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Reversible meiotic arrest in feline oocytes

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Abstract. Increasing intracellular concentrations of cyclic adenosine monophosphate (cAMP) within the cumulus–oocyte complex (COC) inhibits or delays spontaneous oocyte maturation and improves the developmental competence of the oocyte in many species, but information for carnivores is limited. The objectives of the present study were to describe the effects of isobutyl methylxanthine (IBMX), which decreases cAMP degradation, and forskolin, which increases cAMP production, on spontaneous and induced maturation (by equine chorionic gonadotrophin (eCG) and epidermal growth factor (EGF)) of feline oocytes and to evaluate the reversibility of IBMX-induced arrest by measuring the resumption of meiosis and embryonic development following IVF. IBMX decreased ($P < 0.05$) the incidence of spontaneous (6.7% vs 42.0%, metaphase II (MII)) and induced (5.6% vs 66.1% MII) maturation after 24 h of culture. In contrast, forskolin stimulated meiosis (81.7% MII; $P < 0.05$). Following 12 h of culture with IBMX and an additional 24 h with eCG and EGF in the absence of IBMX, the proportions of oocytes reaching MII (66.1%), cleaving (79.9%) and developing to the blastocyst stage (15.3%) were similar ($P > 0.05$) to oocytes cultured continuously with eCG and EGF (70.2%, 83.0% and 18.1%, respectively). These results demonstrate that IBMX reversibly inhibits both spontaneous and eCG+EGF-induced meiosis in feline oocytes without compromising the oocyte's developmental competence.

Additional keywords: assisted reproductive technologies, carnivore, cat.

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Introduction

Numerous studies of oocyte maturation, fertilisation and early embryonic development utilise immature oocytes that have been recovered from antral follicles and cultured *in vitro* until they have reached metaphase II (MII). In many species, the majority of oocytes will reach MII, but less than 50% of these oocytes are capable of developing to the blastocyst stage, and even fewer have the ability to develop to full-term offspring (Papadopoulos *et al.* 2002; Lane *et al.* 2003; Yoshioka *et al.* 2003; Banwell *et al.* 2007; Tang-Pedersen *et al.* 2012). As a result, considerable research has focussed on developing culture systems that produce oocytes that are not only capable of completing meiosis (nuclear maturation), but also pre- and post-implantation development following fertilisation (acquisition of developmental competence or cytoplasmic maturation).

When cumulus–oocyte complexes (COCs) are removed from the follicle and placed in culture, a proportion of the oocytes will spontaneously (in the absence of gonadotrophin stimulation) resume meiosis (Edwards 1965; Schroeder and Eppig 1984; Thomas *et al.* 2002). However, these oocytes would have remained arrested at the germinal vesicle stage of meiosis for several days or weeks if the oocytes had remained in the follicle. Therefore, one strategy to improve the quality of *in vitro*-matured oocytes is to inhibit, or slow, the spontaneous resumption of meiosis after recovery. Oocytes may be able to complete additional cellular processes during this period of arrest that will improve their developmental competence (Gilchrist and Thompson 2007). Similarly, the biochemical pathways involved with gonadotrophin-induced maturation following a period of arrest may better mimic the events occurring in the follicle after the LH surge (Gilchrist 2011). This approach has been tested by a variety of laboratories in pigs, cattle and mice, with some groups reporting significant improvements in embryonic development and others reporting little or no improvement compared with traditional IVM protocols (Funahashi *et al.* 1997; Thomas *et al.* 2004*b*; Gilchrist and Thompson 2007; Kawashima *et al.* 2008; Albuz *et al.* 2010).

Concentrations of cyclic adenosine monophosphate (cAMP) within the oocyte control the resumption of meiosis (germinal vesicle breakdown, GVBD), making cAMP a logical target for strategies to alter the kinetics of meiosis. The intracellular concentration of cAMP is determined by the rate of cAMP synthesis by adenylate cyclases and the rate of cAMP degradation by phosphodiesterases (PDE). There is a constitutively active adenylate cyclase in mammalian oocytes that produces cAMP (Mehlmann 2005). Recent research in mice has shown

that cumulus cells, upon stimulation with natriuretic peptide precursor type C (NPPC), produce cyclic guanosine monophosphate (cGMP), which is transferred into the oocyte via gap junctions and inhibits PDE3 (Norris *et al.* 2009; Zhang *et al.* 2010). The combination of continuous cAMP production and little or no degradation of cAMP by PDE3 results in sufficient concentrations of cAMP within the oocyte to maintain meiotic arrest. When the COC is removed from the follicle and the influence of NPPC, cGMP production by the cumulus cells is decreased, PDE activity within the oocyte increases, cAMP concentrations within the oocyte fall and the oocyte resumes meiosis (Norris *et al.* 2009; Zhang *et al.* 2010). Therefore, maintaining meiotic arrest in isolated COCs requires the use of chemicals that can maintain adequate concentrations of cAMP within the oocyte, either by stimulating production via adenylate cyclase or by inhibiting PDE activity, or both.

