The Relative Centrifugation Force Permits Visualization of the Germinal Vesicle in Pig Oocytes

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ABSTRACT : Pig oocytes contain high levels of lipids in the ooplasm, which reduces the visibility of the germinal vesicle (GV) under microscopic examination. Therefore, the purposes of this study were to investigate the effects of relative centrifugation force (RCF) on the visibility and maturation rates of the GV stage oocytes after centrifugation. In Experiment 1, cumulus-oocyte-complexes (COCs) were collected from slaughterhouse ovaries and randomly allocated to different RCFs (3,000 rpm: 970 *g*; 6,000 rpm: 3,900 *g*; or 10,000 rpm: 10,840 *g*) for 10 or 20 min. Percentages of visible GV were 76-79% in the oocytes centrifuged with 10,000 rpm, which were significantly higher ($p<0.01$) than those with 3,000 and 6,000 rpm. No significant differences in GV visibility were observed among oocytes with different lengths of centrifugation ($p<0.05$) regardless of the RCFs. In esperiment 2, the maturation rate of the oocyte was found significantly lower in the 20 min than in the 10 min group received 10,840 *g* of RCF (30 vs. 75%, $p<0.05$). In conclusion, the GV of porcine oocytes can be clearly visible by centrifugation at 10,840 *g* for 10 min without compromising their subsequent maturation rates and a longer centrifugation time (20 min) had no beneficial influence on the visibility of GV stage pig oocytes. ***(Asian-Aust. J. Anim. Sci. 2004. Vol 17, No. 9 : 1227-1231)***

Key Words : Oocyte, Germinal Vesicle, Pig, Relative Centrifugation Force (RCF)

INTRODUCTION

Germinal vesicle (GV) transfer of the oocytes has been thought to be one of the potential applications for the treatment of human infertility, although many efforts have been made in the mouse (Zhang et al., 1999; Moffa et al., 2002; Liu et al., 2003), rabbit (Li et al., 2001), and other species with limited success. No successful report was available in pig oocytes. However, pig oocytes might be a good model for studying human oocytes due to their similarity in the time required for *in vitro* maturation (38-40 h). However, pig oocytes contain high levels of lipids in the ooplasm, which reduces the visibility of nuclear configuration including the pronuclei and the GV when examined microscopically. This character potentially hinders the GV-related manipulation of pig oocytes. In other studies, pronuclear stage of sheep and pig zygotes were centrifuged at 15,600 g for 3-5 min to reveal the pronuclei for gene microinjection (Hammer et al., 1985; Wall et al., 1985; Wall and Hawk, 1988; Tatham et al., 1996). The viability or subsequent development of the centrifuged zygotes appeared unaffected by the centrifugation (Rho et al., 1998; Han et al., 1999ab). Similarly, to facilitate manipulation of GV transfer, nuclear materials have to be clearly visible during manipulation. However, no information was available for the RCF parameters and their effect on subsequent maturation of GV stage pig oocytes after centrifugation. Therefore, a reliable RCF parameter to permit visualization of the GV without compromising the subsequent maturation or developmental competence of pig oocytes is necessary. In this study, we had optimized the condition for GV visualization in pig oocytes. The effects of different RCFs on subsequent maturation rates were also examined systematically.

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MATERIALS AND METHODS

Experiments were performed to investigate the visibility of porcine GV nucleus and maturation rates after centrifugation in attempt to facilitate future GV transfer and other related studies. All chemicals and reagents used in this study were purchased from Sigma company except otherwise described.

Oocyte collection and IVM culture

Pig ovaries were collected and transported from local abattoirs within 2 h (Song et al., 2002; Ju and Tseng, 2004). The COCs were aspirated from ovarian follicles with an 18G epidermic needle attached to a syringe. Only oocytes completely enclosed by multiple layers of cumulus cells were selected for use. Newly collected COCs were rinsed several times and cultured for 22 h in the NCSU#23 medium supplemented with 10% porcine follicular fluid, cysteine (0.1 mg/mL), EGF (10 ng/mL, E-4127), PMSG (10 IU/mL, G-4877) and hCG (10 IU/mL) as described previously (Ju et al., 2003). The COCs were further cultured for an additional 22 h in the same medium without

