

Cumulus cell co-culture in media drops does not improve rescue in vitro maturation of vitrified-warmed immature oocytes

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Objective: To assess whether co-culture with vitrified-warmed cumulus cells (CCs) in media drops improves rescue in vitro maturation (IVM) of previously vitrified immature oocytes. Previous studies have shown improved rescue IVM of fresh immature oocytes when cocultured with CCs in a three-dimensional matrix. However, the scheduling and workload of embryologists would benefit from a simpler IVM approach, particularly in the setting of time-sensitive oncofertility oocyte cryopreservation (OC) cases. Although the yield of developmentally competent mature metaphase II (MII) oocytes is increased when rescue IVM is performed before cryopreservation, it is unknown whether maturation of previously vitrified immature oocytes is improved after coculture with CCs in a simple system not involving a three-dimensional matrix.

Design: Randomized controlled trial.

Setting: Academic hospital.

Patients: A total of 320 (160 germinal vesicles [GVs] and 160 metaphase I [MI]) immature oocytes and autologous CC clumps were vitrified from patients who were undergoing planned OC or intracytoplasmic sperm injection from July 2020 until September 2021.

Interventions: On warming, the oocytes were randomized to culture in IVM media with CCs (+CC) or without CCs (-CC). Germinal vesicles and MI oocytes were cultured in 25 μ L (SAGE IVM medium) for 32 hours and 20–22 hours, respectively.

Main Outcome Measures: Oocytes with a polar body (MII) were randomized to confocal microscopy for analysis of spindle integrity and chromosomal alignment to assess nuclear maturity or to parthenogenetic activation to assess cytoplasmic maturity. Wilcoxon rank sum tests for continuous variables and the chi square or Fisher's exact test for categorical variables assessed statistical significance. Relative risks (RRs) and 95% confidence intervals (CIs) were calculated.

Results: Patient demographic characteristics were similar for both the GV and MI groups after randomization to +CC vs. -CC. No statistically significant differences were observed between +CC vs. -CC groups regarding the percentage of MII from either GV (42.5% [34/80] vs. 52.5% [42/80]; RR 0.81; 95% CI: 0.57–1.15) or MI (76.3% [61/80]; vs. 72.5% [58/80]; RR 1.05; 95% CI: 0.88–1.26) oocytes. An increased percentage of GV-matured MIIs underwent parthenogenetic activation in the +CC group (92.3% [12/13] vs. 70.8% [17/24]), but the difference was not statistically significant (RR 1.30; 95% CI: 0.97–1.75), whereas the activation rate was identical for MI-matured oocytes (74.3% [26/35] vs. 75.0% [18/24], CC+ vs. CC-; RR 0.99; 95% CI: 0.74–1.32). No significant differences were observed between +CC vs. -CC groups for cleavage of parthenotes from GV-matured oocytes (91.7% [11/12] vs. 82.4% [14/17]) or blastulation (0 for both) or for MI-matured oocytes (cleavage: 80.8% [21/26] vs. 94.4% [17/18]; blastulation: 0 [0/26] vs. 16.7% [3/18]). Further, no significant differences were observed between +CC vs. -CC for GV-matured oocytes regarding incidence of bipolar spindles (38.9% [7/18] vs. 33.3% [5/15]) or aligned chromosomes (22.2% [4/18] vs. 0.0 [0/15]); or for MI-matured oocytes (bipolar spindle: 38.9% [7/18] vs. 42.9% [2/28]); aligned chromosomes (35.3% [6/17] vs. 24.1% [7/29]).

Conclusions: Cumulus cell co-culture in this simple two-dimensional system does not improve rescue IVM of vitrified, warmed immature oocytes, at least by the markers assessed here. Further work is required to assess the efficacy of this system given its potential to provide flexibility in a busy, in vitro fertilization clinic. (Fertil Steril Sci® 2023;4:185–92. ©2023 by American Society for Reproductive Medicine.)

Key Words: rescue in vitro maturation (IVM), fertility preservation, vitrification, cumulus cells, in vitro fertilization (IVF)

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In vitro maturation (IVM) of immature mammalian oocytes was first described in 1935 (1). The first use of IVM for the initiation of pregnancy was in 1991 (2). Retrieval of immature oocytes followed by maturation in vitro has been used in patients with polycystic ovary syndrome to avoid ovarian hyperstimulation syndrome and for patients with previously failed in vitro fertilization (IVF). However, the technology has had limited global application for the rescue of immature oocytes at retrieval (rescue IVM). In rescue IVM, oocytes have been exposed to a human chorionic gonadotropin (hCG) or leuprolide acetate trigger before retrieval, as opposed to the deliberate retrieval of immature oocytes in a natural cycle without the use of an exogenous ovulatory trigger. Meiotic maturation requires complex coordination between the oocyte and its surrounding cells (3). Replicating this process in vitro can be difficult, especially after stripping the critically important surrounding cumulus cells (CCs) from the immature human oocyte, as is routinely performed in preparation for intracytoplasmic sperm injection (ICSI) and oocyte cryopreservation (OC).