Two of the chemicals most commonly used to manipulate intracellular cAMP concentrations are isobutyl methylxanthine (IBMX), a non-specific PDE inhibitor, and forskolin, an adenylate cyclase stimulator. Both of these chemicals have been used extensively in multiple species with consistent, predicted effects (Bilodeau *et al.* 1993; Fan *et al.* 2002; Thomas *et al.* 2002; Chen *et al.* 2009; Downs 2011). In mice, rats, pigs, cattle, macaques and humans, the majority of PDE activity within the oocyte is due to PDE3, which is sensitive to IBMX (Downs *et al.* 1989; Conti *et al.* 1998; Richard *et al.* 2001; Jensen *et al.* 2002; Thomas *et al.* 2002; Liang *et al.* 2005; Nogueira *et al.* 2006; Sasseville *et al.* 2009). By inhibiting PDE3 activity, IBMX maintains meiotic arrest by preventing a decline in cAMP concentrations within the oocyte after recovery (Schultz *et al.* 1983; Luciano *et al.* 2004; Albuz *et al.* 2010) or permitting the accumulation of cAMP produced by intra-oocyte adenylate cyclase(s) (Mehlmann 2005). Although forskolin-sensitive adenylate cyclases are present in both cumulus cells and oocytes (Horner *et al.* 2003; Lastro *et al.* 2006), forskolin increases intracellular cAMP concentrations within cumulus cells, with little or no change in cAMP concentrations within denuded oocytes in mice (Bornslaeger and Schultz 1985), rats (Racowsky 1984), hamsters (Racowsky 1985*a*), pigs (Racowsky 1985*b*) or cattle (Bilodeau *et al.* 1993; Thomas *et al.* 2002). However, forskolin can increase cAMP concentrations within oocytes if the gap-junctional communication between the oocyte and cumulus cells is maintained during forskolin exposure (Racowsky 1984, 1985*a*, 1985*b*; Bornslaeger and Schultz 1985; Bilodeau *et al.* 1993; Thomas *et al.* 2002). Therefore, forskolin maintains meiotic arrest in oocytes by increasing concentrations of cAMP within the cumulus cells, which is then transferred to the oocyte via gap junctions.

Although numerous studies have involved the *in vitro* maturation (IVM) of feline oocytes, surprisingly little is known about the mechanisms controlling meiosis. The objectives of the present study were to: (1) describe the effects of IBMX and forskolin on the spontaneous maturation of feline oocytes, (2) assess the ability of IBMX to inhibit equine chorionic gonadotrophin (eCG)- and epidermal growth factor (EGF)-induced maturation, (3) determine intracellular concentrations of cAMP in feline oocytes exposed to eCG and EGF, IBMX and forskolin, (4) evaluate the reversibility of IBMX-induced meiotic arrest and (5) assess the developmental competence of oocytes that were previously arrested in meiosis by IBMX.

Materials and methods

Medium preparation

All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise specified. Stock solutions were prepared in ultra-pure water (18.2 MΩhm and $<$10 ppb total organic carbon; EMD Millipore, Billerica, MA, USA) and stored at 4°C for either 1 week (pyruvate, bicarbonate, cysteine and cysteamine) or 1 month (basic salt solution, glucose, L-lactate (MP Biomedicals, Costa Mesa, CA, USA) and taurine). Solutions of gentamicin, amino acids (at the same concentrations as those found in MEM; MP Biomedicals), vitamin (at the same concentrations as those found in MEM; MP Biomedicals), fetal calf serum (FCS; Hyclone, Logan, UT, USA) and insulin, transferrin and selenium (ITS) were stored according to supplier instructions and used directly for medium preparation. Stock solutions (100 mM) of IBMX, forskolin, and IBMX + forskolin (100 mM each) were prepared in dimethyl sulfoxide (DMSO) and stored frozen (−20°C) until use. The final concentration of DMSO was 0.1% (v/v) in all treatments and the control medium. Working solutions of all media were prepared for each replicate, filtered (0.22 μm, MillexGV; EMD Millipore), and equilibrated in the appropriate gas atmosphere for at least 4 h before use. All cultures were performed in 50-μL drops of medium (8 to 12 COCs or embryos per drop) covered with embryo-tested mineral oil in standard plastic dishes (Falcon 1007 or 1008; Becton Dickinson Labware, Franklin Lakes, NJ, USA) and maintained at 38.5°C.

In vitro *maturation*

Domestic cat ovaries were recovered from a local veterinary clinic following routine ovariohysterectomies of females $>$6 months old. Immediately after excision, ovaries were placed in 0.9% (w/v) NaCl containing 50 μg mL^{-1} gentamicin and maintained at ~5°C until processing (2 to 3 h). After removing excess tissue, ovaries were repeatedly sliced in a HEPES-buffered (20 mM HEPES and 5 mM $NaHCO_3$) version of feline optimised culture medium (FOCMH; Table 1) containing 40 U mL^{-1} heparin. Cumulus–oocyte complexes containing multiple, compact layers of cumulus cells and an oocyte with a uniformly dark cytoplasm were washed twice in FOCMH before culturing in maturation medium. The medium used for maturation was FOCM (Herrick *et al.* 2007; Table 1) containing 6.0 mM glucose and supplemented with 0.5× MEM essential amino acids, 1.0× MEM vitamins, 0.6 mM cysteine, 0.1 mM cysteamine, 10 μg mL^{-1} insulin, 5.5 μg mL^{-1} transferrin and 5.0 ng mL^{-1} selenium (Herrick *et al.* 2010). Cumulus–oocyte complexes were cultured in 6% CO_2 in air for 6 to 36 h depending on the experiment.

Assesment of nuclear maturation

Cumulus–oocyte complexes were placed into 1.5-mL microcentrifuge tubes containing 100 μL of FOCMH and ~500 U mL^{-1} (0.5 mg mL^{-1}) hyaluronidase and shaken vigorously with a vortex mixer for 2 to 3 min to remove cumulus cells. Denuded

Table 1. Composition of feline optimised culture medium (FOCM)

Component	Concentration (mM)
NaCl	100.0
KCl	8.0
KH_2PO_4	1.0
$CaCl_2$–$2H_2O$	2.0
$MgSO_4$–$7H_2O$	1.0
$NaHCO_3$	25.0
Glucose[A]	1.5 to 6.0
L-lactate	6.0
Pyruvate	0.1
Alanyl-glutamine	1.0
Taurine	0.1
NEAA[B]	1.0×
BSA	4.0 mg mL^{-1}
Gentamicin[C]	20 to 50 μg mL^{-1}

