Increased disruption of sperm plasma membrane at sperm immobilization promotes dissociation of perinuclear theca from sperm chromatin after intracytoplasmic sperm injection in pigs

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Abstract

The effects of sperm-immobilization methods on decondensation of sperm chromatin and retention of subacrosomal sperm perinuclear theca (SAR-PT) after intracytoplasmic sperm injection (ICSI) were examined in pigs. Sperm membrane damage caused by different immobilization methods by rubbing with a micropipette without piezo pulses (R), or with a low (L) or high (H) intensity of piezo pulses while rubbing, was assessed by the time required for staining of sperm heads with eosin Y solution. The average time for staining of sperm heads immobilized by the R, L or H treatments was 76, 41 or 26 s, respectively. The fertilization rate following ICSI was increased by sperm immobilization by piezo pulses compared with R, but increased intensity of pulses from L to H did not cause further improvements (29, 48 and 47%, respectively). An immunofluorescence study revealed that H immobilization promoted the dissociation of SAR-PT from sperm chromatin compared with L and R, and it increased the frequency of male pronuclear formation in which chromatin appeared uniformly decondensed. With *in vitro* fertilization (IVF), SAR-PT disassembled coordinately with sperm chromatin decondensation and it was not detectable around male pronuclei. This was different from most of the oocytes after ICSI in which remnants SAR-PT were detected adjacent to male pronuclei. We concluded that increased damage on the sperm plasma membrane at immobilization improved fertilization rates and decondensation of sperm chromatin after ICSI due to the accelerated dissociation of SAR-PT from the sperm nucleus. Also, the behavior of SAR-PT after ICSI was different from that observed in oocytes after IVF.

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Introduction

Intracytoplasmic sperm injection (ICSI) is an important technology of assisted reproduction, especially in the treatment of severe male-factor infertility in humans (Palermo *et al.* 1992), and has been advocated as a possible technique for the conservation of endangered species where access to semen stocks is limited (Iritani 1991).

Many studies associated with *in vitro* production of pigs have been conducted. However, the progress towards efficient *in vitro* maturation and *in vitro* fertilization (IVF) has been limited by a high incidence of polyspermy (Szöllösi & Hunter 1973, Niwa 1993, Funahashi & Day 1996, Day 2000). Application of ICSI in pigs may provide a useful method for *in vitro* production of monospermic zygotes for embryo transfer. However, fertilization rates after ICSI

vary among the reported studies and litter size after embryo transfer is reduced in pigs (Kim *et al.* 1999, Kolbe & Holtz 1999, 2000, Martin 2000, Lai *et al.* 2001, Nakai *et al.* 2003, Probst & Rath 2003, Kwon *et al.* 2004, Yong *et al.* 2003). The ICSI technique remains to be improved and established for the practical production of piglets.

One of the problems in pig ICSI is the failure or delay of decondensation of sperm chromatins, resulting in a low rate of male pronuclear formation (Kren *et al.* 2003, Lee *et al.* 2003). In the fertilization process, spermatozoa enter the ooplasm by sperm plasma membrane fusion with oolemma (Szöllösi & Hunter 1973, Yanagimachi 1994, Wassarman 1999, Sutovsky & Schatten 2000). Most of the sperm plasma membrane and perinuclear theca are lost during the incorporation process and sperm chromatin, especially in the postacrosomal region, is exposed to

ooplasm soon after the entry into oocytes (Szöllösi and Hunter 1973, Yanagimachi 1994, Sutovsky & Schatten 2000). In the ooplasm, factors supporting chromatin decondensation, such as reduced glutathione and nucleoplasmin, interact with sperm chromatin and induce remodeling of sperm chromatin to form a male pronucleus (Perreault *et al.* 1984, Yanagimachi 1994, Collas and Poccia 1998, McLay & Clark 2003). On the other hand, with ICSI a spermatozoon is introduced directly into ooplasm, bypassing the penetration and demembration process. The ICSI is performed practically in humans using spermatozoa without any pretreatments, except sperm immobilization (Palermo *et al.* 1992). In domestic animals, in which oocytes are usually prepared by *in vitro* maturation, fertilization rates and developmental ability of embryos are low after direct injection of a spermatozoon immobilized in the same manner as in humans. Actually, the retention of acrosome or subacrosomal perinuclear theca seems to prevent decondensation of sperm chromatin in ooplasm after ICSI in rhesus monkeys (Sutovsky *et al.* 1996, Hewitson *et al.* 1999), cattle (Sutovsky *et al.* 1997) and pigs (Katayama *et al.* 2002a).

