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The absence of a DNA replication checkpoint in porcine zygotes

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Summary

It has been demonstrated that in the zygotes of some mammals a unique checkpoint controls the onset of DNA replication. Thus, DNA replication begins in the maternal pronucleus only after the paternal pronucleus is fully formed. In our experiments we have investigated whether this checkpoint also operates in porcine zygotes produced either by *in vitro* fertilization (IVF) or by intracytoplasmic sperm injection (ICSI). Our results show that the onset of DNA replication occurs in the maternal pronucleus even in the presence of an intact sperm head in zygotes produced by ICSI, as well as in polyspermic eggs where some sperm heads are intact or male pronuclei are not yet fully developed. We conclude that in porcine zygotes there is an absence of the DNA replication checkpoint that is typical for some other mammals.

Keywords: Checkpoint, DNA replication, Fertilization, Pig, Zygotes

Introduction

The first zygotic mitotic cell cycle is of special importance for normal embryo development. Among many essential early events, the sperm head protamines are replaced with oocytes histones (Kopecny & Pavlok, 1975) and the paternal chromatin is actively demethylated (Young & Beaujean, 2004). Concomitantly, the oocyte completes meiosis but the maternal chromatin is rather methylated. Thereafter, the maternal and paternal pronuclei are formed and DNA replication begins before the onset of the first mitotic division. Interestingly, the onset of DNA replication in a paternal pronucleus slightly precedes replication in the partner, maternal pronucleus. Chromosome condensation and nuclear envelope breakdown (NEBD) also occur earlier in the paternal pronucleus (Liu *et al.*, 2005). Taken together, all these events must be perfectly orchestrated and well synchronized. It has been documented in non-human primate zygotes that a unique checkpoint controls the onset of DNA replication in a maternal pronucleus, replication being delayed until the paternal pronucleus fully develops (Hewitson *et al.*, 1999). This was subsequently confirmed for bovine zygotes also (Comizzoli *et al.*, 2003). It is not known, however, whether the same checkpoint exists in porcine zygotes. The pig 1-cell stage embryos produced *in vitro* are extremely unusual. Contrary to some other mammalian species they are often polyspermic with spermatozoa in different stages of decondensation. Even after intracytoplasmic sperm injection, when a single sperm is injected into the oocyte, in about half of cases the sperm head remains intact or is only partially decondensed whilst the maternal pronucleus fully develops (Kren *et al.*, 2003). These unique features make porcine zygotes very attractive biological material to test whether a DNA replication checkpoint exists in this species.

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Materials and methods

In vitro maturation

Porcine ovaries from prepubertal gilts were collected at a local slaughterhouse and transported to the

laboratory at 38 °C in phosphate-buffered saline supplemented with 0.01% polyvinyl alcohol (PBS-PVA). Thereafter, the large antral follicles (3–6 mm in diameter) were isolated from the ovaries using surgical blades. Follicles without any signs of atresia were selected and oocytes were released from them. These oocytes were further selected and only good-quality cumulus–oocyte complexes (COCs) were used for culture in 0.5 ml of medium 199 supplemented with 4 mg/ml GPoBS – growth protein of bovine serum (Sevapharma, Prague, Czech Republic), 0.2 mM sodium pyruvate, 0.57 mM L -cysteine, 5 μg/ml LH from ovine pituitary, 5 μg/ml FSH from porcine pituitary and 50 μg/ml gentamicin (Gibco, Prague, Czech Republic) at 38.5 °C in a humidified atmosphere of 5% CO_2 in air for 44–48 h. After this period, the cumulus cells from oocytes were removed by pipetting after a previous short incubation in 0.1% hyaluronidase dissolved in M2.