[A]6.0 mM for IVM only. Media for IVF and IVC contained 1.5 mM and HEPES-buffered medium contained 3.0 mM.
[B]Concentrations of nonessential amino acids (NEAA, 0.1 mM each) found in minimum essential medium (MEM).
[C]HEPES-buffered medium and media for IVM and IVF contained 50 μg mL^{-1}. IVC media contained 20 μg mL^{-1}.

oocytes were transferred to a glass slide in a minimum amount of medium and compressed under a coverslip supported by drops of petroleum jelly and paraffin wax. The coverslip was secured in place with small drops of rubber cement and the slide was placed in fixative (6 parts glacial acetic acid : 3 parts 100% ethanol : 1 part chloroform) for at least 24 h (Herrick *et al.* 2006*a*). Chromatin was stained with 1% (w/v) orcein in 45% acetic acid (v/v in H_2O) and the stage of meiosis was evaluated with phase contrast microscopy (400×).

Measurement of intracellular concentrations of cAMP

Cumulus–oocyte complexes were placed into 1.5-mL microcentrifuge tubes containing 100 μL of FOCMH with 200 μM IBMX and ~500 U mL^{-1} (0.5 mg mL^{-1}) hyaluronidase and shaken vigorously with a vortex mixer for 2 to 3 min to remove cumulus cells. Groups of 8 to 22 denuded oocytes were washed twice in FOCMH with 200 μM IBMX, transferred in 2 μL to a 1.5-mL microcentrifuge tube and stored at −80°C. Prior to analysis, cell lysis was accomplished by freeze–thawing tubes in liquid nitrogen three times, adding 38 μL H_2O to each tube, repeatedly aspirating the oocytes with a pipettor, and then three additional freeze–thaw cycles in liquid nitrogen. Samples were then centrifuged for 10 min at 12 000*g* at 22°C and 30 μL of the supernatant was removed and stored at −80°C until analysis. Intracellular content of cAMP was determined by liquid chromatography, mass spectrometry, mass spectrometry (LC/MS/MS) analysis performed at the Metabolomics Center of University of Illinois at Champaign-Urbana. Samples were analysed with the QTRAP 5500 LC/MS/MS system (AB Sciex, Foster City, CA, USA) with a 1200 series high-performance liquid chromatography (HPLC) system (Agilent Technologies, Santa Clara, CA, USA) including a degasser, an autosampler and a binary pump. The LC separation was performed on a Phenomenex Synergi 4u Polar-RP 80A column (4.6 × 100 mm; Phenomenex, Torrance, CA, USA) with mobile phase A (0.1% formic acid in water) and mobile phase B (0.1% formic acid in acetonitrile). The flow rate was 1 mL min^{-1}. The linear gradient was as follows: 0–1 min, 100% A; 6 min, 10% A; 6.5–9 min, 100% A. The autosampler was set at 5°C. The injection volume was 15 μL. Mass spectra were acquired with positive electrospray ionisation (ESI) with the ion spray voltage at 5500 V. The source temperature was 600°C. The curtain gas, ion source gas 1 and ion source gas 2 were 35, 50 and 65, respectively. Multiple-reaction monitoring (MRM) m/z 330.1 → m/z 136.1 was used to quantify cAMP.

In vitro *fertilisation*

Spermatozoa were collected (artificial vagina) from two domestic cats of proven fertility, pooled and cryopreserved in 0.25-mL plastic straws (AgTech, Manhattan, KS, USA) in liquid nitrogen vapour (Herrick *et al.* 2010). For each replicate, a single straw was thawed in air for 10 s and in a 37°C H_2O bath for 30 s. Thawed spermatozoa were expelled from the straw, immediately diluted with 150 μL FOCMH, and centrifuged for 10 min at 300*g* at 22°C. The resulting pellet of spermatozoa was resuspended in 25–50 μL FOCMH and the concentration of spermatozoa was determined with a haemocytometer. Mature COCs were washed twice and placed in 45-μL drops of FOCM. Spermatozoa were diluted with FOCM to 5×10^6 motile spermatozoa mL^{-1} and 5 μL of this solution was added to each drop containing COCs for a final concentration of 5×10^5 motile spermatozoa mL^{-1}. Gametes were co-incubated for 20–22 h in 6% CO_2 in air. The day of IVF was considered to be Day 0 of culture.

Embryo culture

Loosely bound spermatozoa and remaining cumulus cells were removed from presumptive zygotes as previously described for COCs. Denuded zygotes were washed and placed into FOCM for culture (6% CO_2, 5% O_2 and 89% N_2, 48 h). On Day 3 of culture the proportion of embryos that had cleaved was evaluated and all cleaved embryos were moved to fresh FOCM with 5% (v/v) fetal calf serum instead of BSA and cultured (6% CO_2, 5% O2 and 89% N2) for 96 h. On Day 7 of culture (~168 h after IVF), the proportion of embryos that had developed to the blastocyst stage was determined and all blastocysts were fixed for determination of total cell number (Pursel *et al.* 1985; Herrick *et al.* 2007). Only embryos that were classified as a blastocyst based on morphology and contained at least 50 cells were considered to be blastocysts for calculation of developmental parameters.

