In Vitro Fertilization and Polyspermy in the Pig: Factors Affecting Fertilization Rates and Cytoskeletal Reorganization of the Oocyte

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KEY WORDS IVF; spermatozoa; cumulus; cytoskeleton

ABSTRACT Polyspermy is a common phenomenon in the pig. Extensive information has become available from in vitro studies on not only the quality of oocytes but also the quality of spermatozoa. However, little information is available on the relative penetration rates of fresh and frozen spermatozoa from the same ejaculate from boars of different breeds. The present results, based on a total of 15 boars of three different breeds, revealed that the inter-breed variation in fertilization and polyspermic rates is larger than intra-breed variation. It was also shown that the incidence of polyspermy as well as penetration rate was greatly decreased by freezing and thawing, even if a higher number of sperm was coincubated with cumulus-free oocytes for a longer period compared to fresh sperm of the same ejaculate. This study focuses on the cytoskeletal organization of the oocyte with respect to the status of cumulus investment, and monospermic and polyspermic fertilization. The status of cumulus cells correlated with the density of transzonal cumulus-cell processes and with the maturation rate of oocytes and, to some degrees, the incidence of polyspermy. Polyspermic zygotes formed multiple microtubule domains in association with individual male pronuclei (PN), but in a high degree of polyspermy (more than trispermy), the pronuclear apposition did not proceed. The effect of multiple PN of paternal and maternal origin on the cytoskeletal reorganization is also discussed. Microsc. Res. Tech. 61:327–334, 2003. © 2003 Wiley-Liss, Inc.

INTRODUCTION Polyspermic fertilization occurs more frequently in the pig than in the other species, even for in vivo fertilization under diverse experimental conditions (Hunter, 1967, 1990, 1991). Although techniques for achieving in vitro maturation and in vitro fertilization (IVF) of pig oocytes have been progressively improved in recent years, a high incidence of polyspermy is still one of the major problems in the pig IVF program (Abeydeera, 2001; Nagai and Moor, 1990; Niwa, 1993; Yoshida et al., 1990). Causes for this problem likely include the quality of semen at fertilization (Kim et al., 1996a; Pavlok, 2000; Sirard et al., 1993). Large variations among individual males have been noted in fertilization rates with fresh (Sirard et al., 1993) and frozen sperm (Nagai et al., 1998). Further, because the difficulty of cryopreservation of boar semen, little information is available on the relative penetration rates of fresh and frozen boar sperm from a single ejaculate. The objective of our Experiment 1 was to compare the effects of boars of different breeds on the in vitro fertilizing capacity of fresh and frozen-thawed spermatozoa.

Causes for polyspermic fertilization may also depend on the quality of the matured oocytes in the pig (Cran and Cheng, 1986; Grupen et al., 1997; Wang et al., 1997a). An intercellular communication between the oocyte and cumulus cells are maintained during oocyte growth and maturation, which is necessary for full cytoplasmic maturation (Eppig, 1982; Familiari et al., 1998; Mattioli et al., 1988) and stabilizes the distribution of cortical granules (Galeati et al., 1991). Our previous studies showed that cytoskeletal alteration is involved in the dynamic change of cumulus-oocyte cell communication during oocyte maturation in the pig (Suzuki et al., 2000). Cytoskeletal reorganization is important during oocyte maturation and fertilization in mammals (Kim et al., 1996b,c, 1997b; Long et al., 1993; Maro et al., 1986; Navara et al., 1994; Schatten et al., 1985; Sun et al., 2001; Wang et al., 2000). However, little information is available on a relationship between the oocyte quality and cytoskeletal distribution. Factors affecting in vitro maturation of the oocytes that are associated with the incidence of polyspermy include the effect of cumulus cells on the oocyte. In our Experiment 2, we analyzed (1) the relationship between the status of the cumulus mass of cumulus-oocyte complex (COC gradings) and cytoskeletal distribution of the oocyte, (2) the relationship between COC gradings and the rates of maturation and fertilization in vitro, and...
(3) the cytoskeletal organization in monospermic and polyspermic zygotes.

