Capacitation IVM improves cumulus function and oocyte quality in minimally stimulated mice

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Abstract

Purpose Oocyte in vitro maturation (IVM) is a patient-friendly reproductive technology but lower success rates than IVF have limited its uptake. Capacitation-IVM (CAPA-IVM) is an innovative new IVM system currently undergoing clinical evaluation. This study aimed to determine temporal effects of the pre-IVM phase of CAPA-IVM on cumulus function and oocyte developmental competence in mildly-stimulated mice.

Methods Immature cumulus oocyte complexes (COCs) derived from mildly stimulated (23 h PMSG) 28-day-old mice underwent pre-IVM for 0–24 h in medium containing c-type natriuretic peptide (CNP), E₂, FSH and insulin, prior to IVM (CAPA-IVM). The effect of pre-IVM duration on cumulus cell function and embryo development post-CAPA-IVM/IVF was assessed.

Results Day 6 blastocyst rate increased incrementally with increasing pre-IVM duration: $40.6 \pm 2.0\%$, $45.8 \pm 1.2\%$, $52.2 \pm 3.5\%$, $53.3 \pm 5.9\%$, and $59.9 \pm 2.5\%$ for 0, 2, 6, 12, and 24 h pre-IVM, respectively ($P < 0.01$). DNA content/COC, a measure of cumulus cell proliferation, was significantly higher with 24 h pre-IVM group compared to 0, 2, or 6 h pre-IVM ($P < 0.001$). Pre-IVM for 24 h significantly increased cumulus expansion and mRNA expression of matrix genes *Has2* and *Tnfrsf6* and *Areg* relative to no pre-IVM control ($P < 0.01$). Cumulus-oocyte gap-junctional communication (GJC) was maintained throughout 24 h pre-IVM ($P < 0.0001$), and GJC loss was slowed during the subsequent IVM phase, whilst meiotic resumption was accelerated ($P < 0.05$). Pre-IVM increased COC ATP and ADP content ($P < 0.05$), but not AMP, ATP/ADP, and energy charge.

Conclusion The pre-IVM phase of CAPA-IVM improves the quality of IVM oocytes in a temporally dependent manner and significantly influences cumulus cell function including increased cell proliferation, cumulus expansion, and prolonged cumulus-oocyte GJC.

Keywords Oocyte in vitro maturation (IVM) · Capacitation-IVM (CAPA-IVM) · Pre-maturation (pre-IVM) · c-type natriuretic peptide (CNP) · Cumulus-oocyte gap junction communication · Oocyte quality

Introduction

Oocyte *in vitro* maturation (IVM) is an assisted reproductive technology (ART) that involves collection of immature

cumulus-oocyte complexes (COCs) from unstimulated or short-term stimulated individuals followed by maturation to the metaphase II stage in vitro [1]. Human IVM was first reported in an unstimulated donor cycle in 1991 [2]. Over the subsequent 30 years, improvements to IVM have been incremental and even today IVM is not widely practiced. Nonetheless, numerous advantages to IVM exist over conventional IVF, including reduced or no use of gonadotrophins, lower cost, absence of OHSS, and increased convenience for patients with reduced daily injections of gonadotrophins and less endocrine and ultrasound monitoring. IVM is most widely practiced in East Asia, and in recent years, some significant advances have occurred that are improving the efficiency and uptake of IVM. In 2018, a retrospective study from Vietnam including 921 PCOS patients who underwent hCG-primed

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IVM reported a 32% live birth rate after the first embryo transfer [3]. Walls et al. [4] reported comparable live birth rates per frozen transfer from IVM versus conventional ICSI (34 vs 30%, respectively), although the cumulative live birth rate was higher for ICSI. Hence, in order for patients to benefit from the advantages of IVM, it is worthwhile taking advantage of the advances made in animal IVM to improve the efficiency of human IVM.

The most significant advances made over the past decade in animal IVM involve the use of prematuration culture systems (pre-IVM) prior to IVM (reviewed; [5]). These are well-established IVM technologies in animal IVM and traditionally have involved the use of cAMP modulators in pre-IVM [6, 7], but important recent developments of such approaches have used the cGMP modulator, c-type natriuretic peptide (CNP) [8, 9]. Notably, CNP is the natural follicular factor that maintains oocyte arrest at prophase I of meiosis (GV) within antral ovarian follicles prior to ovulation [10]. CNP is produced by mural granulosa cells and is secreted into follicular fluid, where it binds its receptor, natriuretic peptide receptor 2 (NPR2), which is primarily expressed on cumulus cells (CCs) to induce cGMP production [10–12]. Cyclic GMP enters the oocyte via gap junctions to inhibit phosphodiesterase 3A activity [13, 14], thus preventing the degradation of cAMP and maintaining oocytes under meiotic arrest.