Statistical analysis

For each replicate (day of ovary collection), COCs were randomly allocated to as many treatments (culture condition and time of evaluation) as possible so that each treatment contained 10 to 20 COCs per replicate (incomplete block design). Each treatment or treatment and time combination was replicated three to six times depending on the experiment. For oocyte maturation and embryonic development, each oocyte or embryo

was scored as a 1 or 0 depending on whether or not it achieved the desired stage of meiosis or development (germinal vesicle, metaphase II, cleaved or blastocyst). Data was then analysed using the generalised linear mixed model (GLIMMIX) procedure in SAS with a binomial error distribution and a probit link function (Littell *et al.* 1996). Intracellular concentrations of cAMP (fmol cAMP per oocyte) were analysed with the mixed-model procedure in SAS and with separate variances calculated for each treatment (Littell *et al.* 1996). Blastocyst cell numbers were analysed using the generalised linear mixed model (GLIMMIX) procedure in SAS with a Poisson error distribution and a log link function (Littell *et al.* 1996). In all analyses, treatment, time and/or the treatment × time interaction were considered to be fixed factors, and replicate and replicate × treatment were included in the model as random factors. Pairwise comparisons were made using Fisher's protected least significant difference test and $P < 0.05$ was considered to be a significant difference. All means are presented ± s.e.m.

Results

Experiment 1: spontaneous maturation

Cumulus–oocyte complexes were cultured in maturation medium (control) or maturation medium supplemented with 100 μM IBMX, 100 μM forskolin or a combination of IBMX and forskolin (100 μM of each). After 6, 12 and 24 h of culture the stage of meiosis was evaluated to determine the effects of IBMX and forskolin on spontaneous maturation. Time points in this experiment were chosen to determine the timing of spontaneous GVBD and the proportion of oocytes reaching MII. After 6 h of culture, more than 90% of oocytes contained a germinal vesicle (GV) regardless of the treatment. After 12 h, the majority of control oocytes (82.8 ± 7.0%) and those cultured in IBMX (100.00 ± 0.0%) or IBMX + forskolin (97.7 ± 2.3%) remained at the GV stage; however, 32.3 ± 5.1% of the oocytes cultured with forskolin alone had progressed to metaphase I (MI). After 24 h of culture, forskolin increased (81.7 ± 5.7%, $P < 0.05$) and IBMX decreased (6.7 ± 3.4%, $P < 0.05$) the proportion of oocytes at MII relative to control oocytes (42.0 ± 6.8%, Fig. 1). Although the combination of IBMX and forskolin decreased ($P < 0.05$) the proportion of GV oocytes at 24 h (53.8 ± 10.5%) compared with IBMX alone (84.7 ± 2.1%), the proportion of MII oocytes at 24 h (16.5 ± 1.9%) was still lower ($P < 0.05$) than that observed for control oocytes or oocytes cultured with forskolin (Fig. 1).

Experiment 2: eCG+EGF-induced maturation

Cumulus–oocyte complexes were cultured in maturation medium containing 1.0 IU mL^{-1} eCG and 25 ng mL^{-1} EGF (Herrick *et al.* 2010) without (control) or with 100 μM IBMX. After 18, 24 or 30 h of culture the stage of meiosis was evaluated to determine the effects of IBMX on eCG+EGF-induced maturation. Time points in this experiment were chosen to determine the proportions of oocytes that reached the MII stage of meiosis. At all culture times evaluated, IBMX in the presence of eCG+EGF increased ($P < 0.05$) the proportion of oocytes at the GV stage (Fig. 2*a*) and decreased ($P < 0.05$) the proportion

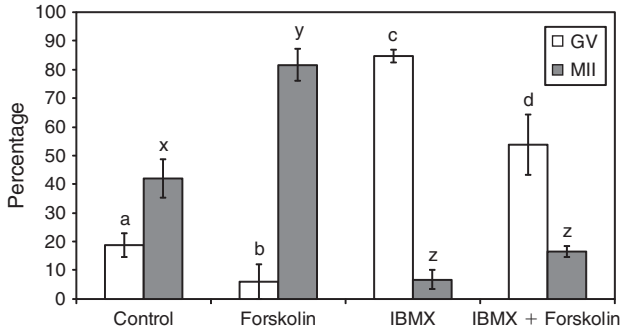

Fig. 1. Percentage of oocytes at the germinal vesicle (GV) or metaphase II (MII) stage of meiosis following 24 h of culture in maturation medium alone (control) or maturation medium supplemented with forskolin (100 μM), IBMX (100 μM) or both IBMX and forskolin (100 μM of each). A total of 41 to 73 oocytes were examined for each treatment (three to six replicates per treatment). Different superscripts indicate significant differences ($P < 0.05$) between treatments within each stage of meiosis.

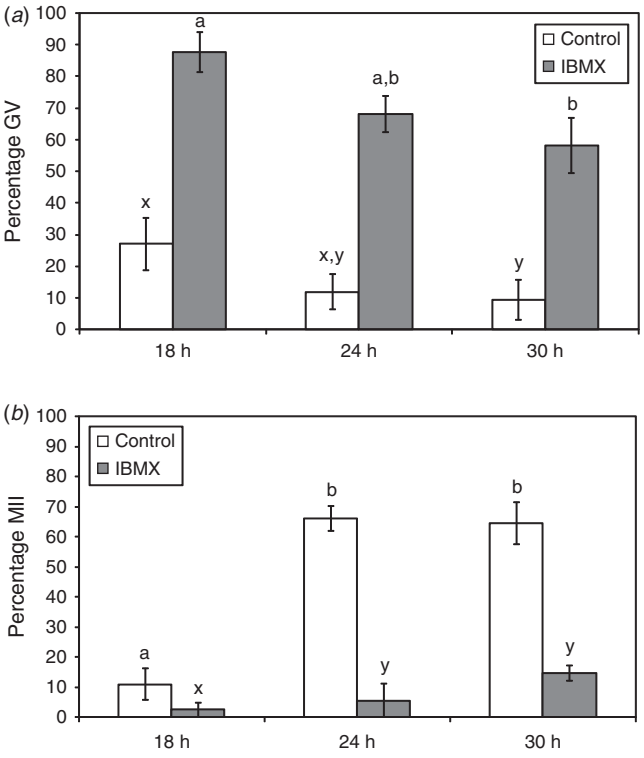

Fig. 2. Percentage of oocytes at (*a*) the germinal vesicle (GV) or (*b*) metaphase II (MII) stage of meiosis following 18, 24 or 30 h of culture in maturation medium containing 1.0 IU mL^{-1} eCG and 25 ng mL^{-1} EGF without (control) or with IBMX (100 μM). A total of 41 to 65 oocytes were evaluated for each treatment and time (four to five replicates). Different superscripts indicate significant differences ($P < 0.05$) between time points for the same treatment. For all time points, the proportion of GV (IBMX > Control) or MII (Control > IBMX) oocytes was different ($P < 0.05$) between treatments.