Table 1. Effects of the relative centrifugation force (RCF) and the lengths of centrifugation on the visibility (%) of the germinal vesicle (GV) of porcine oocytes

RCF / Time	970 *g*/3,000 rpm % (N_1/N_2)	3,900 *g*/6,000 rpm % (N_1/N_2)	10,840 *g*/10,000 rpm % (N_1/N_2)
10 min	30.3 (17/56)[a]	56.5 (39/69)[b]	79.3 (46/58)[c]
20 min	16.0 (4/25)[a]	55.9 (33/59)[b]	76.4 (39/51)[c]

[a,b,c] Different superscripts in the same row represent significant differences ($p<0.01$).
N_1: numbers of oocytes with clearly visible GV. N_2: total numbers of oocytes within the groups.

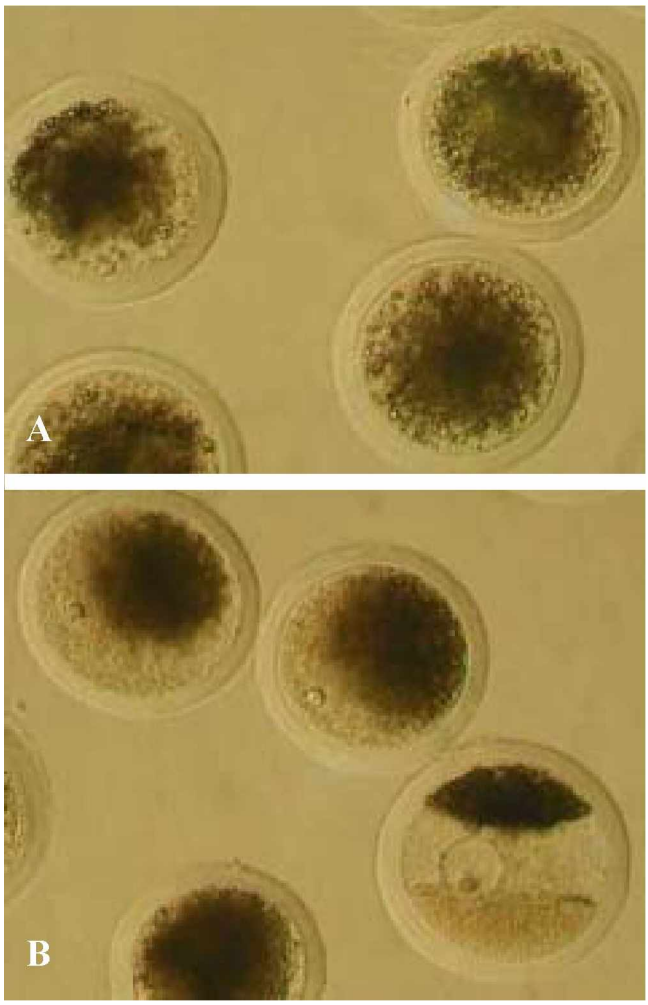

Figure 1. The visibility of the germinal vesicle (GV) in the pig oocyte after centrifugation. (A) Normal GV stage oocytes with their GV nuclei obscured by the dark ooplasm prior to centrifugation. (B) The GV (white arrow) with a nucleolar structure and the stratified ooplasm were clearly visible after centrifugation at 10,840 *g* for 10 min.

hormonal supplementation (Abeydeera and Day, 1998; Wang et al., 1999).

Experiment 1

Visibility of the GV by different RCFs : Porcine COCs isolated from the ovaries were randomly allocated to different RCFs, i.e., 3,000 (970 *g*), 6,000 (3,900 *g*) and 10,000 (10,840 *g*) rpm, for 10 or 20 min, respectively. Prior to IVM, oocytes were removed of cumulus cells and subjected to different RCFs and centrifugation times. Immediately after centrifugation, all groups of oocytes were incubated at 39°C in an incubator containing 5% CO_2 in air. Ten to twenty oocytes in each replicate were fixed at 0, 30, 60, 90 and 120 min after the onset of incubation for GV examination.

Experiment 2

Maturation rates after centrifugation : Freshly collected COCs were designated to different RCFs (970 *g*, 3,900 *g*, and 10,840 *g*) and durations of centrifugation (0, 10 and 20 min) as in Experiment 1. After centrifugation, oocytes were *in vitro* matured for 44 h in NCSU #23 medium and the maturation rates were examined microscopically by the presence of first polar body after removal of cumulus cells (Huang et al., 2002).