**MATERIALS AND METHODS**

**In Vitro Maturation of Oocytes**

COCs were aspirated from antral follicles (2–5 mm in diameter) of ovaries collected from slaughtered prepubertal gilts. After being washed with Dulbecco’s phosphate buffered saline (DPBS) containing 0.1% polyvinyl alcohol, groups of 10–15 oocytes were transferred to North Carolina State University 23 (NCSU23) medium supplemented with 10% (v/v) porcine follicular fluid, 10 i.u./ml eCG (Teikoku Hormone Mfg. Co. Ltd., Tokyo, Japan), and 10 i.u./ml hCG (Mochida Pharmaceutical Co. Ltd., Tokyo, Japan). The oocytes were cultured for 24 hours, then incubated in NCSU23 without hormonal supplements for a total period of 44 hours in an atmosphere of 5% CO₂ at 39°C.

**Semen Preparation and Cryopreservation**

Five boars each from the Large White, Landrace, and Duroc breeds were randomly selected for study and semen collection at Aomori Prefectural Experiment Station of Animal Husbandry, Japan. The sperm-rich fraction of the ejaculate was collected while filtering the gel component by an experienced operator using the glove-hand method. A portion of the fresh ejaculate semen was held in tightly capped tubes at 17°C for 20 hours, and the other portion was frozen by the pellet method and stored at −196°C.

The method of semen cryopreservation was based on the procedures reported by Pursel and Johnson (1975) with minor modifications by Abeydeera and Day (1997). After collection, semen was kept at 17°C for 2 hours and then was diluted (1:1) with Hulsenberg VIII extender and centrifuged to remove the seminal plasma. The sperm pellet was resuspended in BF5 extender at room temperature and cooled to 5°C over 90 minutes. Subsequently, the sperm suspension was diluted (1:1) with BF5 containing 2% glycerol at 5°C and frozen in 100-µl aliquots on dry ice and stored in liquid nitrogen. The final concentration of sperm was 5 × 10⁸ cell/ml.

**In Vitro Fertilization**

Fresh semen was preincubated in modified Tris-buffered medium (Abeydeera and Day, 1997) supplemented with 1 mM caffeine and 0.1% BSA (IVF medium) for 90 minutes. After 44 hours incubation, the COCs were washed and placed in 50-µl droplets of IVF medium. Sperm fraction (50 µl) was introduced to the droplet containing oocytes to make a final concentration of 2 × 10⁶ cells/ml (2,000 motile sperm/oocyte) for 6 hours of coculture. The oocytes were then transferred to 100-µl droplets of NCSU23 medium and cultured for an additional 6 hours at 39°C in an atmosphere of 5% CO₂ in air.

With numerous preliminary experiments, the swim-up procedure was found unsuitable for use with frozen-thawed boar spermatozoa, because of a great reduction of sperm motility during 90 minutes of incubation. In addition, when cumulus-enclosed oocytes were coincubated for 6 hours with frozen-thawed spermatozoa, the total absence of fertilization was observed in 14 out of 15 boars examined. Therefore, frozen-thawed spermatozoa were coincubated with cumulus-free oocytes at a concentration of 5 × 10⁶ cells/ml (12,500 motile sperm/oocyte) for 12 hours without swim-up.

**Fluorescent Observations**

To assess the nuclear configuration and the distribution of microtubules and microfilaments, the eggs were processed as reported previously (Suzuki et al., 2000, 2001). Briefly, denuded oocytes were fixed in a microtubule stabilization buffer (Allworth and Albertini, 1993) at 37°C for 1 hour, washed extensively, and blocked overnight at 5°C in the washing medium (calcium-free DPBS containing 2% BSA, 2% goat serum, 0.2% milk powder, 0.2% sodium azide, and 0.1% Triton X). The fixed samples were then exposed overnight to anti-β tubulin primary antibody (1:200; Sigma Chemical Co., MO) at 5°C, and incubated with fluorescein isothiocyanate (FITC)-conjugated secondary antibody (1:200; Sigma) at 37°C for 2 hours. After rinsing, the samples were stained with rhodamine phalloidin (1:1; 1,000; Molecular Probes, Eugene, OR) for microfilaments for 1 hour, and then stained for DNA with Hoechst 33342 (10 µg/ml) in mounting medium containing PBS and glycerol (1:1). The samples were viewed under an Olympus microscope (BX-FLA, Olympus, Tokyo, Japan): A U-MNIBA filter set (Olympus) was used for FITC, a U-MWIB set (Olympus) was used for rhodamine, and a U-MWU set (Olympus) for Hoechst. A cooled CCD video system (ImagePoint, Photometrics Ltd., Tucson, AZ) was used to obtain images on a computer and color adjustment was performed by IPLab-Spectrum P software (Signal Analytics Corporation, Vienna, VA).