of MII oocytes (Fig. 2*b*) compared with control (eCG+EGF only) oocytes. After 30 h of culture in the presence of eCG+EGF, 64.4 ± 7.0% of control oocytes and 14.6 ± 2.5% of oocytes exposed to IBMX were at the MII stage. In contrast, 9.4 ± 6.2% of control oocytes and 58.1 ± 8.7% of oocytes exposed to IBMX were at the GV stage.

Experiment 3: intracellular cAMP content

Denuded oocytes were recovered from COCs that were cultured for 12 h in maturation medium (control) or maturation medium containing 100 μM IBMX, 100 μM forskolin or 1.0 IU mL^{-1} eCG and 25 ng mL^{-1} EGF. The intracellular concentrations of cAMP in oocytes that had been exposed to IBMX, forskolin or eCG+EGF for 12 h were not different ($P > 0.05$) from control oocytes (Table 2). However, the highest ($P > 0.05$) concentrations of cAMP were found in oocytes that had been exposed to IBMX (62.1 ± 11.7 fmol cAMP per oocyte).

Table 2. Intracellular concentrations of cAMP in feline oocytes after 12 h of IVM

[a]Identical superscripts indicates no significant difference ($P > 0.05$) between treatments

Treatment	n[A]	Cyclic AMP (fmol per oocyte)
Control	6	56.0 ± 10.2[a]
IBMX	6	62.1 ± 11.7[a]
Forskolin	7	46.0 ± 3.3[a]
eCG + EGF	6	41.4 ± 7.0[a]

[A]Pools of 8 to 22 oocytes.

Experiment 4: reversibility of IBMX-induced meiotic arrest

Cumulus–oocyte complexes were cultured for 12 h in maturation medium (control) or maturation medium with IBMX (100 μM), eCG (1.0 IU mL^{-1}) and EGF (25 ng mL^{-1}), or IBMX, eCG and EGF. All COCs were then washed twice in 500 μL of maturation medium and cultured with eCG (1.0 IU mL^{-1}) and EGF (25 ng mL^{-1}) for an additional 12, 18 or 24 h (24, 30 or 36 h of total culture) to determine the reversibility of IBMX inhibition of meiosis. After 12 h of culture in the control medium (no IBMX, eCG or EGF) and an additional 12 h in the presence of eCG+EGF (24 h total culture), 40.9 ± 8.2% of oocytes had reached the MII stage (Fig. 3). After 18 or 24 h of culture with eCG+EGF (30 or 36 h of total culture time), the proportion of MII oocytes increased ($P < 0.05$) to 67.8 ± 6.6% and 86.0 ± 4.9%, respectively. If oocytes were cultured with eCG+EGF for the entire culture period, the proportion of MII oocytes was similar ($P > 0.05$) after a total of 24 (70.2 ± 5.9%), 30 (87.9 ± 3.0%) or 36 h (78.3 ± 5.4%) of culture (Fig. 3). When oocytes were cultured with IBMX for 12 h and then an additional 12 h with eCG+EGF (no IBMX), 26.9 ± 3.3% of oocytes remained at the GV stage and only 6.6 ± 6.6% of oocytes had progressed to the MII stage (Fig. 3). The proportion of MII oocytes continued to increase ($P < 0.05$) after 18 h (49.4 ± 6.8%) and 24 h (66.1 ± 8.2%) of culture with eCG+EGF. When oocytes were exposed to IBMX, eCG+EGF during the first 12 h of culture and then only eCG+EGF (no IBMX) for 12 h, 8.3 ± 4.8% of oocytes were at the GV stage and 13.3 ± 6.7% of oocytes were at the MII stage (Fig. 3), with the majority of oocytes (47.6 ± 14.2%) at the MI stage (data not shown). The proportion of MII oocytes continued to increase ($P < 0.05$) as the total time of culture was extended to

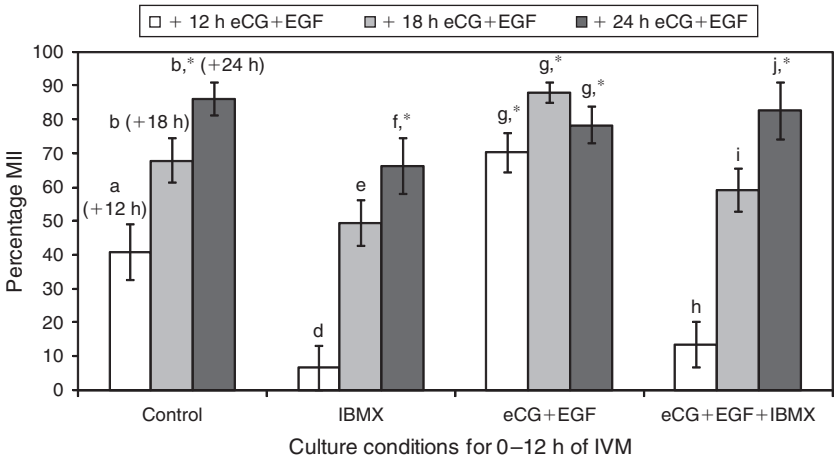

Fig. 3. Percentage of metaphase II (MII) oocytes following 24, 30 or 36 h of culture. Cumulus–oocyte complexes were cultured for 12 h in maturation medium with no supplements (control), IBMX (100 μM), eCG (1.0 IU mL^{-1}) and EGF (25 ng mL^{-1}), or eCG, EGF and IBMX. All COCs were then washed and transferred to medium containing eCG and EGF (no IBMX) for an additional 12, 18, or 24 h of culture. For each treatment and time combination 53 to 68 oocytes were examined (three replicates). [a–j]Different superscripts indicate significant differences ($P < 0.05$) between time points within the same treatment. *Indicates no significant difference ($P > 0.05$) from oocytes cultured continuously with eCG and EGF for a total of 24 h.