Sperm preparation

Czech Meat Pig semen obtained from a local insemination station was used in all experiments. The spermatozoa were stored at 15 °C in the 'long-term' extender Androhep (Minitüb, Tübingen, Germany). After removing the extender by centrifugation, the sperm pellet was separated on two layers (90% and 60%) of Percoll (Amersham Biosciences, Prague, Czech Republic) and subsequently washed twice in PBS-PVA. Finally, the pellet of spermatozoa was resuspended and preincubated for 1 h in mTBM medium (Abeydeera & Day, 1997).

In vitro fertilization (IVF)

Groups of 20–25 denuded oocytes were transferred into 0.5 ml of a sperm suspension (final concentration of 2×10^5 sperm/ml) in mTBM in 4-well dishes (Nunc, Roskilde, Denmark) and cultured for 3 h at 38.5 °C. Then, the oocytes were briefly washed and transferred into PZM3 medium (Yoshioka *et al.*, 2002) supplemented with 100 μM BrdU and cultured for another 16 h.

Intracytoplasmatic sperm injection (ICSI)

ICSI was done in PZM3 microdrops on the preheated stage of an inverted microscope (Leica DMIL) with the help of a mechanical Leica micromanipulator. For micromanipulation, 10 μl drops of PZM3 medium and 5 μl drops of PVP medium (Medicult, Jyllinge, Denmark) were placed separately in the bottom of a 60 mm Petri dish and covered by mineral oil. Only those oocytes with visible polar bodies were used. Sperm suspension prepared as described above was added in a volume of 5 μl into microdrops of PVP (final concentration 5×10^5 cells/ml). A single spermatozoon was aspirated tail-first into an injection pipette with an inner diameter of 8–10 μl and injected directly into the oocyte cytoplasm. Before the aspiration, the sperm tail was rubbed in the midpiece using the micropipette. The oocytes were injected at the 3 o'clock position while the position of the polar bodies was at 12 or 6 o'clock. After ICSI, the oocytes were transferred into PZM3 medium supplemented with 100 μM BrdU and cultured for a further 16 h.

Detection of DNA synthesis

DNA synthesis was assessed immunocytochemically after bromodeoxyuridine (BrdU) incorporation as described by Kim *et al.* (2003). Briefly, after the incubation with BrdU (100 μM, 16 h) and zona pellucida removal, the embryos were fixed in cold methanol (−20 °C, 20 min), then washed three times in PBS containing 1% BSA, permeabilized in 0.1% (v/v) Triton X-100 in PBS (15 min), denatured in 4 M HCl (30 min) and incubated overnight with monoclonal mouse anti-BrdU antibody (DakoCytomation, Brno, Czech Republic; dilution 1:30 in 1% fetal calf serum (FCS in PBS at 4 °C). After extensive washing, embryos were incubated with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG (DakoCytomation) diluted 1:30 in PBS for 2 h in the dark at room temperature. Furthermore, the embryos were also stained with propidium iodide (400 μg/ml) in PBS (15 min, 4 °C) to visualize all the DNA material and mounted on slides in Vectashield antifade mounting medium (Vector, Peterborough, UK). Fluorescence was evaluated under an inverted microscope (Leica DMIL) equipped with appropriate filters.

Unless stated otherwise all chemicals were purchased from Sigma (Prague, Czech Republic).

Results

In total, 169 *in vitro* matured oocytes were used for IVF (91) or ICSI (78) and 124 of them were successfully fertilized, i.e. the oocytes were activated and contained one (maternal) pronucleus along with the paternal pronucleus(i) or intact (decondensing) sperm head(s) (65/91 oocytes after IVF, 75.6%; 59/78 oocytes after ICSI, 71.4%).

