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Procedure used for denuding pig oocytes influences oocyte damage, and development of in vitro and nuclear transfer embryos

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ABSTRACT

The effects of different denuding procedures used during the in vitro culture of porcine embryos on oocyte damage and aspects of porcine embryo development were investigated in a series of studies. Oocytes were denuded by vortexing or pipetting after 44 h in vitro maturation (IVM) or pre-denuded after 22 h IVM. The total oocyte death rate was significantly ($P < 0.05$) higher for pre-denuded (27.3 ± 1.4%) than for vortexed (20.3 ± 1.2%) or pipetted (16.2 ± 2.2%) oocytes. There was no significant difference between the treatments in the percentage of oocytes that extruded the first polar body. The type I cortical granule distribution (reflecting complete maturity) and normal spindle formation rates were significantly lower in the pre-denuding than in the vortexing and pipetting treatments. Blastocyst formation rates were significantly lower for the pre-denuding treatment in PA (25.7 ± 4.5%) and IVF (6.1 ± 1.5%) culture than in the vortexing (PA 42.0 ± 4.5%; IVF 11.2 ± 0.5%) and pipetting (PA 43.4 ± 3.1%; IVF 9.4 ± 1.6%) treatments. The proportion of oocytes developing to blastocysts in SCNT culture was not significantly different between treatments ranging from 9.9 ± 1.8% for pre-denuding to 12.3 ± 2.7% for vortexing. No significant differences in apoptosis or embryonic fragmentation were observed. This study shows that the denuding procedure used for porcine oocytes during the in vitro production of embryos can significantly affect oocyte damage, spindle patterns, oocyte maturation, embryo development but not embryonic apoptosis or the frequency of fragmentation.

1. Introduction

The in vitro production (IVP) of live transgenic animals has been successfully applied to biotechnological and medical applications (Dang-Nguyen et al., 2011; Garrels et al., 2012). The pig is regarded as the primary species for xenotransplantation into humans because their anatomical and physiological characteristics are similar (Lai et al., 2002; Ramsoondar et al., 2003; Ahn et al., 2011; Fan et al., 2013). Pig IVP embryos are developmentally inferior to in vivo produced embryos because the in vitro culture system is suboptimal (Dang-Nguyen et al., 2011; Lee et al., 2013). Previous studies have focused on enhancing the developmental potential of pig IVP embryos by improving the in vitro maturation conditions (Naruse et al., 2007a; Biswas and Hyun, 2011; Somfai et al., 2011; Kwak et al.,

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2012a; Zhang et al., 2012), the in vitro culture conditions (Huang et al., 2011; Kwak et al., 2012b; Ji et al., 2013; Lee et al., 2013), and so on.

The removal of cumulus cells from in vitro matured (IVM) cumulus oocyte complexes (COCs) is called "denuding" and is required prior to oocyte manipulation during IVP. Although simple, the denuding procedure is recognized as an essential procedure for IVP embryos. Traditionally, maturation is completed and then COCs are denuded of cumulus cells by pipetting or vortexing. However, recent reports have indicated that oocytes denuded prior to maturation (pre-denuding method) can support embryonic development following parthenogenetic activation (PA), somatic cell nuclear transfer (SCNT) or in vitro fertilization (IVF) in pig and cattle (Zhang et al., 2010; Jeon et al., 2011, 2012).

Porcine embryos are considered to be more fragile than the embryos of other domestic animals. Vigorous vortexing or pipetting can sometimes injure the oocyte or even cause it to break down, but it is not clear whether this affects developmental competence or other parameters. To the best of our knowledge, no detailed data have been published on the relationship between the denuding procedure and different characteristics of porcine embryos. Therefore, the present study was undertaken to determine the effect of three different denuding methods (vortexing, pipetting and pre-denuding, i.e., prior to the completion of maturation) on porcine oocyte damage, embryonic development, embryonic degeneration, apoptosis, cytoplasmic fragmentation, oocyte nuclear maturation, the position of the first polar body relative to the metaphase spindle, enucleation rate, spindle morphology, and cortical granule distribution.

2. Materials and methods

2.1. Chemicals

Unless otherwise indicated, all the chemicals used in our experiments were purchased from Sigma (St. Louis, MO, USA).

