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GAMETE BIOLOGY

Maturation outcomes are improved following Cryoleaf vitrification of immature human oocytes when compared to choline-based slow-freezing

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Abstract
Purpose The cryopreservation of immature oocytes permits oocyte banking for patients at risk of losing their fertility. However, the optimum protocol for such fertility preservation remains uncertain.
Methods The present study investigated the survival, maturation, cytoskeletal and chromosome organization of sibling immature oocytes leftover from controlled ovarian stimulation cycles, that were either slow-frozen (with choline-substitution) or vitrified. A comparison group included oocytes that were never cryopreserved.
Results Among the three groups, comparable rates were observed for both survival (67-70%) and polar body extrusion (59-79%). Significantly more oocytes underwent spontaneous activation after IVM following slow-freezing compared with either vitrification or no cryopreservation. Likewise, the incidence of spindle abnormalities was greatest in the slow-frozen group, with no differences in spindle morphometrics or chromosome organization.
Conclusions While the overall incidence of mature oocytes with normal bipolar spindles from warmed immature oocytes was low, the yield using Cryoleaf vitrification was slightly superior to choline-based slow-freezing.

Keywords Immature oocyte · Choline-based slow-freezing · Cryoleaf vitrification · Spindle · Chromosomes

Capsule While the incidence of mature oocytes with normal bipolar spindles from warmed immature oocytes was low, the yield using vitrification was slightly superior to slow-freezing.

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Introduction

During the last decade, considerable progress has been made in the development of protocols for cryopreservation of mature oocytes, as evidenced by improved survival and increased implantation rates of embryos derived from such thawed oocytes (reviewed by [1–3]). However, there are compelling and practical applications for freezing immature oocytes including the preservation of fertility for cancer patients when chemotherapy must proceed within a short timeline, the contra-indication of ovarian stimulation (due to hormone-sensitive tumors), and the lack of a male partner. Cryopreservation of immature oocytes may also prove beneficial during routine ART, notably in combination with IVM for patients at risk of ovarian hyperstimulation syndrome. Moreover, the use of immature oocytes for cryopreservation may circumvent some of the limitations associated with the cooling/warming of mature oocytes, specifically relating to the functional integrity of the meiotic spindle and ploidy of resulting embryos (reviewed by [2, 4, 5]).

Many have proposed that the immature oocyte may prove less vulnerable to cryopreservation-induced injuries than the mature, metaphase-II oocyte, due to the absence of the temperature- and chemical-sensitive meiotic spindle in such prophase-I oocytes. Moreover, IVM before freezing

may render the oocyte even more vulnerable to damages associated with cryopreservation, given that IVM per se undoubtedly imposes additional stresses on the oocyte. There is thus an impetus to evaluate cryopreservation protocols of immature oocytes.

The report of a human birth resulting from the slow-freezing of a germinal vesicle (GV)-stage human oocyte [6] provided proof-of-principle that immature oocyte freezing is indeed a tangible option for routine use, should the protocols be optimized and the safety of the procedure be confirmed. Past clinical studies with GV oocytes from a variety of sources as well as freezing protocols have documented wide ranges for survival, maturation, fertilization, and pre-implantation development (slow-freezing: [7–12]; vitrification: [13–16]). For mature oocytes, advancements in protocols have resulted in encouraging outcomes, including slow-freezing with choline-substitution [17–20] and vitrification with the Cryoleaf system [15, 21–24]. However, there are limited prior reports of either choline-based slow-freezing [11, 25] or vitrification with the Cryoleaf [15] in human GV oocytes, with no previous assessments of spindle integrity or chromosome alignment.

Most recently, Fasano et al. [25] reported, for the first time, a side-by-side comparison of GV oocyte cryopreservation by either slow-freezing or vitrification. Outcome measures focused solely on survival, maturation, and fertilization, without a careful and much-needed cytological assessment following cooling and warming. In addition, no comparisons were made to non-frozen oocytes and no maturation occurred in the choline-based slow-freezing group. Taken together, these facts indicate further investigations are needed with GV oocyte cryopreservation, together with an evaluation of cytoarchitecture. Doing so is particularly relevant as much uncertainty resides in the specific tolerance of immature oocytes to cryopreservation.

Our study examined chromosome, microtubule, and microfilament organization in sibling GV human oocytes that were either slow-frozen (with choline-substitution) or vitrified (with Cryoleaf system), with results compared to non-cryopreserved oocytes; all oocytes were analyzed after IVM. Based on an increasing number of reports documenting benefits of vitrification over slow-freezing for the preservation of mature oocytes [23, 26, 27], we hypothesized that vitrification of immature oocytes would result in superior maturation and cytoskeletal outcomes when compared to slow-freezing.

Materials and methods

Source of immature oocytes

With approval from the Partners' Healthcare Institutional Review Board, oocytes were obtained from consenting patients undergoing IVF-ICSI treatment at the Brigham and Women's Hospital. Following controlled ovarian stimulation using routine protocols, cumulus-oocyte-complexes were retrieved, and within 3–5 h of retrieval they were denuded of cumulus cells in preparation for ICSI. Non-degenerate oocytes with an intact germinal vesicle (GV) nucleus were included in the study (n=128 from 46 patients). Only patients with ≤8 GVs retrieved and which represented <50% of the total retrieved cohort, were included. From photographs taken at 400X, oocyte diameters (not including the zona pellucida) were measured prior to cryopreservation or IVM. Oocytes were cryopreserved within 1 h after meiotic stage evaluation for ICSI.