Table 3. Embryonic development of IBMX-exposed oocytes
No significant differences ($P < 0.05$) were observed

IVM	Cleavage (%)	Blastocyst (%)		Blastocyst cell number
		Per oocyte	Per cleaved embryo	
24 h eCG+EGF ($n = 130$)[A]	83.0 ± 6.7	18.1 ± 5.3	22.1 ± 6.4	104.2 ± 7.7
12 h IBMX, 24 h eCG+EGF ($n = 132$)[A]	79.9 ± 8.7	15.3 ± 3.0	19.5 ± 3.6	121.6 ± 9.1

[A]Total number of oocytes cultured (20 to 38 oocytes per replicate) in four replicates.

30 h (59.2 ± 6.4%) and 36 h (82.5 ± 8.4%). Importantly, after 12 h of culture with IBMX (alone or in combination with eCG+EGF) and an additional 24 h of culture with eCG+EGF (no IBMX), the proportion of MII oocytes was not different ($P > 0.05$) from oocytes cultured with eCG+EGF for 24 h.

Experiment 5: effects of IBMX-induced meiotic arrest on embryonic development

Cumulus–oocyte complexes were cultured in maturation medium with IBMX (100 μM) or eCG (1.0 IU mL^{-1}) and EGF (25 ng mL^{-1}) for 12 h. All COCs were then washed twice in 500 μL of maturation medium and cultured with eCG (1.0 IU mL^{-1}) and EGF (25 ng mL^{-1}) for 12 h (COCs that were previously cultured with eCG+EGF) or 24 h (those COCs previously exposed to IBMX). After a total of 24 h in the presence of eCG+EGF, COCs were processed for *in vitro* fertilisation and resulting embryos were cultured to evaluate embryonic development. Exposure to IBMX for 12 h, followed by 24 h of culture with eCG+EGF, did not affect ($P > 0.05$) the proportion of oocytes that cleaved (79.9 ± 8.7%) or the proportion developing to the blastocyst stage (15.3 ± 3.0% of oocytes or 19.5 ± 3.6% of cleaved embryos) following IVF compared with oocytes that were matured for 24 h with eCG+EGF without any exposure to IBMX (83.0 ± 6.7% cleaved, 18.1 ± 5.3% blastocysts per oocyte and 22.1 ± 6.4% blastocysts per cleaved embryo, Table 3). The total number of cells in the resulting blastocysts was also not affected ($P > 0.05$) by exposure to IBMX (121.6 ± 9.1 cells vs control, 104.2 ± 7.7 cells).

Discussion

This series of experiments represents the first attempt to manipulate the kinetics of meiosis in oocytes from the domestic cat, or any other carnivore, using chemicals that alter the production or degradation of intracellular cAMP. Although many of the observed effects were consistent with studies of other species, several unique responses were observed in feline oocytes, including the stimulation of meiosis by forskolin. Such species-specific effects emphasise the need for comparative studies, especially in taxa like *Carnivora*, where a variety of reproductive mechanisms exist and basic information concerning gamete and early embryo biology is limited (Desmarais *et al.* 2004; Songsasen and Wildt 2007; Bateman *et al.* 2009; Wildt *et al.* 2010). Such studies will expand our understanding of reproductive biology, as well as allow for the development of alternative model species for studies of various reproductive processes.

Since the goal of many new *in vitro* maturation systems is to slow or inhibit spontaneous maturation, and the incidence of spontaneous maturation varies between species and culture conditions, it is important to characterise the incidence of spontaneous maturation of feline oocytes in each IVM system. In cattle and mice nearly all oocytes will resume meiosis and the majority (60 to 80%) of those oocytes will progress to MII whether gonadotrophins are present in the medium or not (Sirard and First 1988; Downs *et al.* 2002; Thomas *et al.* 2002; Ali and Sirard 2002). In contrast, reports for porcine oocytes suggest that ~75% of oocytes will undergo GVBD (Laforest *et al.* 2005) but only about half (54%) will achieve metaphase II (Funahashi and Day 1993) in the absence of exogenous gonadotrophins. In our study, only 42% of oocytes cultured in the absence of eCG+EGF reached MII within 24 h of culture, which is similar to the results of Wood *et al.* (1995). Other studies have reported 0 (Godard *et al.* 2009) to 21% (Luvoni *et al.* 2006) of feline oocytes undergoing spontaneous maturation and reaching MII, suggesting that other factors influence spontaneous maturation aside from the presence of gonadotrophins or growth factors. For example, the amount and type of metabolic substrates available in the culture medium influence the rate of spontaneous maturation in rodents and cattle in a species-specific manner (Bilodeau-Goeseels 2006; Downs 2011). In addition, intact COCs can consume a large amount of carbohydrates during *in vitro* maturation (Sutton-McDowall *et al.* 2004; Herrick *et al.* 2006*b*; Stokes *et al.* 2008). These COCs are often cultured in groups in a relatively small volume of medium, so that the metabolism of the COC (e.g. consumption of glucose and production of lactate) can have a significant impact on the composition of the medium and the amount of nutrients available to the COC. Therefore, differences in the rate of spontaneous maturation between laboratories for the same species may depend on the choice of maturation medium, as well as the number of COCs present and the volume of medium used for culture.

