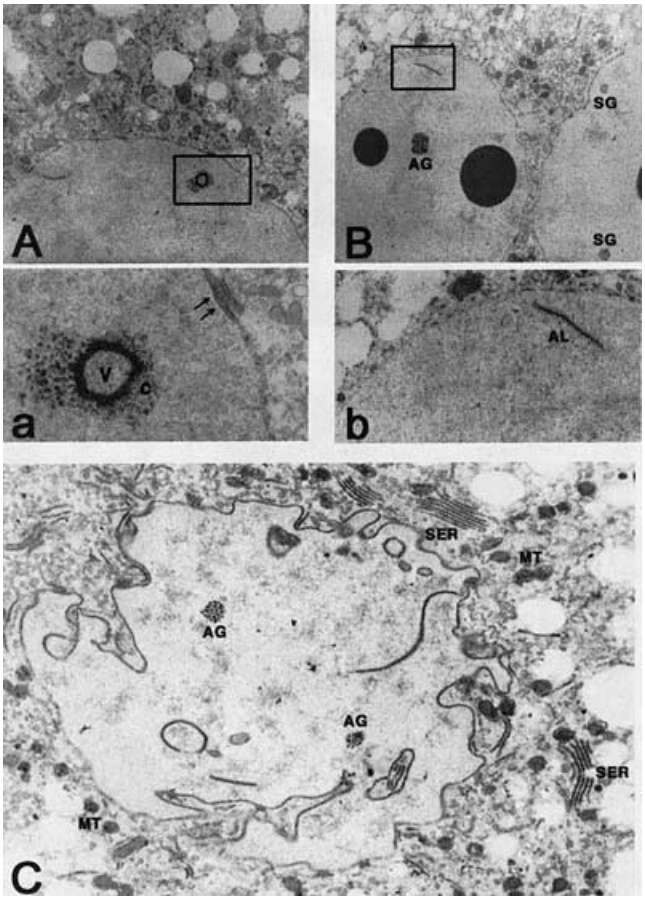

Figure 6 (*A*) Detail of an S-phase pronucleus. (*a*) The boxed area from (*A*) showing a compact, electron-dense fibrillar body with a single large vacuole (V) in contact with chromatin (C). The nuclear envelopes became undulating in this area (arrows). (*B*) Two apposed pronuclei. (*b*) The boxed area from (*B*) showing annulated lamellae (AL). (*C*) Dynamic interchanges between nucleus and cytoplasm can be seen in porcine oocytes 24 h following murine sperm injection. Aggregated granules (AG) were seen in the nucleoplasm. Smooth endoplasmic reticulum (SER) and numerous mitochondria (MT) were also observed in the vicinity of nucleus.

envelope were found in apposed pronuclear regions (Fig. 6*B*, *b*). Dynamic changes in nucleoplasm components were seen in late pronuclear stage zygotes, including well-developed smooth endoplasmic reticulum and numerous mitochondria in the vicinity of the pronucleus, but a nuclear precursor body was not present at this stage. Small granule clusters, and an accumulation of large granules and intranuclear annulate lamellae, were seen (Fig. 6*C*).

Discussion

Previously, Funahashi *et al.* (1995) observed the onset of female and male pronucleus formation in porcine oocytes matured *in vitro* following conventional *in vitro* fertilisation. After insemination, sperm penetration occurred as early as 3 h and female pronuclei had formed by 6 h with complete development by 12 h. Male pronuclei formed between 9 and 12 h after insemination. This result suggests that male pronucleus formation in *in vitro* maturation/fertilisation systems occurs a few hours later than female pronucleus formation (Funahashi *et al.*, 1995). Similarly, in the present study, we observed male pronucleus formation 7–9 h following sperm injection – a few hours later than female pronucleus formation. Since synchronised formation of male and female pronuclei has been reported for *in vivo* fertilised porcine and bovine zygotes (Hunter, 1974; Crozet, 1984), the delay in male pronuclear formation compared with female pronucleus formation has been suggested to be due to inappropriate conditions for *in vitro* maturation/fertilisation in the pig (Funahashi *et al.*, 1995, 1996; Kim *et al.*, 1996). Another explanation for asynchronous pronuclear development is that male pronucleus formation following sperm injection may require additional time for the disassembly of sperm-specific structures such as the acrosome and perinuclear theca (Sutovsky *et al.*, 1997). Ramalho-Santos *et al.* (2000) observed delayed DNA decondensation in Rhesus monkey oocytes following sperm injection, which is probably related to the persistence of the sperm acrosome and perinuclear theca.

