Regulation of sheep oocyte maturation using cAMP modulators

Ryan D. Rose[a], Robert B. Gilchrist[a], Jennifer M. Kelly[b], Jeremy G. Thompson[a] and Melanie L. Sutton-McDowall[ac]

[a]*The Robinson Institute, Research Centre for Reproductive Health, School of Paediatrics and Reproductive Health, The University of Adelaide, Adelaide, South Australia, 5005, Australia.*

[b]*South Australian Research and Development Institute, Turretfield Research Centre, Rosedale, South Australia, 5350, Australia.*

[c]*Corresponding author: E-mail: melanie.mcdowall@adelaide.edu.au*

Telephone: + 61 8 8313 1013

Facsimile: + 61 8 8303 8177

Abstract

Physical removal of mammalian cumulus-oocyte complexes (COCs) from ovarian follicles results in spontaneous resumption of meiosis, largely due to a decrease in cAMP concentrations, causing asynchrony between cytoplasmic and nuclear maturation and decreased oocyte developmental competence. The aim of this study was to modulate cAMP concentrations within ovine COCs to delay spontaneous nuclear maturation and improve developmental competence. Abattoir-derived sheep COCs were cultured for 2 h (pre-IVM) in 100 μM forskolin (FSK) plus 500 μM 3-isobutyl-1-methylxanthine (IBMX). Pre-IVM (100 μM FSK and 500 μM IBMX) culture increased COC cAMP concentrations 10-fold compared to controls ($P<0.05$). With regards to nuclear maturation, +FSK+IBMX/+FSH+cilostamide delayed completion of meiosis (metaphase II, MII) by 3 to 4 h compared to standard IVM (FSH-stimulated induction of meiosis). In this study, pre-IVM (+FSK+IBMX) followed by IVM (+FSH+cilostamide), increased ovine COC cAMP concentrations and delayed, but did not inhibit, completion of nuclear maturation. This did not affect embryo development rates, but increased total cell number of blastocysts compared to IVM with FSH alone (103±6 vs 66±4 cells, respectively, mean ± SEM; $P<0.05$). We inferred that regulation of ovine oocyte cAMP concentrations during IVM improved embryo quality compared to embryos produced by standard IVM methods.

Keywords: Simulated Physiological Oocyte Maturation (SPOM); Sheep IVM; cAMP; Embryo development

1. Introduction

There is accumulating evidence that modulation of cAMP during oocyte in vitro maturation (IVM) may be a solution to the current discrepancy in developmental competence between oocytes produced by IVM and those matured *in-vivo* [1]. It is well established that intra-oocyte cyclic adenosine monophosphate (cAMP) has a major role in regulating mammalian oocyte maturation. A high intra-oocyte cAMP concentration is produced endogenously within the oocyte by adenylate cyclases and constitutively active G-protein-coupled receptors (GPR) [2] and is generated by cumulus cells (CC) and transported to the oocyte via gap junctions [3,4]. Cyclic guanosine monophosphate (cGMP) diffuses from granulosa cells to the oocyte and inhibits cAMP degradation by inhibiting PDE3A [5,6]. In rodents, elevated cAMP concentrations activate protein kinase-A (PKA), followed by complex inhibitory mechanisms involving CDK1 –cyclin B kinase proteins suppressing activation of maturation promoting factor (MPF) and preventing meiosis [7-9]. In vivo, the pre-ovulatory surge of LH decreases cGMP concentrations in granulosa cells as well as oocytes [5,6]; the subsequent activation of PDE3A resulted in degradation of cAMP, activation of MPF, and resumption of meiosis (review; Conti et. al. [7]). The downstream pathways through which cAMP effects meiosis are not fully characterised in larger mammals like cattle [10] or sheep. However, there are major differences among species with regards to cAMP degrading phosphodiesterase (PDE) isoforms [11,12]. Maintenance of cAMP concentrations within an oocyte is achieved through a balance of production (synthesis of cAMP with adenylate cyclases and GPR), degradation (mainly the result of PDEs), and cAMP transfer from CCs.

Discrepancies between IVM and in-vivo matured oocytes in embryo yield and pregnancy outcomes have been reported consistently across mammalian species [13-16]. The ovine oocyte is no different in this respect, with one report citing blastocyst rates of 35 and 75% for *in vitro*- and *in vivo*-matured oocytes, respectively [16]. When the oocyte is mechanically removed from the follicular environment, meiotic resumption occurs spontaneously [17]. This spontaneous resumption of meiosis is accompanied by a rapid decrease in oocyte cAMP and is associated with reduced developmental competence [18]. There is now ample evidence that culturing oocytes in an environment where meiosis is regulated (via cAMP management), either prior to or during IVM, improves developmental competence [19-23]. This can be achieved by preventing the decrease in cAMP concentrations using PDE inhibitors throughout IVM, or by increasing COC cAMP concentrations during maturation with cAMP-elevating agents, e.g. cAMP analogues, adenylate cyclase activators, or PDE inhibitors (reviewed [24]).