To more closely mimic the mechanisms of *in vivo* meiotic arrest, pre-IVM systems consist of two stages: a pre-IVM stage that prevents spontaneous meiotic resumption, prolongs oocyte-CC gap junction communication (GJC) and promotes the acquisition of oocyte developmental competence, and an IVM stage to induce meiotic resumption and oocyte maturation [5]. Pre-IVM systems are specifically designed for oocytes collected from donors that have received minimal or no stimulation [15]. A CNP-mediated pre-IVM strategy has previously been shown to improve the developmental competence of oocytes in animals (mouse, goat, bovine) by maintaining oocyte-CC communication, inducing a shift in oocyte chromatin configuration, increasing oocyte diameter, mtDNA copy number, mitochondrial activity, ROS levels, and decreasing glutathione levels [8, 9, 16–18]. Recently, Sánchez et al. adapted CNP pre-IVM (CAPA-IVM) for use with human oocytes. The CAPA-IVM system improved oocyte maturation and quality leading to increased embryo development on day 3 in PCOS patients [19, 20]. These results suggest that the gap in livebirth rates between IVM and conventional IVF can be closed by pre-IVM systems such as CAPA-IVM. Currently, a large RCT in Vietnam comparing the effectiveness and safety of CAPA-IVM with controlled ovarian hyperstimulation/IVF is underway (Clinical trial number: NCT03405701) [21].

Although much is known about how cAMP-mediated pre-IVM improves oocyte quality [5], CNP signals somewhat differently and the mechanisms underpinning the improvement in the oocyte developmental competence from CNP

pre-IVM require further investigation. This is particularly important given CAPA-IVM is already in clinical use [21] and is likely to be taken up more widely. The quality and developmental competence of an oocyte are reflected in its ability to undergo meiosis, be fertilized, and give rise to a healthy offspring [22]. Oocytes and CCs are interconnected by an extensive network of gap junctions [23]. Bidirectional transfer between the oocyte and CCs via GJC is essential for oocyte growth, for enabling nutrients and other small molecules to transfer between them, together ensuring that the oocyte acquires the molecular machinery required to support early embryo development [5, 24]. Hence, it is clear that the CCs have substantial influence on oocyte biology. However, whilst a number of studies have examined the effects of CNP-mediated pre-IVM strategies on the oocyte [8, 9, 16–18], the consequences for CC function are largely unknown. In this study, we hypothesized that CAPA-IVM would impact cumulus cell function in a temporally dependent manner and that this would be associated with an improvement in oocyte quality. We used an established minimally primed mouse COC model mimicking short-term stimulated patients in the clinic. Focusing on the effect of CAPA-IVM on CC function, we evaluated CC proliferation, apoptosis, cumulus matrix expansion, GJC, and energy metabolism, in association with oocyte developmental competence.

Materials and methods

Unless specified otherwise, all chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

COC collection

All mouse procedures were approved by the UNSW Animal Ethics Committee (ethics number 17/105A) and followed the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. C57BL/6J female mice at 28 days old were minimally stimulated with 5 IU eCG (PMSG; Folligon, MSD, Australia) for 23 h [15]. Ovarian antral follicles were punctured with a 29-gauge needle in HEPES-buffered α minimum essential medium (α MEM, Gibco, Grand Island, NY, USA) supplemented with 3 mg/ml fatty acid-free bovine serum albumin (BSA; MP Biomedicals, Auckland, New Zealand), 50 μ g/ml gentamycin, and 100 μ M 3-isobutyl-1-methylxanthine (IBMX), and COCs were collected.

CAPA-IVM

CAPA-IVM is a novel biphasic IVM system [9, 19, 20] that encompasses pre-IVM and IVM phases, each containing biological additives as follows. For pre-IVM, immature COCs were cultured at 37 °C, 5% CO₂ in humidified air in

bicarbonate buffered α MEM (Gibco) supplemented with 3 mg/ml fatty acid-free BSA (CellMaxx, MP Biomedical, New Zealand), 50 μ g/ml gentamicin, 1 mg/ml fetuin, 2.5 mIU/ml recombinant human FSH (Puregon; Organon, Oss, The Netherlands), 5 ng/ml insulin, 10 nM oestradiol, and 25 nM CNP (Tocris Bioscience, Abingdon, UK) [9]. CNP maintains oocyte meiotic arrest for the duration of the pre-IVM phase of CAPA-IVM. To examine the effect of pre-IVM duration on cumulus cell and oocyte function, COCs were exposed to pre-IVM culture for either 0, 2, 6, 12, or 24 h, depending on the experimental design (Fig. 1). COCs (up to 50 COCs per well) were cultured in pre-equilibrated 500 μ l pre-IVM medium in 4-well culture dishes (NUNC, Thermo Scientific, Roskilde, Denmark) and incubated at 37 °C, 5% CO₂ in humidified air. Following pre-IVM culture, COCs underwent IVM in bicarbonate buffered α MEM supplemented with 3 mg/ml fatty acid-free BSA, 50 μ g/ml gentamicin, 1 mg/ml fetuin, 2.5 mIU/ml recombinant human FSH, 50 ng/ml recombinant mouse amphiregulin (mAREG, R&D Systems, Minneapolis, MN, USA), and 50 ng/ml recombinant mouse epiregulin (mEREG, R&D Systems). Duration of IVM culture depended on the experimental end-point assessed (Fig. 1).

