The impact of vitrification on murine germinal vesicle oocyte In vitro maturation and aurora kinase A protein expression

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Abstract

Purpose Investigate the effect of vitrification on in vitro maturation (IVM) and expression of Aurora kinases A, B, and C in germinal vesicle (GV)-stage oocytes.

Methods GV-stage oocytes from B6D2F1 female mice 7–11 weeks of age were vitrified after collection, thawed, and matured in vitro for 0, 4, 8, and 12 h (hrs). The rate of germinal vesicle breakdown (GVBD), spindle apparatus assembly, and Aurora kinase mRNA and protein expression during IVM was measured.

Results Oocyte vitrification was associated with significant delays in both GVBD and normal spindle apparatus assembly at 4 and 8 h of IVM ($p < 0.05$). There was no difference in mRNA levels between control and vitrified oocytes for any of the Aurora kinases. Aurora A protein levels were reduced in vitrified compared to control oocytes at 0 h ($p = 0.008$), and there was no difference at 4 and 8 h ($p = 0.08$ and 0.69 , respectively) of IVM.

Conclusions Vitrified oocytes have delayed GVBD and normal spindle assembly during in vitro maturation. Reduced levels of Aurora A protein immediately post-thaw may be associated with the impaired oocyte maturation manifested by the delayed progression through meiosis I and II, and the atypical timing of the formation of meiotic spindles in vitrified GV-stage oocytes.

Keywords Vitrification · Aurora kinase · Oocyte · In vitro maturation

Introduction

Over the past decade, the technique of vitrification has emerged as an alternative method to slow freezing in several cryopreservation programs and has expanded clinical opportunities for fertility preservation, oocyte and embryo cryopreservation, and both autologous and donor oocyte IVF cycles. Continued refinement of vitrification techniques is important to expand our understanding of in vitro oocyte and embryonic maturation and development, and to improve clinical outcomes with oocyte cryopreservation.

Vitrification of germinal vesicle (GV)-stage oocytes has several potential advantages over freezing of metaphase II eggs. Firstly, the spindle apparatus has not yet formed in the GV-stage oocyte, while the metaphase II oocyte is arrested in development with an intact spindle. This intact spindle poses a potential vulnerability during oocyte freeze/thaw, as a properly formed and functioning spindle apparatus is prerequisite for normal chromosome segregation, and subsequent euploid embryo formation [1–4]. Re-polymerization of the spindle apparatus in the vitrified metaphase II oocyte does occur after thawing, although the kinetics are altered and there is abnormal localization of the microtubule organizing complex [5]. Collection of GV-stage oocytes is also appealing from a clinical perspective. Specifically, GV stage oocytes collection may be accomplished with less gonadotropins, thus reducing exposure to supraphysiologic hormonal levels and minimizing the risk of development of ovarian hyperstimulation syndrome and hormone-sensitive malignancies [6, 7]. Moreover, during typical stimulation cycles 15–30 % of oocytes obtained are immature (GV and MI) and may provide excess eggs and embryos for research as well as for patient with poor oocyte

Capsule Assessment of the impact of vitrification on murine GV oocyte in vitro maturation and aurora kinase A protein expression

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yields [8, 9]. Finally, higher water (dehydration) and cryoprotectant permeability of GV-stage eggs may reduce the chance of ice-crystal formation-induced damage in the egg and thus improve the overall success of cryopreservation [10, 11].

An important consideration with vitrification of GV-stage oocytes is that in vitro maturation (IVM) of thawed eggs is required to yield fertilizable metaphase II oocytes. Some studies suggested that IVM prior to vitrification is associated with improved spindle organization, mitochondrial function, and reproductive outcomes [12, 13]. GV-stage oocyte maturation involves germinal vesicle breakdown (GVBD, when the nuclear envelope is disassembled), chromosome condensation and alignment on the metaphase plate, meiotic spindle assembly, and segregation of homologous chromosomes during meiosis I. All of these events are pivotal to oocyte fertilization and normal embryonic cleavage. Although multiple studies have demonstrated that vitrification of GV-stage oocytes can contribute to compromised normal cell cycle and aberrant meiotic spindle formation, reduced fertilization rate, which can negatively impacts embryonic development [14, 15, 10], there is limited understanding of the mechanisms by which these alterations take place. Therefore, studies on the impact of vitrification on GV-stage eggs at the molecular level are necessary, so that any vitrification-induced alterations might be identified and vitrification methodologies modified as needed for clinical use.

The processes of GVBD, spindle formation, and chromatin organization are coordinated by the action of several kinases, including, the previously well-described serine/threonine kinases Aurora A, B, and C. Aurora A activation precedes GVBD, and this protein has multiple roles in initiation and successful completion of meiosis I and II, including activation of signaling pathways which mediate mRNA polyadenylation and the transition from a GV to metaphase II oocyte, regulation of microtubule organizing centers that allow accurate chromosome segregation, and proper organization and function of the meiotic spindle [16–22]. Indeed, loss of Aurora A activity reduces the rate of GVBD and is associated with formation of abnormal spindles. Aurora B is part of a chromosome passenger complex (CPC), which has an important role in the regulation of chromosome condensation and alignment, and in homologue segregation during meiosis I [23–25]. Aurora C has a high degree of homology with the catalytic domain of Aurora B, suggesting possible functional redundancy [26]. Inactivation of Aurora C results in multiple oocyte defects, including chromosomal misalignment, abnormal spindle-chromosome attachment, premature chromosome segregation, and failure of cytokinesis during meiosis I [27, 28]. Collectively, these observations indicate that Aurora kinases are critical for successful meiotic progression.