Oocytes are connected to CCs by gap junctions (4). These surrounding cells are responsible for oocyte development, metabolism, and protection from oxidative stress (5, 6). Studies in animal models have shown promise for in vitro oocyte maturation by culturing the oocytes with CCs (7–9). These studies demonstrate increased oocyte maturation and improved embryo quality for oocytes matured in vitro via IVM with CCs over those without CC co-culture. Importantly, all of these studies evaluated conventional IVM with CCs. To our knowledge, there are no animal studies that have evaluated rescue IVM and CC coculture. Research in humans is limited, but some studies demonstrate improved rescue IVM with CC coculture. Combelles et al. (10) showed improved metaphase spindle formation and protein kinase activity in immature oocytes retrieved from stimulated ovaries and then matured with CCs. In a study of fresh rescue IVM after IVF antagonist cycles, Jahromi et al. (11) randomized immature oocytes to culture with or without CCs. The investigators found increased maturation rates and embryo formation rates, as well as improved embryo quality, in the rescue IVM oocytes cocultured with CCs. Virant-Klun et al. (12) demonstrated gene expression more consistent with in vivo matured oocytes when immature oocytes were co-cultured with CCs compared with those without CCs. Importantly, these 3 studies were performed using fresh, retrieved immature oocytes after IVF stimulation.

These findings open the door for more fertility opportunities for patients undergoing fertility preservation with IVF, either for oncologic reasons, inevitable ovarian insufficiency as in Turner syndrome or Fragile-X premutation carriers, or even electively for family planning purposes. These patients often undergo IVF with OC instead of embryo cryopreservation when they are young or unpartnered. Maximizing the number of embryos obtained from cryopreserved oocytes is of utmost importance, particularly for those patients rendered sterile after gonadotoxic therapy.

Many studies have shown decreased maturation and fertilization rates in immature oocytes matured postthaw

compared with those matured before vitrification (13, 14). However, the rescue IVM adds a significant cost, which, when performed before vitrification, is an unnecessary financial burden for most patients who do not return to use their cryopreserved oocytes. Therefore, in this study, we aimed to investigate ways to improve the rescue IVM of previously vitrified immature oocytes. Our primary objective was to determine whether rescue IVM is improved when vitrified, warmed CC are co-cultured with previously vitrified immature oocytes.

MATERIALS AND METHODS

Study Design

This was a randomized controlled trial where 320 immature oocytes (160 germinal vesicles [GVs] and 160 metaphase I [MI]) and autologous CC clumps were vitrified from July 2020 until September 2021. Oocytes and CC were collected from patients who were undergoing planned OC or ICSI, given that it is routine clinical practice to discard immature oocytes for these patient groups. On warming, the oocytes were randomized to culture in IVM media with CCs (+CC) or without CCs (-CC). In vitro matured oocytes metaphase II (MII) was assessed for nuclear maturation using confocal microscopy and for cytoplasmic maturation after parthenogenetic activation.

All samples used in this study were voluntarily donated by patients with informed consent. Study approval was obtained from the Partners HealthCare Institutional Review Board (Protocol number 2019P003802).

Clinical Protocols

Standard controlled ovarian hyperstimulation and monitoring protocols were used for patients with planned OC or ICSI. Gonadotropin doses were determined on the basis of age, serum antimüllerian hormone levels, follicle stimulating hormone levels, antral follicular count, body mass index, and previous response to stimulation. Good prognosis protocols included antagonists and standard-dose luteal Lupron. Poor prognosis protocols included microflare Lupron, estrogen priming with transdermal estradiol, minimal stimulation with clomiphene citrate, and low-dose luteal Lupron. Ovarian stimulation was performed with the use of exogenous gonadotropins (Gonal-F, EMD-Serono, or Follistim, Organon; Menopur, Ferring Pharmaceuticals). Pituitary suppression was attained with the use of gonadotropin-releasing hormone (GnRH) antagonists (Cetrotide, EMD-Serono, or Ganirelix, Ferring Pharmaceuticals) or GnRH agonists (leuprolide acetate, Abbott Laboratories). Gonadotropin dosage was adjusted according to each patient's response to stimulation, which was monitored with the use of transvaginal ultrasounds and serial estradiol levels. When at least 2 follicles reached a mean diameter of 18 mm, final oocyte maturation was triggered with the use of human chorionic gonadotropin (hCG) (Pregnyl, Organon Pharmaceuticals; Novarel, Ferring Pharmaceuticals), a GnRH agonist (leuprolide acetate, Abbott Laboratories), or both. The dose of hCG was tailored on the basis of serum estradiol levels on the day of the trigger and the number of follicles. Patients considered to be at high risk

for ovarian hyperstimulation syndrome were given a GnRH agonist (leuprolide acetate, 40 units), 5,000 units of hCG, or a combination trigger (leuprolide acetate, 40 units, with 1,500 units of hCG). Ultrasound-guided oocyte retrieval was typically performed under intravenous general anesthesia 36 hours after the trigger.