Usually spermatozoa are immobilized by rubbing the sperm tail midpiece with a micropipette before injection. In humans, it is believed that the sperm plasma membrane is damaged sufficiently by rubbing the tail region during the immobilization process to cause male pronuclear formation after ICSI (Dozortsev *et al.* 1995), but probably such a treatment is not sufficient in porcine oocytes matured *in vitro*. Yanagida *et al.* (2001) suggested that sperm immobilization with piezo pulses would cause more severe membrane damage on human spermatozoa than rubbing with a micropipette and showed that calcium oscillations, a hallmark of oocyte activation by the factors released from the sperm perinuclear theca, start more rapidly in oocytes injected with a piezo-immobilized spermatozoon than in those injected with a rubbing-immobilized method. We considered that application of piezo pulses for sperm immobilization would also improve fertilization rates after ICSI in pigs without any additional treatments for artificial activation of oocytes.

In the present study, the same piezo pulses as those used for micropipette penetration of oolemma and zona pellucida during the ICSI process were applied for sperm immobilization. The effects of immobilization methods on male pronuclear formation, decondensation of sperm chromatin and retention of subacrosomal sperm perinuclear theca (SAR-PT) after ICSI were examined.

Materials and methods

Collection of oocyte–cumulus complexes and *in vitro* maturation

Oocytes were aspirated from antral follicles (3–6 mm in diameter) of ovaries collected from slaughtered prepubertal gilts. The oocytes surrounded by compact cumulus mass

(oocyte–cumulus complexes) were collected from the follicular fluid and washed twice. Groups of 50–100 oocyte–cumulus complexes were transferred into a well of a four-well multidish containing 500 µl mTCM-199 (Gibco, Grand Island, NY, USA) including 0.1% (w/v) polyvinyl alcohol (PVA; Sigma Chemical Co, St Louis, MO, USA), 10 ng·ml⁻¹ epidermal growth factor (Sigma), 10 iu·ml⁻¹ follicle-stimulating hormone (Sigma), 10 iu·ml⁻¹ luteinizing hormone (Sigma) and 0.57 mmol·l⁻¹ cysteine (mTCM-199) at 39°C in an atmosphere of 5% CO₂ in humidified air. After 22–24 h, oocyte–cumulus complexes were transferred to mTCM-199 without follicle-stimulating hormone and luteinizing hormone, and then cultured an additional 20 h under these same conditions.

Preparation of boar spermatozoa

Freshly ejaculated sperm rich fraction was collected from fertile boars (one-half Large white × one-quarter Duloc × one-quarter Pietrain) and frozen as described previously (Abeydeera & Day 1997). A sperm pellet was thawed in 2 ml Dulbecco's PBS (calcium- and magnesium-free) supplemented with 0.1% (w/v) PVA (PBS-PVA) at 39°C and centrifuged at 600 g on two layers (80 and 60%) of Percoll (Amersham Biosciences AB, Uppsala, Sweden) for 10 min. Spermatozoa in the resultant pellet were centrifuged once with 5 ml PBS-PVA at 1900 g for 4 min. The spermatozoa were then resuspended in modified Tris-buffered medium (mTBM; Abeydeera & Day 1997) at a sperm concentration of 1 × 10⁶ cells/ml for IVF and (1–10) × 10⁶ cells/ml for ICSI, and cultured at 39°C for 30 min in atmosphere of 5% CO₂ in humidified air until used for IVF, ICSI or eosin Y staining.

IVF

After the completion of maturation of oocytes cultured for 42 h, cumulus cells were removed with 0.1% (w/v) hyaluronidase and washed three times with mTBM. Oocytes showing clear cytoplasmic cortex and plasma membrane with a polar body were selected for IVF. After washing with mTBM, 20–30 oocytes were placed in 50 µl drops of the same medium that was covered with mineral oil in a 35 mm falcon dish. Then, a 50 µl drop of sperm suspended mTBM was added to the 50 µl drop containing oocytes and the mixture gave a final sperm concentration at 5 × 10⁵ cells/ml. Oocytes were coincubated with spermatozoa for 6 h at 39°C in atmosphere of 5% CO₂ in humidified air. The oocytes were then transferred to NCSU23 (Petters & Wells 1993) and cultured for 5–6 h at 39°C in atmosphere of 5% CO₂ in humidified air.

