—Research Note—

Effects of Maturation Conditions on Spindle Morphology in Porcine MII Oocytes

Satoshi UENO¹⁾, Mayuko KUROME¹⁾, Hideto UEDA¹⁾, Ryo TOMII¹⁾,
Katsumi HIRUMA¹⁾, and Hiroshi NAGASHIMA¹⁾

¹⁾Laboratory of Developmental Engineering, Department of Life Science, School of Agriculture, Meiji University, 1-1-1 Higashimita, Kawasaki, 214-8571, Japan

Abstract. Incomplete cytoplasmic maturation of *in vitro* matured (IVM) oocytes has been known to cause microtubule and microfilament alterations, which may result in abnormal pronuclear formation and failed embryonic development. We examined the influences of maturation conditions on meiotic spindle morphology at metaphase of meiosis II (MII) in porcine oocytes. Porcine oocytes were matured under various conditions, *i.e.*, *in vitro* or *in vivo*, with different amounts of cumulus cells, with or without hormonal supplements, and with various exposure durations to the hormones, to examine the effects on spindle morphology in MII oocytes by immunofluorescence under confocal laser microscopy. Interpolar spindle length (μm) and spindle area (μm^2) were compared among these maturation conditions. The spindle length was significantly shorter in IVM oocytes compared to those matured *in vivo*. Oocytes collected from cumulus oocyte complexes (COCs), which were poor in cumulus cells, showed smaller spindle areas than those from cumulus-rich COCs. The spindle length and area were both significantly reduced in oocytes grown without hormonal supplements. When oocytes were grown with hormonal supplements for either 6 or 22 hours for the first half of culture, there was no difference in the spindle morphology between these oocytes. These results suggested that maturation conditions significantly influence morphogenesis of MII spindles in porcine oocytes. Oocytes matured in poor conditions were more likely to have a shorter spindle length (long axis) and smaller spindle areas.

Key words: Pig, Oocyte, IVM, Spindle, Maturation

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Recently, transgenic pigs have been recognized as an essential animal for biological and medical applications [1]. For example, transgenic pigs have been used for production of biologically active compounds and as human disease models [2, 3], and genetically modified cloning of pigs has been attempted to generate organ grafts for xenotransplantation [4].

Production of genetically modified pigs requires advanced reproductive technologies, especially for

micromanipulation of oocytes. Traditionally, transgenic pigs have been produced using *in vivo* derived eggs, but the use of *in vitro* matured (IVM) oocytes has become mainstream in recent years because of the substantial cost and workload involved in obtaining a sufficient number of *in vivo* derived eggs [5].

The developmental potential of porcine IVM oocytes, however, is not as high as that of *in vivo* derived oocytes; the major problem stems from improper cytoplasmic maturation of IVM oocytes [6]. It has been found that incomplete cytoplasmic maturation of IVM oocytes often contains abnormal

microtubules (MTs) and microfilaments, which in turn cause abnormal pronuclear formation and defective embryonic development [7]. The abnormality in MTs profoundly influences formation of the meiotic spindle, a dynamic assembly of MTs [8], and it is often associated with abnormal chromosome segregation after fertilization and reduced developmental rates of embryos as a consequence [9]. Thus, in mice and humans, the spindle morphology at metaphase of meiosis II (MII) has been used as an index of the cytoplasmic integrity of oocytes [10, 11]. Further, the conditions for eggs, such as maturation *in vivo* or *in vitro*, superovulation induction, and aging affect the MII spindle morphology in mice [12, 13].

In this study, we examined the impact of variations in oocyte maturation conditions on spindle morphogenesis at MII in porcine oocytes to determine whether the MII spindle morphology can be used to evaluate the developmental competence of oocytes.

Materials and Method

Chemicals

Unless otherwise indicated chemicals, were obtained from Sigma-Aldrich Corporation (St. Louis, MO, USA).

Collection of ovulated oocytes

Crossbred (Large White/Landrace × Duroc) prepubertal gilts weighing between 100 and 105 kg were used as donors of oocytes. Gilts were treated with a single IM injection of 1500 IU eCG (Teikoku Zouki Co., Tokyo, Japan). Ovulation was induced by an IM injection of 1500 IU hCG (Teikoku Zouki) given 71 h after the first injection of eCG. Oocytes were collected by retrograde flushing of the oviduct under general anesthesia 47 to 50 h after the hCG injection.