Laboratory Protocols

Collection of samples. At the time of oocyte retrieval, the cumulus-oocyte complex (COC) was trimmed to obtain CC clumps, which were placed into 1 mL Global Total Fertilization media (Cooper Surgical, Trumbull, CT) overlaid with 1 mL of oil (Fuji Film, Santa Ana, CA) before their vitrification. Cumulus cell clumps were trimmed to fit into a 10 μ L pipette. Oocytes were cultured in Global Total Culture Media (Cooper Surgical, Trumbull, CT). All oocytes and CC were cultured at 37 °C in a dry incubator under an atmosphere of CO₂ (6%–7%), O₂ (5%), and N₂ (89%–90%) at a pH of 7.3. Oocytes were enzymatically and mechanically denuded of their surrounding cumulus and corona cells 1 hour after retrieval for planned OC and 2–3 hours after retrieval for ICSI. This was done by pipetting COC through 170- to 140- μ m pipettes (Cooper Surgical, Trumbull, CT) after brief exposure to 80 IU/mL of hyaluronidase (Fuji Film), then washed several times in Global Total-HEPES media (Cooper Surgical). The meiotic status of oocytes was determined. Germinal vesicle oocytes were identified by an intact nucleus, and MI oocytes by the absence of a nucleus and polar body. To meet inclusion criteria, patients had to have at least 6 cumulus-oocyte complexes and at least 2 immature oocytes (GV or MI) so the oocytes could be randomized within patient.

Vitrification and warming protocols. Immature oocytes and CC clumps were vitrified within 1–2 hours of oocyte stripping by a standard closed-system vitrification technique (Cryolock straws and vitrification media, Fuji Film). Oocytes were frozen using the OC protocol, although CC clumps were frozen using the embryo cryopreservation protocol. The embryo cryopreservation protocol was chosen because CC clumps consist of multiple cells as opposed to a single cell, as is the case with oocytes. Additionally, this technique has shown excellent viability postwarming with respect to mitochondrial function in a previous study (15). Cumulus cell clumps were vitrified using 100 μ L media droplets and a 10 μ L pipette for loading onto each Cryolock device. Up to 2 oocytes were frozen per Cryolock device.

Immature oocytes and CC clumps were thawed using a standard warming technique (Vit-Kit Warm NX, Fuji Film). On warming, the oocytes were randomized within patients per cycle using a computer-generated randomization scheme to culture in IVM media with autologous CCs (+CC) or without CCs (-CC). Oocytes with or without CCs were cultured in 25 μ L microdrops (SAGE IVM medium, Cooper Surgical) in 12-well μ Drop-GPS dishes (Cooper Surgical). For CC coculture, the oocyte was placed in the media drop first, then the CC clump on top. This was on the basis of pilot work that showed improved adhesion of the CC to the oocyte compared with placing the CC clump in the drop before the oocyte. Germinal vesicles and MI oocytes were cultured for 32 hours

and 20–22 hours, respectively. Oocytes with a polar body (MII) were randomized to confocal microscopy for analysis of spindle integrity and chromosomal alignment or parthenogenetic activation to assess cytoplasmic maturity.

Confocal microscopy. Oocytes randomized to confocal analysis were fixed for 30 minutes at 37 °C in a microtubule-stabilizing buffer (0.1 M PIPES, pH 6.9, 5 mM MgCl₂·6H₂O, 2.5 mM EGTA) containing 2% formaldehyde, 0.1% Triton X-100, 1 μ M taxol, 10 IU/mL aprotinin, and 50% deuterium oxide. Samples were washed and stored at 4 °C in a blocking solution of phosphate-buffered saline (PBS) containing 1% bovine serum albumin, 0.2% powdered milk, 2% normal donkey serum, 0.1 M glycine, and 0.01% Triton X-100 containing 0.2% sodium azide (PBS blocking solution). Microtubules were detected using 5 μ g/mL monoclonal anti- α -tubulin and 5 μ g/mL anti- β -tubulin primary antibodies overnight at 4 °C with shaking, followed by 3 15-minute washes, and 2.5 μ g/mL Alexa-fluor 488 donkey anti-mouse secondary IgG for 2 hours at 37 °C (Life Technologies, Waltham, MA), together with 10 units/mL Texas-red phalloidin (Life Technologies). All antibodies were diluted in a PBS blocking solution. Samples were subsequently washed and labeled with 15 μ g/L DAPI (Sigma Aldrich, St. Louis, MO) for 1 hour before washes and mounting in 50% glycerol and 50% PBS containing 25 mg/mL sodium azide. Imaging was performed using an Olympus FV3000 confocal laser-scanning microscope (funded by National Science Foundation Major Research Instrumentation Award Number 2018114 to Middlebury College), with categorizations of spindle and chromosome arrangements made from 3D reconstructions of z-stacks of optical images taken at 0.33 μ m intervals.