Sperm immobilization and eosin Y staining

The influence of immobilization methods on the sperm plasma membrane integrity was assessed by eosin Y staining. Spermatozoa were cultured in mTBM for 30–90 min at 39°C in atmosphere of 5% CO₂ in 95% humidified air,

and $3.2 \text{ mmol} \cdot \text{l}^{-1}$ progesterone (Sigma) was added to the sperm suspension to induce acrosome reaction (Katayama *et al.* 2002b). For manipulation of spermatozoa, microdrops of $6 \mu\text{l}$ of 10% (w/v) polyvinylpyrrolidone (PVP; Sigma) and 1% (w/v) eosin Y solution in mTBM were placed on the inner side of the lid of the dish and covered with mineral oil. On the stage of an inverted phase-contrast microscope (Zeiss, Oberkochen, Germany) equipped with micromanipulators (Eppendorf, Hamburg, Germany) and a piezo drive unit (Prime Tech, Tsukuba, Japan), $2 \mu\text{l}$ of the sperm suspension were mixed well with a $6 \mu\text{l}$ PVP drop and sperm immobilization was performed there. Using a flat-tipped micropipette with an outer diameter of $8 \mu\text{m}$ prepared with an ultrathin glass capillary (Narishige, Tokyo, Japan), motile spermatozoa were immobilized by rubbing at the midpiece region of the tail and head region (R), by rubbing at the same region while giving a low intensity (at level 2 or 3 at speed 2) of piezo pulses used for penetration of oolemma (L) or by rubbing at the same region while giving a high intensity (at level of 6–8 at speed 5) of piezo pulses used for penetration of the zona pellucida (H). After each immobilization method, some immobilized spermatozoa were aspirated into the micropipette and piezo L and H pulses were applied to the spermatozoa located in the micropipette in the same manner as to when the micropipette penetrated into zona pellucida and oolemma during the ICSI process (+shake). This was done to determine the effect of a set of piezo pulses applied at the zona and oolemma penetration on damage of sperm plasma membrane. Immobilized spermatozoa were then immediately transferred to the drop of 1% eosin Y. As the center of the eosin Y drop had a strong pink color due to the thickness of the drop, a spermatozoon was placed close to the edge of the drop to observe changes in the color of the head region. The time required for staining of the entire sperm head region was recorded.

ICSI driven with piezo pulses

After the completion of maturation culture for 42 h, cumulus cells were removed from oocytes as described above and oocytes showing an acceptable morphology with a polar body were selected for ICSI. For the manipulation, microdrops of $6 \mu\text{l}$ 10% PVP and $20 \mu\text{l}$ HEPES-buffered NCSU23 with adjusted osmolarity (HEPES-NCSU23) were put on the inner side of the lid of the dish and covered with mineral oil. ICSI was performed using the Eppendorf Cell Tram microinjection system equipped with a Prime Tech piezo drill. After sperm immobilization by one of the three different methods described above, a spermatozoon was aspirated into a micropipette and transferred to the microdrop of HEPES-NCSU23. There an oocyte was held by a holding pipette so that the first polar body was located at 12 or 6 o'clock. The micropipette was driven from the 3 o'clock area with a piezo-pulse force at intensity 6–8 and speed 5 to penetrate the zona pellucida, and the spermatozoon was then pushed to the tip of the

micropipette when the tip of the pipette advanced into the perivitelline space. The micropipette was carefully placed against the oocyte without breaching the oolemma and piezo pulses at the intensity 2 or 3 and speed 2 were given to the tensed oolemma through the micropipette. The oolemma was then released from tension at the same time as the pipette penetrated the oolemma. To confirm the penetration of the pipette into the ooplasm, a small amount of ooplasm was aspirated. The micropipette was inserted more deeply to the 9 o'clock area of the oocyte, and then the spermatozoon was released into the oocyte with small amount of ooplasm and PVP solution.

Examination of pronuclear formation after IVF and ICSI

After sperm injection, oocytes were washed twice with NCSU23 and cultured in a $10 \mu\text{l}$ drop of the same medium for 10–12 h at 39°C in an atmosphere of 5% CO_2 in humidified air. Presumed zygotes were mounted on a slide and fixed for 72 h in 25% (v/v) acetic acid in ethanol at room temperature. After being stained with 1% (w/v) orcein in 45% (v/v) acetic acid, presumed zygotes were observed under a phase-contrast microscope at a magnification of $\times 400$. Oocytes released from arrest at the MII stage were recorded as activated oocytes.

