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# Progesterone treatment of boar spermatozoa improves male pronuclear formation after intracytoplasmic sperm injection into porcine oocytes

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## Summary

Boar spermatozoa were prepared for intracytoplasmic sperm injection (ICSI) by two different treatments to facilitate sperm chromatin decondensation and improve fertilisation rates after ICSI in pigs: spermatozoa were either frozen and thawed without cryoprotectants, or treated with progesterone. Morphological changes of the sperm heads after the treatments were examined and then the activation of oocytes and the transformation of the sperm nucleus following ICSI were assessed. After freezing and thawing, the plasma membrane and acrosomal contents over the apical region of sperm head were lost in all the spermatozoa. Following treatment with 1 mg/ml progesterone, the acrosome reaction was induced in 61% of spermatozoa. After injection of three types of spermatozoa, non-treated spermatozoa and progesterone-treated (i.e. acrosome-reacted) spermatozoa induced oocyte activation, but frozen-thawed spermatozoa induced oocyte activation at a significantly lower rate. Sixty-two per cent of sperm heads remained orcein-negative for 6 h, however, resulting in delayed sperm chromatin decondensation and low male pronuclear formation in the oocytes injected with a non-treated spermatozoon. Since the treatments of freezing and thawing and progesterone for spermatozoa accelerated the initial change in sperm chromatin and the latter treatment induced oocyte activation earlier, it is considered that the delay in oocyte activation and decondensation of sperm chromatin after injection of non-treated spermatozoa is caused by the existence of the sperm plasma membrane. These results show that progesterone treatment efficiently induces the acrosome reaction in boar spermatozoa without destroying their potency for oocyte activation, and the induction of the acrosome reaction results in the promotion of male pronuclear formation after ICSI.

Keywords: Acrosome reaction, ICSI, Oocyte activation, Pig, Sperm chromatin decondensation

## Introduction

In intracytoplasmic sperm injection (ICSI), spermatozoa bypass some steps of natural fertilisation, such as the acrosome reaction, penetration into the zona

pellucida and fusion with the oolemma, since intact spermatozoa are introduced directly into the ooplasm. Although the intact plasma membrane and acrosome are brought into the ooplasm by ICSI, male pronuclei are formed from injected spermatozoa and the resultant zygotes develop to term in cattle (Goto *et al.*, 1990), humans (Palermo *et al.*, 1992), mice (Kimura & Yanagimachi, 1995), rabbits (Hosoi & Iritani, 1993), sheep (Catt *et al.*, 1996), rhesus monkeys (Hewitson *et al.*, 2000) and pigs (Martin, 2000).

Polyspermic fertilisation of porcine oocytes has been an unsolved problem and makes it difficult to produce normally fertilised oocytes *in vitro* (Szöllösi & Hunter, 1973). Application of the ICSI procedure is promising for providing *in vitro* fertilised porcine oocytes. However, low fertilisation rates and low

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developmental abilities of embryos produced by ICSI have been reported in pigs (Kolbe & Holtz, 1999; Martin, 2000) as well as cattle (Goto *et al.*, 1990; Keefer *et al.*, 1990; Rho *et al.*, 1998; Li *et al.*, 1999; Wei & Fukui, 1999) and sheep (Catt *et al.*, 1996; Gomez *et al.*, 1997, 1998).

It has been suggested that oocyte activation after ICSI is induced in a different manner compared with *in vitro* fertilisation, since the fusion between spermatozoa and oocytes is bypassed (Tesarik & Mendoza, 1999). The majority of oocytes (65–73%) are activated and form male and female pronuclei after ICSI in humans (Flaherty *et al.*, 1995; Kovacic & Vlasisavljevic, 2000) and mice (Kimura & Yanagimachi, 1995) while the oocyte is not sufficiently activated by injection of an intact spermatozoon in cattle (Keefer *et al.*, 1990; Wei & Fukui, 1999), sheep (Gomez *et al.*, 1997, 1998) and pigs (Kolbe & Holtz, 1999). As the major cause of fertilisation failure after ICSI in humans is failure of oocyte activation (Flaherty *et al.*, 1995), studies of oocyte activation after ICSI are considered important in improving fertilisation. However, oocyte activation after ICSI in pigs has not been examined in detail.

Failed or abnormal decondensation of sperm chromatin occurs frequently after ICSI, especially in domestic species. Most of the sperm heads remain condensed after injection of an intact spermatozoon in cattle (Keefer *et al.*, 1990; Li *et al.*, 1999). Asynchronous changes between female and male chromatin are frequently observed in sheep oocytes after ICSI (Gomez *et al.*, 1997, 1998). Sperm chromatin decondenses heterogeneously under the intact acrosomal cap in mice (Kimura & Yanagimachi, 1993), rhesus monkeys (Hewitson *et al.*, 2000) and pigs (Kim *et al.*, 1998). This heterogeneous decondensation of sperm chromatin under the intact acrosomal cap has been suspected to lead to the anomalies of sex chromosomes in babies derived from ICSI in humans (Hewitson *et al.*, 2000; Leutjens *et al.*, 1999).

To promote decondensation of sperm chromatin in oocytes, artificial modifications of spermatozoa, including removal of the acrosome and tail by sonication (Li *et al.*, 1999), demembranisation of the sperm membrane by freezing and thawing (Goto *et al.*, 1990), partial decondensation of sperm chromatin by dithiothreitol (Rho *et al.*, 1998) and induction of the acrosome reaction (Lacham-Kaplan & Trounson, 1995; Gomez *et al.*, 1997; Lee *et al.*, 1997) have been applied before injection. However, condensed and abnormally enlarged sperm heads, and small-sized male pronuclei are frequently observed (Wei & Fukui, 1999). Since most of the treatments destroy the oocyte activation capacity of the spermatozoa, the potential for embryos to develop to term after injection of these treated spermatozoa remains limited in cattle, even if combined with artificial oocyte activation (Rho *et al.*, 1998).