**Experimental Design**

In Experiment 1, semen collections, from 5 boars each of three different breeds, were repeated so that there were duplicate samples. Each sample was divided into a portion that was used as fresh semen and another fraction that was frozen and thawed. Thus, the experiment consisted of a 3 × 5 × 2 × 2 factorial arrangement.

In Experiment 2, COCs were graded in four categories described by Leibfried and First (1979). Grade 1 COCs are mostly covered with more than three cumulus layers; grade 2 COCs were covered with 2–3 layers; grade 3 COCs were partially covered with clumped cumulus masses and the zona pellucida was partially exposed; and grade 4 COCs were denuded and the oocyte was only enclosed by zona pellucida. Before and after maturation, oocytes for each COC grade were processed for fluorescence staining to evaluate cytoskeletal distribution and oocyte maturation. Some matured oocytes were inseminated with fresh sperm from four (2 Landrace and 2 Large White) boars as described above. At 6 and 12 hours after insemination, oocytes were evaluated on the cytoskeletal distribution with respect to the sperm penetration, polyspermy and the PN formation by fluorescence staining.

Data in both experiments 1 and 2 were assessed by analysis of variance with the help of the BMDP program (BMDP Statistical Software, Inc., Los Angeles, CA). When appropriate, percentage data were arcsin transformed. Differences between the means were de-
Experiment 1. Effects of Boars on IVF

Average sperm motility decreased from 77–84 to 33–57% during semen holding at 17°C (Table 1). Proportion of motile sperm declined greatly after freezing and thawing in all breeds examined ($P < 0.01$): overall mean percentage of post-thawing motility was 36%, ranged from 23 to 49% among boars.

As shown in Table 1, results of ANOVAs showed that the effect of breeds, as well as the effect of sperm condition, was highly significant in terms of the sperm penetration and polyspermy rates. In this study, 98% of the penetrated oocytes showed PN formation. Spermatozoa from Large White boars penetrated at a significantly higher rate than those from Landrace and Duroc boars irrespective of sperm condition. A similar tendency was observed for polyspermic penetration in the oocytes inseminated with fresh sperm ($P < 0.01$), but not with frozen-thawed sperm. There was no penetration of frozen-thawed sperm from 6 out of 15 boars examined (2 boars of Duroc and 4 boars of Landrace). Similarly, the mean number of fresh spermatozoa penetrated per oocyte was significantly higher in Large White than in the other two breeds. Of polyspermic zygotes, about 80% were dispermic or trispermic and the remainder were penetrated by 4 to 8 sperm when using fresh sperm, while almost all polyspermic zygotes (84%) were dispermic when using frozen sperm. The correlation coefficient for penetration rate on polyspermy was significant ($r = 0.68$, $P < 0.01$), indicating that high penetration rates result in high polyspermy rates, when fresh sperm were used.

Experiment 2. The Cytoskeletal Redistirution of the Oocyte After Sperm Penetration

Effect of COC Grading on Cytoskeletal Distribution. Representative pictures of the oocytes before and after maturation are shown in Figure 1. More profound differences were found in the distribution of microfilaments rather than microtubules. At 0 hours of incubation, almost all of the grade 1 oocytes (89%) showed a dense and uniform distribution of the transzonal cumulus-cell processes (CCP, Fig. 1a). However, about 40% of the grade 2 oocytes showed a partial loss of CCP and about half of the oocytes at grade 3–4 had already lost a greater part of their CCP (Fig. 1b). At 44 hours of incubation, all grade-1 oocytes showed strongly stained, thick microfilament bundles at the cell cortex (Fig. 1c), whereas the oocytes of grades 2–4 displayed a decreased density of the cortical microfilaments and uneven distribution of cytoplasmic microfilaments (Fig. 1d).