Parthenogenetic activation. Oocytes randomized to parthenogenetic activation were sequentially exposed to 10 μ M ionomycin (Sigma Aldrich) in Global Total-HEPES media for 5 minutes at 37 °C and 6%–7% CO₂ in the dark (25 μ L microdrops). Oocytes were then washed 3 times and incubated in 25 μ L microdrops of 2 mM 6-Dimethylaminopurine (Sigma Aldrich) in Global Total media for 3 hours at 37 °C and 5%–6% CO₂, after which they were washed 3 times in fresh Global Total media and cultured separately in 25 μ L microdrops of the same medium under mineral oil. The oocytes were analyzed for activation 18–20 hours later, considering the exposure to Dimethylaminopurine as time 0, by assessing for the presence of a single pronucleus within the cytoplasm without a second polar body (16, 17). Parthenotes were washed 3 times and moved to fresh drops of equilibrated Global Total media for culture for 72 hours and 120 hours after activation, checking for cleavage and blastulation at each respective time point.

Outcome Measures

The primary outcome was the number of rescue in vitro matured (MII) oocytes. Secondary outcomes were the number of oocytes with a normal bipolar spindle and the number of oocytes with aligned chromosomes. A normal bipolar spindle was tightly organized as a typical barrel-shaped structure with no apparent irregularities and 2 clearly defined and focused

TABLE 1

Demographic characteristics of patient cycles contributing immature oocytes.

Demographics	GV -CC n (%) N = 48	GV +CC n (%) N = 55	P value	MI -CC n (%) N = 59	MI +CC n (%) N = 66	P value
Producer age (y)			.64			.55
<35	24 (50.0)	29 (52.7)		33 (55.9)	29 (43.9)	
35–37	13 (27.1)	10 (18.2)		12 (20.3)	13 (19.7)	
38–40	6 (12.5)	9 (16.4)		8 (13.6)	16 (24.2)	
41–42	4 (8.3)	7 (12.7)		4 (6.8)	6 (9.1)	
>42	1 (2.1)	0 (0)		2 (3.4)	2 (3.0)	
BMI (kg/m ²)			.97			.69
18.5–24.9	19 (39.6)	20 (36.4)		34 (57.6)	34 (51.5)	
25.0–29.9	12 (25.0)	16 (29.1)		10 (17.0)	16 (24.3)	
30.0–39.9	14 (29.2)	15 (27.3)		12 (20.3)	11 (16.7)	
40+	3 (6.3)	4 (7.3)		3 (5.1)	5 (7.6)	
Stimulation protocol			.94			.39
Good responder	43 (89.6)	49 (89.1)		46 (78.0)	47 (71.2)	
Poor responder	5 (10.4)	6 (10.9)		13 (22.0)	19 (28.8)	
Diagnosis			.91			.98
Diminished ovarian reserve	0 (0)	0 (0)		5 (8.5)	3 (4.6)	
Endometriosis	2 (4.2)	2 (3.6)		2 (3.4)	1 (1.5)	
Male	6 (12.5)	11 (20.0)		13 (22.0)	15 (22.7)	
Male and female	10 (20.8)	7 (12.7)		6 (10.2)	7 (10.6)	
Multiple female	4 (8.3)	4 (7.3)		5 (8.5)	5 (7.6)	
Other	17 (35.4)	18 (32.7)		17 (28.8)	19 (28.8)	
Ovulatory	2 (4.2)	2 (3.6)		4 (6.8)	4 (6.1)	
Tubal	2 (4.2)	2 (3.6)		0 (0)	0 (0)	
Unexplained	5 (10.4)	9 (16.4)		7 (11.9)	11 (16.7)	
Uterine	0 (0)	0 (0)		0 (0)	1 (1.5)	
Gravidity			.91			.48
No	30 (62.5)	35 (63.6)		35 (59.3)	35 (53.0)	
Yes	18 (37.5)	20 (36.4)		24 (40.7)	31 (47.0)	

-CC= culture without cumulus cells; +CC = co-culture with cumulus cells; GV = germinal vesicle; MI - metaphase I.

Protocol: good responder: antagonist, standard-dose luteal Lupron; poor responder: microflare, patch, minimal stimulation, low-dose luteal Lupron

Denominator is cycles

Statistical significance was assessed using Wilcoxon rank sum tests for continuous variables and chi square tests or Fisher's exact test for categorical variables.