Simultaneous detection of SAR-PT, nuclear pore complexes (NPCs) and DNA after IVF and ICSI

At 4–10 h after sperm insemination (IVF), or at 10 h after injection of spermatozoa immobilized by R, L or H (ICSI), oocytes were treated as described previously (Sutovsky *et al.* 1997) to examine the configurations of sperm perinuclear theca, NPCs and DNA. Briefly, the zona pellucida was removed by 0.5% (w/v) protease and oocytes were fixed with 2% (v/v) formaldehyde for 40 min. Zona-free, fixed oocytes were treated in PBS supplemented with 5% normal goat serum and 0.1% Triton X-100 for 30 min at room temperature. To label NPCs and SAR-PT, a mouse monoclonal antibody of mAB414 (BabCo/Covance, Berkeley, CA, USA) and a rabbit antibody against SAR-PT/inner acrosomal membrane protein IAM32 (Oko & Maravei 1994; kindly provided by R O) were diluted at 1/200 and 1/20, respectively in PBS supplemented with 1% (v/v) normal goat serum and 0.1% (v/v) Triton X-100 (labeling medium) and the oocytes were incubated for 1 h at room temperature in the medium. Then, the oocytes were washed and incubated in labeling medium with a 1/80 (v/v) dilution of fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG, a 1/80 (v/v) dilution of tetramethylrhodamine β isothiocyanate (TRITC)-conjugated anti-rabbit IgG and $0.71 \mu\text{mol} \cdot \text{l}^{-1}$ DAPI for 40 min. Labeled oocytes were mounted on glass slides with VectaShield medium (Vector Laboratories, Burlingame, CA, USA) and observed under a Nikon Eclipse 800 epifluorescent microscope (Nikon, Tokyo, Japan) equipped with a CoolSnap CCD HQ camera (Roper Scientific, Tucson, AZ, USA).

and MetaMorph image-acquisition software (Universal Imaging Corp., Downingtown, PA, USA). Figure plates were edited using Adobe Photoshop 5.0.

Statistical analyses

The data obtained from observation of oocytes stained with aceto-orcein were pooled from five replicates. Values in each replicate were analyzed using one-way analysis of variance (ANOVA). Significance of differences was assessed by Student's *t* test. The time required for staining a sperm head after each immobilization method was analyzed using one-way ANOVA and significance of differences was assessed by *t*-test. The data from immunocytochemical study were pooled from three replications, and any significant differences in the values were determined by a χ^2 test.

Results

Membrane damage in immobilized spermatozoa assessed by eosin Y staining

Sperm heads were stained with eosin Y from the postacrosomal region to the entire head region after each immobilization method. In each sperm immobilization by R, L or H treatment, the additional piezo pulses applied for spermatozoa aspirated into a micropipette in the same manner as the ICSI process (+shake) did not increase damage of the spermatozoan plasma membranes (Table 1). Sperm immobilization by treatment H significantly ($P < 0.05$) reduced the time required for complete staining compared with treatments R and L (Table 1).

Pronuclear formation after IVF and injection of a spermatozoon immobilized with R, L or H

A total of 53 oocytes were inseminated by IVF. The sperm penetration rate was 81% (43/53). Monospermic and

polyspermic fertilization was observed in 13% (7/53) and 68% (36/53) of oocytes, respectively. All penetrated oocytes were activated and formed at least two pronuclei accompanied with two polar bodies.

Application of piezo pulses of L or H for sperm immobilization significantly ($P < 0.05$) increased rates of oocyte activation, fertilization and male pronuclear formation compared with R, and there were no differences between L and H (Table 2).