Experimental design

Sibling GV-oocytes from 28 patients (mean±SD: 4.4±2.1/patient) were randomly allocated to either the slow-freezing or vitrification groups (Fig. 1). Following cryopreservation, survival of thawed/warmed oocytes was recorded. Oocytes that were deemed morphologically viable (based on intact GV and contour, a clear cytoplasm, and no signs of degeneration) were matured *in vitro* for 36 h prior to fixation and evaluation of microtubules, microfilaments, and chromatin.

For comparison, oocytes from patients with at least 1 GV-oocyte at retrieval (1.1±0.5/patient; n=18 patients) were *in vitro* matured for 36 h without cryopreservation (henceforth referred to as non-cryopreserved), and then fixed with the same cytological evaluation performed as for cryopreserved oocytes.

Slow-freezing and thawing of immature oocytes

GV oocytes were slow-frozen as previously described [28], using a choline-based slow-freezing protocol with a commercial kit (SAGE® Choline Substituted Cryopreservation or

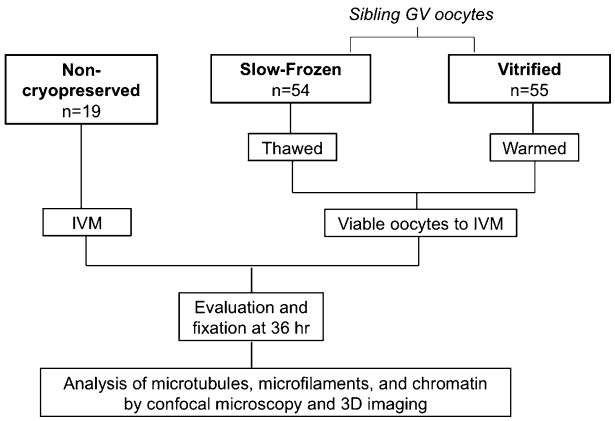

Fig. 1 Experimental design used to test the best cryopreservation protocol for immature germinal vesicle (GV) stage oocytes

CSC Media, ART-8017, CooperSurgical Company, Trumbull, CT, USA). After incubation in 1 ml of CSC Freezing Medium (137 mmol/l choline chloride, 1.5 mol/l 1,2-propanediol, 0.3 mol/l sucrose, and 20% synthetic serum substitute in HTF-HEPES) for 20 min at room temperature, one to two oocytes were loaded per straw. Sealed straws were cooled vertically in a cryobath from 22°C to −6°C at a 2°C/min rate. After holding at −6°C for 5 min, manual seeding was followed by further holding for 10 min. Following cooling down to −35°C at 0.3°C/min, straws were plunged and stored in liquid nitrogen.

Slow-frozen GV oocytes were thawed with the SAGE® CSC Thawing Medium Kit (ART-8018, CooperSurgical Company, Trumbull, CT, USA). The straw was held in air for 40 s and immersed in a 35°C water bath for 40 s until the ice had fully melted. The content of the straw was placed in a dry dish and the oocytes quickly transferred with a minimal amount of medium into 1 ml of CSC Thawing Medium (137 mmol/l choline chloride and 0.5 mol/l sucrose in HTF-HEPES) at room temperature. After incubation for 10 min, oocytes were placed in a second CSC Thawing Medium containing a decreased concentration of sucrose (137 mmol/l choline chloride and 0.2 mol/l sucrose in HTF-HEPES) for another 10 min. Oocytes were then washed twice for 5 min each at 37°C in HTF-HEPES, and thawed oocytes were evaluated morphologically for their survival. Oocytes that survived were then placed in IVM.

Vitrification and warming of immature oocytes

MediCult Vitrification Cooling and Warming kits were used with the McGill Cryoleaf™ system (ORIGIO MediCult, Jyllinge, Denmark). For cooling, GV oocytes were transferred in 1 ml of Equilibration Medium (7.5% v/v ethylene glycol, 7.5% v/v 1,2-propanediol) for a 5 min equilibration period at room temperature. Oocytes were then placed in 1 ml of Vitrification Medium (15% v/v ethylene glycol, 15% v/v 1,2-propanediol, 0.5 mol/l sucrose) where they remained for less than 1 min at room temperature. Oocytes were quickly loaded into the Cryoleaf carrier, the open end of which was heat-sealed prior to immediate plunging in liquid nitrogen for storage.

For warming, oocytes were released into 1 ml of Warming Medium (containing 1.0 mol/l sucrose) at 37°C for a maximum of 3 min. Oocytes were then transferred to a first Dilution Medium with 0.5 mol/l sucrose for 3 min at room temperature followed by a second 3 min incubation at room temperature in Dilution Medium containing 0.25 mol/l sucrose. Following two washes in Washing Medium (2 ml) for 3 min each at room temperature, oocytes were transferred to maturation medium.