TABLE 1. Effects of the breeds and sperm condition on in vitro fertilization of pig oocytes

<table>
<thead>
<tr>
<th>Condition</th>
<th>Breed</th>
<th>Motility (%)</th>
<th>Examined (no.)</th>
<th>Penetrated (%)</th>
<th>Polyspermic (%)</th>
<th>Mean no. spermatozoa in penetrated oocyte</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh</td>
<td>Duroc</td>
<td>77 ± 2</td>
<td>366</td>
<td>48.7 ± 10.3</td>
<td>21.5 ± 7.8</td>
<td>1.53 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>Landrace</td>
<td>84 ± 2</td>
<td>499</td>
<td>49.3 ± 4.7</td>
<td>20.2 ± 4.3</td>
<td>1.23 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>L. White</td>
<td>81 ± 1</td>
<td>691</td>
<td>78.4 ± 5.0</td>
<td>54.1 ± 4.7</td>
<td>2.08 ± 0.08</td>
</tr>
<tr>
<td>Frozen</td>
<td>Duroc</td>
<td>33 ± 2</td>
<td>401</td>
<td>6.5 ± 3.4</td>
<td>12.1 ± 9.9</td>
<td>1.10 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>Landrace</td>
<td>35 ± 4</td>
<td>438</td>
<td>0.9 ± 0.7</td>
<td>0</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>L. White</td>
<td>40 ± 3</td>
<td>461</td>
<td>25.3 ± 7.2</td>
<td>10.5 ± 3.8</td>
<td>1.16 ± 0.03</td>
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</tbody>
</table>

Significance of fresh vs. frozen data within breed

$P < 0.01$ for Duroc

$P < 0.05$ in L. White

$P < 0.01$ for L. White

$P < 0.05$ in Duroc

$P < 0.01$ in L. White

$P < 0.03$ in Duroc

$P < 0.04$ in Landrace

$P < 0.08$ in L. White

$P < 0.05$ between a and b; $P < 0.01$ between a and c.

1Data in parentheses show sperm motility after 20 hours of sperm standing.

*Values with different superscripts within the sperm condition of the same column are significantly different ($P < 0.01$ between a and b; $P < 0.05$ between a and c).
In polyspermic zygotes, there were small multiple domains of microtubules including the male PN derived from each penetrated spermatozoa at 6–12 hours after insemination (Figs. 3 and 4). In dispermic zygotes, the apposition of female and one male PN was occasionally observed (Fig. 4). It seemed not to occur if two male PN located distant from each other. In some cases, the multiple microtubule domains became expanded to merge together over the ooplasm (Fig. 5). However, the union of microtubule domains did not proceed in a high degree of polyspermy (more than 3 spermatozoa penetrated).

**DISCUSSION**

In the pig, polyspermy is dependent on different boars (Sirard et al., 1993; Wang et al., 1991) and on the presence of a high concentration of capacitated spermatozoa during fertilization (Hunter, 1991; Kim et al., 1997a). Xu et al. (1996) reported, based on fresh semen from 3 boars of different breeds, that boars and sperm concentration affect the rates of penetration, polyspermy, and male PN formation. The present study is based on a total of 15 boars and clearly showed that the penetration and polyspermy rates were more

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**Fig. 1.** Fluorescent micrographs of immature and matured pig oocytes. Microtubules are green; microfilaments are red and nuclei/chromosomes are blue; yellow shows the overlap of microtubules and microfilaments. **a:** A GV-stage oocyte assessed at grade 1 showing a dense and uniform distribution of cumulus cell processes consisting of microfilaments within the zona pellucida (Z). Several cumulus cells are attached (arrows). **b:** A GV-stage oocyte assessed at grade 3 showing partial loss of cumulus cell processes within the zona pellucida (Z). Microtubules and microfilaments are not located coincidentally. **c:** An MII-stage oocyte assessed at grade 1. Microfilaments are strongly stained in the cortex and on the contact surfaces of the oocyte and PBI (arrow). The MII spindle is located peripherally, with its long axis perpendicular to the surface of the oocyte, and contains condensed chromosomes at the equatorial plate. **d:** An MII-stage oocyte assessed at grade 3 showing decreased density of cortical and cytoplasmic microfilaments. The MII spindle near PBI (arrow) is not radially oriented with its long axis. Bar = 50 μm.
variable among the breeds rather than among boars within the breed.