Behavior of SAR-PT of spermatozoa, and NPCs in oocytes after IVF or injection of spermatozoa following immobilization with R, L or H

In IVF oocytes, solubilization of sperm SAR-PT of spermatozoa was observed in the cortical area of oocyte cytoplasm and the presence of SAR-PT was not detected around male pronuclei (Fig. 1). Detailed observations of SAR-PT revealed the remnants of SAR-PT signal around the postacrosomal segment of the sperm head and near the implantation fossa (arrowheads in Fig. 1C and D) prior to sperm chromatin decondensation. SAR-PT signals were last detected in anterior part of sperm heads concomitantly with the onset of chromatin decondensation in the postacrosomal region (Fig. 1F). In this case, remnants of SAR-PT were observed around the anterior part of sperm heads when NPCs started to be assembled around sperm chromatin (Fig. 1G and H). No signal of SAR-PT was observed around male pronuclei whereas SAR-PT was detected on the sperm heads that were still being decondensed or did not decondense in polyspermic oocytes (Fig. 1I).

In ICSI oocytes, SAR-PT signal was observed in most oocytes after injection of spermatozoa immobilized by the R, L or H method (Fig. 2). In oocyte cytoplasm, SAR-PT was either (1) positioned close to sperm chromatin on apical surface of the sperm nucleus (associated; Fig. 2A–D), (2) present at the equatorial segment in sperm heads (equatorial; Fig. 2E), in which case sperm heads were kept almost intact, (3) detached from sperm chromatin (dissociated; Fig. 2F and G) or (4) not present at all (missing; Fig. 2H). The immunodetection of SAR-PT closely adjacent to sperm chromatin coincided with sperm head-s/male pronuclei containing an area of condensed chromatin under SAR-PT (Fig. 2B) or an atypical, small male pronucleus (Fig. 2C). The H immobilization significantly increased the percentage of oocytes showing SAR-PT dissociated from sperm chromatin (Table 3) and reduced the percentage of male pronuclei with the area of condensed chromatin to 35% (7/20), which was significantly lower ($P < 0.05$) than those in R and L (87% (13/15) and 60% (9/15), respectively). Also, H immobilization decreased the incidence of oocytes that had spermatozoa labeled at the equatorial segment (Table 3).

There was no difference in the assembly of NPCs around female chromatin in oocytes after IVF and ICSI. In

Table 1 Evalutaion of membrane damage in spermatozoa immobilized by rubbing (R), rubbing with low piezo pulses (L) or rubbing with high piezo pulses (H) using eosin Y stanining.

Method of sperm treatment	No. of spermatozoa examined	Time (s) required for full staining of sperm head (Mean $\pm$ S.E.M.)
None*	15	202 $\pm$ 35 ^a
None + shaket†	15	190 $\pm$ 25 ^a
R	13	76 $\pm$ 8 ^b
R + shake	13	79 $\pm$ 6 ^b
L	14	41 $\pm$ 8 ^c
L + shake	14	41 $\pm$ 3 ^c
H	14	26 $\pm$ 3 ^d
H + shake	14	28 $\pm$ 3 ^d

a,b,c,d Values with different superscripts are significantly different ($P < 0.05$)
* Motile spermatozoa were transferred into eosin Y without any immobilized treatments.
† Spermatozoa were aspirated into a micorpipette and given H and L pulses in the same manner as the injection process.

Table 2 Pronuclear formation in porcine oocytes after intracytoplasmic injection of spermatozoa immobilized by rubbing (R), with low piezo pulses (L) or with high piezo pulses (H).

Sperm immobilization	No. of oocytes examined	No. (%) of oocytes activated*					No. (%) of oocytes with sperm heads†				
		Total	With 2PB		With 1 PB		Intact	Dec.	ESH	MPN	Others
			1 PN	2 PN	2 PN	3 PN					
R	102	58 (57) ^a	10 (10) ^a	30 (29) ^a	0 (0) ^a	6 (6) ^a	28 (27) ^a	20(20) ^a	12(12) ^a	36(35) ^a	6(6) ^a
L	85	56 (66) ^b	4 (5) ^a	41 (48) ^b	0 (0) ^a	4 (5) ^a	8 (9) ^b	16(19) ^a	6(7) ^a	45(53) ^b	10(12) ^a
H	111	72 (65) ^b	7 (6) ^a	52 (47) ^b	1 (1) ^a	5 (5) ^a	11 (9) ^b	10(9) ^a	10(9) ^a	57(51) ^b	23(21) ^a
Sham	36	6 (17) ^c	4 (11) ^a	0 (0) ^c	2 (6) ^a	0 (0) ^a					

^{a,b,c}Values with different superscripts are significantly different ($P < 0.05$)

*Oocytes released from arrest at MII.

†Morphologies of sperm heads were classified into intact decondensed (Dec.), enlarged (ESH), male pronuclei (MPN) or premature condensed chromatins (others).