Statistical analysis

The frequencies (numbers) of ooyctes with visible GV in different treatment groups and *in vitro* maturation of pig oocytes were subjected to Chi-square test at $p<0.05$ or 0.01.

RESULT

Experiment 1

GV visibility of the centrifuged oocytes : When the GV stage oocytes were centrifuged at different RCFs for 10 or 20 min, a clear GV and stratified ooplasm were visible in normal healthy oocytes (Figure 1). The percentages of oocytes with clearly visible GV are presented in Table 1. No significant difference in the percentages of visible GV was found between different lengths of centrifugation. However, percentages of visible GV nucleus of the oocytes increased significantly ($p<0.01$) with the increase of RCFs and reduced with the time after centrifugation (Figure 2). When oocytes were centrifuged at 10,840 *g* (10,000 rpm), the percentages of visible GV were significantly greater than those at other RCFs (76-79% vs. 16-57%, $p<0.01$; Table 1) regardless of the durations of centrifugation. When the centrifuged oocytes were returned to culture and examined for the GV up to 120 min, the percentages of visible GV reduced gradually with time in all the three groups subjected to different RCFs. In the oocytes centrifuged with 1,0840 *g*, the GV appeared in 76-79% of oocytes immediately after centrifugation (0 min). They decreased to 67 and 55-57% at 30 and 60 min after centrifugation.

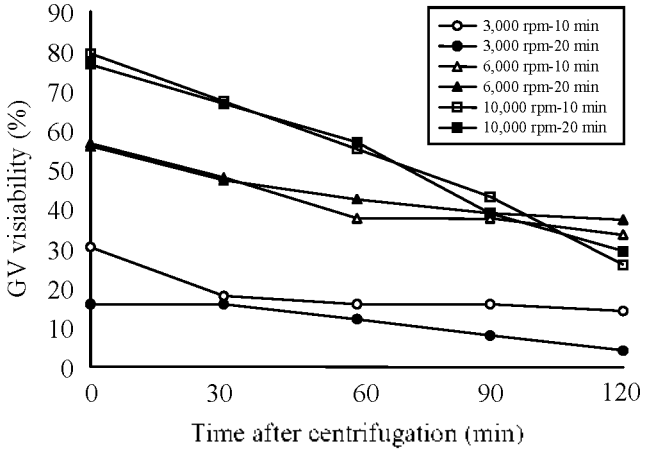

Figure 2. The visibility of germinal vesicle (GV) in porcine oocytes after centrifugation for different durations at different relative centrifugation forces (RCFs).

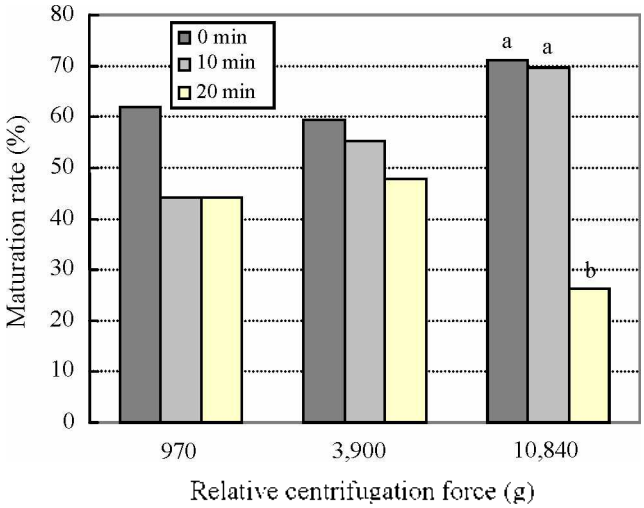

Figure 3. Effects of the relative centrifugation forces (RCFs) and durations of centrifugation on *in vitro* maturation of porcine oocytes. Different superscripts (a, b) on the bar in each RCF group represent significant differences ($p<0.05$).

respectively. At 120 min after centrifugation, only 26-29% oocytes had clearly distinguishable GV (Figure 2). The time of centrifugation (10 or 20 min) did not affect the ratios of oocytes with a visible GV during the recovery periods (Figure 2).

