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Meiotic Competence of Porcine Oocytes after Percoll Sedimentation Treatment for Oocyte Selection

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Abstract: The objective of this study was to evaluate the effects of Percoll sedimentation treatment of porcine oocytes before *In vitro* Maturation (IVM) on the meiotic competence of the oocytes. Cumulus-oocyte Complexes (COCs) with uniform ooplasms and compact cumulus cells obtained from porcine ovaries were placed on the surface of Percoll solutions of various concentrations (5, 10, 15, 20 and 25%) in a petri dish. Only the COCs that settled in the dish within 3 min were transferred into maturation medium. At the end of the IVM culture, the nuclear status of the oocytes was assessed. The proportion of COCs that settled decreased with an increase in the concentration of the Percoll solution. When the COCs were treated with less than 10% Percoll solution, more than half the total number of COCs settled. In contrast, the proportion of COCs that settled was lower ($p<0.01$) in the 20 and 25% Percoll solutions (12 and 1%, respectively) than in Percoll solutions with lower concentrations (49-90%). The proportion of oocytes that underwent germinal vesicle breakdown and reached metaphase II after the Percoll treatment and subsequent IVM culture did not differ among the 5, 10 and 15% Percoll solution groups. Moreover, it also did not differ from that of the control oocytes that were not treated with Percoll. In conclusion, the selection of oocytes by Percoll sedimentation treatment does not improve the rates of nuclear maturation of porcine oocytes and decreases the total number of COCs available for IVM culture.

Key words: Percoll, sedimentation, nuclear status, porcine oocyte

INTRODUCTION

In vitro Production (IVP) of porcine embryos, including *In vitro* Maturation (IVM), *In vitro* Fertilization (IVF) of oocytes and their subsequent *In vitro* Culture (IVC), has vast potential for research and commercial use (Nagai, 2001; Niemann and Rath, 2001). With the growing interest in transgenic pig production, extensive efforts have been made to improve the *in vitro* systems for oocyte maturation and fertilization (Kikuchi, 2004; Nagai *et al.*, 2006). Despite recent developments in the IVM and IVF of porcine oocytes, IVP of porcine embryos remains relatively inefficient compared to the results obtained with *in vivo* matured and fertilized oocytes (Niemann and Rath, 2001; Abeydeera, 2002). Although, the quality of *in vitro* matured porcine oocytes has improved over the last decade (Funahashi and Day, 1993; Funahashi *et al.*, 1997; Abeydeera *et al.*, 1998a; Abeydeera *et al.*, 1998b), IVM/IVF remains to be standardized because the results obtained in different laboratories vary greatly.

The oocyte quality before IVM is the most important factor that determines the success of IVF and *in vitro* development (Nagai *et al.*, 1990; Wongsrikeao *et al.*, 2005). The quality of the cytoplasm and the characteristics of the cumulus cell investment around the oocyte are the most important criteria that have been routinely used to select Cumulus-oocyte Complexes (COCs) for IVM and IVF (Leibfried and First, 1979; Shioya *et al.*, 1988). However, subjective evaluation of oocyte quality based on morphological criteria determined by microscopic observation is thought to differ among evaluators. The establishment of a simple, objective and non-damaging method for the selection of homogeneous and developmentally competent oocytes would advance IVP technology.

Sedimentation is a characteristic of substrates and the migration velocity and equilibrium range depend on

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the mass, buoyant density and friction coefficient (Meselson and Stahl, 1958). Percoll, a colloidal polyvinylpyrrolidone-coated silica medium with low viscosity, is utilized for cell separation by sedimentation via standing or density gradient centrifugation (Pertoft, 2000). Moreover, Percoll has been widely used in methods that are based on the principle of density gradient centrifugation for the rapid and efficient isolation of motile bovine spermatozoa (Henkel and Schill, 2003). Recently, Yotsushima *et al*. (2007) evaluated sedimentation with Percoll as an objective method for the selection of bovine COCs and reported that the Percoll sedimentation method is effective for simple COC selection. However, no report demonstrates the effects of Percoll treatment before IVM on the meiotic competence of porcine oocytes?.

The objectives of this study, were to evaluate the effects of Percoll sedimentation treatment of porcine oocytes before IVM on their meiotic competence and to determine whether or not the Percoll sedimentation method is a reliable and practical method for the selection of porcine oocytes.