2PB1PN, two polar bodies and one pronucleus; 2PB 2PN, two polar bodies and two pronuclei, 1PB2PN, one polar body and two pronuclei; 1PB3PN, one polar body and three pronuclei.

Figure 1 Immunofluorescence localization of SAR-PT and NPCs after IVF. (A) SAR-PT started to disassemble in the cortical area of ooplasm. The sperm head remained condensed and the sperm tail (indicated by an arrow in all panels except I) had not yet entered the ooplasm. (B) The intact sperm head had a strong signal of SAR-PT at the acrosomal region. Note sperm tails. (C) In intact sperm heads, SAR-PT at the acrosomal region was swollen and a spot-like remnant of SAR-PT (arrowhead) was observed near the implantation fossa of postacrosomal region. Note sperm tails. (D) The signal of SAR-PT migrated toward the implantation fossa (arrowhead) and postacrosomal region had the stronger signal than acrosomal region in intact sperm heads. (E) Remnant of SAR-PT in anterior part of sperm head in which chromatin remained condensed. A relatively strong signal of SAR-PT was observed along the decondensed chromatin at posterior part of sperm heads. Note sperm tail. (F) SAR-PT signal as detected in the anterior part of a sperm head with decondensed chromatin in its posterior portion. Note sperm tail. (G) In enlarged sperm heads, NPCs started to be assembled around the anterior part of sperm head, signal of SAR-PT slightly observed there (arrowhead). (H) In the cortical area of ooplasm, NPCs were assembled entirely around enlarged sperm heads and slight signal of SAR-PT was observed at anterior part (arrowhead). Note sperm tail and female nuclei at condensed chromatins and the second polar body (out of focus, small arrows). (I) Supernumerary pronuclei (arrowheads) were observed after polyspermic fertilization. The signal of SAR-PT was not detected around pronuclei but it was observed in sperm heads that remained intact or only partially decondensed (arrows). Scale bar, 10 μ m.

Figure 2 Immunofluorescence localization of SAR-PT and NPCs after ICSI by the R, L or H method. (A) NPCs were assembled around female pronucleus (arrowheads) but not around male pronucleus, which was closely associated with residual SAR-PT (arrow) after injection of L-immobilized spermatozoa. (B) NPCs were observed around both pronuclei, but the smaller male pronucleus displayed an area of condensed chromatin (arrowhead) and the retention of SAR-PT (arrow) after injection of L-immobilized spermatozoa. (C) SAR-PT (arrow) was closely associated with an unusually small male pronucleus surrounded by NPCs after injection of L-immobilized spermatozoa. (D) After injection of H-immobilized spermatozoa, disassembled SAR-PT (arrow) was seen near decondensed sperm head which was not associated with NPCs. This oocyte was activated but arrested at telophase II. Note the assembly of NPCs into annulate lamellae (arrowheads) observed in cytoplasm of this and other activated oocytes. (E) SAR-PT (arrow) was retained at equatorial segment of sperm head which did not decondense after injection of R-immobilized spermatozoa. The oocyte was arrested at MII stage. (F) Two fully decondensed pronuclei surrounded by NPCs after injection of H-immobilized spermatozoa. The disassembled remnants of SAR-PT (arrows) had drifted away from the apposed pronuclei. (G) Two pronuclei of equal size, surrounded by NPCs after injection of H-immobilized spermatozoa. Remnants of SAR-PT (arrow) had drifted away from the apposed pronuclei. (H) In many oocytes forming two fully decondensed pronuclei surrounded by NPCs, no SAR-PT signal was observed after injection of H-immobilized spermatozoa as in IVF oocytes. Scale bar, 10 μ m.

Table 3 Location of SAR-PT signal in porcine oocytes after intracytoplasmic injection of spermatozoa immobilized by rubbing (R), rubbing with low piezo pulses (L) or rubbing with high piezo pulses (H).