Our laboratory recently developed a cAMP-based modulating IVM system for mice and cattle; Simulated Physiological Oocyte Maturation (SPOM) [13] aims to improve developmental competence by attenuating oocyte spontaneous nuclear maturation upon liberating an oocyte from the follicle [25]. This is achieved by increasing cAMP concentrations using cAMP modulating compounds over two intervals: during pre-IVM to generate high COC cAMP concentrations, followed by an induced IVM phase, where re-initiation of meiosis is driven by follicle stimulating hormone (FSH) in the presence of an oocyte-specific PDE inhibitor. The premise of the SPOM system is to delay nuclear maturation by increasing cAMP concentrations in the oocyte, providing a regulated transition of the meiotic stages and more time for oocyte-cumulus cell gap junction communication (GJC), and prolonging the exchange of positive regulatory

molecules and metabolites for improved oocyte cytoplasmic maturation. High cAMP concentrations are generated by addition of an adenylate cyclase activator and maintained by addition of PDE inhibitors (specific and non-specific).

The objective of the present study was to apply the principles of the SPOM system to improve developmental competence of ovine oocytes. Cumulus-oocyte complexes were recovered from peripubertal sheep (6-12 mo), which enabled assessment of the effectiveness of SPOM in oocytes with inherently reduced developmental competence. It is well established that oocytes from prepubertal domestic species have reduced rates of development and poorer embryo quality [26,27]. Nonetheless, Juvenile In Vitro Embryo Transfer (JIVET), namely the in vitro production and subsequent transfer of embryos from prepubertal animals, is a highly attractive means of rapidly multiplying elite animals for genetic improvement [27,28]. As with cattle, exploitation of superior ovine genetics is desirable, but is currently limited due to the poor cost-benefit ratio. Therefore, any advancement in enabling technologies that benefit seed-stock producers would be desirable. In particular, the benefit in developing effective breeding strategies from immature ewe-lambs would enhance the feasibility of applying this technology.

2. Materials and methods

Unless otherwise specified, all chemicals used in the following protocols were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.1. Collection, preparation and culture of oocytes

Ovaries from peripubertal sheep (approximately 6 to 12 mo old), were obtained from an abattoir and transported in warm (29 to 32 °C) saline within 3 to 4 h after slaughter. Antral follicles > 3 mm in diameter were aspirated using a 20-gauge needle and constant suction (1 L/min) into vacutainer tubes containing 2.5 mL of aspiration medium (VitroCollect V1, IVF Vet Solutions, Adelaide, Australia; + 0.2 mg/mL BSA, ICPbio Ltd, Auckland, New Zealand; + 500 μM 3-Isobutyl-1-methylxanthine (IBMX, a non-specific PDE inhibitor) + 50 mIU/mL heparin). Within 15 min after aspiration, the cellular sediment was transferred into collection media (see experimental design). The COCs with more than three cumulus cell layers and a uniform ooplasm were washed once in collection medium and once in corresponding maturation medium. The base maturation medium was VitroMat V1 (IVF Vet Solutions) + 4 mg/mL low fatty acid BSA + 100 mIU/mL recombinant human FSH (Puregon, Organon, Oss, Netherlands). Groups of up to 10 COCs were transferred into pre-equilibrated 100 μL drops of maturation medium overlaid with mineral oil and incubated at 38.5 °C with 6% CO_2 humidified air. Pre-IVM culture occurred at -2 to 0 h (0 h was the onset of IVM culture).

2.2. Oocyte meiotic assessment

At the end of defined periods of oocyte maturation, COCs were mechanically denuded by repeated pipetting and then fixed by placement in 4% paraformaldehyde for > 30 min at 4 °C. Thereafter, fixed oocytes were incubated in permeabalization solution (0.5% triton 100X and 0.5% sodium citrate) for 15 to 30 min, followed by incubation in 3 μM 4',6-diamidino-2-phenylindole (DAPI) solution in the dark for 15 min. Oocytes were washed in 0.01% (w/v) BSA in PBS, and mounted on a slide with

glycerol and antifade (Prolong, Invitrogen, Carlsbad, CA, USA) in a 3:1 ratio. Chromosome configurations were assessed using a Nikon Eclipse TE2000-E Microscope (330 to 380 nm excitation wavelength. Nikon, Melville, NY, USA).