Gordon. IVM of vitrified-warmed oocytes with CC. *Fertil Steril Sci* 2023.

spindle poles at which the microtubules converge in a focal area; chromosomes were defined as aligned when all chromosomes were tightly aligned at the spindle equator; number of parthenogenetically activated oocytes; and proportion of parthenotes that underwent cleavage and blastulation.

Statistical analysis

Statistical significance was calculated using Wilcoxon rank sum tests for continuous variables and chi square or Fisher's exact test for categorical variables. An alpha of 0.05 was considered statistically significant. Relative risks (RRs) and 95% confidence intervals (CIs) were calculated using log-binomial regression. Generalized estimating equations were used to account for oocytes from the same cycle. All statistical analyses were performed with SAS version 9.4 (Cary, NC, USA). Sample sizes for each oocyte group (GV and MI, 80 each) were calculated on the basis of a previous study that found IVM rates of 84% for CC coculture and 65% for nonCC culture for fresh, nonvitrified oocytes (79 oocytes per group) (11).

RESULTS

A total of 320 oocytes from 228 unique patients were included in the study (160 at the GV stage and 160 at the MI stage). Only GV and MI oocytes that survived warming were ran-

domized. The survival rate for GV oocytes was 57.9% and for MI oocytes was 82.2%, both of which remained constant throughout the duration of the study. All CC clumps survived warming, with no degenerated cells noted under light microscopy. Demographic characteristics for patients contributing immature oocytes are shown in Table 1. There was no difference in any demographic characteristic for either the GV or MI groups after randomization to +CC vs. -CC (*P* value > .05).

The meiotic status of oocytes after rescue IVM is shown in Table 2. There was no significant difference in maturation rates (%MII) for GV oocytes randomized to +CC vs. -CC (42.5% [34/80] vs. 52.5% [42/80]; RR 0.81; 95% CI: 0.57–1.15). There was also no significant difference in % MII for MI oocytes between +CC vs. -CC groups (76.3% [61/80] vs. 72.5% [58/80]; RR 1.05; 95% CI: 0.88–1.26).

The results for rescue in vitro matured (MII) oocytes randomized to confocal analysis are shown in Table 3. For GV-matured oocytes, there was no significant difference observed regarding the incidence of bipolar spindles for +CC vs. -CC (38.9% [7/18] vs. 33.3% [5/15]; RR 1.17; 95% CI: 0.50–2.71). There were more oocytes with aligned chromosomes in the GV +CC group compared with -CC group (22.2% [4/18] vs. 0.0 [0/15]), but significance could not be calculated given small sample size. For MI-matured oocytes, there was no difference in the incidence of bipolar spindles for +CC

TABLE 2

Meiotic stage of oocytes after IVM for vitrified-warmed GV and MI oocytes with and without cumulus cell co-culture.

Meiotic Stage after IVM	GV -CC n (%)	GV +CC n (%)	MI -CC n (%)	MI +CC n (%)		
N	80	80	80	80		
# GV (%)	24 (30.0)	31 (38.8)	0 (0.0)	0 (0.0)		
# MI (%)	5 (6.3)	14 (17.5)	21 (26.3)	17 (21.3)		
# MII (%)	42 (52.5)	34 (42.5)	58 (72.5)	61 (76.3)		
# Deg (%)	6 (7.5)	1 (1.3)	0 (0.0)	1 (1.3)		
# Abn (%)	3 (3.8)	0 (0.0)	1 (1.3)	0 (0.0)		
# LT1 (%)	0 (0.0)	0 (0.0)	0 (0.0)	1 (1.3)		
	GV -CC	GV +CC	RR (95% CI)	MI -CC	MI +CC	RR (95% CI)
# MII (%)	42 (52.5)	34 (42.5)	0.81 (0.57–1.15)	58 (72.5)	61 (76.3)	1.05 (0.88–1.26)
# not MII (%)	38 (47.5)	46 (57.5)		22 (27.5)	19 (23.8)	

Abn = abnormal; CC = culture without cumulus cells; +CC = co-culture with cumulus cells; CI = confidence interval; GV = germinal vesicle, IVM = in vitro maturation; Deg = degenerate; LT1 = late telophase I (partial extrusion of polar body); MI = metaphase I; MII = metaphase II; RR = relative risk.

N = # of oocytes; % of N

Referent is -CC

Gordon. IVM of vitrified-warmed oocytes with CC. *Fertil Steril Sci* 2023.

vs. -CC (38.9% [7/18] vs. 42.9% [2/28]; RR 0.91; 95% CI: 0.43–1.91), as also was the case regarding the incidence of aligned chromosomes for these groups (35.3% [6/17] vs. 24.1% [7/29]; RR 1.46; 95% CI: 0.56–3.82). Of note, a total of 99 MII oocytes (identified by extrusion of a polar body) were randomized to confocal analysis. On confocal analysis, however, 4 oocytes were MI stage, 2 were at T1 (telophase of meiosis I), and 9 were abnormal (degenerate or spontaneously activated on the basis of chromatin and microtubule patterns). Three oocytes were lost during processing. Four oocytes were excluded because of detection issues with microtubule or DNA staining (3 MI -CC and one MI +CC). These oocytes were not included in the analysis for their respective groups.