Due to a great reduction of sperm motility after thawing, we realized different IVF conditions for fresh and frozen-thawed sperm from the same ejaculate. By estimating by post-thawing sperm motility, more than 6 times higher number of motile sperm were coincubated with cumulus-free oocytes for as long as 12 hours. Nevertheless, the incidence and degree of polyspermy were consistently lower compared to insemination with fresh sperm. These results imply that polyspermy may not solely be determined by the high sperm:oocyte ratio. In the present study, all the oocytes used for IVF were matured under the identical conditions. Therefore, the effect of the oocyte quality can be eliminated from consideration. Thus, differences in the penetration and polyspermy rates between fresh and frozen-thawed sperm from the same ejaculate depend on the changes in sperm characteristics during treatments.

One of the factors affecting the sperm characteristics may be associated with the incubation conditions before IVF. In the present study, frozen-thawed sperm were neither preincubated nor swim-upped. It is assumed, therefore, that the process of sperm capacitation may be delayed, thereby affecting sperm penetration. Morphological and functional alterations of the acrosome during freezing and thawing might influence the penetration rate and the incidence of polyspermy, although we did not examine the morphological damage.

An alternating possibility may depend on the decreased velocity of motile sperm after thawing. Although the mechanism by which insemination with fresh sperm increases the risk of polyspermy remains unknown, it is likely that a functional block to polyspermy is overridden on the grounds of excessive numbers of capacitated spermatozoa reaching the egg surface more or less simultaneously in vitro. By contrast, a relatively small number of capacitated spermatozoa with decreased velocity after freezing and thawing might positively affect fertilization condition to avoid unphysiological distribution of excessive competent spermatozoa. Xu et al. (1996) described that the optimal sperm:oocyte ratio for the maximal rate of penetration and polyspermy increases when the density of transzonal CCP and that concerning the optimal sperm concentration for successful IVF and development may depend on not only different boars but also sperm condition (fresh vs. frozen semen). Although biological significance of the breed-specific difference is not clear, the focus should not remain simply on boar effects within a single breed on the fertilization parameters.

The present observations demonstrate that the status of cumulus cells when removed from follicles correlates with the density of transzonal CCP and that considerable variation in the distribution of microfilaments already exists among the porcine oocytes assessed at different categories. In addition, the COC gradings correlate well with the maturation of the pig oocytes. However, once the oocytes could mature in vitro, they seemed to be penetrated by sperm with large variation among the breeds of sperm donor. These observations suggest that the cytoskeletal architecture of the oocyte may alter irreversibly during the intrafollicular development, affecting the ability of the oocyte to become mature and fertilized, although gilts of a similar age were immature. Further, the high incidence of decreased cortical microfilaments could account for the decreased rate of PBII expulsion in the denuded (grade 4) oocytes, because microfilaments are required for cytokinesis (Sun et al., 2001).

Polyspermic fertilization occurred more frequently in the lower-grade oocytes, reaching 50–60% when in-

### Table 2. Effect of investment morphology on meiotic maturation of pig oocytes

<table>
<thead>
<tr>
<th>Grade of COC</th>
<th>Examined (no.)</th>
<th>Penetrated (%)</th>
<th>Extruded PBII (%)</th>
<th>Polyspermic (%)</th>
<th>Mean no. spermatozoa in penetrated oocyte</th>
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<tr>
<td></td>
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<tr>
<td>Landrace</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>118</td>
<td>30.4 ± 2.7a</td>
<td>88.8 ± 6.5a</td>
<td>2.3 ± 2.3a</td>
<td>1.40 ± 0.05b</td>
</tr>
<tr>
<td>2</td>
<td>103</td>
<td>35.8 ± 5.8b</td>
<td>88.0 ± 12.0</td>
<td>23.9 ± 3.9</td>
<td>1.57 ± 0.05a</td>
</tr>
<tr>
<td>3</td>
<td>131</td>
<td>30.4 ± 6.5b</td>
<td>68.2 ± 31.8</td>
<td>35.6 ± 17.4</td>
<td>1.76 ± 0.12</td>
</tr>
<tr>
<td>4</td>
<td>60</td>
<td>25.0 ± 6.8b</td>
<td>32.1 ± 17.9b</td>
<td>14.3 ± 14.3</td>
<td>2.13 ± 0.13a</td>
</tr>
</tbody>
</table>

