Transporting cumulus complexes using novel meiotic arresting conditions permits maintenance of oocyte developmental competence

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Abstract

Purpose The aim of this study is to evaluate the effect of a novel bovine cumulus oocyte complex (COC) shipping media designed to arrest meiotic resumption during transport on meiotic arrest, as well as meiotic resumption, subsequent embryonic development, and embryo quality.

Methods Bovine cumulus oocyte complexes were transported overnight from the collection facility to the laboratory. COCs were placed in control in vitro maturation (IVM) or in shipping arrest medium (SAM) containing multiple meiotic inhibitors, and then shipped to our laboratory. Upon arrival, meiotic status was assessed, control COCs were inseminated, and arrested COCs were matured and inseminated the next day. Embryonic development and quality were analyzed.

Results When bovine COC arrived at the laboratory after overnight shipment (21 h) in SAM, the majority of oocytes remained at the GV stage ($75.6 \pm 2.9\%$ GV). Arrested oocytes successfully resumed and completed meiosis during IVM after removal from SAM ($96.8 \pm 0.5\%$ metaphase II compared to control $88.3 \pm 5.0\%$). Moreover, the development of blastocysts per COC was not different from control ($22.3 \pm 2.4\%$ for control and $18.7 \pm 2.1\%$ for SAM), nor was any difference detected in blastocyst quality as determined by cell number and allocation.

Conclusions Our study demonstrates that a physiological system incorporating cyclic adenosine monophosphate and cyclic

guanosine monophosphate modulators can be used to maintain meiotic arrest followed by successful nuclear maturation and pre-implantation embryo development equal to control IVM-derived embryos. Our results offer promising insights for the development of pre-IVM media that may improve oocyte developmental competence in vitro.

Keywords In vitro maturation · Oocyte competence · Meiosis · Transport

Introduction

In 1935, Pincus and Enzmann described in vitro maturation (IVM) of mammalian oocytes for the first time, and showed that spontaneous meiotic resumption occurs as soon as the oocyte is removed from its follicular niche [1]. Since that time, IVM has not become a routine technique in mammalian assisted reproductive technology (ART) outside of the research laboratory, due to reduced embryo development and pregnancy outcomes following IVF of IVM oocytes [2–4]. In bovine, one of the reasons that may explain low developmental competence of in vitro matured oocytes is the use of a heterogeneous population of oocytes collected from antral follicles at different stages of their growth [5]. Another factor contributing to poor results following IVM may be the use of inadequate in vitro maturation conditions that do not support oocyte competence [6, 7]. However, human IVM could be a beneficial alternative to conventional IVF. It reduces the adverse effects of exogenous hormones used to stimulate follicle growth, and is time and cost-effective compared to standard IVF procedures [4, 8, 9].

Because of its potential benefit to infertility patients in human clinic ART, as well as agricultural and biomedical

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applications, much research has gone into the development of protocols and culture systems to improve IVM efficiency, reviewed in [10, 11]. Although IVM promotes nuclear maturation, it fails to support cytoplasmic maturation and thus subsequent fertilization, embryo development, implantation, and pregnancy are compromised [6, 12, 13]. Unfortunately, cytoplasmic maturation remains poorly understood. In bovine, one approach to improve oocyte competence has been to utilize conditions that arrest or delay spontaneous meiotic resumption during a short period, prior to IVM, to potentially synchronize nuclear and cytoplasmic maturation and improve oocyte competence [14–24]. A short period of meiotic arrest prior to IVM using IBMX (a broad phosphodiesterase (PDE) inhibitor) and forskolin (FSK; a broad adenylate cyclase activator), called “SPOM” (simulated physiological oocyte maturation), has been shown to improve developmental competence of bovine oocytes [14]. However, these results have been inconsistent [25], due in part to discrepancies in the SPOM protocol across laboratories [26]. In bovine cumulus oocyte complex (COC), pre-IVM treatment with cyclic adenosine monophosphate (cAMP) activator/modulator (FSK + IBMX) enhances the cumulus cell contribution to oocyte antioxidant defense and improves oocyte quality via gap junction communication [27]. Recently, inhibition of meiotic resumption in the presence of physiologically relevant reagents (C-type natriuretic peptide (CNP), estradiol) instead of synthetic reagents like forskolin and broad PDE inhibitors has been shown to better enable normal functions of the rat COC in vitro [28], maintaining gap junction activity and supporting key gene expression critical for oocyte maturation.