DNA synthesis after IVF

The results are summarized in Table 1. The synthesis of DNA was evaluated 16 h after insemination, i.e. approximately 2–4 h before the first mitotic division. Only 20% (13/65) of zygotes contained two pronuclei, both synthesizing DNA (Table 1A). The remaining zygotes were polyspermic. DNA replication was also

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Table 1 DNA replication in pronuclei of porcine zygotes after IVF

			Type of fertilization	Number of zygotes	%
Monospermy	A		fPN (DNA+) and mPN (DNA+)	13	20
Polyspermy	B		3 ≤ PNs (all DNA+)	9	13.84
	C		fPN (DNA+) and more than 3 sperm heads	1	1.54
	D		fPN (DNA−) and more than 2 sperm heads	1	1.54
	E		2 ≤ PNs (all DNA+) and more than one sperm head	38	58.46
	F		PN (DNA+) and PN (DNA−) and intact sperm head	1	1.54
	G		2 ≤ PNs (all DNA−) and more than 2 sperm heads	2	3.08
			Total	65	

fPN, female pronucleus; mPN, male pronucleus; PNs, pronuclei; DNA+ (green), replicating pronucleus; DNA– (red), non-replicating pronucleus (sperm heads).

detected in all zygotes that contained exclusively fully formed pronuclei (Table 1B). In almost all other polyspermic zygotes we were able to detect at least one DNA replicating pronucleus (presumably maternal) together with either intact sperm head(s) or an incompletely developed paternal pronucleus(i) (Table 1C, E, F). Only exceptionally (in approximately 5% of eggs examined) were we unable to detect DNA replicating pronuclei (Table 1D, G). As in almost all zygotes evaluated at least one replicating pronucleus was detected, our results seem to indicate the absence of a DNA replication checkpoint in the pig. It is possible, however, that the oocytes were first parthenogenetically activated and thereafter fertilized or fertilized with an additional sperm penetration. Thus, DNA replication had already begun and spermatozoa penetrating later on were unable to block it. To exclude this possibility we fertilized porcine oocytes by ICSI.

DNA synthesis following sperm injection

As after IVF the synthesis of DNA was examined 16 h following the injection of the sperm into the oocyte. At that time, about 69% (41/59) of zygotes contained one male and one female pronucleus, both incorporating BrdU (Fig. 1A, Table 2A). Only about 3% (2/59) of zygotes contained also two pronuclei but both without DNA synthesis (Table 2B). In 20% (12/59) of eggs, in spite of the presence of intact sperm heads, female pronuclei replicated DNA (Fig. 1B, Table 2C). Only in about 7% of zygotes we were unable to detect the replicating female pronucleus in the presence of an intact sperm head (Table 2D).

Discussion

It was demonstrated originally in primate zygotes that a unique checkpoint operates during the first mitotic cell cycle and controls the onset of DNA replication in pronuclei (Hewitson *et al.*, 1999, 2003; Ramalho-Santos *et al.*, 2000). Thus, DNA replication starts in the female pronucleus only after the male pronucleus is fully formed. Later, it was shown that this checkpoint also operates in bovine zygotes (Comizolli *et al.*, 2003). Therefore, it might be assumed that this checkpoint represents a universal mechanism(s) controlling the correct progression of the embryonic cell cycle and further embryonic development. However, our results demonstrate a different situation in the pig.

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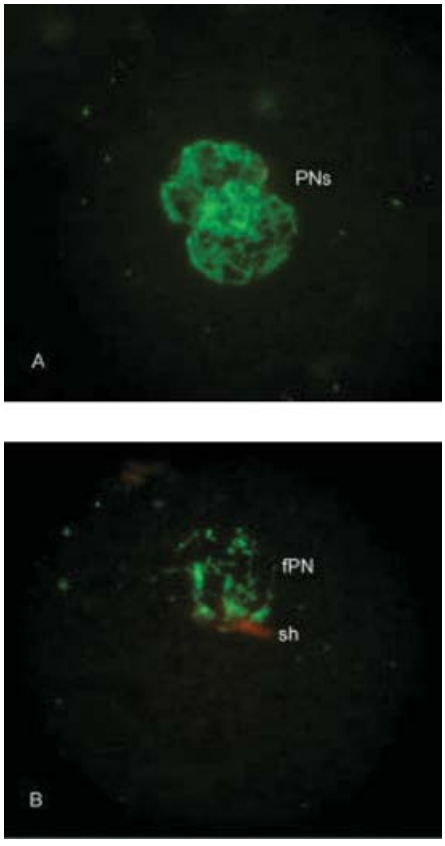

Figure 1 BrdU incorporation into porcine zygotes produced by ICSI. (*A*) Zygote with two pronuclei (PNs) both incorporating BrdU (green). (*B*) DNA replication detected in a female pronucleus (fPN) even in the presence of an intact sperm head (sh). DNA is counterstained by propidium iodide (red).