As described above, vitrification of GV-stage oocytes disrupts spindle assembly and chromosome segregation, and is associated with impaired downstream development potential

of the oocytes and subsequent embryos. Since the Aurora kinases have a well established role in oocyte maturation and spindle formation, we hypothesized that vitrification will impact the expression of Aurora kinases and GV-stage oocyte IVM. To assess this, the effect of vitrification on the rate of IVM and expression of Aurora kinase mRNA and protein in oocytes was investigated using a murine model.

Materials and methods

Animals

All experiments of this study were approved by our Institutional Subcommittee for Research and Animal Care. Animals were housed at the animal facility in accordance with the National Institutes of Health Standards for the care and use of experimental animals.

Superovulation and oocyte collection

Forty to 44 h (hrs) following intraperitoneal injection of 7–11 week old B6D2F1 female mice (Jackson Laboratory, Bar Harbor, ME) with 7.5 IU of pregnant mare serum gonadotropin (Sigma, St Louis, MO), GV oocytes were collected by follicular puncture in human tubal fluid (HTF; Invitrogen, Carlsbad, CA) media supplemented with 10 % fetal bovine serum (FBS; Invitrogen).

Oocyte vitrification and thawing procedures

Vitrification was performed immediately after collection and pooling of GV-stage oocytes. Collected oocytes were vitrified and thawed within 10 to 15 min, prior to use in subsequent experiments. During this time, control oocytes were maintained in HTF with 10 % FBS in a 37 °C incubator. Groups of 10–20 oocytes were vitrified in liquid nitrogen using the Sage Vitrification Kit (Cooper Surgical, Trumbull, CT) and the Cryoleaf™ device (Cooper Surgical) according to the manufacturer protocol. The vitrification method utilized 7.5 % (volume-volume (v/v)) of both DMSO and ethylene glycol. Following vitrification, oocytes were immediately thawed according to the protocol outlined by the Sage Vitrification Warming Kit. All vitrification equilibration and thawing steps were performed at room temperature (RT).

Oocyte In vitro maturation

Control (non-vitrified) and vitrified GV-stage oocytes with a morphologically-normal appearance were pooled separately and divided into different time-matched in vitro maturation time points (0, 4, 8, and 12 h). Oocyte maturation was performed in HTF with 10 % FBS in a 37 °C incubator with an

atmosphere of 5 % CO₂ and 95 % air (Thermo Electron Corp., Madison, WI).

Assessment of germinal vesicle breakdown (GVBD)

The proportion of oocytes that underwent GVBD after 4, 8, and 12 h of IVM was determined with direct visualization by light microscopy. GVBD was defined as the complete dissolution of the nuclear membrane, prior to the emergence of a first polar body. One hundred control and 100 vitrified oocytes were evaluated in groups of 10–20 oocytes by 2 examiners blinded to identifiers. Chi-square analysis was used to calculate statistical significance at the 0.05 level for differences in the proportion of oocytes that had undergone GVBD at the same time point for control and vitrified oocytes.

Assessment of spindle apparatus

The effects of vitrification on spindle formation and chromosome positioning during oocyte maturation was assessed in control and vitrified oocytes at 0, 4, 8, and 12 h time points, according to methods previously described [29]. An oocyte was considered normal if it had a barrel-shaped bipolar spindle with distinct, well-organized microtubule fibers, and tightly aligned chromosomes on the metaphase plate.

To evaluate the spindle apparatus, the zona pellucida was first removed by brief incubation in acidified Tyrode's solution. Oocytes were washed and fixed in 2.0 % neutral-buffered paraformaldehyde with 0.5 % BSA. To permeabilize and block the fixed oocytes, they were incubated in mouse blocking solution (Vector Laboratories) supplemented with 0.5 % BSA, 0.1 % Triton X-100, 0.05 % Tween-20, and 5 % normal goat serum. After washing, oocytes were incubated overnight in a 1:200 dilution of mouse anti- α -tubulin antibody (Sigma) in PBS with 0.5 % BSA. Following an additional wash, oocytes were incubated with a 1:250 dilution of goat anti-mouse IgG conjugated with Alexa Fluor-488 (Invitrogen). After final washing, oocytes were mounted using Vectashield™ containing DAPI (Vector Laboratories, Burlingame, CA). Analysis was performed using confocal microscopy by two examiners blinded to the identifiers.

Forty to 70 vitrified oocytes were individually examined in groups of 10 oocytes for each time point. Chi-square analysis was used to calculate statistical significance at the 0.05 level for differences in the proportion of oocytes with a normal spindle apparatus at the same time point for control and vitrified oocytes.

