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Effect of removing cumulus cells from porcine cumulus-oocyte complexes derived from small and medium follicles during IVM on the apoptotic status and meiotic progression of the oocytes

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ABSTRACT

The present study was undertaken to examine the apoptotic status and meiotic progression of oocytes from small follicle (SF; 0.5–2 mm in diameter) and medium follicle (MF; 3–6 mm in diameter) when the oocytes were denuded before, during, and after IVM. Cumulus-oocyte complexes (COCs) were collected from SF or MF of prepubertal gilt ovaries. Before or 20 hours after the start of IVM culture, some oocytes were denuded and cultured for IVM. At the end of IVM, apoptotic status and meiotic progression of the oocytes were compared with oocytes matured in the presence of cumulus cells (CCs) by Annexin-V/PI assay and 4′,6-Diamidino-2-phenylindole staining. Apoptotic status of the oocytes was only affected by time when the oocytes were denuded. In both oocytes from SF and MF, although the incidence of early and late apoptotic oocytes was significantly higher when the CCs were removed before IVM, the rate was significantly lower when CCs were removed 20 and 44 hours after the start of IVM. The incidence of mature oocytes was significantly affected by both the origin of COCs and time when oocytes were denuded from the COCs. Although the percentage of mature oocytes was higher in MF than SF, maturation rates were significantly higher when oocytes were denuded 20 hours, as compared with 0 and 44 hours after the start of IVM. However, the percentage of mature oocytes with a morphologically normal spindle was significantly higher when oocytes were denuded 44 hours, rather than 22 hours of IVM. In conclusion, removing CCs 20 hours after the start of IVM seems to promote meiotic progression of the oocytes to the metaphase-II stage even when the COCs were collected from SF, although factor(s) from or communication with CCs during IVM may need to obtain a morphologically normal spindle in mature oocytes.

1. Introduction

Usually pigs for pork production have been slaughtered before the onset of puberty in developed countries. The ovaries are a common resource of follicles and oocytes for their use in reproductive biotechnologies [1,2], and many studies have shown that oocytes in the cumulus-oocyte complexes (COCs) obtained from prepubertal gilts have significantly lower meiotic and cytoplasmic competence than those from cycling sows. For *in vitro* embryo production, routinely COCs from medium follicles (MF) with 3 to 6 mm in diameter of prepubertal gilt's ovaries are used because oocytes from MF have a relatively higher rate of meiotic competence as compared with those from small

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follicles (SF; with <2 mm in diameter), though the number of MF in the ovary is significantly lower than that of SF [3–6].

The oocytes recovered from antral follicles are surrounded by multiple layers of cumulus cells (CCs), which are in deep communication with the oocyte via cytoplasmic projections that go through the zona pellucida and contact with the oolema via gap junctions forming a structure known as COC [7]. It has been well-known that COCs not only secrete paracrine factors but they also interact through the gap junctions (passing molecules only smaller than 1 kDa, [8]) in a bidirectional manner, which is essential for oocyte nutrition, growth, and regulation of meiotic progression [9–11]. Also, CCs contribute partially to maintain the oocyte into meiotic arrest by the production and transfer of cAMP via gap junctions, which accumulates into the ooplasm [12]. The communication through the gap junctions between the oocyte and CCs decreases progressively during IVM [13,14]. This disconnection between the cells seems to be responsible for meiotic resumption in fully grown oocytes, due to a reduction in the intracellular cAMP level [15,16], which will activate CDC2/CDK1 and MAP kinases [17,18], and consequently, permit the oocytes to achieve maturation to the metaphase-II stage *in vitro* [19,20] and *in vivo* [16]. In the pig, this communication between the oocyte and CCs during the first 4 hours of IVM appears to be most important when the COCs were collected from MF [21].

However, there are no studies about if removing CCs or breaking off the intercellular communications in COCs affects the nuclear maturation of oocytes derived from SF. Therefore, the aims of the present study were to compare the viability and meiotic ability of porcine oocytes derived from SF and denuded during IVM with those derived from MF.