### Table 3. Effects of the breed and investment morphology on in vitro fertilization of pig oocytes

<table>
<thead>
<tr>
<th>Breed</th>
<th>Grade of COC</th>
<th>Examined (no.)</th>
<th>Penetrated (%)</th>
<th>Extruded PBII (%)</th>
<th>Polyspermic (%)</th>
<th>Mean no. spermatozoa in penetrated oocyte</th>
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</tr>
</tbody>
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*Values with different superscripts in the same column are significantly different (P < 0.05 between a and b; P < 0.01 between others).
Figs. 2–5.
seminated with sperm from Large White boars. The reason for this may be due to a delayed and incomplete exocytosis of the cortical granules (Cran and Cheng, 1986; Wang et al., 1997b) or premature cortical granules release in poor-grade oocytes, causing a weaker block to polyspermy. Some poor-grade oocytes had shown GV breakdown at 0 hours of incubation, thus aging of the oocytes could be another factor. These observations suggest that cytoplasmic maturation may not proceed in the grade 3–4 oocytes so much as in the higher-grade oocytes. The fact that the highest proportion of monospermic fertilization was derived from grade-1 oocytes suggests that grade-1 oocytes were the most synchronous group with respect to nuclear and cytoplasmic maturation.

It has been known that the paternal centrosome is active during fertilization in farm animals (Long et al., 1993; Navara et al., 1994). Sperm aster enlarged during sperm head decondensation and extended throughout the cytoplasm during PN formation (Kim et al., 1996c, 1997b). Recent reports describe the origin of the active microtubule-organizing center as paternally derived and its role in PN migration and apposition (Kim et al., 1996c, 1997b; Sun et al., 2001). In monospermic zygotes, the male and female PN were included in two different domains of microtubules, respectively. These microtubule domains were anchored to the microfilament architecture. During polyspermy, multiple male PN were found in separated microtubule domains, associated with individual penetrated sperm. A similar situation was described by Szollosi and Hunter (1973) and Kim et al. (1996c). From these observations, the sperm aster may develop to a microtubule-rich domain including the male PN as seen in the monospermic and polyspermic zygotes. On the contrary, pronucleate parthenogenotes show one domain of microtubules in the cytoplasm even if they are digynic (Suzuki et al., 2002a). Furthermore, in pig oocytes of which PBI was electrically fused back, the PBI's chromatin was easily incorporated into the microtubule networks of the oocytes, subsequently transforming into one “extra” nuclear-like structure (Suzuki et al., 2002a). From these results, it is assumed that an interphase network of microtubules, originated from the maternal centrosome and retained after the second meiotic division, is distributed in the ooplasm and includes preferentially the chromatin of the maternal origin, such as female PN and also PBI chromatin.

In the monospermic zygote, paternal and maternal microtubule domains unified during PN apposition. Interaction with microfilaments may be important for merging of two microtubule domains. Multiple microtubule domains in the polyspermic zygote became merged, but some delay or interruption was noted in the oocyte showing a higher degree of polyspermy (usually over trispermy). These observations suggest that the assemblies and interaction of microtubules and microfilaments may be disturbed in the presence of more than three male PN. The centration of the microtubule-rich domain as well as PN apposition may depend on the location and the number of male PN. A recent report has shown that the correction of ploidy can occur at the zygote stage before the first cell division, depending on the PN location (Han et al., 1999). More recently, Xia et al. (2001) suggested that the extra chromatin that does not affect the genome may suffer from the phagocytic activity of lysosomes in the ooplasm. The feasibility that antipolyspermic mechanism of the oocyte functions physiologically might depend on the location and the number of multiple male PN in the polyspermic zygote.

In conclusion, there is a breed-specific variation in fertilization and polyspermic rates and the freezing and thawing treatment may affect the sperm characteristics. The status of cumulus investment correlates with microfilament distribution during maturation, which may affect the rates of maturation and polyspermy. Multiple male PN derived from polyspermy may exert different effects on cytoskeletal reorganization during PN formation and migration as compared to the monospermic zygotes.