2.3. cAMP assay

Concentrations of cAMP were measured with a validated radioimmunoassay [29]. Groups of 10 COCs were collected in minimal media, snap frozen in liquid N_2, and stored at -80 °C. Prior to quantification, 0.5 mL of cold 100% ethanol was added to the COCs, vortexed and stored at -20°C overnight. Samples were centrifuged at 3,000 g for 15 min at 4 °C, air dried and re-suspended in assay buffer (0.05 M sodium acetate, pH 6.2) and acetylated in triethylamine:acetic anhydride solution (2:1 ratio). The cAMP antibody and ^{125}I-cAMP tracer were added to samples and incubated overnight at 4 °C. Cold ethanol was added to samples and centrifuged at 3000 g for 10 min at 4 °C. Supernatant was discarded, samples were air dried, and cAMP quantified using a gamma counter. Samples were measured in duplicates and compared to a standard curve of known cAMP concentration (1024 to 0 fmol cAMP). Intra- and inter-assay CVs were 7.1 and 10.4%, respectively.

2.4. Experimental design

2.4.1. Experiment 1. Effect of type-3 PDE inhibitor (cilostamide) on nuclear maturation

A cilostamide dose response experiment was performed to determine the optimal concentration of the PDE3 specific inhibitor to delay nuclear maturation during the IVM phase. Treatments included control (VitroMat + 4 mg/mL BSA + 100 mIU/mL FSH) 0.1, 1, 10, and 100 μM cilostamide (Enzo Life Sciences, Farmingdale, NY, USA). The COCs were removed at 24 h, denuded, and nuclear maturation was assessed using DAPI chromatin staining. The experiment consisted of four replicates (average of 20 oocytes per treatment).

2.4.2. Experiment 2. Effect of FSK and IBMX supplementation on COC cAMP concentrations after pre-IVM

The effect of cAMP modulators during pre-IVM treatment on cAMP concentrations at the end of pre-IVM was determined. Aspirate sediment (2 mL) was randomly allocated to 10 mL of: 1) control = VitroCollect + 0.2 mg/mL BSA; 2) control + 50 μM FSK; 3) control + 100 μM FSK; 4) control + 500 μM IBMX; 5) control + 500 μM IBMX + 50 μM FSK; and 6) control + 500 μM IBMX + 100 μM FSK. Groups of 10 COCs were collected at the end of the 1.5 to 2 h pre-IVM phase, washed twice, and snap frozen in liquid N_2 for cAMP radioimmunoassay. The experiment consisted of three replicates with 10 COCs per treatment, per replicate.

2.4.3. Experiment 3. Effect of pre-IVM treatment and cilostamide during IVM on in vitro nuclear maturation

This experiment was conducted to determine the optimal concentration of cilostamide during IVM when combined with a pre-IVM treatment. Aspirate sediment (2

mL) was randomly allocated to 10 mL: 1) control = VitroCollect + 0.2 mg/mL BSA, or 2) pre-IVM = control + 500 μM IBMX + 50 μM FSK and incubated at 38.5 °C for 1.5 to 2 h. After the pre-IVM phase, COCs were transferred to IVM culture media: A) control = VitroMat + 4mg/mL BSA + 100 mIU/mL FSH; B) control + 1 μM cilostamide; or C) control + 20 μM cilostamide. The COCs were removed at 20 h, denuded and nuclear maturation assessed using DAPI chromatin staining. The experiment consisted of four replicates, each averaging 22 oocytes per treatment.

2.4.4. Experiment 4. Effect of pre-IVM and IVM conditions on COC cAMP concentrations after IVM

The effects of cAMP modulating agents on COC cAMP concentrations after IVM were determined. Aspirate sediment (2 mL) was randomly allocated to 10 mL: 1) control = VitroCollect + 0.2 mg/mL BSA, or 2) pre-IVM = control + 500 μM IBMX + 100 μM FSK and incubated at 38.5 °C for 1.5 to 2 h. After the pre-IVM phase, COCs were transferred to IVM culture media: A) control = VitroMat + 4 mg/mL BSA; B) control + 100 mIU/mL FSH; C) control + 1 μM cilostamide; or D) control + 100 mIU/mL FSH + 1 μM cilostamide. Groups of 10 COCs were collected at 20 h IVM and snap frozen in liquid N_2 for the cAMP radioimmunoassay. The experiment consisted of two replicates with 10 COCs per treatment, per replicate.