2. Materials and methods

2.1. Chemicals and culture media

The reagents NaCl, NaOH, $MgCl_2 \cdot 6H_2O$, $CaCl_2 \cdot 2H_2O$, KH_2PO_4, gentamicin sulfate, and paraffin liquid were purchased from Nacalai Tesque Inc. (Kyoto, Japan). Potassium chloride, $NaH_2PO_4 \cdot 2H_2O$, and $MgSO_4 \cdot 7H_2O$ were purchased from Ishizu Pharmaceutical Co., Ltd. (Osaka, Japan). Equine chorionic gonadotropin (eCG; Serotropin) and human chorionic gonadotropin (hCG; Gonatropin) were purchased from ASKA Pharmaceutical Co., Ltd. (Tokyo, Japan). Unless specified, all the other reagents were obtained from Sigma–Aldrich Japan K. K. (Tokyo, Japan).

The medium used for the collection and the washing of the COCs was modified TL-HEPES-PVA medium composed of 114-mM NaCl, 3.2-mM KCl, 2.0-mM $NaHCO_3$, 0.34-mM KH_2PO_4, 10.0-mM sodium lactate, 0.5-mM $MgCl_2 \cdot 6H_2O$, 2.0-mM $CaCl_2 \cdot 2H_2O$, 10.0-mM HEPES, 0.2-mM sodium pyruvate, 12.0-mM sorbitol, 0.1% (w:v) polyvinylalcohol, 25-μg/mL gentamycin, and 65-μg/mL potassium penicillin G [22]. The medium used for IVM culture was a BSA-free chemically defined medium, porcine oocyte medium (Research Institute for the Functional Peptides, Yamagata, Japan), supplemented with 50-μM β-mercaptoethanol (mPOM) [23]. This IVM medium has been shown to support successful development to the blastocyst stage after IVF [24] and piglet production [23]. The TL-HEPES-PVA medium was kept at room temperature for its utilization, whereas the mPOM medium was equilibrated under paraffin liquid at 39 °C in a wet atmosphere of 5% CO_2 in air overnight before use.

2.2. Preparation of COCs

Ovaries without any evidence of corpora lutea were collected at a local abattoir and placed in a 0.9% (w:v) NaCl solution supplemented with 75 mg/L of potassium penicillin G and 50 mg/L of streptomycin sulfate. They were transported to the laboratory within 2 hours at 25 °C. Cumulus-oocyte complexes were aspirated from SF (0.5–2 mm in diameter) and MF (3–6 mm in diameter) located on the ovarian surface using a disposable 10-mL syringe with an 18-gauge needle and collected into a 50-mL centrifuge tube. The pooled follicular contents were washed three times with modified TL-HEPES-PVA medium at room temperature to remove any traces of follicular fluid and thus any endogenous gonadotropic activity and growth factor activity. Thereafter, only COCs from SF and MF were selected if they had a uniform ooplasm and a compact cumulus cell mass of at least three layers of CCs.

2.3. In vitro maturation and decumulation of the oocytes

Cumulus-oocyte complexes derived from SF and MF were randomly distributed into groups (30–35 COCs per group). One group from each, SF and MF, was used to analyze the apoptotic status and nuclear stage of the oocytes before IVM. The oocytes were denuded with TL-HEPES-PVA containing a 0.1% (w:v) hyaluronidase by using a glass narrow-bore pipette, washed 3 times with TL-HEPES-PVA, and then used for fluorescence assays.

The remaining groups of COCs from SF and MF were cultured separately in 300 μL of mPOM supplemented with 1-mM dibutyryl cyclic AMP (dbcAMP), 10-IU/mL eCG, and 10-IU/mL hCG for 20 hours at 39 °C in an atmosphere of 5% CO_2 in air. The COCs were then washed 3 times in equilibrated mPOM without dibutyryl cyclic AMP and gonadotropins, transferred to 300 μL of the same medium and continued culture for an additional 24 hours at 39 °C in an atmosphere of 5% CO_2 in air. Before (0 hours) and after the start 20 hours of IVM, the oocytes from SF and MF COCs were denuded as previously described and continued the IVM culture after washing three times with mPOM. At the end of IVM culture (totally 44 hours from the start), the remaining oocytes were also denuded, washed three times, and then used for the fluorescence assays.

2.4. Fluorescence assays for the apoptotic status, viability, and meiotic progression of oocytes

Denuded oocytes were processed for an apoptotic and/or viability assay before (0 hours) and after IVM for totally 44 hours of culture, according to the manufacturer's instructions by using the Alexa Fluor 488 Annexin V/Dead Cell Apoptosis Kit (Molecular Probes, Eugene, USA). Briefly,