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Effects of cilostamide and/or forskolin on the meiotic resumption and development competence of growing ovine oocytes selected by brilliant cresyl blue staining

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ABSTRACT

The relevance of low developmental competence of *in vitro*-matured oocyte to the incomplete/delayed cytoplasmic maturation, and the heterogeneity of retrieved oocytes is well established in several species. A short phase of prematuration culture was used to allow better oocyte cytoplasmic maturation. The preselection of growing and fully grown oocytes has been proposed to improve developmental competency. This study investigated the effects of phosphodiesterase type 3–specific inhibitor, cilostamide, and adenylate cyclase activator, forskolin, on the resumption of meiosis and developmental competence of growing ovine oocytes selected by brilliant cresyl blue (BCB) staining. Results indicate that cilostamide, forskolin, and their combination significantly ($P < 0.05$) increased the percentage of growing (BCB−) oocytes maintained at the germinal vesicle stage. However, only forskolin significantly ($P < 0.05$) increased the yield and quality of blastocysts derived from BCB− oocytes compared with non–BCB-treated oocytes. We conclude that a short prematuration culture with forskolin may improve the *in vitro* developmental competency of growing oocytes in ovine.

1. Introduction

Oocyte maturation is a well-orchestrated process that results in the ovulation of one or a few oocytes (depending on species) from a cohort of developing oocytes [1]. A promising technique for infertility treatment or fertility preservation is IVM of immature oocytes obtained from small antral follicles [2–4]. However, developmental competence of *in vitro*–matured oocytes is lower than *in vivo*–matured oocytes [5]. Therefore, the provision of a cohort population of competent oocytes is a major challenge in the field of *in vitro* production (IVP) [5,6].

There is good evidence that the low developmental competence of *in vitro*–matured oocytes is related firstly to incomplete/delayed cytoplasmic maturation of oocytes and secondly to the heterogeneity in developmental stage of retrieved oocytes [2]. It takes several days for a small antral follicle to progress to the preovulatory stage. During this long-growth phase, cytoplasmic maturation of antral follicle proceeds while the nucleus is arrested at the germinal vesicle (GV) stage [7–9]. However, antral follicles retrieved for IVM have a substantially shorter time for cytoplasmic maturation, more importantly, oocyte retrieval triggers meiosis resumption due to the lack of inhibitory action of follicular fluid and granulosa cells to maintain the level of

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cAMP in the oocyte [10,11]. Retrieved oocytes for IVM are from donors with different reproductive performance and estrus cycle stages [12]. Even, antral follicles of a single donor encompassed oocytes that are at two distinct developmental phases, namely fully grown and growing. Therefore, it would be desirable to establish a novel method to select a more homogenous group of oocytes with high developmental competence.

cAMP is well recognized as a major regulator of the meiotic arrest in mammalian oocytes [13]. A high intra-oocyte level of cAMP is essential for meiosis arrest as any decrease in intraoocyte content of cAMP can induce meiotic resumption [14]. The compounds that increase intracellular levels of cAMP by either inhibiting phosphodiesterase such as cilostamide [15–17], or activating the adenylate cyclase such as forskolin have been used to inhibit or attenuate spontaneous meiotic maturation in several species [18–27].

Mangia and Epstein [28] first found that growing and fully grown oocytes encompass higher and lower activities of glucose-6-phosphate dehydrogenase, respectively. Notably, Palmer and Jackson [29] showed that the nicotinamide adenine dinucleotide phosphate–oxidase generated by glucose-6-phosphate dehydrogenase can reduce brilliant cresyl blue (BCB) stain, a phenoxazine compound and transform it to a colorless compound (leuco-phenoxazine). Therefore, BCB staining has been widely used to select competent oocytes in goats, pigs, cattle, sheep, and mice [30–36]. Most studies have shown that the embryos derived from BCB+ cumulus–oocyte complexes (COCs) had higher developmental competency than embryos derived from BCB− COCs [37–43].