In vitro maturation of oocytes

Ovaries were recovered from gilts at a local slaughterhouse and transported to the laboratory in Dulbecco's phosphate-buffered saline (PBS, Nissui Pharmaceutical, Tokyo, Japan) containing 0.1% (w/v) polyvinylalcohol (PVA), 75 µg/ml penicillin G, and 50 µg/ml streptomycin.

Cumulus oocyte complexes (COCs) were aspirated from follicles (diameter 3–6 mm) using a

20-gauge needle attached to a 10-ml disposable syringe and were washed twice with Hepes-buffered Tyrode's medium containing 0.3% (w/v) polyvinylpyrrolidone medium (Hepes-TL-PVP). Groups of 20–25 COCs were cultured for 44 h in a 100 µl droplet of NCSU23 medium [14] supplemented with 0.6 mM cysteine, 10 ng/ml epidermal growth factor, and 10% (v/v) porcine follicular fluid, and covered with paraffin oil in a 35 mm petri dish (Falcon no. 1008, Becton Dickinson Labware, Franklin Lakes, NJ, USA) at 38.5 C under 5% CO₂ in air. Hormonal supplements of 10 IU/ml eCG and 10 IU/ml hCG were added to the IVM media according to the experimental design described below.

In vitro matured oocytes with expanded cumulus cells were briefly treated with hyaluronidase (0.01% in Hepes-TL-PVP) and denuded of cumulus cells using a finely drawn glass capillary pipette. Oocytes extruding the first polar body were used for the experiments.

Immunofluorescence

Spindle MTs were immunostained according to Kim *et al.* [7] and Liu *et al.* [15]. Denuded oocytes were fixed with 3% paraformaldehyde in PBS for 90 min on ice. Subsequently, oocytes were incubated in PBS containing 0.5% Triton-X100 and 30 mg/ml bovine serum albumin (BSA, Fraction V) for 20 min and then with anti- α -tubulin monoclonal antibody (T-5168, 1:300 in PBS) for 90 min. To prevent nonspecific cross reactions, oocytes were treated with blocking solution containing 0.1 M glycine (Wako Pure Chemical, Osaka, Japan), 0.01% Triton-X100, 10 mg/ml powdered milk (Wako), 5 mg/ml BSA, 0.015 mM sodium azide and 10 mg/ml goat serum for 60 min. Oocytes were then incubated with FITC-labeled goat anti-mouse antibody (F-4018, 1:100 in PBS) for 60 min. Chromosomes were stained with 0.05 mg/ml propidium iodide in PBS for 60 min. All procedures were performed at 39 C. Oocytes were mounted on glass slides and covered with anti-fading mounting media (Vectashield, Vector Lab. Burlingame, CA, USA). Meiotic spindles were examined using a confocal laser microscope (LSM-510, Carl Zeiss Inc., Jena, Germany).

Measurements of spindle length and area

Ten z-section images per oocyte were acquired to reconstruct a spindle morphology using the

Fig. 1. Immunofluorescence images of spindles in MII oocytes matured under various conditions. Spindle length and area were measured as shown in (a). The arrow indicates the length of the spindle and the dashed line shows the area measured. The spindle in the *in vivo* matured oocyte is shown in (b). The spindle in IVM oocytes matured with or without hormonal supplements were shown in (c) and (d). Green: Microtubules; Red: chromosomes. Bar=10 μ m.

accompanying software of the LSM-510. The length (long axis) and area of each spindle were measured from reconstructed spindle images using the scale tool on the microscope (Fig. 1a).

Experiment 1: Comparison between in vivo and in vitro matured oocytes

Spindle morphology was compared between oocytes matured either *in vitro* or *in vivo* in this experiment. Cumulus-rich COCs, which had compact multiple layers of cumulus cells, were used to prepare IVM oocytes. Hormonal supplements were given for 22 h during the first half of maturation.

Experiment 2: Influence of amounts of cumulus cells on spindle morphology in IVM oocytes

COCs were divided into two groups based on the amount of cumulus cells. Cumulus-rich COCs were selected as described in Experiment 1, and those with a single or double layer of cumulus cells were selected as cumulus-poor COCs. IVM oocytes were produced under the same conditions as

Experiment 1, and spindle morphology was compared between these COCs groups.

Experiment 3: Effects of hormonal deprivation on spindle morphology in IVM oocytes

Spindle morphology was compared between IVM oocytes matured with or without hormonal supplements. COCs were selected as described in Experiment 1 for IVM oocyte preparation. For oocytes matured with hormonal supplements, hormones were present in media throughout the maturation process.