Boar spermatozoa for ICSI were prepared by two different treatments in the present study to facilitate the decondensation of sperm chromatin. Spermatozoa were frozen and thawed without cryoprotectants to remove the plasma membrane and acrosome, or treated with progesterone, which has been shown to initiate the acrosome reaction in capacitated boar spermatozoa physiologically (Melendrez *et al.*, 1994). The morphological changes in the sperm heads after the treatments, and the activation of oocytes and morphological changes in injected sperm heads following ICSI, were examined.

## Materials and methods

### Maturation of porcine oocytes *in vitro*

Prepubertal porcine ovaries were collected from local slaughterhouses and transported to the laboratory at 20 °C. The method for collection of follicular oocytes was based on Kure-bayashi *et al.* (1996). Briefly, the ovaries were rinsed in Dulbecco's phosphate-buffered saline containing 0.1% polyvinyl alcohol (PBS-PVA), and the oocyte-cumulus-granulosa cell complexes (OCGCs) were picked with forceps from the inner surface of healthy antral follicles 4–6 mm in diameter without detachment of the oocyte and granulosa cells. Groups of 50 OCGCs were matured in 2 ml of TCM-199 (Earl's salt; Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% (v/v) heat-treated fetal calf serum (FCS; Biocell, Carson, CA), 0.1 mg/ml sodium pyruvate, 0.08 mg/ml kenamycin sulfate (Sigma Chemical, St Louis, MO) and 0.1 IU/ml human menopausal gonadotropin (Pergonal; Teikokuzoki, Tokyo, Japan), and co-cultured with two thecal shells from healthy follicles 4–6 mm in diameter that had been freed of follicular fluid and granulosa cells in a CO<sub>2</sub> incubator under 5% CO<sub>2</sub> in humidified air at 38.5 °C for 47–49 h with gentle agitation.

### Sperm preparation for IVF

The sperm-rich fraction was collected from a healthy, fertile boar by the gloved hand method, and centrifuged at 1500 g on two layers (80% and 55%) of Percoll (Amersham Pharmacia Biotech, Uppsala, Sweden) for 10 min. The pellets were resuspended in PBS-PVA and centrifuged at 1500 g for 5 min twice. Spermatozoa in the resultant pellets were adjusted to a concentration of  $2 \times 10^8$  cells/ml and stored at room temperature for about 30 min before insemination.

### Treatments of boar spermatozoa

As described in the previous section, the pellets of

spermatozoa were obtained after centrifugations on a discontinuous gradient of Percoll and PBS-PVA. Spermatozoa in the resultant pellets were divided into three aliquots, one of which was used as non-treated spermatozoa for injection. The second aliquot of the spermatozoa was frozen ( $-80^{\circ}\text{C}$ ) and thawed three times without cryoprotectants, and kept at  $-80^{\circ}\text{C}$  until use (frozen-thawed spermatozoa). To induce the acrosome reaction, the third aliquot of spermatozoa was resuspended in Brackett and Oliphant solution (BO solution; Brackett & Oliphant, 1975) at the concentration of  $2 \times 10^8$  cells/ml, and incubated in a  $\text{CO}_2$  incubator under 5%  $\text{CO}_2$  in humidified air at  $38.5^{\circ}\text{C}$ . After 6 h of preincubation, the culture medium was supplemented with progesterone (Sigma) to give a final concentration of  $10\ \mu\text{g}/\text{ml}$  or  $1\ \text{mg}/\text{ml}$ , and the spermatozoa were cultured for a further 10 min in a  $\text{CO}_2$  incubator.

### Transmission electron microscopy

The three types of spermatozoa described above were fixed in 4% glutaraldehyde in PBS. The specimens were postfixed in 2%  $\text{OsO}_4$  for 1 h, dehydrated in a graded ethanol series, and embedded in Araldite (TAAB Laboratories Equipment, Berks, UK). Ultrathin sections of the specimens were cut with a diamond knife and poststained with 1% uranyl acetate and 1% lead citrate. The ultrastructure of the spermatozoa was observed by a transmission electron microscope (H7200, Hitachi, Tokyo, Japan). Spermatozoa with swollen plasma membranes were frequently observed in the non-treated control group, and were classified as intact spermatozoa, since a swollen plasma membrane has been reported to be an artifact of fixation (Jones, 1973).

### *In vitro* fertilisation

After the maturation culture, OCGCs were put into 2 ml of modified BO solution supplemented with 5 mM caffeine in a 35 mm dish (Falcon no. 1008; Becton Dickinson Labware, Franklin Lakes, NJ) and inseminated with spermatozoa at a concentration of  $5 \times 10^5$  cells/ml. After 3–15 h culture, the cumulus cells were removed by pipetting and oocytes fixed by acetic ethanol (acetic acid:ethanol = 1:3).

### Sperm injection into matured oocytes

Non-treated, frozen-thawed and progesterone-treated spermatozoa were resuspended in PBS-PVA at a concentration of  $1 \times 10^6$  cells/ml, and each sperm suspension was diluted in 10% polyvinylpyrrolidone (PVP) (Irvine Scientific, Santa Ana, CA) solution (1:1). Microdrops of  $10\ \mu\text{l}$  of each suspension and a drop of

$25\ \mu\text{l}$  of Hepes-buffered TCM-199 (pH 7.4) containing 0.1% PVA (Hepes-199) were placed in the same culture dish (Falcon no. 1007) on the stage of an inverted phase contrast microscope equipped with micromanipulators (Narishige, Tokyo, Japan).