In vitro fertilization and embryo culture

COCs exposed to pre-IVM for either 0, 2, 6, 12, or 24 h, followed by 17 h IVM were fertilized using sperm from CBB6F1 mice (≥ 10 weeks old). Sperm were first expelled from the cauda epididymis of both testes into a pre-equilibrated 900 μ l drop of fertilization medium (Research Fert, IVF Vet Solutions, Adelaide, Australia) supplemented with 4 mg/ml BSA for capacitation. After 1 h capacitation (37 °C, 5% CO₂, 5% O₂, and N₂ balance), 10 μ l of sperm was added to pre-equilibrated 90 μ l drops of fertilization medium supplemented with 4 mg/ml BSA containing COCs (up to 20 COCs per drop). Gametes were co-incubated for 4 h in a humidified atmosphere (37 °C, 5% CO₂, and 5% O₂ and N₂ balance), thereafter presumptive zygotes were denuded, washed three times in wash medium (Research Wash, IVF Vet Solutions) supplemented with 4 mg/ml BSA. Ten zygotes

were then placed into a 20- μ l drop of cleavage medium (Research Cleave, IVF Vet Solutions) supplemented with 4 mg/ml BSA under paraffin oil (Merck, Germany) in a humidified atmosphere (37 °C, 5% CO₂, 5% O₂, and N₂ balance). Cleavage rate was evaluated 24 h after IVF on day 2 (D2) and uncleaved oocytes were removed. Morula, blastocyst, and hatching rates were assessed on D4, D5, and D6 (Fig. 1). Between 38 and 90 COCs were used per time point in each experimental run and the experiment was replicated six times, totaling 383–441 COCs per time point assessed. IVM and embryo culture was performed under best practice culture conditions, including the use of serum-free media and atmospheric oxygen in the IVM phases and low oxygen for embryo culture.

Blastocyst differential staining

To assess the effect of pre-IVM treatments on subsequent quality of the embryos produced, blastocyst cell allocation (inner cell mass (ICM) and trophectoderm (TE)) was assessed by differential staining. On day 6 post-IVF, blastocysts in each group were processed using a differential staining method to determine ICM and TE cell numbers (Fig. 1), as previously described [25]. In brief, blastocysts were exposed to 0.5% Triton X-100 for 12–15 s, then incubated in 25 μ g/mL Hoechst 33342 in absolute ethanol overnight at 4 °C, before mounting in a glycerol drop on a siliconized slide. The number of ICM and TE cells was assessed using an Olympus BX51 fluorescence microscope (Olympus Corporation, Tokyo, Japan), whereby ICM stain blue and the TE stain pink.

Assessment of DNA content in COC

Following pre-IVM, COCs were placed in a microcentrifuge tube (15–20 COCs per sample), immediately snap frozen, and stored at –80 °C. DNA was extracted from thawed COCs by incubation 30 min at room temperature (with vortexing every 10 min) in an extraction buffer (400 μ l/sample, 50 mM phosphate buffer pH 7.5, 1.5 M NaCl, 1 mM phenylmethylsulfonyl fluoride). Samples were then centrifuged at 16,900g for 30 min at 4 °C and the supernatant was immediately used

Fig. 1 Outline of the study design. 23 h-primed COCs were either not subjected to pre-IVM (control, standard IVM) or to varying durations of pre-IVM culture medium containing CNP, E₂, FSH, and insulin (CAPA-

IVM). Study experimental end-points assessed in response to treatments are shown below the time-line

for DNA quantification. A separate quality control sample of mouse COCs was collected and prepared using the same extraction procedure. DNA concentration in COC samples was determined as a measure of cell count using a fluorometric analysis based method [26]. Calf thymus DNA (Invitrogen, Carlsbad, California, USA) was used as a standard for DNA quantification. The assay was performed using a 96-well clear polystyrene flat bottom microplate (Corning, New York, USA). The standard and quality control were assayed in triplicate and samples were assayed in duplicate. All samples were added (150 µl/well) to the plate first, followed by Hoechst 33342 (50 µl/well, 4 µg/mL). Fluorescence was read at 350 nm and 455 nm excitation and emission wavelengths, respectively, using the EnSpire Multimode Plate Reader (PerkinElmer, MA, USA). A third order polynomial (cubic) standard curve was used to interpolate the sample DNA concentration (GraphPad Prism (GraphPad Software, La Jolla, CA, USA)). The DNA concentration of each sample within respective groups was normalized to the number of COCs per sample and expressed as ng of DNA per COC. Between 8 and 11 DNA samples per time point were examined with each sample consisting of 15–20 COCs.