Table 2 DNA replication in pronuclei of porcine zygotes after ICSI

	Type of fertilization	Number of zygotes	%
A	fPN (DNA+) and mPN (DNA+)	41	69.49
B	fPN (DNA−) and mPN (DNA−)	2	3.39
C	fPN (DNA+) and intact sperm head	12	20.34
D	fPN (DNA−) and intact sperm head	4	6.78
	Total	59	

fPN, female pronucleus; mPN, male pronucleus; PNs, pronuclei; DNA+ (green), replicating pronucleus; DNA– (red), non-replicating pronucleus (sperm heads).

After IVF, DNA replication in the maternal pronucleus began even in the presence of intact sperm heads or a developing paternal pronucleus which did not replicate DNA. Only in about 5% of zygotes evaluated were we unable to detect replicating nuclei at all. In the rest, we detected at least one pronucleus with replicating DNA. Although the labelling procedure does not allow us to distinguish between the male and female pronucleus we suppose that the replicating pronucleus is of maternal origin. This is supported by the evaluation of those zygotes where a single replicating pronucleus was detected in the cytoplasm along with one or more incompletely decondensed sperm heads (Table 1C).

As DNA replication starts in porcine zygotes approximately 6 h after fertilization it may be argued that some oocytes were first parthenogenetically activated and started DNA replication before being penetrated by spermatozoa. To exclude this possibility we produced zygotes also by ICSI. Under our conditions, the handling of oocytes does not activate them parthenogenetically. The situation in the ICSI group was basically the same as that in the IVF oocytes: DNA replication could be detected in the maternal pronucleus even in the presence of an intact sperm head or a decondensing pronucleus.

These results seem to suggest that the situation in the pig is somewhat different from that in other species. On the other hand, Kim *et al.* (2003) observed a delay in DNA replication in porcine zygotes into which either porcine or murine spermatozoa were injected. In their experiments, replication of DNA began only after complete sperm head decondensation. However, DNA replication was evaluated within first 12 h after ICSI. Hence, it is possible that in our experiments the onset of DNA replication was delayed too and started much later after the oocyte passed the critical period. After the injection of human spermatozoa into hamster oocytes Terada *et al.* (2000) observed some oocytes in which the maternal pronuclei started to replicate DNA even in the presence of decondensing sperm heads which showed no BrdU incorporation. With prolonged culture and advanced sperm head decondensation replication was detected in almost all (maternal, paternal) pronuclei.

The reason why the DNA replication checkpoint does not operate in porcine zygotes in the same way as in other species is unknown. When fertilized *in vitro*, porcine zygotes are often polyspermic and in some cases sperm head decondensation does not occur. In polyspermic zygotes the pronuclear location before the first mitotic division may determine the further ploidy of embryos (Han *et al.*, 1999). Thus, it is also possible that those pronuclei without DNA replication are eliminated. Nevertheless, we cannot fully eliminate the possibility that certain technical factors can also influence the control of DNA replication in porcine

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1-cell stage embryos. The replication of DNA is a complex and sensitive process in which the disruption of some pathways may lead to its block (Gonzalez *et al.*, 2005). It has also been demonstrated in cattle that the origin of spermatozoa influences the onset of DNA replication (Comizzoli *et al.*, 2000). It must also be noted that the checkpoint control mechanisms do not fully operate in maturing mammalian oocytes or in very early embryos (Dai *et al.*, 2003).

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