2.4.5. Experiment 5. Effect of SPOM on meiotic progression to MII

Based on the results of the previous four experiments, an ovine-adapted SPOM system was defined as: pre-IVM (VitroCollect containing 0.2 mg/mL BSA + 500 μM

IBMX + 100 μM FSK) followed by IVM (VitroMat containing 4 mg/mL BSA + 100 mIU/mL FSH + 1 μM cilostamide). To assess the effects of SPOM on the kinetics of meiotic progression, COCs were randomly allocated to: 1) control (Standard IVM); collection in VitroCollect + 0.2 mg/mL BSA followed by IVM in VitroMat + 4 mg/mL BSA + 100 mIU/mL FSH; or 2) SPOM (as defined above). Approximately 20 COCs were collected at 21, 24, 27 and 30 h; denuded and nuclear maturation was assessed using DAPI chromatin staining. The experiment consisted of five replicates, with each replicate averaging 20 oocytes per treatment.

2.4.6. Experiment 6. Effect of SPOM on oocyte developmental competence

To assess the effects of ovine-SPOM on oocyte developmental competence, oocytes were fertilized after 24 h (control) or 27 h (SPOM) of maturation. Thereafter, cleavage and blastocyst rates and blastocyst cell numbers were determined. After oocyte maturation (Day 0), excess CC were removed by gentle pipetting (corona radiata remained intact) in 400 IU/mL hyaluronidase, washed twice in IVF medium (as described [30]), and 25 to 30 COCs were transferred into 450 μL of pre-equilibrated IVF medium overlaid with oil. Motile sperm were obtained using a "swim-up" method; 150 μL of frozen-thawed sperm, pooled from two rams of proven fertility, were placed under 1 mL of IVF medium in a 14-mL tube. Motile sperm were retrieved from the upper portion after 30 to 60 min, approximately $1x10^6$ sperm/mL was then added to the COCs, which were incubated at 38.5 °C in humidified 5% CO_2 air.

After 24 h, remaining CC were removed by gentle pipetting, presumptive zygotes were washed three times in *in vitro* culture (IVC) media [30] and 25 to 30 presumptive zygotes were cultured in 600 μL of IVC medium overlaid with oil at 38.5 °C in

humidified 5% CO_2, 5% O_2, and 90% N_2 air. Non-cleaved oocytes were removed on Day 1 and cleavage was recorded. Embryo development was subsequently assessed on Day 7. The experiment consisted of five replicates, each averaging ~70 oocytes per treatment. To assess the effects of SPOM on blastocyst cell numbers, in three of the five replicate experiments, Day-7 blastocysts were mounted on a slide in drops of Hoechst, and incubated overnight at 4 °C. Embryo cell number was counted using a Nikon Eclipse TE2000-E Microscope (excitation wavelength = 330 to 380 nm).

2.5. Statistical analyses

All experimental data were analysed using the SPSS Statistics Program (PASW Stats V.17, SSPSS Inc., Chicago, IL, USA). One way ANOVA (Experiments 1 and 5) with post-hoc Bonferroni comparison and nonparametric analysis (Experiment 5 – total cell number) were used to determine significance (Experiments 1 to 5). For all analyses, $P < 0.05$ was deemed significant.

3. Results

3.1. Experiment 1. Effect of the type-3 PDE inhibitor (cilostamide) on nuclear maturation

A cilostamide dose-response experiment was conducted to determine its effect during IVM on nuclear maturation. Cilostamide supplementation attenuated the spontaneous resumption of meiosis by COCs in a dose-dependent manner following 24

h incubation ($P < 0.001$, Fig. 1). The presence of 1 μM cilostamide reduced ($P < 0.05$) the proportion of oocytes completing nuclear maturation, and at 100 μM, 3.5-fold less COCs reached MII (compared to COCs cultured in the absence of cilostamide).

3.2. Experiment 2. Effect of FSK and IBMX supplementation on COC cAMP concentrations after pre-IVM

The effect of adding IBMX and FSK to a pre-IVM treatment on initial COC cAMP concentrations after 2 h of culture was assessed. The COCs cultured in the absence of cAMP modulators had lower cAMP concentrations, compared to other treatment groups (Fig. 2). There was no significant difference between cAMP concentrations in COCs treated with 50 versus 100 μM FSK. The presence of 500 μM IBMX with 100 μM FSK resulted in a 10-fold increase in cAMP concentrations (176 ± 33 fmol/COC) compared to the control group (17 ± 15 fmol/COC, $P < 0.05$, Fig. 2).