Results for the parthenogenetic activation analyses are presented in Table 4. More parthenogenetically activated oocytes were derived from the GV-matured MIIs in the +CC group compared with the -CC group (92.3% [12/13] vs. 70.8% [17/24]), but the difference did not reach statistically significant (RR 1.30; 95% CI: 0.97–1.75). Similar proportions of MI-matured oocytes underwent parthenogenetic activation in the 2 CC groups (74.3% [26/35] vs. 75.0% [18/24], CC+ vs. CC-; RR 0.99; 95% CI: 0.74–1.32). When parthenotes were assessed for development in culture, no significant differences were observed between +CC vs. -CC groups for those derived from GV-matured oocytes for either the proportion that cleaved (91.7% [11/12] vs. 82.4% [14/17]; RR 1.11; 95% CI: 0.84–1.48) or blastulated (0 for both). There was also no difference in parthenote cleavage rate for MI-matured oocytes for +CC vs. -CC (80.8% [21/26] vs. 94.4% [17/18]; RR 0.86; 95% CI: 0.69–1.06). More parthenotes derived from MI-matured oocytes blastulated in the -CC vs. +CC group (16.7% [3/18] vs. 0 [0/26]) but again significance could not be calculated given small sample size.

DISCUSSION

In this study, we investigated rescue IVM rates of GV and MI oocytes postvitrification and warming with and without

cumulus cell coculture. We found no significant differences in the maturation rate of immature oocytes when cultured with or without CC. There was a higher parthenogenetic activation rate and more oocytes with aligned chromosomes in GV-matured MIIs with CC coculture, but these differences did not reach statistical significance. There were no significant differences in parthenote development (cleavage and blastulation rates) or in the incidence of bipolar spindles for GV- or MI-matured MIIs.

Currently, in our practice, immature oocytes are being vitrified with mature MII oocytes in patients with oncology undergoing fertility preservation with the hope of an eventual increase in the number of usable oocytes for these unfortunate patients. However, for patients undergoing planned oocyte banking, we only vitrify MIIs. Only a few patients return to use their vitrified oocytes after banking them. One study reported a 5% return rate for patients with oncofertility (18), although another reported a 38% return rate for elective and planned OC patients (19).

Because of the high financial cost of rescue IVM and the low return rate for using these samples, many institutions cryopreserve immature oocytes before performing rescue IVM. Given the critical importance of CCs for the acquisition of oocyte developmental competence (20) and the improved rescue IVM rate of fresh oocytes with CC co-culture (10, 11), we hypothesized that CC co-culture would improve rescue IVM postvitrification and warming. Given that vitrification did not alter the structure of CC organelles (21) and the vitrification technique chosen here has been shown to result in high viability rates and good mitochondrial function post-warming (15), the use of vitrified-warmed CC was felt to be feasible and safe. Considering we only objectively evaluated CC clumps under light microscopy after vitrification and warming, we cannot comment on how cumulus cell function was preserved through this process. However, we did not note any degenerate cells in the warmed clumps. Future studies may consider evaluating the warmed CC function in more detail. Another option to consider is cryopreserving COCs

TABLE 3

Confocal microscopy analysis of spindle integrity and chromosomal alignment for in vitro matured vitrified-warmed GV and MI oocytes cultured with and without cumulus cells.

Confocal microscopy finding	GV -CC n/N (%)	GV +CC n/N (%)	RR (95% CI)	MI -CC n/N (%)	MI +CC n/N (%)	RR (95% CI)
Bipolar spindle	5/15 (33.3)	7/18 (38.9)	1.17 (0.50–2.71)	12/28 (42.9)	7/18 (38.9)	0.91 (0.43–1.91)
Chromosomes aligned	0/15 (0.0)	4/18 (22.2)	n/a	7/29 (24.1)	6/17 (35.3)	1.46 (0.56–3.82)

-CC = culture without cumulus cells; +CC = co-culture with cumulus cells; CI = confidence interval; GV = germinal vesicle; MI = metaphase I; MII = metaphase II; RR = relative risk.

N = # of MII oocytes; % of N

Referent is -CC

n/a - algorithm did not converge because of small sample size

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for fertility preservation patients instead of stripped oocytes. The problem with this approach is twofold. Without stripping the oocytes of their surrounding cells, one cannot determine the meiotic state of the oocyte, which is helpful in patient counseling regarding expectations, especially when they have time to complete another OC cycle before starting gonadotoxic therapy. Additionally, animal models have shown ultrastructural alterations in COCs after vitrification and warming, including separation of CCs from the oocytes, interrupted gap junctions, reduced microvilli, and fractured zona pellucida (22, 23). Although vitrifying extra samples CC at the time of OC adds some time and effort, the potential financial benefit to patients cannot be overlooked.