MATERIALS AND METHODS

Preparation of oocytes: Ovaries from prepubertal crossbred gilts, approximately 6 months old, were collected from a local slaughterhouse and transported to the laboratory in physiological saline [0.85% (w v^{-1}) NaCl] at 35°C within 3 h. To release the COCs from the antral follicles, the cortex of each ovary was sliced repeatedly with a scalpel blade in a 90-mm culture dish containing modified PBS (mPBS; Embryotech, Nihon Zenyaku Kogyo, Fukushima, Japan). COCs with 2 or more dense layers of cumulus cells were collected and washed twice with mPBS. Only oocytes with uniform ooplasms and compact cumulus cells were used for the experiment.

Selection of COCs by percoll sedimentation: Percoll sedimentation treatment was carried out according to the method described by Yotsushima *et al*. (2007). Briefly, pure Percoll (Amersham Pharmacia Biotech, Uppsala, Sweden) was diluted beforehand with mPBS solution (1:1) and stored at 4°C until use. Then 5, 10, 15, 20 and 25% Percoll solutions were prepared by diluting 50% Percoll with mPBS. The COCs were placed on the surface of the Percoll solutions (2 mL) of each concentration in 35×10 mm petri dishes (Falcon, Franklin Lakes, NJ, USA) such that the distance between the surface of the solution and the bottom of the dish was approximately 0.5 cm. Only the COCs that settled in the Percoll solution within 3 min were harvested and washed twice with mPBS. Control oocytes were not treated with the Percoll solution.

Maturation culture of oocytes: After the COCs were washed with mPBS, they were transferred into maturation medium; a modified North Carolina State University (NCSU)-37 solution supplemented with 10% (v v^{-1}) porcine follicular fluid, 0.6 mM cysteine (Sigma, St. Louis, MO, USA), 1 mM dibutyryl cyclic AMP (dbcAMP; Sigma), 50 μM β-mercaptoethanol (Wako Pure Chemicals, Osaka, Japan), 10 IU mL^{-1} equine chorionic gonadotropin (Kawasaki-Mitaka, Tokyo, Japan), 10 IU mL^{-1} human chorionic gonadotropin (Kawasaki-Mitaka) and 50 μg mL^{-1} gentamicin (Sigma). Approximately 10 COCs were cultured for 22 h in a 100 μL drop of the maturation medium covered with a layer of mineral oil (Sigma) in a petri dish. They were then transferred to maturation medium without hormones and dbcAMP and cultured for an additional 22 h. All cultures were performed at 38.5°C in a humidified incubator and 5% CO_2.

Assessment of oocyte nuclear status: After 44 h of maturation culture, the cumulus cells surrounding the oocytes were mechanically denuded following treatment with 1 mg mL^{-1} hyaluronidase (Sigma) in PBS (Invitrogen, Carlsbad, CA, USA). The denuded oocytes were fixed and permeabilized for 15 min at room temperature in PBS supplemented with 3.7% (w v^{-1}) paraformaldehyde and 1% (v v^{-1}) Triton X-100 (Sigma) and they were then suspended in PBS containing 0.3% (w v^{-1}) polyvinylpyrrolidone for 15 min at room temperature. The oocytes were transferred to a small drop comprising PBS supplemented with 90% (v v^{-1}) glycerol (Wako Pure Chemicals) and 1.9 μM bis-benzimide (Hoechst 33342, Sigma) on a slide. Subsequently, they were overlaid with a coverslip supported by 4 droplets of vaseline/paraffin and incubated overnight at 4°C. The maturational stage of each oocyte was precisely determined based on the changes in its chromosome configuration and nuclear membrane. Oocytes that were fragmented or distinctly irregular in shape were classified as degenerated.

Statistical analysis: Data were expressed as means±SEMs. The proportions of oocytes that reached each stage of meiosis and settled after Percoll treatment were subjected to arcsin transformation prior to Analysis of Variance (ANOVA). The transformed data were then tested by a post-hoc, Fisher's Protected Least Significant Difference (PLSD) test conducted using the STATVIEW program (Abacus Concepts, Inc, Berkeley, CA, USA). Probability values (P) of 0.05 or less were considered significant.