In vitro maturation

A commercial kit from SAGE In-Vitro Fertilization (ART-1600, CooperSurgical Company, Trumbull, CT, USA) was used for IVM. Within 1 to 4 h after retrieval for non-cryopreserved oocytes or immediately after thawing or warming, GV-oocytes were washed and cultured in 1 ml of Oocyte Maturation Medium supplemented with 75 mIU/ml FSH and 75 mIU/ml LH (Menopur®, Ferring Pharmaceuticals, Parsippany, NJ, USA) at 37°C in a humidified atmosphere comprised of 5% CO_2, in air for 36 h. This duration of maturation was chosen to achieve a maximal number of oocytes reaching metaphase-II, with fixation prior to 36 h yielding a high proportion of telophase-I oocytes (unpubl. observation).

Fixation and processing for immunofluorescent labeling

After 36 h in IVM, mature oocytes (*i.e.* oocytes with a polar body by light microscopy; +PB), as well as those lacking a PB (*i.e.* GV and metaphase I [MI] oocytes) were fixed at 37°C for 30 min with 2% formaldehyde, 0.1% Triton X-100 in a microtubule-stabilizing buffer [29, 30]. Following fixation, oocytes were washed, stored, blocked, and processed for immunofluorescence by the simultaneous labeling of microtubules, microfilaments, and DNA. Oocytes were incubated overnight at 4°C with shaking in a 5 μg/ml mixture of mouse anti-α and anti-β-tubulin (Sigma-Aldrich, St Louis, MO, USA). Following washes, oocytes were exposed to Alexa Fluor 488 donkey anti-mouse (Invitrogen, Carlsbad, CA, USA) at 2.5 μg/ml together with Texas-red phalloidin (Invitrogen, Carlsbad, CA, USA) at 10 units/ml for 2 h at 37°C. Oocytes were then incubated in 5 μg/ml of DAPI (Sigma-Aldrich, St Louis, MO, USA) for 1 h prior to final washes and mounting in a 50% glycerol in PBS medium with minimal compression.

Analysis by laser-scanning confocal microscopy and three-dimensional imaging

Each oocyte was imaged with a Zeiss LSM 510 META confocal laser microscope. Fluorescent signals were captured after excitation with 405-, 488-, and 543 nm laser lines, using 63X or 100X objectives. For each oocyte, optical sections were obtained at 0.4 μm intervals, and a z-stack of 26–30 sections projected into a single image as shown in Figs. 2 and 3. Based on the organization of microtubules, microfilaments, and DNA, the meiotic stage of each oocyte was determined, as well as unexpected stages including activation (identified by the presence of interphase microtubules) in the absence of a PB (activated –PB, *i.e.* no polar body, no intact GV,

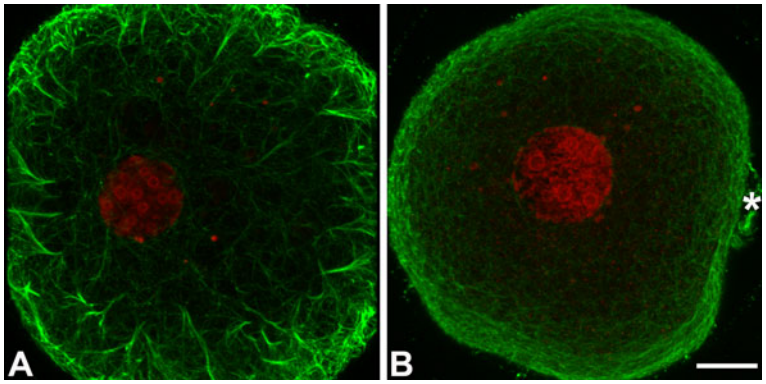

Fig. 2 Patterns of spontaneous oocyte activation in oocytes that failed to mature to metaphase-II by 36 h IVM. Shown are confocal projections of optical z-sections for microtubule (*green*) and chromatin (*red*) labeling. Panel A is a representative example of an oocyte without a polar body (Activated –PB) and with signs of spontaneous activation that is based on cytoplasmic microtubules typical of interphase with the formation of a pronuclear-like structure with decondensed chromatin. Shown in B is a representative oocyte with extrusion of a polar body (Activated + PB) but with cytoplasmic microtubules and a pronucleus, both hallmarks of oocyte activation. The asterisk marks the polar body in B. Scale bar: 10 μm

condensed or decondensed chromatin, and cytoplasmic microtubules), or the presence of a PB (activated + PB, *i.e.* with a polar body, pronucleus, and interphase microtubules) (Fig. 2).

For each M-II oocyte (+PB and single spindle), spindle and chromosome organizations were evaluated based on high-resolution rendering of multi-channel 3D datasets (in all spindle orientations) using the Volocity® Acquisition 3D imaging software (Perkin-Elmer, Branford, CT, USA). Spindles with two identifiable poles were defined as bipolar, either with defined and focused poles (BP) or with any irregularities (BP*) that included splaying of microtubule fibers at the poles or equatorial region. Non-bipolar (NBP) spindles were defined as monopolar, tripolar, or any other structures without apparent organization. Chromosomes were categorized as: aligned (A) with all chromosomes tightly disposed at the equatorial region of bipolar spindles, slightly dispersed (D*) with most chromosomes aligned at an equatorial metaphase plate and 1 to 6 chromosomes away from the plate, and dispersed (D) with >6 chromosomes not associated with the metaphase plate (Fig. 3). Pole-to-pole length and equatorial width (for BP and BP* spindles), and volumes (for BP, BP*, and NBP spindles) were obtained from 3D-reconstructions using Volocity®.