Microinjection of a spermatozoon into the ooplasm was performed in a microdrop of Hepes-199. After the maturation culture, oocytes were denuded of cumulus cells by treatment with 0.01% (w/v) hyaluronidase (Sigma) and by pipetting with a small-bore pipette. Oocytes expelling a polar body were selected and transferred into the microdrop.

Each of the oocytes was held according to the position of the first polar body at 6 or 12 o'clock and injected from the 3 o'clock position. A single spermatozoon in the PVP solution was aspirated tail-first by a microinjection pipette (6–7  $\mu\text{m}$  inner diameter; Humagen, Charlottesville, VA) after the tail just below the mid-piece had been rubbed with a microinjection pipette. The pipette was injected into the oocyte and a small amount of the ooplasm was drawn into the pipette, and then the spermatozoon together with the cytoplasm and a small volume of PVP solution were expelled into the oocyte. Some oocytes were injected in the same manner without a spermatozoon (sham injection).

After injection of a spermatozoon or sham injection, the oocytes were cultured in TCM-199 supplemented with 10% (v/v) FCS and 0.1 mg/ml sodium pyruvate for 3–15 h, and then fixed with acetic ethanol.

### Examination of the nuclear stage of oocytes and the morphology of sperm heads

After fixation with acetic ethanol, the oocytes were stained with 1% aceto-orcein. The morphology of injected sperm heads and the nuclear stage of oocytes were examined under a light microscope equipped with differential interference contrast optics. Since multiple spermatozoa penetrated into the ooplasm after IVF, only the morphology of sperm head at the most advanced stage of male pronuclear formation was recorded in Table 2. The morphologies of sperm heads were classified into five groups: (1) sperm heads not stained with aceto-orcein (orcein-negative SH), such as spermatozoa in the perivitelline space in IVF; (2) sperm heads stained with aceto-orcein without a change in their original size and shape (orcein-positive SH); (3) enlarged sperm heads (enlarged SH); (4) male pronuclei (MPN) and (5) orcein-positive and slightly decondensed sperm heads associated with a filamentous structure (SH associated with filaments) that were observed in particular on sperm heads after ICSI.

## Immunostaining of $\alpha$ -tubulin

To examine the filamentous structures around the sperm head, some of the oocytes injected with a frozen-thawed spermatozoon were observed after immunostaining as described by Lee *et al.* (2000). Briefly, the oocytes were fixed in 4% paraformaldehyde in PBS-PVA containing 0.2% Triton X-100 15 h after injection. The fixed oocytes were washed twice in PBS-PVA for 15 min each, then stored in PBS-PVA supplemented with 1% bovine serum albumin (Intergen, Purchase, NY) (PBS-BSA) for at least overnight. The oocytes were blocked with 10% goat serum (Dako, Glostrup, Denmark) in PBS-BSA, and incubated with rat monoclonal anti- $\alpha$ -tubulin antibody (1:100 dilution, MAS 078, Harlan Sera Lab, Sussex, UK) at 4 °C overnight. After being washed three times in PBS-BSA, the oocytes were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-rat IgG (1:40, Dako) for 40 min at room temperature. After being washed three times in PBS-BSA, DNA was counterstained with 400  $\mu$ g/ml propidium iodide (Sigma) for 15–20 min. After being washed, the oocytes were mounted with a mounting medium (Vector Laboratories, Burlingame, CA) and observed under a confocal laser scanning microscope (MRC 1024 system; Bio-Rad, Hercules, CA).

## Statistical analyses

Differences in the values in Table 1 were determined by a chi-square test. The data from experiments on ICSI

**Table 1** Morphological changes in boar spermatozoa caused by freezing and thawing or progesterone treatment

Treatments	No. of spermatozoa examined	No. (%) of spermatozoa		
		intact <sup>d</sup>	OAM swollen <sup>e</sup>	acrosome reacted <sup>f</sup>
None	53	50 (94) <sup>a</sup>	0	3 (6) <sup>a</sup>
Freezing-thawing	50	0	50 (100)	0
Preincubation <sup>c</sup>	52	50 (96) <sup>a</sup>	1 (2) <sup>a</sup>	1 (2) <sup>a</sup>
Progesterone				
10 $\mu$ g/ml	51	43 (84) <sup>a</sup>	3 (6) <sup>a</sup>	5 (10) <sup>a</sup>
1 mg/ml	57	9 (16) <sup>b</sup>	13 (23) <sup>b</sup>	35 (61) <sup>b</sup>

<sup>a,b</sup>Values with different superscripts within each column are significantly different ( $p < 0.05$ ).

<sup>c</sup>Preincubation for 6 h in BO solution.

<sup>d</sup>Spermatozoa which showed morphologically intact spermatozoa except for a swollen plasma membrane (PM) over the acrosomal region.

<sup>e</sup>Spermatozoa which lost the PM over the acrosomal region and showed a swollen outer acrosomal membrane (OAM).

<sup>f</sup>Spermatozoa which showed vesiculation of the PM and OAM over the acrosomal cap region and fusion between the PM and OAM.

and IVF were pooled from at least four replications. Values were subjected to an arcsine transformation in each replication, and were analysed by one-way ANOVA. Significance of differences was assessed by *t*-test.