We chose a simple system of coculture in media drops because our intention was to identify a system that would be easy to replicate across embryology laboratories. Although we did not find a difference in rescue IVM rates with CC coculture in our simple system, perhaps another model of CC coculture may lead to improved outcomes. Cumulus cells provide numerous paracrine factors that support oocyte development and metabolism, including glucose and pyruvate for adenosine triphosphate production and energy maintenance within the oocyte (3). Nicotinamide adenine dinucleotide phosphate, which plays a role as an antioxidant by neutralizing reactive oxygen species, and phosphoribosyl pyrophosphate, which is required for nucleotide synthesis for DNA repair and meiotic regulation, are also transferred from the CC to the oocyte (3). A coculture system using a microfluidic chip may improve rescue IVM rates by enhancing the exposure of the immature oocytes to these paracrine factors, either with CC and immature oocytes in the same well or cultured

separately with IVM media passing over oocytes and CC through the channels. Studies have shown that the addition of certain antioxidants to IVM media may reduce reactive oxygen species and improve maturation rates and embryo yield in animal models (24). Further research on optimizing rescue IVM media components with CC would be possible with a microfluidic chip platform. Another culture model to consider is a collagen gel matrix, similar to that used in the 2005 Combelles et al. (10) study, which may allow for better adhesion of CC to the immature oocytes. We found that after coculture, the CC clumps appeared densely adherent to the oocytes. This was solely on the basis of difficulty with mechanically stripping the CCs from the oocyte after culture, but future research could more objectively evaluate the cohesion of the CC to the oocytes and assess for the reformation of gap junctions using confocal microscopy, the transfer of injected Lucifer yellow dye from oocytes to the CC or vice versa (25), or the use of microphotometry to assess for fluorescent dye (calcein-AM) transfer between cells (26). Rescue IVM rates may be also improved simply by extending the time in culture. In vivo maturation from GV to MII-stage takes approximately 36 hours after the onset of the luteinizing hormone surge (27). Although prior rescue IVM studies cocultured fresh oocytes with CC for 24 hours (10, 11), perhaps additional time is needed when using warmed oocytes and CC. We noted nonsignificantly lower maturation rates for GVs +CC (42.5% vs. 52.5%). This may be because of delayed maturation in the setting of vitrified-warmed CC, which, when monitored longer, may have resulted in higher rescue IVM rates. Perhaps the reformation of gap junctions after warming requires additional time. However, this is speculation given the paucity of

TABLE 4

Parthenogenetic activation, cleavage and blastulation rates for in vitro matured vitrified-warmed GV and MI oocytes cultured with and without cumulus cells.

Warmed rescue IVM oocytes	GV -CC n/N (%)	GV +CC n/N (%)	RR (95% CI)	MI -CC n/N (%)	MI +CC n/N (%)	RR (95% CI)
# Activated (%)	17/24 (70.8)	12/13 (92.3)	1.30 (0.97–1.75)	18/24 (75.0)	26/35 (74.3)	0.99 (0.74–1.32)
# Cleaved (%)*	14/17 (82.4)	11/12 (91.7)	1.11 (0.84–1.48)	17/18 (94.4)	21/26 (80.8)	0.86 (0.69–1.06)
# Blastulated (%)*	0/17 (0.0)	0/12 (0.0)	n/a	3/17 (16.7)	0/21 (0.0)	n/a

-CC = culture without cumulus cells; +CC = co-culture with cumulus cells; CI = confidence interval; GV = germinal vesicle; MI = metaphase I; RR = relative risk.

N = # of MII oocytes, % of N

Referent is -CC

n/a - algorithm did not converge because of small sample size

* % of activated

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data in the literature, and it is possible that additional time in culture would not result in a different outcome. Finally, leaving the tightly bound corona cells on immature oocytes before vitrification through less aggressive stripping may also improve rescue IVM rates.

Despite the lack of improved rescue IVM with CC co-culture in our study, our overall maturation rates of both GV-stage oocytes (40%–50%) and MI oocytes (70%–75%) were higher than those of previous studies assessing maturation postvitrification (24%–33%) (13, 14). These previous studies were published in 2012 and 2014, shortly after vitrification became the mainstay cryopreservation method. Perhaps with refined techniques and improved vitrification and thawing media, oocyte quality postwarming has also improved. Our findings highlight an option for a potential increase in oocyte yield for long-term banking patients, who typically only have one opportunity to undergo an OC cycle before starting gonadotoxic therapy, and for patients undergoing IVF who have low yields of mature oocytes retrieved, such as those with diminished ovarian reserve because of premature ovarian insufficiency, chromosomal and genetic abnormalities (Turner syndrome and Fragile-X premutation carriers), or postponed fertility.

