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Effects of coculture with cumulus-derived somatic cells on *in vitro* maturation of porcine oocytes

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ABSTRACT

In the process of IVM, cumulus-oocyte complexes (COCs) separate from the follicular microenvironment, leading to the loss of endocrine interactions between follicular mural somatic cells and COCs. To restore the microenvironment, a coculture system was established using cumulus-derived somatic cells (CSCs) for IVM. The CSCs were cultured in Dulbecco's modified Eagle's medium for 48 hours with varying numbers of CSCs (0.0, 2.5×10^4, 5.0×10^4, and 10.0×10^4) and then cultured in tissue culture medium 199 (TCM 199) for 4 hours before adding the oocytes. Cumulus-oocyte complexes from 3- to 6-mm follicles were matured in 500 μL of TCM 199 with eCG and hCG for 22 hours and then cultured in TCM 199 without hormones for 22 hours. After IVM, the group with 2.5×10^4 CSCs showed a significant increase in intracellular glutathione levels compared with the control group. In the evaluation of sperm penetration, efficient fertilization was increased in the groups with 2.5×10^4 and 5.0×10^4 CSCs compared with controls (44.9 and 46.5 vs. 32.1, respectively). The mRNA expression pattern analysis in matured COCs showed a significant upregulation of *PCNA, COX-2, Has2, Ptx3,* and *Nrf2* in the 2.5×10^4 CSC group compared with controls. During COC maturation at 0, 11, 22, 33, and 44 hours, the 2.5×10^4 and 5.0×10^4 CSC groups showed a significantly altered mRNA expression of *BMP15* and *GDF9*. The developmental competence of the matured oocytes in all groups was evaluated after IVF and parthenogenetic activation (PA). After IVF, the 2.5×10^4 CSC group showed significantly higher cleavage, blastocyst formation rate, and total cell numbers compared with controls (60.0%, 35.7%, and 127.3 vs. 43.2%, 21.1%, and 89.3, respectively). After PA, the 2.5×10^4 CSC group had significantly higher blastocyst formation rate and total cell number than the control group (52.0% and 120.4 vs. 35.4% and 90.9, respectively). In conclusion, these results suggest that the presence of a population of 2.5×10^4 CSCs during IVM synergistically improved the developmental potential of IVF- and PA-derived porcine embryos by increasing the intracellular glutathione level *via* changing of a specific gene expression pattern during oocyte maturation.

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1. Introduction

Various transgenic animals have been generated through assisted reproductive technologies, including IVM and IVF, using gametes [1–3]. In particular, pigs have been used to develop some of the most important large animal models for biomedical research [4–6]. For translational

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research, the development of IVM, which can be attributed to improved culture conditions for the embryo, has improved fertilization efficiency in transgenic pigs. Nonetheless, compared with oogenesis *in vivo*, IVM conditions are still suboptimal for oocyte maturation, because numerous factors, including maturation time, temperature, and culture medium composition, affect oocyte maturation *in vitro* [7]. Therefore, a greater understanding of the *in vivo* environment will allow the modifications of culture conditions necessary to improve the developmental potential of oocytes matured *in vitro* [8].

The successful development of *in vivo* follicles requires bidirectional communication between oocytes and follicular somatic cells, such as granulosa cells and theca cells [9,10]. During the progression of follicular growth, the expressions of bone morphogenetic protein 15 (BMP15) and growth differentiation factor 9 (GDF9) have been demonstrated to be positive regulatory paracrine factors in a variety of species, including rodents, humans, and ruminants [11–13]. Oocyte-secreted *GDF9* and *BMP15* regulate the oocyte microenvironment, for instance, by promoting granulosa cell proliferation and modulating FSH-dependent follicular function [14]. In response to *GDF9* and *BMP15*, follicular somatic cells secrete their own paracrine factors, such as anti-Mullerian hormone, inhibin, activin, and BMP2, -5, -6, which affect oocyte intracellular maturation [15]. However, because cumulus-oocyte complexes (COCs) separate from the follicular microenvironment for maturation during typical IVM, they are not able to respond to factors secreted by follicular somatic cells.