## Results

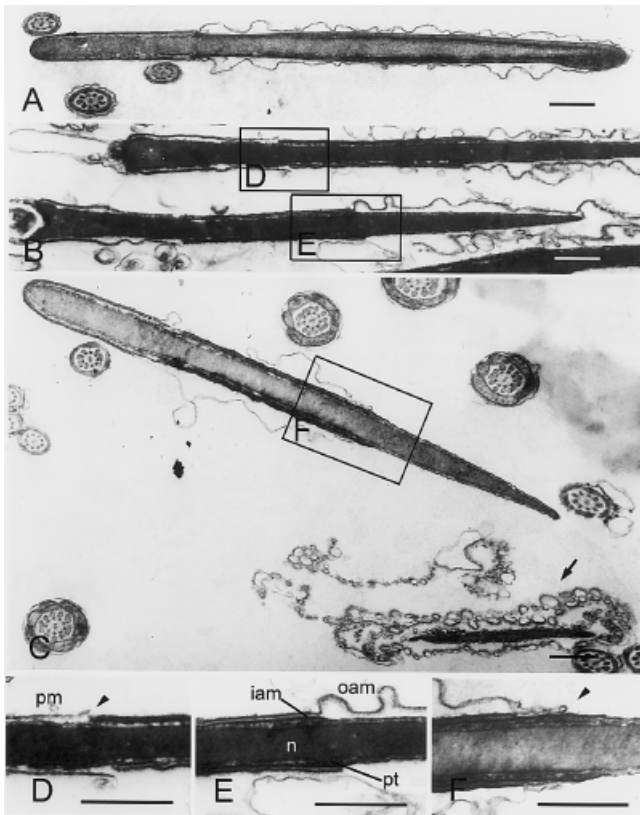
### Sperm morphology after freezing-thawing and progesterone treatment

Table 1 shows the morphology of the head region of non-treated, frozen-thawed, preincubated spermatozoa, and preincubated and progesterone-treated spermatozoa. After washing, 94% of non-treated boar spermatozoa showed intact morphology except for swelling of the plasma membrane over the acrosome (Table 1, Fig. 1A). All frozen-thawed spermatozoa lost the plasma membrane overlying the acrosome, and the plasma membrane remained only over the postacrosomal region (Fig. 1B, D, arrowhead; E). Moreover, the outer acrosomal membrane at the acrosomal cap region was swollen, and most of acrosomal contents over the acrosomal cap region had disappeared (Fig. 1B, E).

After 6 h of preincubation in BO solution, 96% of the spermatozoa showed intact morphology (Table 1). Only 10% of the spermatozoa treated with 10  $\mu$ g/ml progesterone showed vesiculation of the plasma membrane and outer acrosomal membrane over the acrosomal cap region and the loss of the acrosomal contents, while treatment with 1 mg/ml progesterone induced the vesiculation in 61% of spermatozoa (Fig. 1C, arrow). The vesiculation halted at the equatorial segment where the plasma membrane and outer acrosomal membrane fused, resulting in the formation of a fold (Fig. 1C; F, arrowhead). Because the morphology of these spermatozoa was similar to the acrosome reaction in boar spermatozoa that penetrated oocytes (Szöllösi & Hunter, 1973), they were classified as acrosome-reacted spermatozoa.

### Oocyte activation and morphological changes in sperm heads after IVF

For IVF experiments, a total of 167 OCGCs after maturation culture were inseminated with spermatozoa. The fertilisation rates were 0, 45%, 81%, 89% and 92% at 3, 6, 9, 12 and 15 h, respectively. The number of spermatozoa penetrating oocytes increased time-dependently and after 12 h most of the oocytes had been penetrated by multiple spermatozoa (Table 2). All the oocytes penetrated by spermatozoa were activated and a female pronucleus was formed in 19%, 75% and 92% of oocytes at 9, 12 and 15 h, respectively. At 6 h,



**Figure 1** Transmission electron micrographs of non-treated, frozen-thawed and 1 mg/ml progesterone-treated boar spermatozoa. (A) Non-treated spermatozoa had an intact morphology except for the swelling of the plasma membrane overlying the acrosome. (B) In frozen-thawed spermatozoa, the plasma membrane overlying the acrosome was lost and remained only over the postacrosomal region (arrowhead in D). Moreover, the outer acrosomal membrane at the acrosomal cap region showed swelling, and the acrosomal contents had disappeared (E). Perinuclear theca was observed between the nucleus and the inner acrosomal membrane (E). (C) In progesterone-treated spermatozoa, the loss of the acrosomal contents and vesiculation of the plasma membrane and outer acrosomal membrane were observed (arrow in C), and the two membranes fused at the anterior point of the equatorial segment (arrowhead in F). Scale bar represents 0.5  $\mu$ m. iam, inner acrosomal membrane; n, nucleus; oam, outer acrosomal membrane; pm, plasma membrane; pt, perinuclear theca.

**Oocyte activation and morphological changes in sperm heads after ICSI**

After maturation culture, oocytes with a first polar body were used for the ICSI experiments after removal of expanded cumulus cells. The maturation rate of oocytes for ICSI and sham injection was 90.0% (691/768) before injection. The rate of oocytes successfully injected with a spermatozoon was 91.7% (548/597).

The rates of oocyte activation at 3 h were not different among sham and sperm-injected groups (Table 3). The rate of oocytes with an orcein-negative sperm head (Fig. 2A) was higher (80%) in oocytes injected with a non-treated spermatozoon than in oocytes injected with a frozen-thawed (30%) or a progesterone-treated (46%) spermatozoon ( $p < 0.05$ ). On the other hand, the rate of oocytes with an orcein-positive sperm head was significantly higher in the oocytes injected with a frozen-thawed (70%) or a progesterone-treated spermatozoon (54%,  $p < 0.05$ ; Fig. 2B).