3.3. Experiment 3. Effect of pre-IVM treatment and cilostamide supplementation during IVM on in vitro nuclear maturation

The effect of treating COCs with FSK (an adenylate cyclase activator) and IBMX (a non-specific PDE inhibitor) during a pre-IVM phase followed by IVM culture with various concentrations of cilostamide on nuclear maturation status after 20 h maturation was assessed. Cilostamide at 20 μM (without pre-IVM) compared to pre-IVM only (-cilostamide) had more oocytes at MI ($P <0.05$). There were no significant differences between treatments in anaphase I and teleophase I stages. However, the

combination of 20 μM cilostamide supplementation during IVM and pre-IVM culture, tended to reduce oocytes at MII, with approximately 3-fold less reaching MII compared to control (P = 0.081, Fig. 3).

3.4. Experiment 4. Effect of pre-IVM and IVM conditions on COC cAMP concentrations after IVM

The effect of pre-IVM treatment and FSH and cilostamide supplementation during the IVM phase, on COC cAMP concentrations after 20 h of IVM was assessed using a multi-factorial experimental design. The COCs within the control group, with no cAMP modulators (- pre-IVM, - FSH, - cilostamide), had cAMP concentrations of 0.33 ± 0.04 fmol/COC, whilst standard IVM conditions (- pre-IVM, + FSH, - cilostamide) yielded COCs with 1.00 ± 0.24 fmol/COC (Fig. 4). The combined components of the SPOM system had the highest COC cAMP concentrations of all treatments (Fig. 4).

3.5. Experiment 5. Effect of ovine-adapted SPOM on meiotic progression to MII

The extent of delay in completing nuclear maturation in oocytes cultured in an ovine-adapted SPOM system (pre-IVM with 500 μM IBMX + 50 μM FSK and IVM with 100 mIU/mL FSH + 1 μM cilostamide) compared to IVM conducted with 100 mIU/mL FSH, with no pre-IVM treatment was assessed. In standard IVM (+ FSH), 77.9 ± 1.3 % of COCs reached the MII stage by 21 h of maturation, compared to 54.1 ± 8.3 % of COCs cultured in the SPOM system (P = 0.001, Fig. 5). By 27 h, SPOM oocytes reached approximately the same rates of MII as standard IVM at 24 h (the standard insemination time for ovine IVM). All COCs cultured in the IVM (with FSH) and SPOM systems reached

MII by 30 h of culture, indicating no prevention of maturation in either culture system (Fig. 5). Based on these data, we inferred that the optimal, comparable time for insemination of SPOM oocytes was 27 to 28 h post-maturation.

3.6. Experiment 6. Effect of ovine-adapted SPOM on oocyte developmental competence

The developmental competence of COCs cultured in SPOM versus standard IVM (with FSH) was determined by comparing cleavage rates, on-time embryo development on Day 7 and blastocyst cell numbers, following IVF and IVC. There was no significant difference in cleavage rates between treatments, with approximately 79% of oocytes successfully cleaving in both treatments (Table 1). On Day 7 of culture, approximately 54% of all cleaved zygotes developed into blastocysts in both standard IVM and SPOM treatments, with no significant differences in blastocyst yield. However, SPOM blastocysts had substantially more cells (1.6-fold) per embryo compared to standard IVM ($P < 0.001$, Table 1).

4. Discussion

In-vivo, the surge of gonadotrophins prior to ovulation overrides natural meiotic inhibition and specific epidermal growth factor (EGF)-like peptides initiate cascades that induce oocyte maturation [31]. In comparison, mechanical removal of the oocyte from the follicular environment results in spontaneous resumption of meiosis [17]. In this study, we investigated the effect of using modulators of cAMP to control meiotic

maturation within ovine oocytes from 6 to 12 mo-old lambs (a less-competent oocyte model), utilizing a new approach to cAMP-regulated meiosis (SPOM). Similar to that reported for the murine and bovine COC [13], in an ovine-adapted SPOM model, the aim was to increase cAMP concentrations within the COC at the time of collection from the follicle, thereby preventing spontaneous meiotic resumption (pre-IVM). This initial increase in COC cAMP concentrations was followed by inhibition of PDE-mediated cAMP degradation using a type-3 specific PDE inhibitor and FSH-stimulated induction of meiosis during an extended IVM phase [1] .