Statistical analysis

When the data were normally distributed with homogeneity of variance, means were compared using One-Way ANOVA. Otherwise, non-parametric tests were used. Fisher's Exact Chi-square tests were used to compare proportions (PASWStatistics 18.0, IBM SPSS Inc., Chicago, IL, USA). A p-value less than 0.05 was considered significant.

Results

Patient and oocyte characteristics

There were no differences (mean±SD) in patient age (non-cryopreserved: 36.5±5.6 yr; slow-frozen: 35.6±5.4 yr; vitrified: 36.0±5.2 yr), or oocyte diameters (non-cryopreserved: 114.3±8.4; slow-frozen: 118.3±5.0; vitrified: 118.8±4.9 μm) across the three groups. In addition, groups did not differ for the proportions of patients distributed among infertility diagnoses or stimulation protocols (data not shown).

Oocyte survival and maturation to + PB upon cooling and warming

Oocyte survival was comparable between the slow-freezing and vitrification groups (70.4% and 67.3% survival, respectively; Table 1). Based on the presence of a PB by 36 h under light microscopy, the incidence of + PB oocytes (of those oocytes that survived cooling/warming) was not statistically different whether oocytes were never cryopreserved (78.9%), slow-frozen (60%), or vitrified (58.8%) (Table 1).

Spontaneous activation as evaluated by fluorescence microscopy

The incidence of oocytes that underwent spontaneous activation after 36 h IVM, with or without polar body extrusion, was quantified in each group (as described above and shown in Fig. 2). There were more Activated –PB oocytes in the slow-frozen when compared to the vitrified group (17.1% versus 0%, $p=0.014$), with a similar trend being observed when compared with non-