COC RNA extraction and RT-qPCR

Following pre-IVM + 6 h IVM, COC samples (50 COCs per treatment per replicate with 6 replicate experiments) were collected for quantification of gene expression. RNA was extracted using miRNeasy Micro Kit (QiaGen, Hilden, Germany) according to the manufacturer's instructions. The final RNA concentrations were determined by absorbance using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Scoresby, Australia). An equal amount of RNA was reverse transcribed across all samples. Cumulus expansion genes (*Has2*, *Tnfrsf6*, *Ptgs2*, and *Ptx3*), EGF signalling genes (*Egfr*, *Areg* and *Ereg*) and apoptosis genes (*Bax* and *Bcl-2*) were assessed by RT-qPCR, as previously described [27]. The relative gene expression values were calculated using the comparative Ct (ΔCt) methods and normalized to the geometric mean of two housekeeping genes (*Mpl19* and *Ppia*). The sequences of the primers are listed in Table 1.

Cumulus matrix expansion scoring

At the end of 17 h IVM culture following 0 h or 24 h pre-IVM, blinded scoring of COC cumulus expansion was performed using the system reported by Vanderhyden, et al [28]. A score of 0 indicates no detectable response, + 1 indicates the minimum observable response, with a score of + 2 the outer layers of CCs have begun to expand, + 3 all layers have expanded except the corona radiata, and a score of + 4 indicates expansion of all CC layers. 25–46 COCs were scored individually per treatment per replicate and the experiment was replicated twice.

Assessment of CC-oocyte GJC

The functionality of GJC between the oocyte and the CCs was assessed as previously described [29]. A 3% (w/v) solution of the fluorescent dye lucifer yellow in 5 mM lithium chloride was pressure injected into the ooplasm of oocytes surrounded by several complete layers of CCs. After 10 min incubation, the spread into the surrounding CCs was monitored under a Leica DMI3000 B inverted fluorescence microscope (Leica Microsystems, Wetzlar, Germany). GJC was scored by a blinded assessor using previously described criteria [29]. A semi-quantitative GJC index was calculated from the scores [30]. As a control for the GJC assay, COCs were also treated with pre-IVM plus the GJ blocker carbenoxolone (CBX, 200 µM, Tocris Bioscience, Abingdon, UK). For examination of both the pre-IVM phase and the IVM phase, 10–15 COCs were scored individually per treatment per time point in each replicate experiment, with individual COCs assessed at one time point only. The experiment was replicated 3 times.

Assessment of ATP, ADP and AMP in COC after pre-IVM culture

COCs were exposed to 0 h pre-IVM, 24 h pre-IVM, or 24 h arrest in IBMX (50 µM). COC ATP, ADP, and AMP content was extracted and measured by liquid chromatography with tandem mass spectrometry detection (LC-MS/MS) as previously described [31]. Briefly, COCs were placed in 80% methanol and sonicated on ice using a probe sonicator for 3×20 s and then incubated at -30°C for 20 min. Samples were centrifuged at 14,000g for 10 min at 4°C and supernatants were then stored at -80°C prior to spiking with 20 µL of 400 nM isotope-labelled internal standards ($^{13}\text{C}_5$ -AMP, $^{15}\text{N}_5$ -ADP, and $^{13}\text{C}_{10}$ $^{15}\text{N}_5$ -ATP) and evaporating to dryness using a Savant SpeedVac vacuum centrifuge. Samples were then reconstituted in 100 mM ammonium acetate. Analytes were separated and quantified by LC-MS/MS using a porous graphite Hypercarb column and a Quantum Vantage triple quadrupole mass spectrometer as detailed by [31]. $N = 6$ biological replicates were performed (61–110 COCs per treatment group per biological replicate). COC energy charge was calculated as previously described [32].

$$\text{Energy charge} = \frac{[\text{ATP}] + \frac{1}{2}[\text{ADP}]}{[\text{ATP}] + [\text{ADP}] + [\text{AMP}]}$$

Statistical analysis

Data were analyzed using GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA). Proportional data for embryo development and blastocyst differential staining were arcsine transformed before statistical analyses. Treatment

Table 1 Sequences of PCR primers used for RT-qPCR

Gene	GeneBank accession no.	Forward primer (5' → 3')	Reverse primer (5' → 3')	PCR size (bp)
<i>Has2</i>	U52524	AAG ACC CTA TGG TTG GAG GTC TT	CAT TCC CAG AGG ACC GCT TAT	167
<i>Tnfrsf6</i>	NM_009398	CCT TCC TCC CGT ACG AGA TG	ATG AAC TCT CTC CGT AGA AGA ACC TT	111
<i>Ptx3</i>	X83601	GGA CAA GCA AAT AGA CAA TGG ACT T	CGA GTT CTC CAG CAT GAT GAA C	109
<i>Ptgs2</i>	NM_011198	CCTTCCTCCCGTAGCAGATG	ATGAACTCTCTCCGTAGAAGAACCTT	111
<i>Areg</i>	NM_009704.3	TTGGTGAACGGTGTGGAGAA	CGAGGATGATGGCAGAGACA	111
<i>Ereg</i>	NM_007950.2	AGACGCTCCCTGCCTCTTG	TTCTCCTGGGATGCATGATG	104
<i>Egfr</i>	BC023729.1	TCTGGAAACCGAAATTTGTGCTA	ACGGCCTTGCACTTTCTC	116
<i>Bax</i>	NM_007527	TGCTAGCAAACCTGGTGCTCA	GGCCTTCCCAGCCACCC	134
<i>Bcl-2</i>	NM_009741	GGAGGCTGGGATGCCTTTGT	ATGACCCCAGAGTGATGCAG	127
<i>Mrp119</i>	NM_026490	GAAAGGTGCTTCCGATTCCA	TGATCGCTTGATGCAAATCC	116
<i>Ppia</i>	NM_008907	TGGCAAATGCTGGACCAA	CCTTCTTTCACCTTCCCAAAGA	106

effects were assessed by one-way ANOVA followed by Tukey's multiple-comparison post hoc test. Two sample *t* tests were used where only two sample means were compared. Statistical significance was considered at $P < 0.05$.