Rescue IVM postvitrification should be considered for patients who freeze immature oocytes after they have exhausted their pool of MII oocytes. Patients undergoing elective and planned OC may prefer to cycle again when they have a low number of MII oocytes, as opposed to freezing immature oocytes, but can consider vitrification of MIs. An important consideration for counseling is that not all immature oocytes will survive warming. In our study, the survival rate for GV oocytes was 57.9% and for MI oocytes was 82.2%. Other studies report higher GV survival rates (70%–84%) (14, 28). This difference could be because of the relative inexperience of the researcher performing vitrification and thawing (a research fellow, not a trained embryologist) or because of varying techniques across different embryology laboratories. As a tertiary referral center, we use relatively aggressive stimulation protocols, and our providers often aspirate small follicles at the time of retrieval, which may result in more immature oocytes of poorer quality being retrieved. Oocyte survival rates are known also to vary across clinical laboratories. Providers who offer rescue IVM postthawing should counsel patients on their laboratory-specific survival rates for the thawing of immature oocytes to set expectations. Another important consideration for counseling is that not all *in vitro* matured oocytes will lead to an embryo and a healthy pregnancy. Only some of these oocytes will have acquired nuclear maturity, and of those that do emit a polar body, not all will be euploid; our confocal microscopy analyses revealed that only 30%–40% of GV-matured MIIs and 40% of MI-matured MIIs had bipolar spindles, as stringently defined in this study. It is important to note that in the group with bipolar spindles, we did not include bipolar spindles with irregularities (e.g., having 2 poles but with splayed microtubule fibers at either end pole or equatorial region). This contrasts a previous study that counted all bipolar spindles as representing nuclear maturity and noted 40%–50% bipolar spindle rates (29). In addition, it is important to consider that not all oocytes will

have acquired cytoplasmic maturity, which is essential for embryonic development. Parthenogenetic activation in our study showed rates of 70%–90% for GV-matured MIIs and 75% for MI-matured MIIs, which were similar to activation rates noted in previous rescue IVM studies of fresh or vitrified oocytes without CC culture (30, 31). Cleavage and blastulation rates in our study were also similar to those in previous studies (approximately 80% and < 3%, respectively) (30, 31). Although these previous studies did not include oocyte coculture with CCs, the consistency in embryonic development rates reassures us that parthenogenetic activation is a reasonable assessment of cytoplasmic maturity. Low blastulation rates in parthenogenetically activated oocytes are expected given that development from cleavage to the blastocyst stage requires activation of the embryonic genome, not just the maternal genome (27).

There are many strengths in our study. The study was powered to detect a difference in rescue IVM rates between the 2 treatment groups, and all oocytes were randomized within patients. Oocyte and cumulus manipulation was performed by one researcher, and there were no changes in culture conditions throughout the study period. Although this allowed for consistency, scheduling capacity limited our ability to extend the duration of the culture. As mentioned previously, a longer time in culture may have resulted in improved maturation rates.

However, our study had some limitations. It was not powered to detect a difference in nuclear maturation rates by confocal analysis or cytoplasmic maturation rates by parthenogenetic activation. Despite finding higher activation rates and a higher incidence of bipolar spindles in GV-matured MII oocytes cultured with CC, these differences were not significant. Whether increasing the sample sizes would have allowed statistical significance to be reached is unknown. In addition, our study was limited in that we did not confirm that the CC were from *in vivo* mature oocytes, although we did choose only expanded CC clumps for study. Rescue IVM rates with CC co-culture are improved when immature oocytes are cultured with CC clumps from mature oocytes, improving development through transcriptional regulation (12). We also did not directly assess CC function after vitrification and warming in this study. In future studies, we would plan to assess warmed CC viability using Trypan Blue with a 90% threshold for inclusion, as was done in a previous study (10), and would consider assessing CC function either through gene expression profiling (32, 33) or metabolic profiling (15). Finally, we did not randomize parthenogenetic activation or confocal analysis within the oocyte group (GV vs. MI at culture start). Metaphase II oocytes, regardless of their prescued IVM meiotic state, were randomized at the time of the maturation check to confocal analysis or parthenogenetic activation. This led to unequal numbers of oocytes in each group, and small sample sizes in some groups, limiting what conclusions could be drawn.

CONCLUSION

In this simple system of media drop co-culture of CC with oocytes, culture with CC does not appear to improve nuclear and

cytoplasmic maturity of immature oocytes after ovarian hyperstimulation, at least by the markers assessed here. Further work is required to assess the efficacy of this system given its potential to provide flexibility in a busy IVF clinic. Alternatively, more complex culture systems may lead to improved rescue IVM rates postvitrification and thaw.

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