Oocyte developmental competence is compromised in spontaneous IVM due to many factors, including presumably an absence of the full ovulatory molecular EGF cascade in CC and premature breakdown of oocyte-cumulus cell gap junctions; together this reduced exchange of positive regulatory molecules and metabolites from the follicular compartment [32]. Furthermore, meiotic resumption occurs in a rapid and uncontrolled manner [25]. However, the SPOM and related IVM models using elevated cAMP concentrations attenuated spontaneous meiosis, thereby resulting in a more gradual cessation of oocyte transcription and transition into the M-phase and promoting and prolonging oocyte-cumulus cell gap junction communication (GJC) [13,32-35]. Part of the rationale for these approaches were *s*ubstantial increases in cAMP occurred in vivo following LH-induction of the ovulatory cascade in porcine [36] and murine COCs [13,37]. In contrast, liberation of the oocyte from the follicle for spontaneous IVM drastically decreases COC cAMP [13,34,38]. In the current study, the 1.5 to 2 h pre-IVM treatment with cAMP modulators, FSK and IBMX in the SPOM system, resulted in a > 10-fold increase in COC cAMP concentrations compared to COCs exposed to pre-IVM conditions in the absence of FSK and IBMX. Comparatively, SPOM pre-IVM in bovine generated a 100-fold increase in cAMP concentrations [13]. Apparent differences

between the two species may be due to the presence and expression of different PDE and adenylate cyclase isoforms [11]. However, since cAMP concentrations do not substantially differ between ovine and bovine COCs in the presence of IBMX and FSK (< 20 fmol/COC), perhaps peripubertal ovine COCs have greater endogenous production of cAMP, or ovine PDEs degrade cAMP at a slower rate than in bovine COCs. Nonetheless, IBMX and FSK together generated high COC cAMP concentrations (compared to the absence of cAMP modulators).

The pre-IVM phase in the SPOM system primarily targets CC to increase cAMP concentrations in CC and also serves to maintain CC-oocyte GJC [13,32]. These two effects combine to load the oocyte with cAMP from the CC [39]. In parallel, increases in CC cAMP induce expression of EGF-like peptides and subsequent activation of the EGF receptor and MAPK pathway, thereby stimulating cumulus expansion and oocyte maturation [40-42]. It is widely reported that cAMP modulators in cattle IVM extended CC-oocyte GJC and increased oocyte developmental competence [13,19,20,32,43,44]. Therefore, the ovine SPOM system increases COC cAMP concentrations, preventing the spontaneous resumption of meiosis, likely by maintaining GJC between the oocyte and cumulus cells and maintaining higher cAMP concentrations in cumulus cells and oocytes.

In previous studies, PDE inhibitors have been used extensively in various experimental IVM systems in an effort to improve oocyte developmental competence [45]. However, they have apparently not yet been examined in ovine IVM. In the current study, 1 μM cilostamide delayed completion of nuclear maturation by 30% compared to the control. Furthermore, the full ovine-SPOM system delayed completion of meiosis by 3 to 4 h. Concentrations of PDE inhibitors used in the ovine-SPOM system differed from

those used in the bovine and murine SPOM models [13]. For example, during IVM, optimal cilostamide concentrations were 1 μM (sheep, current study) 20 μM (cattle) and 0.1 μM (mouse) [13]. This was expected due to differences among species in PDE kinetics, the presence of other PDE subtypes within the oocyte, and varying concentrations of endogenous oocyte cAMP production [46]. In the pre-IVM phase, 10-fold higher concentrations of IBMX were needed in bovine compared to murine models (500 versus 50 μM, respectively), perhaps due to expression of PDE8 in bovine COCs, which accounted for more than 20% of total PDE activity and is not inhibited by IBMX [11]. Conversely, murine cumulus cells did not express PDE8, but expressed the IBMX-sensitive PDE4 as its major cumulus cell PDE [12]. Hence, lower concentrations of IBMX are required during the murine pre-IVM phase, compared to the bovine system. Expression of different PDEs within ovine cumulus cells and oocytes are unknown and requires further investigation.

The combined actions of cAMP modulators in pre-IVM and during the IVM phase prevented degradation of COC cAMP, and at the end of IVM, SPOM had the highest cAMP concentration out of all experimental groups. The meiotic inducing effects of FSH present in IVM allowed the attenuated meiosis to continue with an approximate delay of 3 to 4 h. However, the sheep SPOM system did not cause notable increases in blastocyst and cleavage rates previously reported in cattle and mice [13]. There is currently no clear explanation for this difference, but it could be the result of the age of the sheep used. Nonetheless, in the current study, the SPOM IVM system significantly increased total cell number of blastocysts, which has been reported to reflect developmental competence, as shown by increased capacity to develop into a viable fetus following transfer to donors, regardless of blastocyst formation and hatching rates [47].

Based on this study, in combination with other working SPOM models [13], we inferred that use of SPOM improved outcomes from IVM. Hence, SPOM may have an application in domestic animal artificial breeding programs, where there is a need to improve the efficiency of in vitro embryo production methodologies. Since it is widely recognized that the IVM phase is rate-limiting, particularly when COCs from juvenile animals are used to produce embryos in vitro (JIVET), the SPOM system described herein may be beneficial.