24% of oocytes had orcein-positive sperm heads and 21% of them had enlarged sperm heads. An enlarged sperm head was formed in 72% of oocytes at 9 h and a male pronucleus was formed in 75% and 89% of oocytes at 12 h and 15 h, respectively. Although no orcein-negative spermatozoa were observed on the most advanced sperm head, orcein-negative sperm head(s) were observed with a male pronucleus(ei) in some of polyspermic oocytes later than 12 h.

**Table 2** Oocyte activation and morphological changes of sperm heads in ooplasm following *in vitro* fertilisation in pigs

Time (h) after insemination	No. of oocytes examined	No. (%) of oocytes penetrated	No. (%) of oocytes activated		No. (%) of oocytes					Mean no. of spermatozoa in penetrated oocytes
			Total <sup>d</sup>	FPN <sup>e</sup>	Orcein-negative SH <sup>f</sup>	Orcein-positive SH	SH associated with filaments	Enlarged SH	MPN	
3	29	0	0	0	0	0	0	0	0	–
6	33	15 (45) <sup>a</sup>	15 (45) <sup>a</sup>	0	0	8 (24)	0	7 (21) <sup>a</sup>	0	1.0
9	32	26 (81) <sup>b</sup>	26 (81) <sup>b</sup>	6 (19) <sup>a</sup>	0	0	0	23 (72) <sup>b</sup>	3 (9) <sup>a</sup>	1.9
12	36	32 (89) <sup>b</sup>	32 (89) <sup>b</sup>	27 (75) <sup>b</sup>	0	0	0	5 (14) <sup>c</sup>	27 (75) <sup>b</sup>	2.7
15	37	34 (92) <sup>b</sup>	34 (92) <sup>b</sup>	34 (92) <sup>b</sup>	0	0	0	1 (3) <sup>c</sup>	33 (89) <sup>b</sup>	3.0

<sup>a-c</sup>Values with different superscripts within each column are significantly different ( $p < 0.05$ ).

<sup>d</sup>The number of oocytes at second anaphase or second telophase or which had formed a female pronucleus.

<sup>e</sup>Female pronucleus.

<sup>f</sup>Sperm head; the morphological classification of sperm heads is as described in Materials and Methods.

**Table 3** Oocyte activation and morphological changes of sperm heads following intracytoplasmic sperm injection into porcine oocytes

Time (h) after injection	Type of spermatozoa injection	No. of oocytes successfully injected <sup>d</sup>	No. (%) of oocytes activated		No. (%) of oocytes with				
			Total <sup>e</sup>	FPN <sup>f</sup>	Orcein-negative SH <sup>g</sup>	Orcein-positive SH	SH associated with filaments	Enlarged SH	MPN
3	Sham injection	28	5 (18)	0					
	Non-treated	40	8 (20)	3 (8)	32 (80) <sup>a</sup>	8 (20) <sup>a</sup>	0	0	0
	Frozen-thawed	27	7 (26)	0	8 (30) <sup>b</sup>	19 (70) <sup>b</sup>	0	0	0
6	Progesterone-treated	39	8 (21)	0	18 (46) <sup>b</sup>	21 (54) <sup>b</sup>	0	0	0
	Sham injection	28	5 (18) <sup>a</sup>	5 (18)					
	Non-treated	37	15 (41) <sup>b,c</sup>	13 (35)	23 (62) <sup>a</sup>	11 (30) <sup>a</sup>	0	1 (3) <sup>a</sup>	2 (5)
9	Frozen-thawed	53	15 (28) <sup>b</sup>	13 (25)	8 (15) <sup>b</sup>	16 (30) <sup>a,b</sup>	24 (45) <sup>a</sup>	5 (9) <sup>a</sup>	0
	Progesterone-treated	37	27 (73) <sup>c</sup>	15 (41)	3 (8) <sup>b</sup>	20 (54) <sup>b</sup>	1 (3) <sup>b</sup>	13 (35) <sup>b</sup>	0
	Sham injection	28	4 (14) <sup>a</sup>	4 (14) <sup>a</sup>					
12	Non-treated	35	15 (43) <sup>b,c</sup>	14 (40) <sup>b</sup>	15 (43) <sup>a</sup>	11 (31) <sup>a</sup>	0	2 (6) <sup>a</sup>	7 (20)
	Frozen-thawed	42	12 (29) <sup>a,b</sup>	11 (26) <sup>b</sup>	4 (10) <sup>b</sup>	2 (5) <sup>b</sup>	25 (60) <sup>a</sup>	5 (12) <sup>a,b</sup>	6 (14)
	Progesterone-treated	37	25 (68) <sup>c</sup>	21 (57) <sup>c</sup>	12 (32) <sup>a,b</sup>	2 (5) <sup>b</sup>	3 (8) <sup>b</sup>	14 (38) <sup>b</sup>	6 (16)
15	Sham injection	31	7 (23) <sup>a</sup>	7 (23) <sup>a</sup>					
	Non-treated	35	24 (69) <sup>b</sup>	22 (63) <sup>b</sup>	10 (29)	8 (23) <sup>a</sup>	0	8 (23)	9 (26) <sup>a</sup>
	Frozen-thawed	26	5 (19) <sup>a</sup>	3 (12) <sup>a</sup>	2 (8)	1 (4) <sup>b</sup>	19 (73) <sup>a</sup>	2 (8)	2 (8) <sup>a</sup>
15	Progesterone-treated	35	24 (69) <sup>b</sup>	24 (69) <sup>b</sup>	4 (11)	2 (6) <sup>a,b</sup>	6 (17) <sup>b</sup>	6 (17)	17 (49) <sup>b</sup>
	Sham injection	28	5 (18) <sup>a</sup>	5 (18) <sup>a</sup>					
	Non-treated	35	32 (91) <sup>b</sup>	30 (86) <sup>b</sup>	5 (14)	4 (11)	0	15 (43) <sup>a</sup>	11 (31) <sup>a</sup>
15	Frozen-thawed	34	9 (26) <sup>a</sup>	9 (26) <sup>a</sup>	1 (3)	0	24 (71) <sup>a</sup>	6 (18) <sup>a,b</sup>	3 (9) <sup>a</sup>
	Progesterone-treated	36	28 (78) <sup>c</sup>	28 (78) <sup>b</sup>	5 (14)	4 (11)	1 (3) <sup>b</sup>	3 (8) <sup>b</sup>	23 (64) <sup>b</sup>