Experiment 4: Effects of duration of hormonal exposure on spindle morphology in IVM oocytes

Hormone supplements were given either for 22 h (as in Experiment 1) or 6 h during the first part of maturation, and spindle morphology was compared between the oocytes prepared under these conditions.

Statistical analysis

Differences between measured spindle lengths and areas were tested for significance with the Student's *t*-test. The level of significance was set at $P < 0.05$.

Results

Fig. 2 summarizes the result of comparisons for spindle length and area. When *in vivo* and *in vitro* matured oocytes were compared (Fig. 2a), the spindle length was significantly shorter in IVM oocytes ($p < 0.05$). Spindle shape in these IVM oocytes were rather round and reduced in size (Fig. 1b and c).

The result of comparison of spindle morphology between oocytes derived from cumulus-rich and cumulus-poor COCs is shown in Fig. 2b. Oocytes prepared from cumulus-poor COCs showed a reduction in spindle areas compared to those from cumulus-rich COCs ($p < 0.05$, Fig. 2b).

The effect of hormonal deprivation on spindle morphology in IVM oocytes is shown in Fig. 2c. Oocytes matured without hormones showed significant reduction in both spindle length and area compared to those matured with hormones. As shown in Fig. 1d, the MII spindle in oocytes matured without hormonal supplements had a remarkably shorter length and a round shape.

Fig. 2. Comparison of spindle morphology between oocytes matured under various conditions. (a): Spindle morphology comparison between *in vivo* and *in vitro* matured MII oocytes; (b): the influence of amounts of cumulus cells on spindle; (c): the effect of hormonal deprivation during *in vitro* oocyte maturation on spindle morphology; (d): the effect of changes in hormonal exposure duration on spindle morphology of IVM oocytes. Length= μm , Area= μm^2

Fig. 2d shows the result of comparison of spindle morphology between oocytes matured in hormone-supplemented media for 6 and 22 h. There was no statistical difference in spindle length and area between these two groups.

Discussion

Maturation of mammalian oocytes has two aspects: nuclear maturation and cytoplasmic maturation. Nuclear maturation refers to the completion of meiotic chromosomal division, which results in a haploid DNA content, while cytoplasmic maturation is the process in which oocytes acquire developmental competence [16]. In porcine oocytes, nuclear maturation can be efficiently induced *in vitro* by culturing in gonadotropin-supplemented media for about 44 hours. On the other hand, *in vitro* cytoplasmic maturation of porcine oocytes is very vulnerable to various factors, such as medium composition and oxidative stress [17]. For example, follicular fluid factors [18], gonadotropin [19], growth factor [20], cysteine [21], cysteamine [22] and hyaluronic acid [23] have been reported as medium components

that influence cytoplasmic maturation.

Recent advancements in porcine IVM systems have successfully produced normal offspring from IVM and *in vitro* fertilized (IVF) oocytes [24]. NCSU23, which was used in this study, is one of the most frequently used media for porcine IVM. We have also produced a significant number of pigs from oocytes matured in NCSU23 medium using cloning technology [25] and intracytoplasmic sperm injection (Kurome *et al.*, submitted for publication). In this study, we showed that spindle length of IVM oocytes was clearly shorter than that of *in vivo* derived oocytes. This suggests that the current IVM conditions not yet reached the quality of the *in vivo* conditions. In fact, we found that the rate of embryonic development to blastocysts was significantly lower in artificially activated IVM oocytes than for *in vivo* ovulated eggs (data not shown).

Reduction in spindle length and area was similarly observed in oocytes derived from cumulus-poor COCs and in those matured without gonadotropins. We also found that *in vitro* developmental competence to the blastocyst stage was lower in the IVM oocytes derived from cumulus-poor COCs, when compared with those from cumulus-rich COCs (data not shown). IVM oocytes matured without gonadotropins also showed extremely compromised developmental competence to blastocysts (data not shown). On the other hand, the different durations of culture in hormone-supplemented media, 6 or 22 hours, during the first half of maturation did not affect spindle length and area. In our preliminary study, these conditions also did not influence the ability of parthenogenetic development (data not shown).

Collectively, our study showed that suboptimal maturation conditions caused significant reduction in both spindle length and area. Therefore, spindle morphology might also be a useful index for pigs that reflects the cytoplasmic quality along the course of oocyte maturation. Sunfins *et al.*, however, has reported in mice that *in vivo* derived oocytes have a larger reduction in both spindle length and area than those of IVM oocytes [13]. Further studies are required to determine whether improper cytoplasmic maturation is linked to shorter and smaller spindle formation in other animals.

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