Results

Pre-IVM culture duration affects subsequent blastocyst yield

There was no effect of pre-IVM duration on cleavage or morula rates (Fig. 2(A, B)). Increasing duration of pre-IVM tended to lead to incremental increases in D5 and D6 blastocyst development, with 24 h of pre-IVM significantly ($P < 0.05$) improving D5 and D6 blastocyst (Fig. 2(C–F)) and hatching blastocyst rates (Fig. 2(G, H)), compared to 0-h pre-IVM. There were no differences in blastocyst TE cell number, ICM cell number, total cell number, ICM/TE ratio, or ICM/total number following IVM, IVF, and embryo culture after pre-IVM treatment for 0, 2, 6, 12, and 24 h (Table 2).

Pre-IVM culture affects COC DNA content, expansion and matrix formation

There was a significant increase in COC DNA following 24 h of pre-IVM compared to 0 h control ($P < 0.05$) (Fig. 3). This pre-IVM system does not induce cumulus expansion by the end of pre-IVM [20]. Cumulus expansion was assessed at 17 h of IVM following 0 h or 24 h pre-IVM. Pre-IVM significantly increased cumulus expansion in the subsequent IVM phase relative to control (Fig. 4(A); $P < 0.05$), and significantly increased mRNA expression of *Areg* and cumulus matrix genes *Has2* and *Tnfrsf6* at 6 h of IVM (Fig. 4(G, B, and C)). There were no significant differences in *Ptx3*, *Ptgs2*, *Egfr*, *Ereg*, *Bax*, and *Bcl-2* gene expression between treatment groups (Fig. 4).

Effect of pre-IVM on CC-oocyte GJC

CC-oocyte gap junctional communication (GJC) was measured to determine (1) whether the pre-IVM system was capable of preserving GJC during the pre-IVM phase, and (2) whether pre-IVM affected the subsequent rate of loss of GJC during the IVM phase. GJC was assessed by LY dye transfer from the oocyte to the surrounding cumulus vestment (Fig. 5(A)). Pre-IVM culture preserved GJC for the 24 h of pre-IVM with GJC at the end of pre-IVM being not significantly different ($P > 0.05$) to the beginning of culture (Fig. 5(B)). By contrast, in controls (media lacking CNP/E₂/FSH/insulin), GJC fell significantly ($P < 0.05$) within 2 h of culture and was significantly lower than pre-IVM from 6 h onwards. As assay validation, addition of the gap-junction blocker CBX rapidly and progressively blocked CC-oocyte GJC during the 24 h of culture (Fig. 5(B)).

To assess the effect of pre-IVM on oocyte maturation in the IVM phase, COCs were treated with 0 h or 24 h pre-IVM followed by 4 h of IVM (Fig. 5(C, D)). During the first 4 h of the IVM phase, GJC remained significantly ($P < 0.05$) higher than control when COCs had been previously exposed to pre-IVM culture for 24 h (Fig. 5(C)). Interestingly, despite retained GJC, most COCs in 24 h pre-IVM group resumed meiosis rapidly between 0 and 2 h in the IVM phase, whilst COCs in the control group resumed meiosis at a slower rate ($P < 0.05$; Fig. 5(D)).

Effect of pre-IVM on COC energy metabolism

An experiment was conducted to determine the effect of pre-IVM on COC energy metabolism as it is an important determinant of oocyte developmental competence [33]. Relative to levels at collection from follicles (control), COC ATP and ADP levels increased significantly after 24 h pre-IVM culture ($P < 0.05$, Fig. 6(A, B)). To determine if this effect was due to the specific combination of active ingredients in pre-IVM (i.e.,

Fig. 2 Effect of different durations of pre-IVM on subsequent embryonic development. 23 h primed COCs were subjected to 0, 2, 6, 12, or 24 h pre-IVM culture, followed by 17 h IVM culture and IVF. Oocyte developmental competence was subsequently assessed. 383–441 COCs

per time point were assessed over 6 replicate experiments. Data are presented as mean $\pm$ SEM. Means with non-common letters are significantly different ($P < 0.05$; one-way ANOVA)

CNP/E₂/FSH/insulin), or due to meiotic inhibition per se, adenosine nucleotides were also measured in COCs treated with base medium with IBMX, a well-established meiotic inhibitor. COCs meiotically arrested for 24 h with IBMX also had elevated ATP, but not ADP levels, compared to the 0 h control (Fig. 6(A, B)). A similar pattern of treatment effects was

observed for AMP, although there were no significant differences (Fig. 6(C)). Compared to the control, there was no effect of treatment on the ATP:ADP ratio (Fig. 6(D)). Despite notable pre-IVM treatment effects on ATP and ADP, there was no effect of any treatment on overall energy charge of COCs (Fig. 6(E)).