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Table 1: Comparison (means ± SEM) of Standard-IVM to SPOM on development of ovine embryos.

Treatment	Cleaved (%)	Blastocyst/cleaved (%)			Total no. cells
		Total	Expanded	Hatched	
Standard- IVM	78.7 ± 3.1	54.2 ± 2.3	38.1 ± 4.2	14.5 ± 3.2	63.5 ± 3.9[a]
SPOM	79.4 ± 2.2	53.5 ± 3.2	36.8 ± 5.9	12.4 ± 3.4	103.3 ± 6.0[b]

[a,b]Means without a common superscript differed (P = 0.001).

Figure Legends

Fig. 1. Mean + SEM effects of increasing concentrations of cilostamide on nuclear maturation of peripubertal ovine oocytes following 24 h of culture.

[a,b, w-z]Within a stage of maturation, means without a common superscript differed ($P < 0.05$).

GV = germinal vesicle stage, MII = metaphase II.

Fig. 2. Mean + SEM effects of FSK and IBMX on COC cAMP concentrations in peripubertal ovine oocytes following 2 h pre-IVM treatments.

[a,b]Means without a common superscript differed ($P < 0.05$).

Fig. 3. Mean + SEM effects of pre-IVM treatment and cilostamide (CIL) during IVM on the nuclear maturation of peripubertal ovine oocytes following 20 h of culture. Pre-IVM: 500 µM IBMX + 50 µM forskolin (FSK).

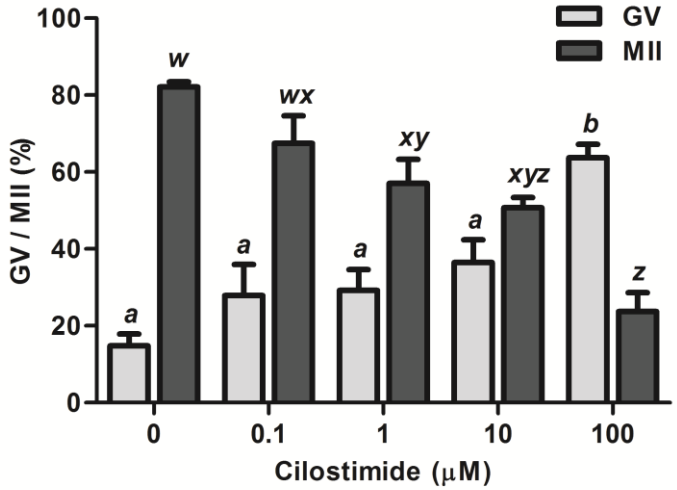
*Means differed ($P < 0.05$).

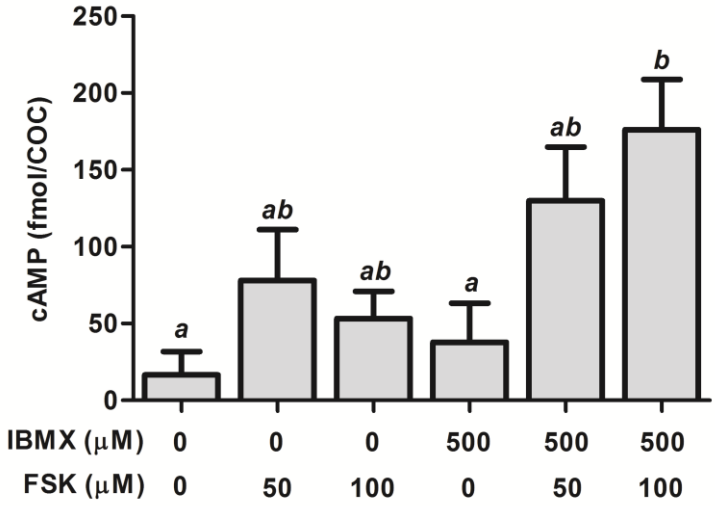
GV = germinal vesicle stage, AI+TI = anaphase I + telophase I, MII = metaphase II.