The objective of our experiments was to develop a shipping medium for bovine COC in which nuclear maturation of the oocyte would be arrested using a physiological approach. We hypothesized that considering the reported beneficial effect of short pre-IVM systems, arresting meiosis during shipment of COC may improve oocyte developmental competence.

Materials and methods

Both oocyte collection and shipping medium contain an inhibitor of cAMP degradation (a PDE3 specific inhibitor: cilostamide) and an inhibitor of cyclic guanosine monophosphate (cGMP) degradation (a PDE5 specific inhibitor: sildenafil) as well as CNP and estradiol. CNP stimulates the production of cGMP and estradiol help maintains CNP receptors on cumulus cells in many species [29, 30]. After collection and selection, COCs were placed into a defined (no follicular fluid or serum) shipping medium in the presence of CNP, estradiol, BMP15, and a low concentration of FSH. Estradiol has been shown to maintain CNP receptors on cumulus/granulosa cells in different species; CNP

stimulates the production of cGMP; and BMP15 and low concentration of FSH help keep gap junctions open [29–32], which is critical to promote transfer of molecules that prevent meiotic resumption (reviewed in [33]). Cilostamide, sildenafil, and hypoxanthine (a naturally occurring cAMP-phosphodiesterase inhibitor [21, 34, 35]) were also included.

Unless specified otherwise, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Bovine COC were cultured in 5% CO₂ in air during pre-IVM shipment, and 7.5% CO₂ in air for subsequent IVF followed by 7.5% CO₂ and 6.5% O₂ for embryo culture. The gas concentrations used for embryo culture (7.5% CO₂ and 6.5% O₂) are increased to compensate for the elevation of our laboratory (1830 m above sea level) and are equivalent to 6% CO₂ and 5% O₂ at sea level. All incubations were conducted 38.5 °C.

COC shipment and maturation

Bovine COCs were recovered from 2 to 8 mm follicles on abattoir derived ovaries (DeSoto Biosciences, Inc.; Seymour, TN). Control COCs were selected and washed in HEPES-buffered medium. Fifty COCs were placed in 2 ml of equilibrated IVM medium (Table 1) in 2.5-ml glass tubes. Tubes were sealed with parafilm and placed into a temperature controlled (38.5 °C) shipping container (Minitube International®) for overnight shipment. For meiotic arrest, COCs were collected, selected, and washed in HEPES-buffered medium in the presence of inhibitors of meiotic resumption (100 µM cilostamide, 100 µM sildenafil, 100 nM estradiol, 100 nM CNP). Fifty COCs were then placed into 2 ml of equilibrated shipping arrest medium (SAM) in 2.5 ml tubes, and shipped overnight with control COC. SAM is a modified version of the bovine IVM medium including meiotic inhibitors (100 µM hypoxanthine, 100 nM CNP, 100 µM cilostamide, 100 µM sildenafil, 100 nM estradiol, 100 ng/ml BMP15; 1 × 10^{−5} IU/ml rh-FSH) and without EGF. After 24 h of maturation, control COCs were fertilized in vitro. After 21 h of arrest, COCs in SAM were placed into IVM medium (5 COC/50 µl drops under oil) for 24 h in the laboratory, and fertilized the next day. Composition of IVM, IVF, in vitro culture (IVC) 1, and IVC 2 medium is presented in Table 1.

Oocyte nuclear maturation

To determine nuclear status, oocytes were fixed and stained using aceto-orcein as previously described [36]. Oocytes were classified into three groups: germinal vesicle (GV)-stage oocytes, corresponding to oocytes that did not mature; germinal vesicle breakdown (GVBD)/metaphase I (MI) stage oocytes (GVBD/MI stage), corresponding to oocytes that did resume meiosis but did not complete nuclear maturation; and metaphase II (MII) stage

oocytes, corresponding to oocytes that matured successfully. A total of 148 oocytes in control IVM, 157 in SAM 21 h, and 131 in SAM 21 h followed by 24 h IVM were studied in 3 replicates.