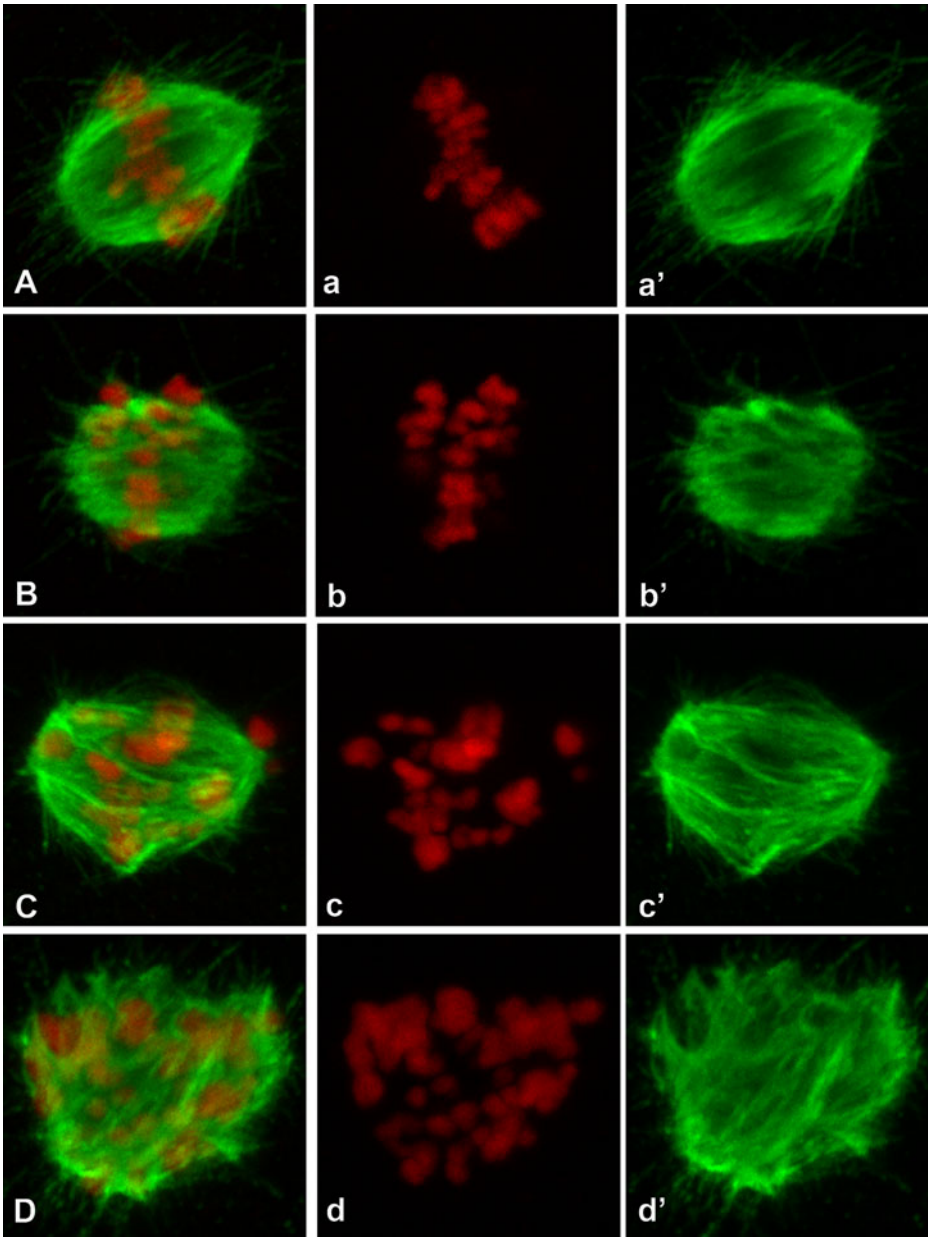

Fig. 3 Spindle and chromosome organization in matured metaphase-II oocytes. Confocal three-dimensional reconstructions of microtubules (*green*) and chromosomes (*red*) are showing representative patterns used in the classification of spindles as bipolar (A, a'), bipolar with irregularities at either the spindle poles (B, b') or equator (C, c'), or non-bipolar, including mono- or multi-polar instances (D, d'). Similarly, chromosomes were classified as aligned (a), mostly aligned with ≤6 chromosomes away from the spindle equatorial region (b), and dispersed with >6 chromosomes away or all chromosomes located throughout the spindle (c, d). Scale bar: 2 μm

cryopreserved oocytes (0%, $p=0.06$) groups (Table 2). The incidence of activation in oocytes that extruded a polar body was similar in non-cryopreserved (5.3%), slow-frozen (5.7%), and vitrified (14.7%) oocytes (Table 2).

Table 1 Survival and maturation of GV oocytes that were non-cryopreserved or cryopreserved by either slow-freezing or vitrification protocols

	Survival		Maturation to + PB	
	N	%	N	%
Non-cryopreserved	-	-	19	78.9[a]
Slow-frozen	54	70.4[a]	35	60.0[a]
Vitrified	55	67.3[a]	34	58.8[a]

Percentages that share a superscript are not statistically different.

Table 2 Incidence of spontaneous activation (with or without a polar body) after IVM for 36 h in non-cryopreserved, slow-frozen, or vitrified oocytes

	N	Activated -PB	Activated + PB
Non-cryopreserved	19	0[a,b]	5.3[a]
Slow-frozen	35	17.1[a,c]	5.7[a]
Vitrified	34	0[b]	14.7[a]

Percentages that share a superscript are not statistically different. For Activated –PB: (b,c), $p=0.014$. Note that comparison between non-cryopreserved and slow-frozen groups had a p value of 0.06 with the limitation of a small sample size in the non-cryopreserved group.

Chromosome arrangement, spindle organization and morphometric analysis

Although not significant, more MII oocytes exhibited bipolar spindles in non-cryopreserved oocytes, compared with those in either of the cryopreserved groups. Significantly more bipolar spindles with only slight irregularities were observed in the vitrified group as compared with those slow-frozen (76.5% versus 30.8%; $p=0.012$). Concomitantly, fewer mature oocytes had non-bipolar spindles in the vitrified when compared to the slow-frozen (17.6% versus 53.8%; $p=0.037$; Figs. 3 and 4a) group. The incidence of non-bipolar spindles was similar between non-cryopreserved and vitrified groups, but significantly lower in the non-cryopreserved as compared with slow-frozen oocytes ($p=0.037$; Fig. 4a).