From the time of first trial of porcine IVM, to improve the IVM environment, the coculture system has been modified to contain diverse types of somatic cells, such as granulosa cells, theca cells, sections of the follicle wall, oviductal epithelial cells, and cumulus clumps, from various mammalian species [16–19]. In one case, denuded buffalo oocytes were embedded in cumulus cell clumps to restore the three-dimensional environment and allow maturation. The embedded denuded oocytes had significantly higher maturation rates and blastocyst formation after parthenogenetic activation (PA) [17]. Analogously, ovine oocytes cocultured with somatic cells of a cumulus origin had altered expression levels of the Transforming Growth Factor-beta (TGF-β) ligand and receptor and higher maturation rates than controls [20]. In porcine models, prematured oocytes cocultured with oviductal epithelial cell monolayers during IVM resulted in increased oocyte cytoplasmic maturation and blastocyst development [19]. Another study revealed that cocultures with ovarian cortex cell monolayers during porcine oocyte maturation enhanced the maturation quality and rate of porcine oocytes as well as the blastocyst formation rate on subsequent embryonic development after IVF [21].

To restore the follicular microenvironment, many studies have applied coculture systems using various cell types during porcine IVM [19,21–24]. However, until now, no information has been available on the effect of cumulus-derived somatic cells (CSCs) on porcine oocyte maturation, and the concentration of feeder cells has not yet been determined. In this study, the effects of coculture with CSCs were investigated on porcine oocyte IVM and subsequent embryonic development after IVF and PA. Oocyte nuclear maturation, intracellular levels of glutathione (GSH) and reactive oxygen species (ROS), embryonic cleavage, blastocyst formation, and blastocyst cell numbers were analyzed. In addition, the expression patterns of *GDF9* and *BMP15* during oocyte maturation and embryonic developmental marker and apoptosis-related genes, proliferating cell nuclear antigen *(PCNA), POU domain, class 5, transcription factor 1 (POU5F1), Bcl-2-associated X protein (Bax), Bcl-2 homologous antagonist/killer (Bak), caspase-3 (cas3)*, and B-cell lymphoma 2 (*Bcl-2)*, in IVF- and PA-derived blastocysts were measured.

2. Materials and methods

2.1. Chemicals

Unless otherwise indicated, all chemicals and reagents used in the present study were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA).

2.2. Oocyte collection and IVM

Porcine ovaries (mixed Yorkshire, Landrace, and Duroc breeds, predominantly from 6-month-old gilts) were collected from a local abattoir and transported to the laboratory in physiological saline supplemented with 100 IU/L of penicillin G and 100 mg/mL of streptomycin sulfate at 32 °C to 39 °C within 2 hours. The ovaries were washed twice with physiological saline. The COCs were aspirated from follicles 3 to 6 mm in size using an 18-gauge needle attached to a 10 mL syringe, collected in 15 mL conical centrifuge tubes, and allowed to settle to the bottom of the tube. After 10 minutes, the supernatant was removed, a conical tube was filled with HEPES-buffered Tyrode's medium containing 0.05% (wt/vol) polyvinyl alcohol (TLH-PVA), and the COCs were observed under a stereomicroscope. More than three layers of COCs comprising compact cumulus cells and homogenous cytoplasm were selected from the collected fluid for IVM and washed three times in TLH-PVA. The 50 to 60 randomly selected COCs were transferred into a four-well dish (Nunc, Roskilde, Denmark) containing 500 μL of maturation medium (tissue culture medium 199; Invitrogen Corporation, Carlsbad, CA, USA) supplemented with 0.6 mM of cysteine, 0.91 mM of sodium pyruvate, 10 ng/mL of epidermal growth factor, 75 μg/mL of kanamycin, 1 μg/mL of insulin, and 10% (v:v) porcine follicular fluid. The porcine follicular fluid was extracted from 3- to 6-mm follicles of prepubertal gilt ovaries, prepared according to Hyun et al. [25] and stored at −70 °C until used. The transferred COCs were cultured with 10 IU/mL of eCG and 10 IU/mL of hCG for 22 hours at 39 °C in a humidified atmosphere of 5% CO_2 and 95% air. After 22 hours of maturation with the hormones, the COCs were washed twice in a hormone-free maturation medium and subsequently cultured in that medium for an additional 20 hours. During IVM, COCs were incubated with varying CSC numbers of 0, 2.5×10^4, 5.0×10^4, and 10.0×10^4 CSCs.

2.3. Isolation of CSCs

The isolation of CSCs for coculture with COCs was performed according to Kyasari et al. [20] with a few

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