<sup>a-c</sup>Values with different superscripts within each column and time after injection are significantly different ( $p < 0.05$ ).

<sup>d</sup>The oocytes which had a spermatozoon in the ooplasm without degeneration after injection.

<sup>e</sup>The number of oocytes at second anaphase or second telophase or which had formed a female pronucleus.

<sup>f</sup>Female pronucleus.

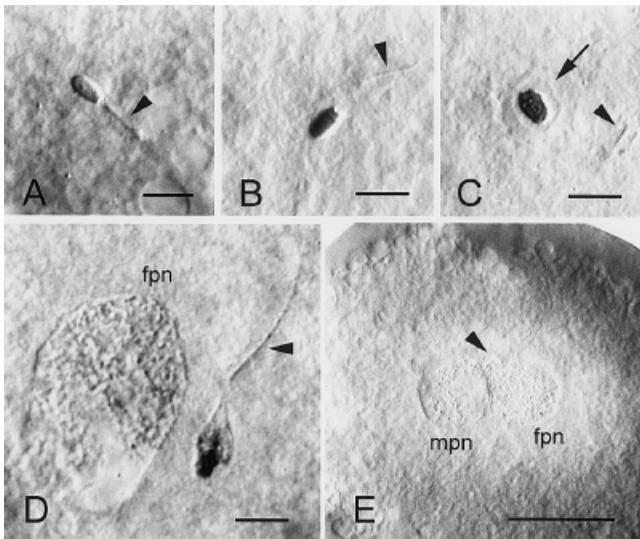
<sup>g</sup>Sperm head; the morphological classification of sperm heads is as described in Materials and Methods.

The highest activation rates were obtained in oocytes injected with a progesterone-treated spermatozoon at 6 h (73%) and 9 h (68%), and these values were significantly higher than those of oocytes sham-injected or injected with a frozen-thawed spermatozoon (Table 3;  $p < 0.05$ ). In addition, an enlarged sperm head was observed in 35% of oocytes injected with a progesterone-treated spermatozoon at 6 h (Fig. 2D). Sperm heads associated with a filamentous structure (Fig. 2C, arrow) were observed first 6 h after injection in oocytes injected with a frozen-thawed (45%) or a progesterone-treated spermatozoon (3%), but not in oocytes injected with a non-treated spermatozoon at any culture duration.

Oocytes with a male pronucleus were observed 9 h after sperm injection (Table 3; 14–20%). The rate of oocytes with a male pronucleus increased up to 15 h in oocytes injected with a progesterone-treated spermatozoon, and was significantly higher than in oocytes injected with a non-treated or a frozen-thawed spermatozoon at 12 h (49% vs 26% and 8%;  $p < 0.05$ ) and 15 h (64% vs 31% and 9%;  $p < 0.05$ ). Twenty-three per

cent and 43% of injected non-treated spermatozoa had an enlarged head at 12 h and 15 h, respectively. Sixty-nine per cent and 91% of oocytes injected with a non-treated spermatozoon were activated, and 63% and 86% of them had formed a female pronucleus(ei) 12 and 15 h after injection, respectively.

In the oocytes injected with a frozen-thawed spermatozoon, the oocyte activation rate was consistently low (19–29%) throughout the experimental period and 45–73% of oocytes had a slightly decondensed and orcein-positive sperm head, which was associated with a filamentous structure later than 6 h (Fig. 2C, Table 3). These sperm heads were observed only in oocytes arrested at the second metaphase. To examine the filamentous structure in the vicinity of a sperm head, 32 oocytes were subjected to immunostaining with an anti- $\alpha$ -tubulin antibody 15 h after injection of a frozen-thawed spermatozoon. Microtubules associated with the second metaphase spindle were observed in all the oocytes. In 75% (23/32) of the oocytes, some microtubules had assembled and formed astral array-like structures at the base of the sperm head (Fig. 3, arrow),