Sperm immobilization	No. of oocytes examined	No. (%) of oocytes with SAR-PT signal located* in sperm chromatin			
		Associated	Equatorial	Dissociated	Missing
R	27	10 (37) ^a	10 (37) ^a	6 (22) ^a	1 (4) ^a
L	19	5 (26) ^a	6 (32) ^a	7 (37) ^a	1 (5) ^a
H	25	2 (8) ^b	3 (12) ^a	16 (64) ^b	4 (16) ^a

^{a,b}Values with different superscripts are significantly different ($P < 0.05$)

* The classification of signal location: associated, positioned close to sperm chromatin on apical surface of the sperm nucleus; equatorial, present at the equatorial segment in sperm heads; dissociated, detached from sperm chromatins; missing, not present.

oocytes both after IVF and ICSI, NPCs were associated with female condensed chromatin after the second polar body was extruded. In some oocytes after ICSI, NPC association was observed only in female and not in male pronuclei (Fig. 2A), but most of male pronuclei formed after ICSI contained NPCs (80% (12/15), 80% (12/15) and 95% (19/20) of male pronuclei in the R, L and H groups, respectively), even when the male pronucleus had an area of condensed chromatin (Fig. 2B).

Discussion

It has been proposed that sperm damage caused by sperm immobilization before injection promotes breakdown of intact sperm plasma membrane, which is required for the release of the sperm factors involved in oocyte activation (Swann *et al.* 1994) and for the disassembly of the sperm nuclear envelope that allows access of cytoplasmic factors associated with decondensation of sperm chromatin during the ICSI procedure in humans (Dozortsev *et al.* 1994, 1995, Tesarik & Mendoza 1999). Our results show that sperm heads of boars treated with piezo pulses were stained with membrane damage marker, eosin Y, faster than sperm heads treated by rubbing, suggesting that piezo pulses cause more severe damage to the sperm plasma membrane. As membrane damage increased, fertilization rates and activation rates were improved by L immobilization; but they were not improved further by H immobilization. These results are consistent with the studies that show the beneficial effects of piezo pulses to immobilize spermatozoa (Yanagida *et al.* 2001) and to increase sperm membrane damage in humans (Swann *et al.* 1994, Dozortsev *et al.* 1994, 1995, Tesarik & Mendoza 1999). The observation that oocyte activation was significantly increased by sperm immobilization by piezo pulses suggests that the increased damage to sperm membranes may contribute to the release of factors involving oocyte activation from spermatozoa injected into the ooplasm.

Even though the injection of an intact spermatozoon permits sperm head decondensation in humans, non-human primates and rodents, the decondensation of sperm chromatin does not occur in cattle after injection

of intact bull spermatozoa, possibly due to the higher rigidity of their perinuclear theca (Sutovsky *et al.* 1997). The perinuclear theca of sperm heads is composed of three parts: the subacrosomal region (SAR-PT), the outer layer at the equatorial segment and the postacrosomal region. Ultrastructural studies show that the perinuclear theca at postacrosomal region becomes solubilized and disappears soon after sperm entry into oocytes (Yanagimachi 1994, Sutovsky & Schatten 2000, Sutovsky *et al.* 2003). On the other hand, SAR-PT remains around sperm chromatin temporarily after IVF (Sutovsky & Schatten 2000, Sutovsky *et al.* 2003). In rhesus monkeys, a male pronucleus is formed after ICSI but the area of condensed chromatin in male pronuclei is observed in the anterior part of the sperm nucleus because SAR-PT is retained there and remains closely associated with male chromatin for several hours (Sutovsky *et al.* 1996, Hewitson *et al.* 2000). Heterogeneous decondensation of male chromatin could be considered as one of reasons for the delay of the initiation of DNA synthesis prior to the first mitosis and low developmental capability of embryos derived from ICSI in rhesus monkeys (Hewitson *et al.* 1999). These studies suggest that disassembly of SAR-PT is one of the key requirements for successful fertilization after ICSI. Our immunocytochemical study with the antibody against SAR-PT revealed that H immobilization promoted the dissociation of SAR-PT from sperm chromatin, which also decreased the frequency of male pronuclei containing an area of condensed chromatin. Recently it was shown that disruption of sperm tail midpiece in human sperm caused mechanical damage along the plasma membrane to the postacrosomal perinuclear theca and the outer acrosomal membrane (Takeuchi *et al.* 2004). There are several studies to have suggested the beneficial effects of membrane damage of spermatozoa on fertilization in humans (Palermo *et al.* 1992, Swann *et al.* 1994, Dozortsev *et al.* 1994, 1995, Tesarik & Mendoza 1999, Yanagida *et al.* 2001). The present results indicate that a similar mechanism of membrane damage at sperm immobilization improves fertilization and sperm chromatin decondensation after ICSI in pigs.