As expected, IBMX effectively inhibited spontaneous maturation in feline oocytes, such that 85% of the oocytes remained at the GV stage after 24 h of culture and only ~12% of oocytes had progressed to MI or MII. Similarly, IBMX significantly inhibited eCG+EGF-stimulated meiosis with 68% of oocytes remaining at the GV stage and only 22% of oocytes reaching MI or MII by 24 h of culture. Culturing bovine oocytes with PDE inhibitors only slows GVBD and the progression of meiosis by ~2 h (Thomas *et al.* 2002, 2004*a*, 2004*b*). However, culturing feline oocytes for an additional 6 h (30 h total) with IBMX, eCG and EGF did not increase the proportion of MII oocytes,

suggesting that meiosis was arrested. Feline oocytes also appeared to be highly sensitive to IBMX since 100 μM maintained the majority of oocytes at the GV stage, similar to observations in murine oocytes (Downs *et al.* 1988; Downs 2011). This is in contrast to studies of bovine and porcine oocytes, in which much higher concentrations of IBMX (500 to 5000 μM) had less pronounced effects on meiosis (Bilodeau *et al.* 1993; Fan *et al.* 2002; Laforest *et al.* 2005). The lower concentration of IBMX needed to maintain meiotic arrest may be indicative of relatively low PDE activity within the feline oocyte, which would be consistent with the relatively low rate of spontaneous maturation in feline oocytes.

In contrast to the expected results achieved with IBMX, forskolin significantly stimulated the resumption of meiosis and progression to MII in feline oocytes. After 12 h of culture, forskolin had stimulated GVBD in half of the oocytes, while the majority (>80%) of control or IBMX-exposed oocytes remained at the GV stage. By 24 h, the proportion of forskolin-exposed oocytes that reached MII was similar to the results of subsequent experiments using eCG and EGF. To the best of our knowledge, this response is unique to feline oocytes. In mice (Sato and Koide 1984; Chen *et al.* 2009), rats (Dekel *et al.* 1984; Racowsky 1984), hamsters (Racowsky 1985*a*), pigs (Racowsky 1985*b*) and cattle (Sanbuissho *et al.* 1992; Bilodeau *et al.* 1993) continuous exposure to forskolin, in doses similar to those tested in our study, significantly inhibits GVBD and the progression of meiosis in intact COCs. These differences between species are likely to be related to differences in adenylate cyclase and PDE activity within the oocyte. In most species, exposure to gonadotrophins or forskolin increases production of cAMP within the cumulus cells, which activates type II protein kinase A (PKA) and stimulates cumulus expansion (Bornslaeger and Schultz 1985; Downs and Hunzicker-Dunn 1995; Thomas *et al.* 2002; Lastro *et al.* 2006). Increased cAMP production and activation of type II PKA within the cumulus cells can also lead to the resumption of meiosis, but this stimulatory effect is dependent on the concentration of cAMP and the associated activity of type I PKA within the oocyte (Downs and Hunzicker-Dunn 1995). If the adenylate cyclase in the oocyte is highly sensitive to forskolin, or PDE activity in the oocyte is low, continuous exposure to forskolin would lead to elevated cAMP within the oocyte, stimulation of type I PKA, and meiotic arrest. In contrast, if the adenylate cyclase in the oocyte is only minimally responsive to forskolin or PDE activity in the oocyte is high, the concentration of cAMP in the oocyte would remain low, type I PKA activity would be low, and the stimulatory signal from the cumulus cells could lead to GVBD. This type of compartmentalised response is evident in the differential effects observed after exposing feline COCs to forskolin or eCG+EGF alone or in combination with IBMX. Both forskolin and eCG+EGF stimulated meiosis when used alone, presumably by stimulating cAMP production and type II PKA activity in the cumulus cells. However, when these chemicals were used in combination with IBMX, which maintained or elevated intra-oocyte concentrations of cAMP, meiosis was only minimally stimulated. To further support this model, significant cumulus cell expansion was observed (data not shown) in feline COCs exposed to IBMX and forskolin or IBMX and eCG+EGF, indicating that these chemicals did elicit a response in the COC even though the majority of the oocytes remained arrested in meiosis.

Despite consistent, significant effects of IBMX and forskolin on meiosis, measured concentrations of cAMP in oocytes that were exposed to IBMX, forskolin or eCG+EGF for 12 h were not significantly affected. However, cAMP concentrations were numerically higher in oocytes exposed to IBMX, in which meiosis was arrested (>80% GV), compared with oocytes exposed to forskolin or eCG+EGF, in which a large portion of oocytes had undergone GVBD (<52% GV). Such discrepancies between effects on meiosis and intracellular concentrations of cAMP are not uncommon. Thomas *et al.* (2002) reported that 50 μM milrinone (PDE3 inhibitor) decreased the proportion of denuded oocytes reaching metaphase II and increased the proportion of oocytes arrested at the GV stage, although the same concentration of inhibitor failed to significantly alter cAMP concentrations within the oocytes. Similarly, Bilodeau *et al.* (1993) demonstrated that 500 μM IBMX increased the proportion of GV bovine oocytes after 8 h of culture and 2000 μM IBMX decreased the proportion of MII oocytes after 24 h, but neither dose of IBMX had an effect on the cAMP content of COCs or denuded oocytes. It may be that oocytes are highly sensitive to cAMP and even small changes in intracellular concentrations of cAMP that are not statistically significant can have biologically significant effects on the oocyte. Detecting these subtle differences may be further complicated by the large variability in COC quality and cAMP content when oocytes or cumulus cells are recovered from follicles at different stages of maturation (Lastro *et al.* 2006; Bagg *et al.* 2009; Sasseville *et al.* 2009). Finally, it is necessary to maintain cAMP concentrations within the oocyte during sample collection and processing before the assay. This is often accomplished by including IBMX in the collection medium, since the majority of PDE activity in mammalian oocytes is due to PDE3, which is sensitive to IBMX (Downs *et al.* 1989; Conti *et al.* 1998; Richard *et al.* 2001; Jensen *et al.* 2002; Thomas *et al.* 2002; Liang *et al.* 2005; Nogueira *et al.* 2006; Sasseville *et al.* 2009). Although IBMX was capable of inhibiting enough PDE activity to maintain meiotic arrest in feline oocytes, it is possible that other IBMX-insensitive PDEs are present and active in feline oocytes and capable of degrading some cAMP before analysis. Additional research to characterise the type and activity level of all PDEs in feline oocytes may allow for better selection of PDE inhibitors. Collecting oocytes from a more homogeneous population of follicles and the use of alternative PDE inhibitors could facilitate more accurate measurements of cAMP concentrations.

