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Supplement of cilostamide in growth medium improves oocyte maturation and developmental competence of embryos derived from small antral follicles in pigs

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ABSTRACT

This study was conducted to evaluate the effects of cyclic AMP (cAMP) modulator cilostamide (CIL) and forskolin (FSK) treatment during *in vitro* growth (IVG) on growth, maturation, and embryonic development of cumulus-oocyte complexes (COCs) derived from small antral follicles < 3 mm in diameter (SAFCOCs). SAFCOCs were untreated (control) or treated with 20 μM CIL and/or 50 μM FSK for 2 days for IVG. Next, IVG oocytes were cultured for maturation and then induced for parthenogenesis (PA) or used as recipient ooplasts for somatic cell nuclear transfer (SCNT). Nuclear maturation of oocytes was significantly lower in the control (49.6 ± 9.3%) group than in other groups (67.2 ± 5.0–79.8 ± 7.9%). The cumulus expansion score after IVG-IVM was significantly higher in the control and CIL group than in the FSK and CIL + FSK groups. CIL significantly increased mean diameter SAF-derived oocytes (120.0 ± 0.5 μm) compared to the control, FSK, and CIL + FSK (114.8 ± 0.5–116.7 ± 0.6 μm) and showed a comparable level of intracellular glutathione (GSH) contents (0.84 ± 0.07 pixels/oocyte) to medium antral follicle (MAF)-derived oocytes (1.00 ± 0.08 pixels/oocyte), but was higher than those of oocytes treated with FSK and CIL + FSK (0.29 ± 0.05 and 0.37 ± 0.05 pixels/oocyte, respectively). CIL treatment significantly increased blastocyst formation (55.1 ± 4.7%) after PA relative to the control (29.4 ± 6.4%), FSK (34.8 ± 7.1%), and CIL + FSK (41.1 ± 5.2%). A higher proportion of oocytes treated with CIL, FSK, and CIL + FSK (73.3 ± 1.7–82.8 ± 9.1%) remained at the germinal vesicle stage after IVG culture than control oocytes (40.0 ± 5.0%). Following SCNT, blastocyst formation of SAFCOCs treated with CIL (22.4 ± 6.3%) was higher than that of oocytes (0–10.4 ± 5.3%) in control, FSK, and CIL + FSK, but similar to that (25.3 ± 3.5%) of MAF-derived COCs not cultured for IVG. The cAMP level of SAFCOCs before IVG was 0.1 ± 0.03 fmol/oocyte. After 2 days of IVG culture, cAMP level was increased significantly by treatment with FSK and CIL + FSK (3.0 ± 0.57 and 12.1 ± 0.62 fmol/oocyte, respectively) relative to the control and CIL treatment (0.1 ± 0.03 and 0.3 ± 0.04 fmol/oocyte, respectively). Our results demonstrate that CIL treatment during IVG improves the low developmental competence of SAFCOCs to levels comparable to MAFCOCs by allowing oocyte growth while inhibiting premature meiotic maturation, probably via maintenance of cAMP concentrations at appropriate levels.

1. Introduction

Pigs have long been considered a useful animal model for human diseases because their organ features are of similar size and physiology to those of humans. Currently, somatic cell nuclear

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transfer (SCNT) technique using *in vitro*-matured oocytes and somatic cells is commonly being used to produce transgenic pigs with specific purposes such as bio-organ donors for xenotransplantation and animal diseases models [1]. Although great advances have been achieved in the field of assisted reproductive technologies, including *in vitro* maturation (IVM) and SCNT in pigs, *in vitro*-produced (IVP) oocytes and embryos still have lower developmental competence than their *in vivo* counterparts [2]. Thus, improving the developmental competence of IVP oocytes and embryos is prerequisite to increasing the efficiency of piglet production using these techniques.

Generally, oocytes are collected from slaughtered ovaries and used for further purposes such as IVP of embryos following *in vitro* fertilization (IVF) and SCNT in livestock. In the pig ovary, there are approximately 450 growing follicles of various sizes, with about 85 follicles 1–8 mm in diameter present and visible on the ovarian surface [3]. In another study, the proportion of medium antral follicles (MAFs) 3–8 mm in diameter was approximately 38%, while other small antral follicles (SAFs) with a diameter of less than 3 mm comprise 62% of visible follicles on the slaughtered ovaries of prepubertal gilts [4]. It is well known that *in vitro* maturational and developmental competence of oocytes is associated with various morphological characteristics such as size of follicles and thickness of cumulus cell layer [5–7]. Cumulus-oocyte-complexes (COCs) can be retrieved from both SAFs and MAFs, but it is common to use COCs from MAFs (MAFCOCs) for IVM and IVP of pig embryos because COCs derived from SAFs (SAFCOCs) show lower maturational and developmental competence than those from MAFs [4,8,9]. As a result, more than half of total oocytes from antral follicles are discarded because of their low developmental competence, which in turn results in valuable genetic materials being wasted. Thus, if it is possible to establish an *in vitro* growth (IVG)-IVM system to produce high-quality oocytes from SAFCOCs, more oocytes can be produced and used for production of animals using various reproductive technologies.