Despite wide application of cAMP-elevating agents and BCB test to improve IVM efficiency, the combined effect of both approaches has not been evaluated so far. Moreover, in ungulates, unlike in human and mice, exposure of COCs to these compounds during a short phase before IVM called prematuration culture (PMC) has resulted in partial improvement of meiotic arrest, nuclear and cytoplasmic synchronization, and subsequent embryo development both in terms of stage and quality [23,25,27]. Therefore, in an attempt to extend oocyte cytoplasmic maturation *in vitro*, cilostamide and/or forskolin were used to extend PMC of growing ovine oocytes, selected by BCB test, to improve synchronization between nuclear and cytoplasmic maturation.

2. Materials and methods

2.1. Preparation and in vitro maturation of cumulus–oocyte complexes

The procedure of COCs preparation and IVM was as described elsewhere [44]. In brief, ovine ovaries were collected from the slaughterhouse and were transported in saline (28 °C–32 °C) to the laboratory [45]. Cumulus–oocyte complexes were retrieved from 2 to 6-mm antral follicles into HEPES-buffered tissue culture medium-199 (HTCM-199) supplemented with 10% fetal bovine serum (FBS), 50 IU/mL of heparin, 100 IU/mL of penicillin, 100 μg/mL of streptomycin (control) or cilostamide and/or forskolin (1 μM, 50 μM, respectively). Only COCs consisting of an oocyte surrounded by more than three layers [46,47] and intact cumulus cells were selected for the experiments.

2.2. Brilliant cresyl blue staining

Cumulus–oocyte complexes were incubated with 26 μM of BCB diluted in modified Dulbecco's PBS for 45 minutes at 38.5 °C in humidified air [44]. Then, COCs were examined and sorted under a stereomicroscope according to their cytoplasm color to BCB+ (COCs with blue-colored cytoplasm) and BCB− (COCs with colorless cytoplasm). BCB+ COCs were directly used for IVM, whereas BCB− COCs were used for either PMC before IVM or direct IVM.

2.3. Prematuration culture with cilostamide and/or forskolin

The PMC medium was composed of TCM supplemented with 10% FBS and further supplemented with cilostamide (1 μM), forskolin (50 μM), or cilostamide + forskolin (1 μM, 50 μM, respectively). BCB− COCs were incubated in PMC for different durations (6, 8, 10, and 22 hours). The concentrations of cilostamide and forskolin were based on our previous study and the literature [44,48–52]. At the end of the PMC period, COCs were washed three times in HTCM and were transferred to maturation medium (MM) for 22-hour IVM in absence of cilostamide and/or forskolin. To assess the effects of these compounds on embryos' quality and development, BCB− COCs were only exposed to PMC for 6 hours.

2.4. In vitro oocyte maturation

BCB+ COCs were washed three times in HTCM and were incubated in MM composed of TCM-199 supplemented with 10% FBS, 100 ng/mL of EGF, 100 ng/mL of insulin-like growth factor, 2.5 mM of Na pyruvate, 100 IU/mL of penicillin, 100 μg/mL of streptomycin, 10 μg/mL of FSH, 10 μg/mL of LH, 1 μg/mL of estradiol-17β, and 0.1 mM of cysteamine [45] for 22 hours.

2.5. Assessment of the nuclear status

To assess the nuclear status of the oocytes, a proportion of COCs from each treatment group were treated with hyaluronidase (300 IU/mL in HTCM, 10 minutes), vortexed to remove cumulus cells, fixed for 30 minutes in 4% paraformaldehyde and subsequently stained with 10 μg/mL of Hoechst 33342. Stained oocytes were mounted on microscope slides and were viewed under a fluorescence microscope. Nuclear status was classified as GV, GV breakdown, metaphase I (MI), and metaphase II (MII) [44].

2.6. Parthenogenetic activation and embryo development

Oocytes were denuded in hyaluronidase (300 IU/mL in HTCM) and then were activated by incubation in 5 μM of ionomycin in HTCM supplemented with 1 mg/mL of BSA for 5 minutes. Then, oocytes were washed in HTCM supplemented with 30 mg/mL of BSA and were treated for 2 hours with a serine/threonine kinase inhibitor, 2 mM of 6-dimethyl aminopurine in HTCM supplemented with