Bovine IVF and embryo culture

Cryopreserved bull spermatozoa were thawed and separated on a 45/90% discontinuous gradient (PureSperm®100; Nidacon). Spermatozoa were washed, counted, and diluted to a final concentration in the fertilization drop of 2×10^6 spermatozoa/ml. Ten COCs were added to the 50 µl drop of

fertilization medium (Table 1) under oil and coincubated with spermatozoa for 22 h.

After fertilization, COC were denuded by vortexing and cultured in IVC 1 medium (10 zygotes per 20 µl drop). Ninety-six hours post insemination, zygotes were checked for cleavage. Embryos with more than four cells were moved to IVC 2 medium (5 embryos/20 µl). After 7 days of culture (D7; 174 h), development to the blastocyst stage was assessed and hatching/hatched blastocysts were fixed for subsequent determination of cell number and allocation. A total of 263 COCs in control and 362 COCs in SAM 21 h were studied in 3 replicates. The total number of blastocyst produced in the

Table 1 Composition of media used for bovine maturation, fertilization, and embryo culture

Reagent	Concentrations (mM)			
	IVM	IVF	IVC 1	IVC 2
NaCl	100	100	100	100
KCl	5	5	5	5
KH ₂ PO ₄	0.5	0.5	0.5	0.5
CaCl ₂ ·2H ₂ O	1.7	1.7	1.7	1.7
MgSO ₄	1.2	0.2	1.2	1.2
Bicarbonate	25	25	25	25
Glucose	5	0.5	0.5	–
Fructose	–	–	–	3
Pyruvate	0.4	0.4	0.3	0.1
L-Lactate	6	6	6	6
Ala-Gln	1	1	1	1
Citrate	0.5	–	0.25	0.5
Cysteamine	0.5	–	–	–
Cysteine	0.6	–	–	–
Taurine	5	0.1	0.1	0.1
Glycine	2.0	–	–	2
MEM-NEAA	1×	1×	0.25×	1×
MEM-EAA	0.5×	–	0.25×	0.5×
MEM vitamins	1×	–	–	1×
EDTA	–	–	5 µM	–
Insulin, transferrin, selenium (ITS)	(0.5 µg/ml I, 0.275 µg/ml T, 0.25 ng/ml S)	–	(0.5 µg/ml I, 0.275 µg/ml T, 0.25 ng/ml S)	(0.5 µg/ml I, 0.275 µg/ml T, 0.25 ng/ml S)
myo-Inositol	–	–	–	0.075
rh-FSH ^a	0.1 IU/ml	–	–	–
rm-EGF ^b	50 ng/ml	–	–	–
Caffeine	–	2	–	–
Heparin	–	7.5 µg/ml	–	–
Hyaluronan	0.25 mg/ml	–	0.125 mg/ml	0.125 mg/ml
rHSA ^c	2.5 mg/ml	–	–	–
FAF BSA	–	8 mg/ml	8 mg/ml	8 mg/ml
Gentamicin	50 µg/ml	50 µg/ml	25 µg/ml	25 µg/ml

^a Recombinant human FSH

^b Recombinant mouse epidermal growth factor

^c Recombinant human serum albumin

control treatment was 80 and in SAM 116. The total number of hatching/hatched blastocysts produced in the control treatment was 39 and in SAM was 48.

Embryo quality

Quantification of inner cell mass (ICM) and trophectoderm (TE) cells was performed on hatching or fully hatched blastocysts at D7 of development. Blastocysts were fixed in 4% paraformaldehyde. Antibodies against SRY (sex determining region Y)-box 2 (SOX2; AN579; Biogenex, Fremont, CA, USA) to detect ICM cells and CDX2 (caudal type homeobox 2) (MU392A-UC; Biogenex) to detect trophectoderm cells were used, as described previously [37]. Stained embryos were analyzed using an Olympus BX52 microscope and MetaMorph Microscopy Automation and Image Analysis Software. A total of 83 blastocysts for control IVM and 40 for SAM 21 h were used in 3 replicates.

Statistical analysis

Data were analyzed using NCSS software (Number Cruncher Statistical System, Kaysville UT, USA). For maturation, blastocyst development and cell number, a one-way ANOVA was performed with treatment as a fixed factor. A Bonferroni multiple comparison test was used. Differences were determined to be significant when $p < 0.05$.