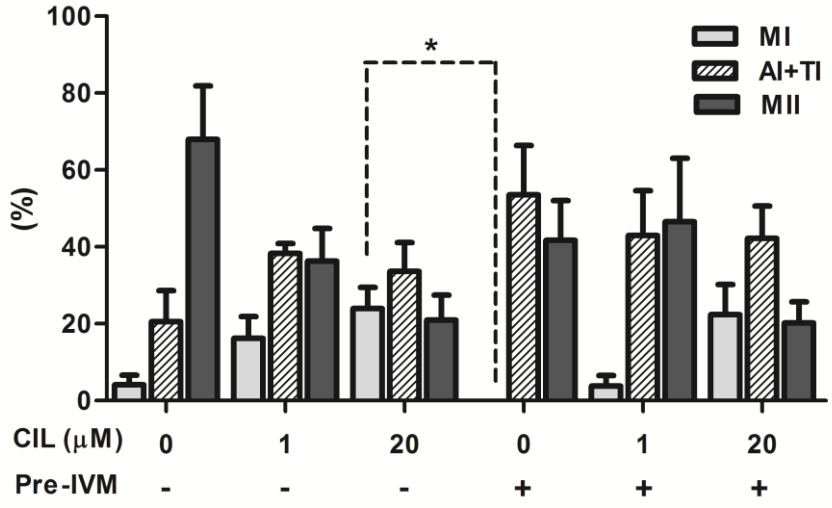
Fig. 4. Mean + SD effects of pre-IVM treatment, FSH and cilostamide (CIL) on cAMP concentrations in peripubertal ovine COCs following 20 h of culture. Pre-IVM, 500 µM IBMX + 100 µM forskolin; FSH, 100 mIU/mL; cilostamide, 1 µM.

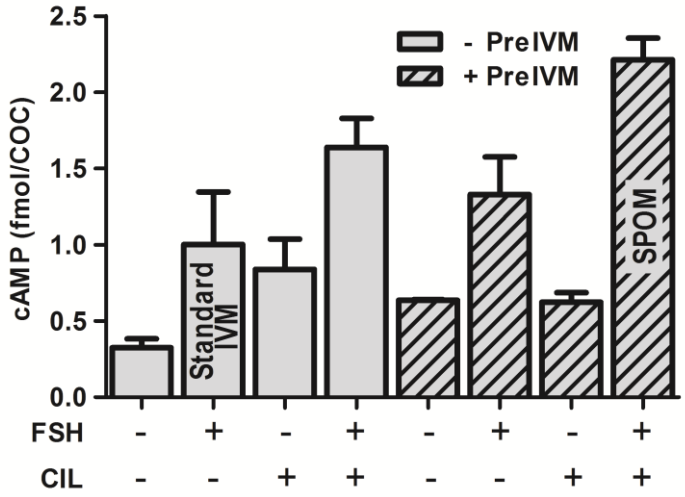
Fig. 5. Mean + SEM kinetics of maturation of peripubertal ovine oocytes in standard IVM and SPOM.

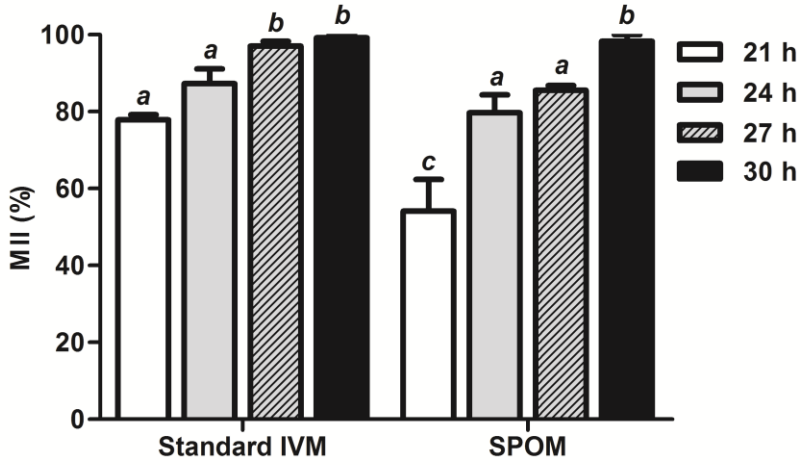
[a-c]Means without a common superscript differed ($P < 0.05$).


GV
MII
GV / MII (%)
100
80
60
40
20
0
w
wx
xy
xyz
b
a
a
a
a
z
0
0.1
1
10
100
Cilostimide (μM)


250
200
150
100
50
0
cAMP (fmol/COC)
a
ab
ab
a
ab
b
IBMX (μM) 0 0 0 500 500 500
FSK (μM) 0 50 100 0 50 100


100
80
60
40
20
0
(%)
*
MI
AI+TI
MII
CIL (μM)
0
1
20
0
1
20
Pre-IVM
-
-
-
+
+
+


2.5
2.0
1.5
1.0
0.5
0.0
cAMP (fmol/COC)
- PreIVM
+ PreIVM
Standard IVM
SPOM
FSH - + - + - + - +
CIL - - + + - - + +


MII (%)
100
80
60
40
20
0
a
a
b
b
c
a
a
b
Standard IVM
SPOM
21 h
24 h
27 h
30 h