Howlett & Bolton (1985) reported that under conventional *in vitro* fertilisation conditions, murine meiosis is completed within 2 h post-insemination, and male and female pronuclei form 4–7 h post-insemination and 5–8 h post-insemination, respectively. In contrast to what is observed for the mouse, in the pig, sperm penetration and pronucleus formation appear to occur a few hours later (9–12 h). In the present study we observed that the mean time of male pronucleus formation following porcine sperm injection was longer than that following murine sperm injection. This suggests that species-specific components present in sperm may be important in initiating for sperm nuclear decondensation and pronucleus formation. The mechanism whereby sperm components induce pronuclear formation is elusive at present. Successful sperm decondensation and male pronucleus formation requires glutathione for the reduction of sulfate bonds (Sutovsky *et al.*, 1996) and nucleoplasmin from germinal vesicle (Philpott *et al.*, 1991; Maeda *et al.*, 1998) to mediate the replacement of sperm nuclear protamine with histone. Different levels of glutathione and/or nucleoplasmin in porcine and murine oocytes may explain why murine sperm triggers the decondensation of sperm chromatin more quickly than does porcine sperm.

The onset and duration of zygotic DNA synthesis has been studied in mice (Luthardt & Donahue, 1973) and cattle (Eid *et al.*, 1994) as well as in the pig

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(Laurincik *et al*., 1995). In porcine zygotes produced by *in vivo* fertilisation, S-phase occurs about 55–62.5 h after the injection of hCG, and comparable extents of autoradiographic labelling are observed for both pronuclei in almost all zygotes, suggesting that the maternal and paternal S-phase are synchronised (Laurincik *et al*., 1995). In the present study we observed that S-phase is initiated in both male and female pronuclei after the full development of the male pronucleus. Although a fully formed female pronucleus is present in oocytes before sperm decondensation, DNA synthesis is not seen in either nucleus. This result suggests that only the paternal component influences the timing of the onset of replication. Similar research results in Rhesus monkey showed that the factor or factors contributed by paternal components are effective only after pronucleus formation, and they affect both pronuclei (Ramalho-Santos *et al*., 2000). However, it is unclear which component in sperm initiates DNA synthesis in porcine zygotes. As shown for mice (Howlett & Bolton, 1985), we also observed that in the absence of paternal components activated oocytes could initiate DNA replication at a specific interval after pronucleus formation. This finding supports the theory that endogenous oocyte programming, set in train by the terminal events of oocyte maturation, may regulate the 'house-keeping' of the eggs while sperm penetration superimposed on the oocyte programme initiates subsequent embryogenesis (Howlett & Bolton, 1985; Ramalho-Santos *et al*., 2000).

Ultrastructural studies showed that dense spherical nucleus precursor bodies are observed in both female and male pronuclei of porcine oocytes after the injection of murine sperm. It has been proposed that human nuclear precursor bodies are formed by the aggregation of small dense bodies, which arise in association with condensed chromatin (Tesarik & Kopecny, 1989). However, this phenomenon is not seen in porcine zygotes (Laurincik *et al*., 1995). In the present study we found intranuclear granules of varying size and shape as early as the pronuclear stage, similar to those observed in the pronuclei of porcine zygotes *in vivo* (Laurincik *et al*., 1995).

Conspicuous intranuclear particles or granules have been seen in pronuclei during mammalian fertilisation (Szollosi *et al*., 1990; Laurincik *et al*., 1995), and are thought to be involved in RNA/ribonucleoprotein metabolism in early embryos (Fakan & Odartchenko, 1980; Szollosi *et al*., 1990). The present study revealed that annulate lamellae develop in mature pronuclei. Intranuclear annulate lamellae have been reported in normally fertilised human (Soupart & Strong, 1974), bovine (Crozet, 1984) and porcine (Szollosi & Hunter, 1973) zygotes. In human ova, annulated lamellae are predominantly found in proximity to the pronuclei (Soupart & Strong, 1974), and they are more frequent in oocytes with polyspermic pronucleus development. In rabbit oocytes they are not seen until the fully developed pronuclei migrate to the oocyte centre. As suggested previously, lamellae may result from the overproduction of nuclear envelope fragments, with which they share structural similarities (Soupart & Strong, 1974; Thompson *et al*., 1974).

As observed for fertilisation with isogenic species sperm (Laurincik *et al*., 1995), characteristic 'undulation' of the nuclear envelope is seen in porcine oocytes following murine sperm injection. This structural change may be required for specialised nucleocytoplasmic transport. Extensive development of Golgi complex, cisternae of endoplasmic reticulum and annulated lamellae in the vicinity of the apposed pronuclei is seen following murine sperm injection (Szollosi & Hunter, 1973), which is a common feature of mammalian zygotes. Collectively, in the present study we did not see any critical ultrastructural differences between male and female pronuclei following murine sperm injection, suggesting that non-species-specific nuclear cytoplasmic interactions take place during pronuclear formation and apposition.

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