Results

Meiotic arrest

When bovine COCs were shipped overnight (21 h) from the collection facility to the laboratory in SAM, most but not all of the oocytes remained at the GV stage ($75.6 \pm 2.9\%$ GV versus $1.4 \pm 0.9\%$ for control; Fig. 1). When matured in control IVM medium after shipment in SAM, arrested oocytes successfully resumed and completed meiosis ($96.8 \pm 0.5\%$ MII compared to control $88.3 \pm 5\%$; Fig. 1).

Embryo development and quality

Cleavage was not different from control ($59 \pm 1.7\%$ for control and $64 \pm 2.9\%$ for SAM). Development of bovine blastocysts on D7 of culture following meiotic arrest for 21 h in SAM and IVM/IVF was not different than development of control embryos produced following IVM during shipment (18.7 ± 2.1 and $22.4 \pm 2.4\%$ blastocyst per COC; 13.2 ± 0.9 and $14.5 \pm 2.8\%$ hatching/hatched blastocyst per COC, respectively) (Fig. 2a). Development per cleaved oocyte was also not different (30.3 ± 2.9 for control and 25.9 ± 2.1 for SAM; Fig. 2b).

Fig. 1 Analysis of bovine oocyte nuclear maturation after shipment in IVM medium or in shipping arrest medium (SAM) followed by 24 h IVM (control IVM ($n = 148$); 21 h SAM ($n = 157$); 21 h SAM + 24 h IVM ($n = 131$); three replicates). Data are presented as mean $\pm$ S.E.M.

To assess the quality of embryos produced after meiotic arrest, cell number and allocation were determined in D7 hatching/hatched blastocysts (Fig. 3a). There was no difference in blastocyst TE (CDX2 positive) or ICM (SOX2 positive) cell number between blastocysts derived from arrested or control oocytes (Fig. 3b). The proportion of ICM cells was also not significantly different between treatments (18.95 ± 1.2 versus $21.15 \pm 1.4\%$, respectively; Fig. 3c).

Discussion

Here, we described for the first time the impact of a shipping medium arresting oocyte meiotic resumption that targets cumulus cells, cAMP/cGMP signaling, and gap junctions on developmental competence of bovine oocytes. We show that the shipping arrest medium maintained meiotic arrest during overnight shipment without negatively affecting developmental competence. Our results will facilitate shipment of COC between production facilities and research laboratories as well as offer insights for the development of maturation systems that can improve oocyte developmental competence in vitro.

Modulation of cAMP concentrations within mammalian COC during maturation modestly improves oocyte developmental competence in several species [14, 17, 19, 24, 27, 38–47]. Modulation of cAMP before IVM, in a short pre-IVM period of meiotic arrest, also improves developmental competence [14, 20, 24, 27], although the mechanisms involved are unclear. To date, the use of pre-IVM culture systems to improve developmental competence has yielded inconsistent results, and this technology is not yet used routinely [25, 26]. In addition to the cAMP pathway, the CNP/cGMP pathway is a major player in the regulation of meiosis [31, 48–52]. Addition of physiologically relevant granulosa cell-

Fig. 2 Effect of meiotic arrest in shipping arrest medium (SAM), followed by IVM, IVF, and IVC on subsequent bovine embryo development, compared to control embryos matured during shipment. Development is reported per COC entered into SAM or control IVM

(a) and per cleaved embryo (b). Data is presented as mean $\pm$ S.E.M (SAM, $n = 362$ COC; Ctl, $n = 263$ COC; three replicates). No differences were detected between treatments

derived reagents like CNP and estradiol, rather than supplementation with pharmaceutical chemicals such as forskolin and PDE inhibitors, better enables normal physiological functions of the COC during meiotic arrest in vitro [28].