One reason for manipulating the kinetics of meiosis during *in vitro* maturation is the potential to improve subsequent embryonic development of the oocytes following *in vitro* fertilisation and embryo culture. This technique is perhaps most widely used for porcine oocytes in which dibutyryl cAMP or PDE inhibitors are used for the first 20 to 24 h of *in vitro* maturation (Funahashi *et al.* 1997; Shimada and Terada 2002; Somfai *et al.* 2003; Kawashima *et al.* 2008). For bovine oocytes, improved embryonic development is observed when COCs are exposed to conditions that elevate intracellular cAMP during the recovery and selection of the COCs and the first few hours of

in vitro maturation (Luciano *et al.* 2004; Thomas *et al.* 2004*b*; Albuz *et al.* 2010). Exposure to PDEs before *in vitro* maturation also delays the initiation of meiosis and improves the proportion of oocytes reaching metaphase, metaphase spindle morphology and embryonic development in mouse and human oocytes (Nogueira *et al.* 2006; Vanhoutte *et al.* 2008; Albuz *et al.* 2010). In general, inclusion of PDEs, adenylate cyclase stimulators or cell-permeable cAMP analogues tends to improve embryonic development by maintaining gap-junction communication between the oocyte and the cumulus cells and temporarily arresting or slowing GVBD (Shimada and Terada 2002; Luciano *et al.* 2004; Thomas *et al.* 2004*a*; Ozawa *et al.* 2008; Albuz *et al.* 2010). Although exposing feline COCs to IBMX was effective at blocking the resumption of meiosis, no improvements were observed for embryonic development (cleavage, blastocyst development or blastocyst total cell number) following IVF. However, it is important to point out that no negative effects on embryonic development were observed either.

There are several possible reasons for different effects of meiotic arrest on embryonic development between our study and those of other species. Since many of the benefits of increased intracellular cAMP are attributed to a temporary arrest or delay of meiosis, it would not be surprising if these methods had more pronounced benefits on species with higher rates of spontaneous maturation, like the mouse and the cow, compared with the cat. Alternatively, it may be necessary to determine the most appropriate amount of time to arrest or delay meiosis for each species. In pigs, COCs are typically exposed to cAMP for 20 to 24 h, which is half of the total maturation period (Funahashi *et al.* 1997; Kawashima *et al.* 2008). In contrast, the beneficial effects of increased intracellular cAMP in bovine oocytes have been attributed to delaying GVBD for only 2 to 4 h (Thomas *et al.* 2004*a*, 2004*b*; Albuz *et al.* 2010). Another important variable is the presence or absence of gonadotrophins and growth factors (eCG and EGF in our system) during the period of meiotic arrest. Some protocols require moving the COCs from a medium containing the meiosis arresting agent(s) to a fresh medium without these chemicals at a specific time of culture (Funahashi *et al.* 1997; Somfai *et al.* 2003; Kawashima *et al.* 2008; Shu *et al.* 2008). Other protocols expose COCs to meiosis-arresting agents at the same time as gonadotrophins or growth factors and maturation proceeds as the stimulatory signal of the gonadotrophins and growth factors overcomes the inhibitory cAMP signal (Downs *et al.* 1988; Thomas *et al.* 2004*b*; Albuz *et al.* 2010). The presence of eCG and EGF during the 12 h of exposure to IBMX did not affect the proportion of feline oocytes reaching MII after 24 h of culture with eCG+EGF, but effects on embryonic development were not assessed. Finally, the most appropriate time for fertilisation relative to the time the oocyte reaches MII must be identified. In bovine oocytes, the majority of cAMP-exposed oocytes have reached MII by 24 h of culture, but improved embryonic development is only observed when IVF is delayed until 28 to 30 h of culture (Thomas *et al.* 2004*b*; Albuz *et al.* 2010). Improving the developmental competence of feline oocytes by manipulating cAMP concentrations and the kinetics of meiosis during IVM may require optimisation of these additional variables.

In conclusion, this study has demonstrated that a non-specific PDE inhibitor, IBMX, effectively inhibits spontaneous and eCG+EGF-induced meiosis in feline COCs. In contrast, exposure to an adenylate cyclase stimulator, forskolin, stimulates the resumption of meiosis in feline COCs. The inhibitory effects of IBMX are completely reversible after 12 h of exposure, with the majority of arrested oocytes reaching metaphase II within 24 h of removal of IBMX. Finally, embryonic development of arrested, matured oocytes following IVF was not different from oocytes matured without exposure to IBMX. These results provide an effective protocol for reversible inhibition of meiosis in feline oocytes, which may lead to improved embryonic development with further optimisation of the culture conditions used for the period of meiotic arrest.

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