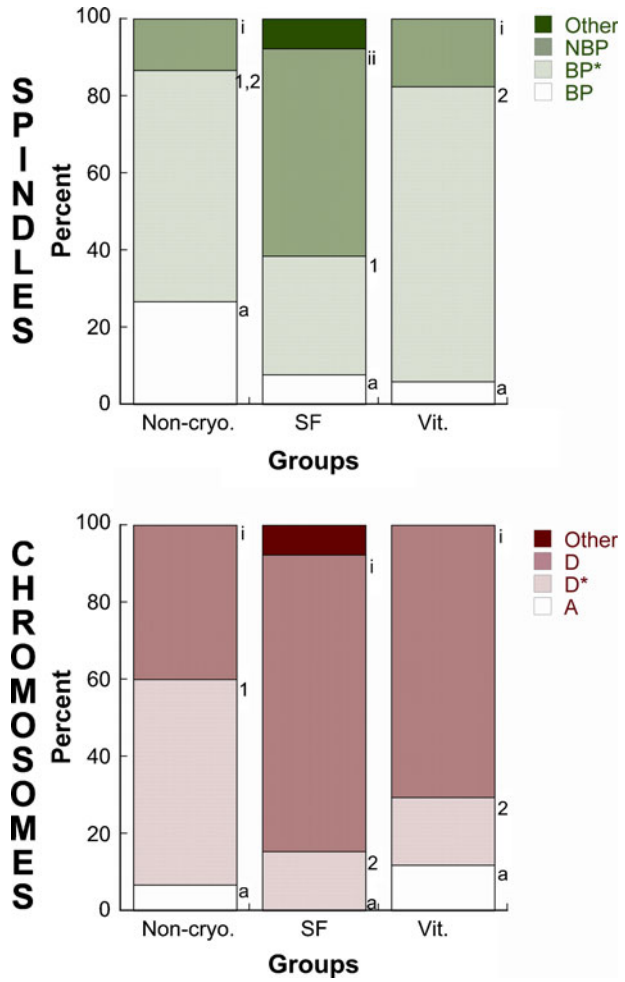

Fig. 4 Quantification of spindle and chromosome organizational patterns in metaphase-II oocytes that were *in vitro* matured without cryopreservation (Non-cryo) or after cooling/warming at the germinal vesicle stage with either a slow-freezing (SF) or vitrification (Vit) protocol. Stacked bars represent the percent of oocytes with each type of spindle (**a**) or chromosome (**b**) patterns (BP, bipolar; BP*, bipolar with irregularities; NBP, not bipolar; A, aligned; D*, mostly aligned; D, dispersed; Other, no microtubules associated with a tight cluster of chromosomes; see Fig. 3). Different letters or numbers denote statistical difference within a spindle or chromosome patterns and across the three experimental groups

While the proportions of completely aligned chromosomes in M-II oocytes were similar among the three groups, there were more oocytes with only slightly dispersed chromosomes (i.e. < 6 chromosomes away from the metaphase plate) in the non-cryopreserved group when compared to slow-frozen ($p=0.037$) or vitrified ($p=0.034$) groups. There was also a tendency towards higher proportions of oocytes with dispersed chromosomes (i.e. > 6 chromosomes off the plate) in both the slow-frozen ($p=0.049$) and vitrified ($p=0.082$) groups, compared with those not cryopreserved (Fig. 4b).

Morphometric measurements (spindle length, width, and microtubule volume) of M-II bipolar spindles were not statistically different across the three groups (data not shown).

Cortical actin organization

Two patterns of actin organization (either continuous or discontinuous) were observed in the cortex of metaphase-II oocytes after IVM (Fig. 5). The incidence of discontinuous cortical actin varied across groups, with 7%, 15%, and 35% in non-cryopreserved ($n=15$), slow-frozen ($n=13$), and vitrified ($n=17$). The difference in discontinuous actin approached significance when comparing vitrified to non-cryopreserved groups ($p=0.051$).

Overall success of cryopreserving immature oocytes by either slow-freezing or vitrification

When computing overall yields from slow freezing versus vitrification, a comparable percentage of oocytes survived (67.3% versus 70.4%) and matured (36.4% versus 38.9%) (Fig. 6). However, slow-freezing yielded significantly fewer M-II oocytes with a bipolar spindle as compared to vitrification (9.3% and 25.5%, respectively; $p=0.042$). In contrast, significantly more oocytes matured in the non-cryopreserved group (78.9%; $p=0.003$, against both freezing groups), and more MII oocytes exhibited bipolar spindles (68.4%; $p<0.0001$, and $p=0.002$ versus slow-freezing, and vitrification, respectively).

Discussion

In this study, we directly compared cryopreservation by choline-based slow-freezing withor Cryoleaf vitrification in sibling GV oocytes. In oocytes that failed to mature *in vivo*, our data demonstrate that both oocyte survival and subsequent extrusion of a polar body were similar between the two groups. However, our detailed confocal analysis

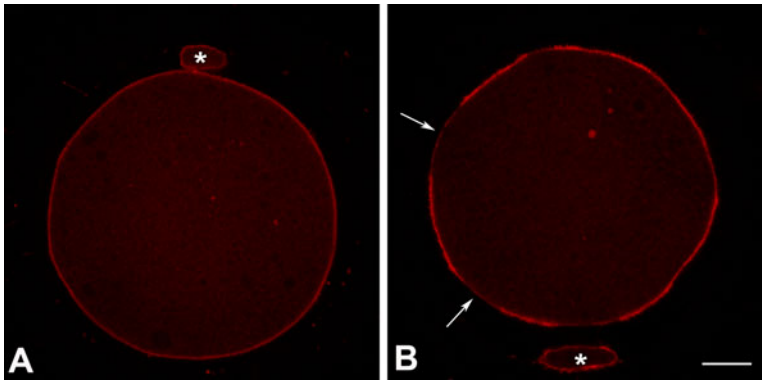

Fig. 5 Cortical actin organization in matured metaphase-II oocytes. Panel A shows a normal homogenous cortical actin network, in contrast to a discontinuous pattern with patches of cortical microfilaments intermittent with a reduction of them (B, arrows). Polar bodies are denoted with an asterisk. Scale bar: 20 μm

revealed that vitrification resulted in a higher proportion of mature oocytes with a normal bipolar spindle, as compared with slow-freezing. We conclude, therefore, that vitrification may be a slightly superior protocol over slow-freezing for GV oocytes. Nevertheless, the overall yield of oocytes with bipolar spindles is lower when compared to that of oocytes that were never frozen, thereby indicating the need for further optimization of vitrification protocols for immature oocytes.