RESULTS

The proportions of porcine oocytes that settled after treatment with different concentrations are shown in Fig. 1. The proportion of settled COCs decreased with

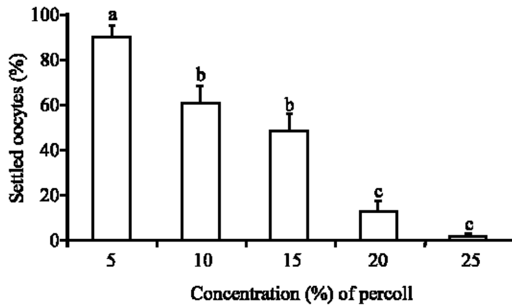

Fig. 1: The proportion of porcine oocytes that settled after treatment with different Percoll concentrations. Porcine oocytes were treated for 3 min with 5% (n = 159), 10% (n = 181), 15% (n = 208), 20% (n = 164) and 25% (n = 165) Percoll solutions. Bars with different letters differ significantly (p<0.01)

Table 1: Meiotic competence of porcine oocytes settled after treatment with different Percoll concentrations

Concentration (%) of Percoll solution	No. of oocytes examined*	Means±SEMs (No. of oocytes)	
		GVBD	MII
Control	185	90.8±3.3 (163)[a]	47.6±3.0 (90)[a]
5	142	89.2±2.8 (126)[a]	28.7±4.2 (37)[a,c]
10	114	94.4±1.4 (107)[a]	33.6±6.1 (39)[a,c]
15	96	96.2±1.7 (92)[a]	36.2±3.7 (35)[a,c]
20	19	87.4±8.5 (15)[a]	63.7±15.2 (9)[b]
25	2	20.0±20.0 (2)[b]	10.0±10.0 (1)[c]

GVBD, germinal vesicle break down; MII, metaphase II. *Control oocytes were not treated with Percoll. Only oocytes that settled after Percoll treatment were cultured and their meiotic status was then examined. [a-c]Values with different superscripts in the same column are significantly different (p<0.01)

an increase in the Percoll concentration. When the COCs were treated with less than 10% Percoll solution, more than half the total number of COCs settled. In contrast, the proportion of COCs that settled in 20 and 25% Percoll solutions (12.6 and 1.0%, respectively) was significantly lower (p<0.01) than that in Percoll solutions of lower concentrations (49-90%).

As shown in Table 1, the proportion of oocytes that underwent Germinal Vesicle Breakdown (GVBD) and reached Metaphase II (MII) after Percoll treatment and the subsequent IVM culture did not differ among the 5, 10, 15 and 20% Percoll solution groups. Moreover, it did not differ from that of non-treated control oocytes (p>0.05). The proportion of oocytes that reached MII was significantly higher (p<0.05) in the 20% Percoll solution group than in the other groups.

DISCUSSION

For successful *in vitro* development of IVM/IVF porcine oocytes, it is necessary for the oocytes to complete nuclear maturation during IVM and have the ability to be fertilized normally (Nagai, 2001). Oocyte quality has been suggested to be the most important factor for successful IVF and *in vitro* development (Nagai *et al*., 1990; Wongsrikeao *et al*., 2005). Yotsushima *et al*. (2007) reported that Percoll sedimentation can be used for the simple selection of bovine COCs and that the superior class of COCs generally has higher sedimentation values than those of the inferior classes. In their study, when the concentrations of Percoll used for the sedimentation of bovine COCs were evaluated, the mean concentration for the sedimentation of Class A COCs, which have compact and dense cumulus cell layers, was found to be 21.9±1.5%. In the present study, when a similar concentration (20%) of Percoll was used for the selection of porcine COCs, the proportion of oocytes reaching MII was higher than that of the control oocytes. However, the sedimentation rate in this group was very low (Fig. 1). These results indicate that sedimentation treatment with high concentrations of Percoll decreased the total number of COCs available for IVM. Moreover, in the other groups, the proportion of oocytes that matured to MII after Percoll treatment was not higher than that of the control oocytes.

Mullen *et al*. (2007) reported that osmotic stress has a detrimental effect on the developmental competence of porcine oocytes. They suggested that when the osmolality of the solution in which the oocytes were equilibrated differed from that of an isotonic solution, an increasing proportion of oocytes displayed abnormal MII spindle morphology. It is known that exposing cells to solutions containing high concentrations of solutes can be damaging because of the resulting osmotic effects. Moreover, the osmolality of the culture medium has been shown to have a critical effect on the meiosis of porcine oocytes (Yamauchi *et al*., 1999). In the present study, we did not examine the osmolality of each Percoll solution used for the sedimentation treatment. Percoll sedimentation treatment might have a negative effect on the meiotic competence of oocytes due to the osmotic stress resulting from exposure to Percoll solution.

CONCLUSION

The selection of oocytes by using the Percoll sedimentation method does not improve the rate of nuclear maturation in porcine oocytes. Percoll sedimentation treatment is not a reliable and practical method for the selection of porcine oocytes for IVM.

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