During the *in vivo* maturation process, oocytes grow along with follicular growth and maintain open gap junctional communication (GJC) until the pre-ovulatory surge of luteinizing hormone occurs. In addition, nuclear maturation of immature oocytes is delayed during the growth phase, which is regulated by follicular environments such as changes in the cyclic adenosine monophosphate (cAMP) level and follicular cell metabolism. In contrast, once oocytes are removed from follicles *in vitro* and exposed to hormones in IVM medium, GJC is closed and meiotic resumption occurs. Conversely, oocytes in growing SAFs are smaller in diameter and have thinner cumulus cell layers than those from MAFs. The low developmental competence of SAFCOCs may be attributed to the precocious initiation of meiotic resumption without accompanying sufficient cytoplasmic maturation after full growth during the maturation process [10]. To date, various studies to improve low developmental competence of SAFCOCs have been conducted in mice, cattle, and pigs [11–13]. For pigs, it has been reported that 5-day culture of SAFCOCs in an IVG medium supplemented with dibutyryl cAMP (dbcAMP) and follicle stimulating hormone (FSH) induces oocyte growth while inhibiting meiotic resumption and improving developmental competence of parthenogenesis (PA) embryos [14]. In cows, IVG culture of SAFCOCs with low competence for 14 days in medium containing hypoxanthine increased developmental capacity of IVF embryos, and calves were produced after transfer of IVF embryos to recipient cows [13]. In pigs, Wu et al. [15] reported that an oocyte growth-maturation system could facilitate the final stage of oocyte growth, which resulted in better nuclear and cytoplasmic maturation of SAFCOCs than conventional IVM. Despite many studies being conducted, oocytes and embryos derived from SAFs still show lower developmental competence than those derived from MAFs; accordingly, it is imperative to establish an efficient IVG-IVM system to produce mature oocytes with high developmental capacity from SAFCOCs in pigs.

Cyclic AMP is a key molecule regulating meiotic resumption of mammalian oocytes [16,17]. A high level of cAMP prevents immature oocytes from initiating their meiotic resumption until oocyte growth is completed [18,19]. Cilostamide (CIL) is a phosphodiesterase type3 (PDE3) inhibitor that maintains or increases the cAMP level within oocytes by inhibiting the hydrolysis of cAMP [20]. Forskolin (FSK) activates adenylyl cyclase and increases cAMP levels in oocytes by stimulating cAMP synthesis [17,18]. It has been reported that SAFCOCs show lower competence of accumulating cAMP and thus the cAMP level in SAFCOCs is lower than that in MAFCOCs [21]. Therefore, it is considered that maintaining cAMP level at an appropriate level to induce meiotic arrest is required for allowing IVG of SAFCOCs while premature meiotic resumption is prevented when they are cultured *in vitro*. In this study, we investigated whether IVG culture using cAMP modulators to allow oocyte growth but arrest meiotic progression at the germinal vesicle (GV) stage before IVM would increase cytoplasmic maturation of oocytes and then improve developmental competence of pig embryos derived from SAFs. To accomplish this, SAFCOCs were untreated or treated with 20 μM CIL and/or 50 μM FSK for 2 days before IVM. After IVG and IVM, oocyte growth, nuclear maturation, intracellular cAMP and glutathione (GSH) contents were evaluated, as was the developmental competence of PA and SCNT embryos. We found that CIL treatment during IVG improved the low developmental competence of SAFCOCs to levels comparable to MAFCOCs by allowing oocyte growth while inhibiting premature meiotic maturation via maintenance of intraoocyte cAMP concentration at an appropriate level.

2. Materials and methods

2.1. Oocyte collection and IVG of SAFCOCs

All reagents used in this study were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise noted. Ovaries from prepubertal gilts were obtained at a local abattoir. COCs were aspirated from MAFs and SAFs. Follicular contents of MAFs and SAFs were aspirated and put into a 15-mL centrifuge tube for 10 min. COCs with unexpanded cumulus cells (Fig. 1A and D) were selected and washed in HEPES-buffered Tyrode's medium (TLH) containing 0.05% (w/v) polyvinyl alcohol (PVA) (TLH-PVA).

The SAFCOCs were cultured for 0 or 2 days in 500 μL IVG medium in a 4-well multidish (Nunc, Roskilde, Denmark) at 39 °C under a humidified atmosphere of 5% CO_2 and 95% air. The base medium for IVG culture for SAFCOCs was Minimum Essential Medium alpha medium (α-MEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% (v/v) fetal bovine serum (FBS) (Hyclone, Thermo, UT, USA), 0.91 mM pyruvate, 75 μg/mL kanamycin, and 8 μg/mL FSH (Antrin R-10; Kyoritsu Seiyaku, Tokyo, Japan). To elucidate the effects of CIL and/or FSK, the base medium was supplemented with none (control), 20 μM CIL (BML-PD125; Enzo Life Science, Farmingdale, NY, USA) and/or 50 μM FSK (BML-CN100; Enzo Life Science). The concentrations of CIL and FSK to maintain cAMP level and inhibit precocious meiotic resumption in this study was chosen from the previous results obtained in pigs [22,23]. CIL and FSK were dissolved in dimethyl sulfoxide at 74.0 mM and 12.2 mM, respectively, stored at −20 °C, and diluted into IVG medium before use. An equal amount of carrier was added to the control medium.

2.2. IVM of SAFCOCs and MAFCOCs

The COCs were placed into each well of a 4-well multidish