Our results for the survival and maturation of oocytes after slow-freezing and vitrification are consistent with prior reports that evaluated either of the two protocols separately. Indeed, we obtained a 70% and 67% survival for slow-freezing and vitrification, which compare favorably with previously published ranges (43-73%: [7–12, 31]59-100%: [13–16]. Our findings regarding proportions of oocytes that extruded a polar body (60% for slow-freezing and 69% for vitrification) are also consistent with the aforementioned reports (58-83% for slow-freezing and 51-72% for vitrification)

Only one prior study directly compared the efficacy of slow-freezing and vitrification of human GV oocytes [25], and the results differed strikingly to those presented here. In the Fasano et al. study, maturation rates of 0% and 11% were achieved for slow-freezing and vitrification, respectively, in contrast to our 60% and 59%, for choline-based slow-freezing and Cryoleaf vitrification, respectively. Such differences are puzzling, although variation in exact protocols and media used may provide a partial explanation. In addition, perhaps of relevance is the lack of a sibling oocyte study design in Fasano et al. (2010). Particularly encouraging is thus the high survival and maturation rates we obtained with either cryopreservation protocol.

It is important to note that our study represents a first report evaluating the cytoarchitecture (microtubules, microfilaments, and chromatin) of oocytes following IVM, together with an assessment of oocytes that failed to extrude a polar body. The latter focus permitted the relevant comparison of potential influences of the two distinct freezing protocols on cell cycle control, including spontaneous activation. Of significance was an elevated incidence of activation in oocytes that were slow-frozen at the GV stage. Specifically, these oocytes had not extruded a polar body and displayed signs of activation with respect to the nuclear and/or cytoplasmic compartments. Such cell cycle asynchrony likely reflects disturbances in the proper control and coordination of nuclear and cytoplasmic maturation. Previous studies in ovulated mouse M-II oocytes described disturbances in calcium rises in association with the use of cryoprotectants, including ethylene glycol and 1,2-propanediol [32, 33], or following vitrification [34]. Given the pivotal roles of calcium during female meiosis [35], premature influxes of calcium could explain some of the cell cycle-related defects reported herein. Future studies should thus evaluate spontaneous activation in maturing oocytes that were cooled and warmed at the GV stage.

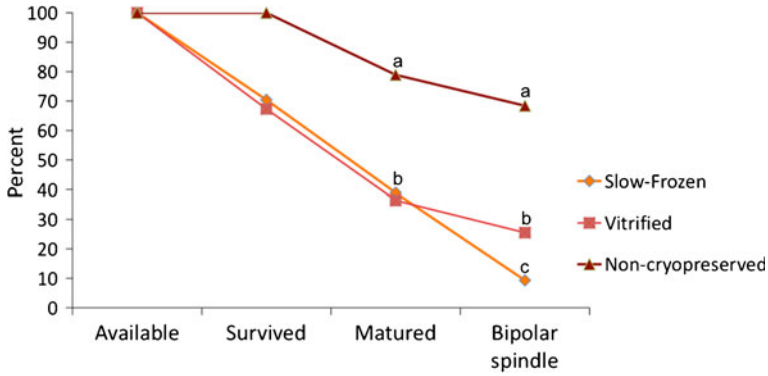

Fig. 6 Summary of the proportions of immature oocytes in each group (non-cryopreserved, slow-frozen, or vitrified) with respect to survival, maturation, and formation of metaphase-II bipolar spindles. Different letters denote statistical significance (for maturation, (a,b): $p=0.003$; for bipolar spindles, (a,b): $p=0.002$; (a,c): $p<0.0001$; (b,c): $p=0.042$)

Another interesting cellular change caused by cryopreservation pertains to the organization of the cortical actin cytoskeleton, which showed discontinuous patterns after maturation, notably in the vitrified group (35% versus 15% after slow freezing). In a previous study analyzing various slow-freezing protocols in M-II human oocytes, an interrupted cortical actin network was reported in 11-26% of oocytes [36]. Nottola et al. [24] also documented that 30% of oocytes (vitrified, human M-IIs) displayed a non-uniform distribution of microvilli at the oolemma. This abnormality was discussed in relation to possible disturbances in the sub-oolemma cytoskeletal network, and with the microfilament-richness of microvilli [24]. Patchy regions that were poor in microvilli were reported in human M-II oocytes that were slow-frozen with ethylene glycol and, interestingly, microvilli distribution at the oolemma was uniform with propanediol [37]. Lastly, a study in mouse 2-cell embryos reported on discontinous actin staining after freezing/thawing [38]. Membrane fluidity was also measured, with some interesting correlates inferred between lipid dynamics, membrane fluidity, and the integrity of the cortical actin network following freezing/thawing. Future studies of cooled/warmed oocytes should thus consider the actin cytoskeleton together with the biological significance of variants in its organization.

Aberrations in oocyte maturation were also reflected in the spindle and chromosome organization of both slow-frozen and vitrified oocytes. Of note, vitrification of GV oocytes resulted in more normal spindle structures after IVM than slow-freezing. For M-II oocytes, vitrification appears superior to slow-freezing with respect to viability and developmental outcomes [23, 26, 27]. Therefore, it is interesting that vitrification also performs better for GV oocytes, at least in the present study employing a single vitrification protocol.