The removal and disassembly of SAR-PT has been studied during bovine IVF and suggests that the

interaction of SAR-PT with microvilli on the oolemma at sperm–oocyte fusion is necessary for the removal of SAR-PT from sperm chromatin at early stages of fertilization (Sutovsky *et al.* 1997, 2003). Disassembly and/or solubilization of SAR-PT after IVF in pigs was also observed in the cortical area of ooplasm in the present study. The interaction of SAR-PT with microvilli and sperm–oocyte fusion are bypassed in the ICSI procedure where spermatozoa are injected directly into the ooplasm after sperm immobilization. It is considered that some spermatozoa were kept intact in the ooplasm although spermatozoa were damaged by immobilization before injection. As the increased damage on the sperm plasma membrane by application of piezo high pulses decreased the frequency of spermatozoa with a SAR-PT signal at the equatorial segment and promoted the dissociation of SAR-PT from sperm chromatin, the direct interaction of SAR-PT with the ooplasm after breakdown of sperm plasma membrane might be involved in the dissociation process in ICSI oocytes.

The IVF results in the present study showed that SAR-PT was disassembled as it was distributed in the area in which sperm chromatin became decondensed and gradually disappeared from the anterior area of enlarged sperm heads or early male pronuclei. In contrast, most of oocytes after ICSI had detectable remnants of SAR-PT as late as the pronuclear apposition stage of zygotic development. The signal of SAR-PT observed in association with male pronuclei at apposition was relatively strong and appeared to be just separated from sperm chromatins in ICSI oocytes. It is considered that the removal of SAR-PT after ICSI was delayed and/or its mechanism was not the same as in IVF oocytes, where SAR-PT disassembled coordinately with the sperm incorporation into ooplasm and decondensation of sperm chromatin. Acrosome reaction and interaction of SAR-PT with microvilli, which are bypassed in the ICSI procedure, may cause the different behavior of SAR-PT after IVF.

Recently it has been reported that SAR-PT contains unique proteins, such as a variant of histone protein SubH2Bv (Aul & Oko 2002) and core somatic histones (Tovich & Oko 2001), which implies an involvement of perinuclear theca or its proteins in fertilization and zygotic development. Although little is known about the role of SAR-PT, disassembly of SAR-PT coordinated with decondensation of sperm chromatin after IVF as observed in the present study also suggest a contribution of sperm SAR-PT to an early stage of the fertilization process. If so, the altered characteristics of male pronuclei formed after ICSI as compared with those after IVF may be due to the lack of SAR-PT association with sperm chromatin during decondensation.

An IVF study in cattle showed that the demembration of the sperm chromatin soon after sperm penetration into ooplasm is almost immediately followed by the *de novo* assembly of pronuclear envelope and

NPCs. The assembly of NPCs from recruited, ooplasmic NPC proteins is required for normal development of pronuclei (Sutovsky *et al.* 1998, Payne *et al.* 2003). The failure of male pronucleus to attract nucleoporins from the cytoplasm of an activated oocyte was observed after ICSI in rhesus monkeys (Sutovsky *et al.* 1996). In the present study, some male pronuclei were not decorated with NPCs whereas female pronuclei in the corresponding ICSI ova did contain NPCs at 10 h after ICSI. The sperm immobilization methods did not seem to affect the association of NPCs with male pronucleus. Even when male pronuclei had an area of condensed chromatin, NPCs were observed around the male pronucleus in the activated ova with a female pronucleus. As almost all pronuclei formed at 10 h after IVF in this study were surrounded with NPCs, the lack of such a pattern could be considered an abnormality after ICSI. The nucleo-cytoplasmic transport through NPCs is required for many cellular activities, such as DNA replication (Hanover 1992, Harel *et al.* 2003). Thus, the formation of male pronuclei without NPC association may cause failure or delay of the first cleavage of zygotes after ICSI.

In conclusion, application of piezo pulses for sperm immobilization increased sperm membrane damage and improved fertilization rates following ICSI compared with simple sperm rubbing during the piezo-driven ICSI procedure in pigs. Increased membrane damage caused by high-intensity piezo pulses promoted dissociation of perinuclear theca of spermatozoa which allowed uniform decondensation of chromatin in male pronuclei. Also, the behavior of SAR-PT after ICSI deviated from that observed in oocytes after IVF by the virtue of prolonged SAR-PT association with the male pronuclei.

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