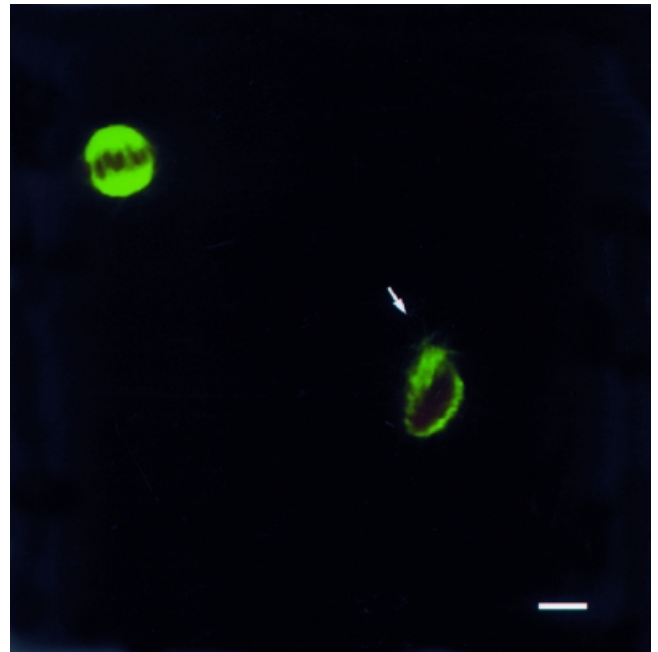


**Figure 2** Differential interference micrographs of non-treated and frozen-thawed sperm heads after microinjection into the ooplasm in pigs. (A) An orcein-negative sperm head 3 h after injection of a non-treated spermatozoon. No morphological change was observed in the sperm head. (B) An orcein-positive sperm head 6 h after injection of a non-treated spermatozoon. It was weakly stained with aceto-orcein while no obvious change in size and shape was observed. (C) A sperm head associated with filamentous structure derived from a frozen-thawed spermatozoon 6 h after injection. It was strongly stained with aceto-orcein. Note the filamentous structure surrounding the swollen sperm head (arrow). (D) An enlarged sperm head derived from a non-treated spermatozoon 15 h after injection. The anterior tip of the sperm head swelled insufficiently. A female pronucleus had already formed near the enlarged sperm head. (E) A male pronucleus derived from a non-treated spermatozoon in the vicinity of a female pronucleus 15 h after injection. Arrowheads show the tail of the injected spermatozoon. Scale bar represents 50  $\mu\text{m}$ . fpn, female pronucleus; mpn, male pronucleus.

and other microtubules surrounded the sperm head (Fig. 3). While the sperm nucleus was slightly decondensed in these oocytes, microtubules were not detected around the sperm head that kept their original size and shape in the other 8 oocytes.

## Discussion

Melendrez *et al.* (1994) have reported that 14–15% of capacitated boar spermatozoa initiated the acrosome reaction following a 10 min treatment with 1  $\mu\text{g}/\text{ml}$  progesterone, and 60–80% of them were motile. Progesterone induced a morphologically normal acrosome reactions in boar spermatozoa in the present study, and the rate of acrosome-reacted spermatozoa increased to 61% following treatment with 1 mg/ml progesterone in spite of the severe motility loss. Since



**Figure 3** Laser-scanning confocal micrograph of a frozen-thawed boar spermatozoon 15 h after injection into the ooplasm in the pig. Microtubules associated with the second metaphase spindle were observed. Some microtubules assembled to form an astral array-like structure at the base of the sperm head (arrow), and other microtubules surrounded the sperm head, which showed slight decondensation. Scale bar represents 10  $\mu\text{m}$ .

sperm motility and even the viability of spermatozoa are not important for the ICSI procedure (Goto *et al.*, 1990), 1 mg/ml progesterone treatment was used here.

In IVF, sperm penetration into oocytes was observed from 6 h after insemination, and the resumption of meiosis was observed in all penetrated oocytes that had orcein-positive or enlarged sperm heads. Oocytes with an enlarged sperm head were most frequently observed, and the rate of activated oocytes increased to 81% 9 h after insemination. The formation of the male pronucleus was observed in 75% of oocytes concomitant with female pronuclear formation at 12 h. These results indicate that oocyte activation and the initial change in sperm chromatin are undergone quickly and the male pronucleus is formed around 6 h after sperm penetration, and that the transformation of female and male nuclei proceeds synchronously.

The stimulus produced by puncturing the oolemma with a micropipette has been reported to induce oocyte activation in sheep (Gomez *et al.*, 1997, 1998), hamsters (Uehara & Yanagimachi, 1976) and pigs (Catt *et al.*, 1995). In the present study there was no difference in the frequencies of oocyte activation at 3 h (18–26%) among sham-injected and sperm-injected groups, and no increase in the activation rate of sham-injected oocytes at later times. It is considered that the activa-



tion rate of oocytes induced by the stimulus of injection is about 20% in pigs.

Injection of non-treated and progesterone-treated spermatozoa, into mature oocytes easily induced activation. The rate of oocyte activation reached a plateau in oocytes injected with a progesterone-treated spermatozoon at 6 h and increased to 15 h after sperm injection in those injected with a non-treated spermatozoon. On the other hand, frozen-thawed spermatozoa induced oocyte activation at a significantly low rate equivalent to that after sham injection. Removing the sperm membrane by repeated freezing and thawing has been reported to result in loss of a sperm's ability to induce oocyte activation in humans (Rybouchkin *et al.*, 1996). The present results show that boar acrosome-reacted spermatozoa retained their oocyte activating ability, but not frozen-thawed spermatozoa as in humans. Thus, oocyte activation is accelerated by the progesterone treatment of spermatozoa, although the rate of activation is still lower compared with the case of IVF.