Experiment 2

Maturation rates of pig oocytes after centrifugation : When the oocytes were subjected to IVM after centrifugation with different RCFs, no significant differences ($p>0.05$) in maturation rates among different durations of centrifugation (0, 10 or 20 min) were observed in low RCF treatment groups (970 *g* and 3,900 *g*). However, in the oocytes centrifuged with high RCF (10,840 *g*), a reduced maturation rate was found in the 20 min (71 vs. 26%, $p<0.05$), but not in the 10 min (71 vs. 70%, $p>0.05$), treatment group when compared to the control oocytes (Figure 3).

DISCUSSION

The GV and pronuclei can be observed in mouse and rabbit oocytes under a bright field or Normaski-equipped microscope due to their transparent ooplasm. In contrast, pronuclei and nuclei of a zygote or embryo in many species including sheep, goats (Baldassarre et al., 2003), cattle (Loskutof et al., 1986) and dogs are obscured. In these oocytes, routine micromanipulation, such as pronuclear gene injection or GV transfer, was not possible without visualization of the nuclei. To overcome this problem, centrifugation (RCF>10,000 *g*, for 3-7 min) of the fertilized ooyctes has been applied to localize the opaque ooplasm and reveal the nuclear structures. This process has been proven to be not detrimental to the subsequent development of the centrifuged zygotes (Wall and Pursel, 1985; Wall and Hawk, 1988; Rho et al., 1998; Han et al., 1999ab).

The ooplasm of pig oocytes is very similar to that of canine oocytes (our unpublished observations), which possess, presumably, high lipid content and, possibly, other dense cytosolic materials. When examined under a stereomicroscope, the dark ooplasm dominates the majority of the central ooplasm with somewhat clearer or lighter peripheral ooplasm (Figure 1A). Therefore, the GV in the ooplasm is also difficult to locate without centrifugation. For this purpose, a similar RCF might be applicable to the GV stage oocyte. In the cows, the pronuclei in 73% out of 106 centrifuged zygotes were detected by Wall and Hawk (1988) using differential interference-contrast microscopy. When subsequent development was evaluated by transferring into rabbit oviducts, the transferred embryos showed no significant difference in blastocyst formation between the centrifuged (15,600 *g*, 3 min) and non-centrifuged zygotes. However, no direct evidence is available for the effect of RCF on their subsequent maturation or development of the GV stage pig oocytes. Furthermore, it is not known whether a longer duration of centrifugation or a lower RCR would enhance visualization of the GV without causing reduction in maturation rate of pig oocytes. In this study, we found that low RCFs only revealed a poor visualization of the GV and that no differences in maturation rates of the centrifuged oocytes were observed regardless of the lengths of centrifugation. The GV of porcine oocytes appeared clearly between the stratified ooplasm in the 10 min centrifugation groups (10,840 *g*; Figure 1) without compromising their maturation rates (Figure 3). However, a prolonged centrifugation time (20 min) at 10,840 *g* did not improve GV visibility (Table 1) but significantly reduced subsequent maturation rate of

pig oocytes compared to the control and other 10 min groups (26% vs. 70 and 71%, $p<0.05$; Figure 3). The causes of the impairment were not clear yet. However, the organelles and/or cytoskeletal structures (unpublished observations) in the ooplasm were relocated or disrupted temporally. Recovery of these cellular structures might occur during *in vitro* culture after centrifugation. We speculated that a longer time with higher RCF (10,840 *g*, 20 min) might cause rupture of organelle structures or some other irreversible damages to the oocytes, which requires further examinations of the cellular and/or subcellular ultrastructures of the centrifuged oocytes.

In conclusion, we have established an adequate condition for visualization of pig germinal vesicle by centrifugation. Combination of this protocol with potent kinase inhibitors, such as roscovitine (Meijer et al., 1997; Mermillo et al., 2000; Krischek and Meinecke, 2001; Ju et al., 2003), 6 dimythylaminopurine (Fulka et al., 1991; Liu et al., 1998ab) butyrolactone I (Kitagawa et al., 1993; Motlik et al., 1998; Kubelka et al., 2000), or 3-isobutyl-1-methylzanthine (IBMX; Liu et al., 2000; Moffa et al., 2002), would be helpful to prevent germinal vesicle breakdown and to facilitate the GV-related micromanipulation in pig oocytes.

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