2.2. Cumulus oocyte complex (COC) collection and in vitro maturation (IVM)

The entire experimental procedure was approved by the Institutional Animal Care and Use Committee of Chungnam National University. Porcine ovaries were obtained from a local slaughterhouse (NH Livestock Cooperation Association, Nonsan City, Chungnam Province, Korea) where we had acquired permission to use porcine ovaries, and transported to the laboratory within 2 h in phosphate-buffered saline (PBS) solution supplemented with 75 μg/mL potassium penicillin G and 50 mg/mL streptomycin sulfate, at 35 °C. Follicles of 3–6 mm in diameter on the ovarian surface were selected, and the contents were aspirated using an 18-gauge needle attached to a 10-mL disposable syringe. Oocytes with a uniform ooplasm and compact cumulus cell mass were selected for IVM. The selected COCs were allocated at random to three denuding treatments (vortexing, pipetting and pre-denuding).

For IVM, groups of approximately 50 COCs with several layers of cumulus cells were cultured in 500 μL TCM-199 supplemented with 10% porcine follicular fluid (PFF), 10 IU/mL pregnant mare serum gonadotropin (PMSG), 10 IU/mL human chorionic gonadotropin (hCG), and 10 ng/mL epidermal growth factor (EGF) in each well of a four-well multi dish. The culture environment consisted of a saturated-humidity atmosphere of 5% CO_2 in air at 38.5 °C. After 22 h of culture, the oocytes were transferred to fresh TCM-199 plus 10% PFF and 10 ng/mL EGF, and cultured under the same conditions for an additional 22 h.

2.3. Denuding methods

Cumulus cells were removed from the COCs by three different denuding methods. For the vortexing method, COCs were cultured for 44 h, transferred into denuding medium [TL-HEPES supplemented with 0.1% (w/v) PVA (polyvinyl alcohol) and 0.3% (w/v) hyaluronidase] in a 1.5-mL centrifuge tube, and vortexed for 3–4 min at 1500–1700 rpm. For the pipetting method, the COCs were cultured for 44 h, and then gently dispersed with a 200-μL disposable pipette tip attached to a corresponding pipettor. For the pre-denuding method, COCs were matured for 22 h and vortexed in denuding medium, and then the denuded oocytes were cultured in IVM medium for an additional 22 h.

2.4. PA and embryonic culture

Oocytes were washed three to five times with an activation solution containing 0.3 M D-mannitol, 0.1 mM $MgSO_4$, 0.05 mM $CaCl_2$, and 0.01% PVA. Activation was induced with a direct current-pulse of 1.5 kV/cm for 100 μs using an Electro Cell Manipulator 2001 (BTX, San Diego, CA, USA). After activation, oocytes were immediately transferred into PZM-3 containing 3 mg/mL bovine serum albumin (BSA) and 7.5 μg/mL cytochalasin B for 6 h. The embryos were washed three times in PZM-3 plus BSA culture medium, and transferred into 500 μL of the same culture medium in a four-well multi dish, covered with mineral oil, and incubated at 38.5 °C for 7 days in a 5% CO_2 atmosphere. The day of PA was designated as day 1; cleavage and blastocyst formation were assessed at days 3 and 7 after activation, respectively.

2.5. IVF and embryo culture

IVF was carried out as described previously (Lin et al., 2013). Briefly, oocytes were washed three times in mTBM (modified Tris-buffered medium containing 113.1 mM NaCl, 3 mM KCl, 7.5 mM $CaCl_2$, 11 mM glucose, 20 mM Tris, 2 mM caffeine, 5 mM sodium pyruvate, and 2 mg/mL BSA). After washing, groups of 10–12 oocytes were transferred into 45-μL droplets of mTBM and covered with warm mineral oil in a 35 mm × 10 mm Petri dish. A fresh semen sample was obtained each week from the Darby Pig Artificial Insemination Center (Yeongi-gun, Korea). Each semen sample was washed three times by centrifugation at 2000 × *g* for 3 min in Dulbecco's phosphate buffered saline (DPBS) supplemented with 0.1% BSA. After washing, the