Table 2 Effect of pre-IVM culture duration on day 6 blastocyst cell allocation

	0 h	2 h	6 h	12 h	24 h	<i>P</i> value
ICM cells (<i>n</i>)	16.4 ± 1.3	16.7 ± 0.9	15.5 ± 1.1	14.9 ± 1.0	16.3 ± 0.9	0.710
TE cells (<i>n</i>)	59.9 ± 3.4	58.8 ± 2.3	54.3 ± 2.7	49.8 ± 2.9	53.7 ± 2.4	0.067
Total cells (<i>n</i>)	74.2 ± 4.0	69.0 ± 3.1	64.8 ± 3.0	62.4 ± 3.1	67.1 ± 2.5	0.148
ICM/TE (%)	29.3 ± 2.6	29.4 ± 1.6	30.0 ± 2.0	31.7 ± 2.2	33.0 ± 2.3	0.653
TE/total (%)	21.6 ± 1.4	22.1 ± 0.9	22.3 ± 1.1	23.2 ± 1.2	23.8 ± 1.2	0.632

Data are presented as means ± SEM. In total, 45–74 blastocysts were assessed per treatment

Discussion

The primary objective of IVM is to minimize patient stimulation with gonadotrophins whilst simultaneously producing high-quality mature oocytes which are able to sustain healthy embryo and fetal development. This presents the core conundrum for IVM, as superovulation increases follicle size which is also the principal determinant of oocyte developmental competence (reviewed; [33]). A potential solution to this conundrum is pre-IVM culture systems (e.g., CAPA-IVM), which are designed to support the on-going acquisition of oocyte developmental competence in vitro in meiotically arrested oocytes during the pre-IVM phase, rather than that which would occur in vivo during superovulation (reviewed; [5]). Whilst the beneficial effects of CNP based pre-IVM on oocyte developmental competence are well supported by in vitro experiments in mouse, bovine, and human [8, 9, 17, 18, 20, 34, 35], the effect on CC function and the optimal duration of pre-IVM in relation to the extent of in vivo gonadotrophin-priming are not clear.

In the present study, we used a short 23 h PMSG-primed mouse model that generates oocytes a lower developmental

competence than full stimulation [15], in order to approximate the 2- to 3-day minimal stimulation protocol most commonly used for IVM infertility patients [3]. We observed that 24 h pre-IVM culture was most effective at improving oocyte developmental competence (blastocyst rate and hatching rate) when compared with shorter pre-IVM durations. Likewise, we have previously shown that extending cAMP-mediated pre-IVM duration is beneficial in zero-stimulation bovine IVM [30]. In zero-stimulation mouse IVM, it has been demonstrated that 48 h CNP pre-IVM culture improves oocyte developmental competence [9]. Interestingly, in full stimulation mouse models (46–48 h PMSG-primed), CNP pre-IVM for only 2 h was more effective than 0 h or 24 h pre-IVM [17], and 2–4 h cAMP pre-IVM was more effective than no pre-IVM [36], in terms of blastocyst and pregnancy rates. These findings are consistent with the two human CNP pre-IVM reports to date, which showed that in patients receiving 3 days of FSH-priming, 24 h CNP-mediated pre-IVM improved oocyte quality and blastocyst yield compared to no pre-IVM, and that 48 h pre-IVM was not better than 24 h [19, 20]. Collectively these results support the hypothesis that different durations of pre-IVM culture could be used in accordance with starting developmental status of the COC, as determined by the degree of donor stimulation: (i) 2 h pre-IVM culture for fully stimulated donors; (ii) 24 h pre-IVM culture for minimally stimulated donors, and (iii) 48 h pre-IVM culture for zero-stimulation IVM. This is compatible with the current RCT comparing CAPA-IVM versus conventional IVF, which uses a pre-IVM duration of 24 h in minimally stimulated IVM patients [21]. Furthermore, these observations suggest that the idea of zero-stimulation ART may be a real possibility in the near future if appropriate pre-IVM conditions are used.