It has been reported that the initial change in sperm chromatin can be judged on the basis of its reaction with dyes (aceto-orcein in the present study) and that the staining with dyes reflects the stability, of sperm chromatin, especially the condition of the disulfide bonds (Miller & Masui, 1982; Yanagimachi, 1994). Orcein-negative sperm heads were observed in oocytes injected with all kinds of spermatozoa 3 h after injection. Orcein-negative sperm heads were still in the majority in oocytes injected with a non-treated spermatozoon, while orcein-positive sperm heads were dominant in those injected with frozen-thawed and progesterone-treated spermatozoa. These results show that the initial change in sperm chromatin after ICSI is accelerated by either freezing and thawing or progesterone treatment. It has been explained that the breakdown of the intact plasma membrane is required for the release of sperm factors associated with oocyte activation (Swann *et al.*, 1994) and the nuclear envelope disassembly that allows access of cytoplasmic glutathione (GSH) and nucleoplasmin to the sperm chromatin during the ICSI process (Dozortsev *et al.*, 1994; Tesarik & Mendoza, 1999). In the present study electron microscopy showed that the plasma membrane over the acrosomal region was lost completely after both treatments of spermatozoa. Taken together, the present results showing the acceleration of both oocyte activation and the initial change in sperm chromatin demonstrate that the loss of the plasma membrane of spermatozoa after both treatments facilitates the access of cytoplasmic factors such as GSH and nucleoplasmin of oocytes to the sperm chromatin and the release of sperm factors in progesterone-treated spermatozoa. The difference in oocyte activation capacity between frozen-thawed spermatozoa and progesterone-treated

spermatozoa may be explained by the morphological differences between them. The plasma membrane was broken at the anterior site in frozen-thawed spermatozoa, while it was fused with the outer acrosomal membrane at the equatorial segment in progesterone-treated spermatozoa. As sperm factors have been reported to localise in the equatorial segment in cytosolic form (Swann & Lai, 1997), the present results suggest that these factors are at least partially lost in frozen-thawed spermatozoa.

Although the rate of oocytes with an orcein-negative spermatozoon decreased time-dependently, the rate of oocytes with an orcein-positive sperm head was always higher in oocytes injected with a non-treated spermatozoon than the other types of spermatozoa. At 6 h an enlarged sperm head was observed in 35% of oocytes injected with a progesterone-treated spermatozoon, but in only 3% and 9% respectively of those injected with a non-treated or a frozen-thawed spermatozoon. The formation of a male pronucleus was observed in oocytes injected with any kind of spermatozoon at 9 h, although the rates were low (14–20%). The rate of male pronuclear formation was increased slightly in oocytes injected with a non-treated spermatozoon later and showed no increase in those injected with a frozen-thawed spermatozoon. Forty-three per cent of oocytes injected with a non-treated spermatozoon still had enlarged sperm heads in spite of the completion of female pronuclear formation in 86% of them at 15 h. On the other hand, in oocytes injected with a progesterone-treated spermatozoon, the rate increased from 16% at 9 h to 64% at the end of culture. These results strongly suggest that the existence of an intact plasma membrane of spermatozoa results not only in the delay of initiation but also in the slow decondensation of sperm chromatin. Although no orcein-negative sperm head was observed in monospermic oocytes, and the most advanced sperm head in polyspermic oocytes was never orcein-negative, orcein-negative sperm heads were observed together with orcein-positive sperm head(s) and/or a male pronucleus in oocytes penetrated by multiple spermatozoa later than 12 h, suggesting that sub-optimal conditions, such as a delayed penetration, lead to failure and/or delay of the initiation of sperm chromatin decondensation in the ooplasm in IVF. The delay in initiation and the slow progress in the transformation of the sperm nucleus caused the asynchronous decondensation of male and female nuclei, and the low rate of male pronuclear formation in the oocytes injected with a non-treated spermatozoa.

A particular form of sperm head classified as an orcein-positive and slightly decondensed sperm head associated with a filamentous structure (SH associated with filaments) was observed only in oocytes injected with a frozen-thawed or a progesterone-treated sper-

matozoon later than 6 h after injection. This type of sperm head was observed especially in the oocytes injected with a frozen-thawed spermatozoon. The rate of oocytes with this type of sperm head increased up to 12 h and did not decrease 15 h after injection of a frozen-thawed spermatozoon. These results indicate that the process of male pronuclear formation ceased or remained the same for a long duration at this stage. Double staining using anti- $\alpha$ -tubulin and propidium iodide clearly showed that the sperm heads underwent slight chromatin decondensation associated with the assembly of microtubules in the oocytes at the second metaphase. Partially decondensed sperm chromatin together with microtubules in oocytes at the second metaphase was also reported in human oocytes which failed to resume meiosis after ICSI (Flaherty *et al.*, 1995; Kovacic & Vlasisavljevic, 2000). The concomitance of sperm chromatin decondensation with microtubule assembly around the sperm chromatin is not at odds with the hypothesis that the mammalian sperm centrosome 'priming' to nucleate microtubules is generated by the disulfide bonds reduction of chromatin and/or detachment of the sperm tail (Simerly *et al.*, 1999). These findings also show that the initiation of sperm chromatin decondensation does not depend on oocyte activation, whereas the assembly of the male pronuclear envelope, including the enlargement of sperm heads, requires oocyte activation after ICSI.

Although most oocytes injected with a non-treated spermatozoon were activated, as previously reported (Kim *et al.*, 1999), the timing of oocyte activation was delayed and the chromatin of most sperm heads remained unchanged 6 h after injection in the present study. Since both freezing and thawing of spermatozoa and progesterone treatment accelerated the initial change in sperm chromatin and the latter treatment induced oocyte activation earlier, it is considered that the delay in the oocyte activation and decondensation of sperm chromatin after injection of non-treated spermatozoa is caused by the existence of the sperm plasma membrane. It has been reported that the fertilisation rate is improved by treatments of spermatozoa. In most reports, however, additional treatments of oocytes, namely oocyte activation, are performed, even when spermatozoa induced to acrosome-react by calcium ionophore were injected in mice (Lancham-Kaplan & Trounson, 1995) and cattle (Wei & Fukui, 1999). The present results clearly show that progesterone treatment efficiently induces the acrosome reaction of boar spermatozoa without causing the loss of their oocyte activation ability, and the acrosome reaction results in the promotion of male pronuclear formation after ICSI without treatments for oocyte activation.

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