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Fig. 2. Fluorescent micrographs of a monospermic zygote at 6 hours after insemination. a: Three components: microtubules are green; microfilaments are red and nuclei/chromosomes are blue; yellow shows the overlap of microtubules and microfilaments. b,c: Microtubule and microfilament labeling, respectively. The first and second polar bodies are shown by small and large arrowheads, respectively. Female (large arrow) and male pronuclei (small arrow) are included with different domains of microtubules, respectively (a,b). Microtubules are located between two microtubule domains (c). Bar = 50 μm.

Fig. 3. Fluorescent micrographs of a dispermic zygote at 12 hours after insemination. a: Three components: microtubules are green; microfilaments are red and nuclei/chromosomes are blue; yellow shows the overlap of microtubules and microfilaments. b,c: Microtubule and microfilament labeling, respectively. The first and second polar bodies are shown by small and large arrowheads, respectively. Female (large arrow in a) and male pronuclei (small arrows in a) are found in three domains of microtubules (asterisks in b). Microfilaments are concentrated around the microtubule domain (c).

Fig. 4. Fluorescent micrographs of another dispermic zygote at 12 hours after insemination. a: Three components: microtubules are green; microfilaments are red and nuclei/chromosomes are blue; yellow shows the overlap of microtubules and microfilaments. b,c: Microtubule and microfilament labeling, respectively. The first polar body is out of focus. The second polar body is shown by a large arrow (c). Microfilaments are distributed in the ooplasm and includes preferentially the chromatin of the maternal origin, such as female PN and also PBI chromatin.

In the monospermic zygote, paternal and maternal microtubule domains unified during PN apposition. Interaction with microfilaments may be important for merging of two microtubule domains. Multiple microtubule domains in the polyspermic zygote became merged, but some delay or interruption was noted in the oocyte showing a higher degree of polyspermy (usually over trispermy). These observations suggest that the assemblies and interaction of microtubules and microfilaments may be disturbed in the presence of more than three male PN. The centration of the microtubule-rich domain as well as PN apposition may depend on the location and the number of male PN. A recent report has shown that the correction of ploidy can occur at the zygote stage before the first cell division, depending on the PN location (Han et al., 1999). More recently, Xia et al. (2001) suggested that the extra chromatin that does not affect the genome may suffer from the phagocytic activity of lysosomes in the ooplasm. The feasibility that antipolyspermic mechanism of the oocyte functions physiologically might depend on the location and the number of multiple male PN in the polyspermic zygote.

In conclusion, there is a breed-specific variation in fertilization and polyspermic rates and the freezing and thawing treatment may affect the sperm characteristics. The status of cumulus investment correlates with microfilament distribution during maturation, which may affect the rates of maturation and polyspermy. Multiple male PN derived from polyspermy may exert different effects on cytoskeletal reorganization during PN formation and migration as compared to the monospermic zygotes.

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Fig. 2. Fluorescent micrographs of a monospermic zygote at 6 hours after insemination. a: Three components: microtubules are green; microfilaments are red and nuclei/chromosomes are blue; yellow shows the overlap of microtubules and microfilaments. b,c: Microtubule and microfilament labeling, respectively. The first and second polar bodies are shown by small and large arrowheads, respectively. Female (large arrow) and male pronuclei (small arrow) are included with different domains of microtubules, respectively (a,b). Microtubules are located between two microtubule domains (c). Bar = 50 μm.

Fig. 3. Fluorescent micrographs of a dispermic zygote at 12 hours after insemination. a: Three components: microtubules are green; microfilaments are red and nuclei/chromosomes are blue; yellow shows the overlap of microtubules and microfilaments. b,c: Microtubule and microfilament labeling, respectively. The first and second polar bodies are shown by small and large arrowheads, respectively. Female (large arrow in a) and male pronuclei (small arrows in a) are found in three domains of microtubules (asterisks in b). Microfilaments are concentrated around the microtubule domain (c).

Fig. 4. Fluorescent micrographs of another dispermic zygote at 12 hours after insemination. a: Three components: microtubules are green; microfilaments are red and nuclei/chromosomes are blue; yellow shows the overlap of microtubules and microfilaments. b,c: Microtubule and microfilament labeling, respectively. The first polar body is out of focus. The second polar body is shown by a large arrow (c). Microfilaments are distributed in the ooplasm and includes preferentially the chromatin of the maternal origin, such as female PN and also PBI chromatin.

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