With either of the protocols, oocytes present more abnormalities when subject to cryopreservation in comparison to oocytes also matured *in vitro* but with no prior cryopreservation. Overall, the success post-freezing (as gauged by the proportion of M-II oocytes with a bipolar spindle) remains low, indicating the current inefficiency in the cryopreservation of GV oocytes, at least from this source (*i.e.* the pool of oocytes that failed to mature *in vivo*) (Fig. 5). It is thus relevant to consider why GV-stage oocytes may remain challenging to freeze. There may be multiple reasons for an acute sensitivity of GV oocytes to freezing-associated injuries. Currently, GV cryopreservation relies on protocols developed for embryo freezing, and at best adapted for mature M-II oocytes. If membrane permeability differs between such distinct developmental stages as GV and MII, cryoprotectants that are best for M-II freezing may not even get a chance to act effectively in GVs. Notably, a recent report in bovine oocytes demonstrates significant differences between GV and M-II oocytes, with decreased water and solute permeability at the GV when compared to the M-II stage [39]. Also in bovine, the lipid composition of membranes underscores the cryotolerance of cells [40]; GV and M-II oocytes vary in their lipid content [41], and therefore GVs may be particularly susceptible to the osmotic and oxidative stresses associated with cryopreservation.

GV oocytes are arrested in prophase-I, a time when chromosomes are in the midst of meiotic recombination and chromosome condensation is not yet completed. This stage may be particularly susceptible to damage; of relevance are studies performed in cat immature oocytes for which a pre-treatment with a drug promoting chromatin compaction proved beneficial for subsequent developmental competence [42]. While we aimed to freeze oocytes during GV arrest, it is also possible that some of the oocytes began the process of spontaneous meiotic resumption in the time between follicle aspiration and freezing (about 2–4 h) [43]. If so, the oocytes may actually be undergoing germinal vesicle breakdown (GVBD), a time of considerable cellular remodeling, including microtubules that become more dynamic [44]. With decreased stability, the turnover rate is higher and the polymerization/depolymerization equilibrium is favored towards depolymerization; slow-freezing would thus render these microtubules even less stable, unless sufficiently protected with cryoprotectants. With warming and the dilution of tubulin monomer concentrations with water entry, the balance is also shifted towards depolymerization and new microtubules must thus form de novo. In contrast, ultra-rapid cooling rates and the solidification state reached with vitrification may minimize microtubule depolymerization.

Adequate recovery with slow-freezing may pose even more of a challenge given that GVBD is a critical period during early spindle formation when microtubules become organized around the condensed chromosomes; microtubules also promote the spatial rearrangement of organelles that is needed for nuclear and cytoplasmic maturation [45]. Freezing during GVBD may thus be a particularly risky proposition, as supported in the bovine with improved outcomes when vitrifying M-II compared to GVBD oocytes [46]. Even if oocytes were true GVs at the time of cooling, it is relevant to note that in human GV, cytoplasmic microtubules are not acetylated [43], with acetylation of α-tubulin itself an indicator of heightened microtubule stability. This lack of microtubule acetylation could perhaps contribute to a particular sensitivity of GVs to cooling, with vitrification thus here again expected to minimize the negative effects of cooling.

Given the current and well-recognized limitations of human oocyte IVM, it is perhaps not surprising that GV cryopreservation is still in an investigative phase. Even

without freezing, *in vitro* matured oocytes are still not up to par with their *in vivo* counterparts [47, 48], and the currently low efficiency of GV freezing may thus reflect additive negative effects of IVM, as well as cryopreservation. In an effort to compensate or perhaps even remedy some of the stresses associated with cryopreservation, IVM culture media may need to be tailored for use with cooled/warmed GV oocytes. Future studies should also test whether our findings apply to the use of other slow-freezing and vitrification protocols.

The source of oocytes is a limitation of our study that is worth noting. Indeed, all GV oocytes were failed to *in vivo* mature cumulus-free oocytes, and thus our study design did not make use of the exact IVM system that would be employed clinically. However, a study like ours informs future optimization efforts with respect to cryopreservation protocols and the use of relevant oocyte markers. Human studies also need to consider the influences of cumulus cell vestment (cumulus-oocyte-complex versus cumulus-free) or oocyte origin (stimulated versus unstimulated cycles) during the cryopreservation of immature oocytes. It may be that the oocyte should be surrounded by an intact cumulus oophorous when vitrifying immature oocytes but studies to date point to the challenges and cellular disruptions resulting from cryopreserving intact cumulus-oocyte-complexes [11, 49–56]. Yet, promising or improved results with the cryopreservation of cumulus-free feline [57] and bovine [58, 59] immature oocytes (obtained without hormonal stimulation) warrant further comparison of outcomes when freezing intact versus denuded cumulus-oocyte-complexes.

In conclusion, the cryopreservation of GV oocytes can permit survival of oocytes capable of maturation with bipolar spindles, albeit at low efficiencies. Cryoleaf vitrification appears a superior protocol over choline-based slow-freezing, and future efforts should focus not only on optimization but also on systematic study of targets downstream of cooling or warming. There is no doubt that fertility preservation and even routine ART can benefit from an ability to freeze immature oocytes reliably and safely. With encouraging survival and maturation rates, the ground is paved for future breakthroughs in the cryopreservation of immature oocytes.

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