The arresting shipping system we developed is composed of both an HEPES-buffered medium for collection and selection of COC and a pre-equilibrated bicarbonate-buffered medium for shipment. The HEPES-buffered medium used to collect and select COC has been designed to maintain cAMP at high levels within the oocyte, taking advantage of the role of cGMP in this process, using PDE3 and PDE5 specific inhibitors (cilostamide and sildenafil), as well as preserve the signaling that maintains meiotic arrest with CNP and estradiol. In the shipping arrest medium (SAM), meiotic resumption was prevented by physiological signaling molecules that maintain meiotic arrest via gap junction-mediated communication between the cumulus cells and the oocyte (CNP

and estradiol with low concentrations of FSH and BMP15). In addition to these physiological arresting molecules, low concentrations of cilostamide, hypoxanthine, and sildenafil were also included in the shipping medium, as our preliminary studies found that multiple inhibitors were required to efficiently and consistently maintain arrest and preserve developmental competence in bovine COC. It is interesting to note that cAMP activators like Forskolin are not needed to maintain meiotic arrest in our system. Because the presence of high levels of cAMP in the oocyte are necessary to keep the oocyte in meiotic arrest [53–55], and most of the oocytes in our study were arrested after 21 h, this suggests that cAMP is maintained at elevated levels in oocytes during collection and shipment. The oocytes that did not maintain meiotic arrest may have been collected from follicles that were regressing or atretic, due to the heterogeneous population of follicles present on abattoir ovaries in the bovine. These COCs would have

Fig. 3 Cell number and allocation in D7 hatched/hatching bovine blastocysts produced after 21 h meiotic arrest in SAM followed by IVM, IVF, and IVC, compared to those produced from control IVM oocytes matured during shipment. Embryos were stained for SOX2 (ICM cells, green) and CDX2 (TE cells, red) using specific antibodies (a). The number of ICM and TE cells was determined (b), and the percentage of SOX2 positive (ICM) cells was calculated (c). Data are expressed as mean $\pm$ SEM ($n = 83$ for control and 40 for SAM; three replicates)

likely already lost gap junction communication between the cumulus cells and the oocyte, resulting in a decrease of cGMP/cAMP and initiating germinal vesicle breakdown prior to collection that could not have been reversed [43, 44].

To our knowledge, this is the first report using a pre-IVM medium incorporating physiological ligand/receptor molecules in the context of shipping COC. Our results demonstrate that oocytes can be maintained for 21 h in meiotic arrest during shipment without compromising oocyte quality. We specifically targeted PDE5 and PDE3 using the inhibitors cilostamide and sildenafil. In the bovine, PDE8 participates in cAMP regulation; however, the commercially available PDE8 inhibitor dipyridamole inhibits multiple other PDEs including PDEs 6, 7, 10, and 11. Moreover, in our system, dipyridamole was not able to consistently maintain meiotic arrest (data not shown).

In the SAM medium we have developed, the majority of bovine oocytes remain arrested for 21 h. We did observe that bovine oocyte arrest is exquisitely sensitive to perturbations in temperature and pH; improvements in the percentage of arrested oocytes in this medium were possible when these parameters were more carefully maintained in a steady state (data not shown). Although not an end point, we examined that arresting medium can impact chromatin condensation. Keeping the gap junctions open helps to maintain chromatin in a decondensed state [56, 57], which may help promote developmental competence of the oocyte. In this system, developmental competence of the oocyte is maintained, although it was not improved. Because these oocytes were obtained from abattoir derived ovaries and likely come from a heterogeneous population of follicles, it would be of interest to explore whether use of the SAM system in oocytes derived from known follicles by OPU would be more effective at maintaining meiotic arrest and/or improving oocyte quality. The ability to arrest oocytes from genetically valuable females during transportation from the farm to the laboratory is of particular interest for the bovine industry, even without quality enhancement. Also, because about 25% of the oocytes were not arrested in GV after shipment, it is possible that these oocytes undergo in vitro aging prior to IVF, and in that sense do not participate or participate poorly in the development of blastocysts [58]. If these non-arrested oocytes are numerically excluded from the analysis (about 25% of the oocytes) and only arrested oocytes considered, the shipping arrest medium may indeed be beneficial for development of the arrested oocytes following IVF. Further studies examining the effects, if any, of SAM on epigenetic modifications and fetal development are warranted as characterization and use of this approach increases.

In conclusion, our study demonstrates that a physiological system incorporating cAMP and cGMP modulators can be

used to maintain meiotic arrest during bovine oocyte shipment followed by successful nuclear maturation and pre-implantation embryo development equal to control IVM-derived embryos. Our results offer promising insights for the development of shipping media that may improve oocyte developmental competence in vitro, as well as suggesting a physiological approach to pre-IVM systems in general that could be applied to laboratory and agricultural species, as well as human ART.

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