In light of the clear potential clinical applications of this technology, it is important to gain a further understanding of how pre-IVM culture more broadly, and CAPA-IVM specifically, regulate COC function, and in this study, we focused on CCs and associated key events in oocyte development. As the principle of pre-IVM is to support continued ex vivo growth and development of the COC prior to meiotic maturation, as exemplified by an increase in oocyte diameter achieved using CAPA-IVM [9], we investigated whether CCs continue to proliferate during pre-IVM culture by assessing COC DNA

Fig. 3 Effect of different durations of pre-IVM culture on COC DNA content. 23 h-primed COCs were cultured in pre-IVM medium for 0, 2, 6, 12, or 24 h. At the end of pre-IVM culture, DNA content per COC was assessed (8–11 samples per treatment/15–20 COCs per sample). Data are presented as mean ± SEM. Means with no common letters are significantly different ($P < 0.05$, one-way ANOVA)

Fig. 4 Effect of pre-IVM on cumulus expansion and cumulus cell gene expression. 23 h-primed COCs were placed in pre-IVM culture for 0 h (control) or 24 h pre-IVM, followed by 6 h IVM culture for gene expression assessment, or 17 h IVM culture for cumulus expansion assessment. (A) Cumulus matrix expansion was scored after 17 h of IVM (71 and 68 COCs in control and pre-IVM, respectively, were scored from 2 replicate experiments). (B–J) COC mRNA expression was quantified after 6 h of IVM using RT-qPCR and normalized to the geometric mean of the *Ppia* and *Mrpl19* housekeeper genes (6 replicate experiments each consisting of 50 COCs per treatment). Data are presented as mean $\pm$ SEM. Asterisk indicates significant difference ($P < 0.05$, t test)

content over the course of pre-IVM. Our results demonstrate that DNA content per COC was greater at the end of 24 h pre-IVM culture. We also assessed expression of the well characterized apoptosis genes *Bax* and *Bcl-2* in CCs, but 24 h pre-IVM culture had no influence on the expression of these genes. Sánchez et al. [20] showed a low rate of CC caspase-3/7 activation after 24 h pre-IVM using human COCs, but a higher rate of activation after 46 h pre-IVM. Hence, it appears that 24 h pre-IVM culture supports on-going growth of CCs surrounding the oocyte, which is likely to be beneficial to post-IVM oocyte function.

The most important functions of pre-IVM culture systems are to prevent spontaneous resumption of meiosis upon removal of the COC from the follicle and to preserve the integrity of the CC-oocyte GJC network (reviewed; [5, 37]). The pre-IVM phase of CAPA-IVM used in this study achieved both these objectives. We observed that pre-IVM culture fully maintained GJC functionality in COCs for 24 h, in contrast to the loss of GJC that occurred in untreated COCs. This result is consistent with previous CNP- [8] and cAMP-mediated [30, 38] pre-IVM studies examining GJC. Retained CC-oocyte communication would allow continued transfer of CNP-induced cGMP from CCs to the oocyte, inhibiting oocyte PDE3A activity on cAMP, and thus preventing meiotic resumption during pre-IVM [10]. Following pre-IVM culture, we assessed the function of GJC in the first 4 h in IVM culture. The effect of pre-IVM on sustained GJC persisted a further 2 h into the IVM phase and slowed the closure of GJC during the first 4 h of IVM culture. Gap junction communication within the developing COC, both between oocytes and CCs and among the CCs themselves, maintains the COC in a functionally integrated state. Ions and molecules passing from the CCs into the oocyte are beneficial for oocyte growth and survival [39]. The prolongation of functional intercellular communication between oocytes and CCs is believed to be beneficial for oocyte developmental competence due to the maintenance of exchange of metabolites, ions, pyruvate, and regulatory molecules [22]. The other benefit of sustained CC-oocyte communication in the last phase of oocyte development, before meiosis restarts, is the ordered cessation of oocyte transcription and appropriate remodeling of chromatin in preparation for meiosis. Such remodeling of oocyte chromatin is achieved

Fig. 5 Effect of pre-IVM on cumulus-oocyte gap junction communication and oocyte maturation. As shown in the representative images (A), gap junctions were classified as open, partially closed or closed, and scored as 2, 1, or 0 respectively, and the gap junction index was calculated. (B) COCs were cultured in basic culture medium + BSA (control), pre-IVM culture medium containing supplements (pre-IVM) or pre-IVM with CBX (pre-IVM + CBX). GJC was assessed throughout the 24 h pre-IVM culture phase (10–15 COCs per treatment per time point in each of 3 replicate experiments). (C) COCs were treated without (control) or with 24 h pre-IVM, and then GJC was assessed at 0–4 h of IVM (10–15 COCs per treatment per time point in each of 3 replicate experiments). (D) COCs were treated without (control) or with 24 h pre-IVM, and oocyte meiotic resumption rate (GVBD) was assessed at 0–4 h of IVM (4 replicate experiments each consisting of 28–42 COCs per time point). Means with non-common letters are significantly different ($P < 0.05$; one-way ANOVA) between time points within treatment. Asterisks indicate a significant difference ($P < 0.05$) between different treatments at the same time point (B, *treatment vs pre-IVM + CBX, **pre-IVM vs control and pre-IVM + CBX, one-way ANOVA; C, D, *pre-IVM vs control)

with superovulation [40], but also with pre-IVM culture [8, 9, 41], including with CAPA-IVM [20]. Therefore, arresting oocytes at the GV stage in vitro and extending CC-oocyte functional communication at the same time had a positive effect on the pre-IVM system.

Pre-IVM affected the kinetics of subsequent meiotic resumption, whereby COCs treated with 24 h pre-IVM resumed meiosis faster than the control COCs. Interestingly, such faster resumption of meiosis in pre-IVM treated COCs occurred simultaneously with a greater degree of CC-oocyte GJC. Whilst it is now clear that GVBD occurs with open CC-oocyte GJs and as a result of efflux of intra-oocyte cGMP to the CC mass [42], it is nonetheless noteworthy that GVBD occurred faster in oocytes that were in the pre-IVM treatment that seeks to elevate COC cGMP. This suggests that the pre-IVM phase of CAPA-IVM effectively primes these oocytes for meiotic resumption, and may be consistent with the observation that pre-IVM causes chromatin remodeling associated with cessation of oocyte RNA synthesis, which normally only occurs in oocytes from preovulatory follicles [8, 9, 20, 41]. This is consistent with CNP pre-IVM culture improving meiotic maturation of animal [9, 35] and human oocytes [20]. Such improvements in oocyte maturation are reflected in better developmental competence of the oocytes treated with pre-IVM.

The oocyte must meet its energy requirements during development by modulating a number of metabolic pathways that generate ATP [33]. In some species, lower ATP concentrations in oocytes are associated with poor developmental competence [34, 43, 44]. A previous study showed that intra-oocyte ATP levels are increased with cAMP-mediated pre-IVM [45]. Recently, another study showed that 2 h or 24 h CNP-mediated pre-IVM did not increase the ATP concentration in oocytes [17]. In our present study, we observed 24 h pre-IVM culture in the CAPA-IVM system or 24 h IBMX culture increased COC ATP and ADP but not AMP.

Fig. 6 Effect of 24 h pre-IVM on energy metabolism of the COC. 23 h-primed COCs were either untreated (no culture, 0 h control) or cultured for 24 h with indicated treatments. Whole COC ATP, ADP, and AMP were measured by LC-MS/MS. Data are presented as means $\pm$ SEM, from 6 replicate experiments each consisting of 60–110 COCs per treatment. Means with non-common letters are significantly different ($P < 0.05$; one-way ANOVA)

This suggests notable changes in COC energy metabolism in response to pre-IVM although the overall energy charge of the COC was unchanged. In addition, the increased ATP and ADP can be principally attributed to the effect of culture of meiotically arrested COCs per se, and not attributable specifically to CNP and the other additives in pre-IVM, as the same effect was observed with IBMX alone.

Cumulus expansion is imperative for normal oocyte development and in vivo is induced by the EGF-like peptides [46]. However, developing COCs from growing antral follicles have an underdeveloped EGF receptor signalling system and hence a poor capacity to respond to EGF and/or the EGF-like peptides [47, 48]. EGF receptor functionality and EGF-like peptide responsiveness can be induced in vitro in COCs from small antral follicles by pre-treatment with a combination of cAMP modulators and oocyte-secreted factors (reviewed; [49]). This has usually been achieved with agents such as dibutyryl cAMP (dbcAMP), forskolin or FSH [36, 47, 50]. The CAPA-IVM system intentionally prevents cumulus expansion during pre-IVM but promotes expansion during the IVM phase using amphiregulin and epiregulin, and not by FSH [20]. In this study, CAPA-IVM led to increased expression of *Has2*, *Tnfrsf6*, and *Areg* and improved amphiregulin/epiregulin-induced cumulus expansion in the IVM phase. Hence, differing pre-IVM strategies seem to be able to induce functionality of the EGF receptor signalling system in COCs from minimally stimulated animals, including the use of dbcAMP+GDF9+BMP15 [50] and the use of CNP with other pre-IVM additives used in the current study. This adds further weight to the notion that prematuration culture systems are capable of inducing appropriate development and differentiation of COCs in vitro, prior to IVM, and that these events lead to enhanced oocyte quality and developmental capacity (reviewed; [5]).

In conclusion, this study shows that CAPA-IVM improves the quality and developmental competence of COCs from minimally stimulated mice. Such improvements were associated with notable changes in CC functions, including increased proliferation, altered adenine nucleotide metabolism, prolonged GJC between CCs and the oocyte, and improved cumulus matrix expansion. This adds to the growing body of evidence that pre-IVM systems provide benefit to the oocyte in terms of its capacity to support preimplantation embryo development. We found that the duration of the pre-IVM phase affected the benefit gained from pre-IVM, and it is possible that the length of pre-IVM required is inversely related to the extent of oocyte donor stimulation, which suggests

that with further development of pre-IVM methodologies the notion of further reducing or eliminating gonadotrophin-priming for IVM is a possibility.

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Author contributions This study was funded by RBG, and designed by RBG, DR, and YZ. YZ performed all experiments under guidance from DR and MJB and with assistance from XL, DR, and MJB. AEK performed COC DNA analyses and DR performed the LC-MS/MS. YZ and RBG wrote the manuscript which was edited and approved by all authors.

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Compliance with ethical standards All mouse procedures were approved by the UNSW Animal Ethics Committee (ethics number 17/105A) and followed the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Conflict of interest The authors declare that they have no conflict of interest.

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