RNA isolation, reverse transcription, and real-time PCR

RNA was isolated from groups of 25 oocytes at each developmental time point using the RNeasy Plus Micro Kit™ (Qiagen Sciences, Hilden, Germany) per manufacturer

instructions. cDNA was synthesized from 4 µl of isolated RNA using random hexamer primers according to manufacturer's instructions for SuperScript III VILO cDNA Synthesis Kit™ (Invitrogen). Primers for mouse *Aurora A*, *B*, *C*, and *beta-Actin* were used with the SYBR Green PCR method (sequences available from www.origene.com). Real-time PCR (RT-PCR) was performed on a Real-time PCR System (Cepheid, Sunnyvale, CA). PCR reactions were performed by adding 2 µl of cDNA to the SYBR Green PCR Universal Mix (Invitrogen). Reverse transcriptase negative and sterile water controls were utilized.

Beta-Actin mRNA was used as an internal loading control for standardization, and fold differences in gene expression of vitrified oocytes were quantified and compared relative to control oocytes at the same time point using the $\Delta\Delta CT$ method. Experiments were repeated in triplicate using three different collections of oocytes with all vitrified and control time points. A paired *t*-test was used to calculate statistical significance at the 0.05 level for differences in mRNA levels at the same time point in control relative to vitrified oocytes.

Electrophoresis and western blot analysis

Groups of 50 oocytes were prepared for each developmental time point for Western blot analysis, and experiments were repeated in triplicate using three different collections of oocytes with all vitrified and control time points. Oocytes were placed in 10 µl of 1× NuPAGE LDS Sample Buffer (Invitrogen) containing reducing agent. The sample was vortexed for 1 min, and stored at −80 °C until Western blotting was performed. Thawed samples were again vortexed, denatured at 70 °C for 10 min, and total protein lysates were loaded on a NuPAGE 4–12 % Bis-Tris Gel for electrophoresis. 293 T and Jurkat cell lysates (BD Biosciences, San Jose, CA) served as a positive control for Aurora A and Aurora B; Aurora C lysate (Novus Biologicals, Littleton, CO) was a positive control for Aurora C per manufacturer instructions. Gels were equilibrated and transferred to PVDF transfer membranes (Millipore, Billerica, MA). Following transfer, the blots were blocked with 5 % nonfat milk in Phosphate-buffered saline with 0.5 % Tween (PBST-M) at RT for 90 min, followed by overnight incubation with the appropriate antibody at 4 °C on a rocking platform. Blots were then washed in PBST, and incubated at RT for 1 h with the appropriate horse-radish-peroxidase-labeled IgG secondary antibody (1:5,000 dilution). Following washing in PBST, the blot was developed with ECL Plus reagents (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions.

Primary antibodies used were rabbit polyclonal Aurora A (1:2,000 dilution; Abnova, PAB0359, Walnut, CA), mouse monoclonal Aurora B (1:2,000 dilution; BD Biosciences, #611082), rabbit polyclonal Aurora C (1:3,000 dilution; Novus Biologicals, Littleton, CO, NBP1-54994), and beta-

Actin (1:4,000 dilution; Thermo Scientific, MS-1295, Waltham, MA). Blots were stripped between different primary antibodies by agitating with stripping solution (1 M glycine, pH 2.5) for 1 h at 37 °C. Following stripping, blots were re-blocked with PBST-M for 1 h at room temperature, followed by overnight incubation with the appropriate primary antibody at 4 °C. Each blot was sequentially probed with Aurora A, B, C, and lastly beta-Actin. The beta-Actin antibody was used to verify equal protein loading for each sample. Densitometric analysis of bands was assessed using Kodak 1D 3.6 software.

A paired *t*-test was used to calculate statistical significance at the 0.05 level for differences in protein levels at the same time point in control relative to vitrified oocytes. Results were expressed in percent change of vitrified oocyte protein levels compared to controls.

Results

To investigate the rate of oocyte maturation *in vitro*, we first assessed the rates of GVBD in control and vitrified-thawed oocytes using standard morphological criteria. Following initiation of cultures, the oocytes were assessed for the presence of germinal vesicle and the first polar body every 4 h. Comparison of the percentage of GV-stage oocytes that underwent GVBD during IVM showed a reduced percentage in vitrified compared to control GV-stage oocytes after 4 h (50.7 ± 8.1 % vs 75.0 ± 3.2 %, respectively, $p=0.002$) and 8 h (75.7 ± 3.9 % vs 89.3 ± 3.2 %, respectively, $p=0.04$) of oocyte maturation. The proportion of eggs with GVBD was similar for vitrified and control eggs at 12 h, with 92.9 % and 87.2 % of oocytes that had resumed meiosis at that time point, respectively (Fig. 1).

Fig. 1 Impact of vitrification of germinal vesicle (GV) oocytes on germinal vesicle breakdown (GVBD) during *in vitro* maturation (IVM). Percent of (total oocytes in respective groups) of GVBD of control (solid line) and vitrified (dashed line) GV oocytes is depicted (100 oocytes in each group). Vitrified oocytes demonstrate significant delay in GVBD compared to control oocytes during first 8 h of maturation. ** Indicates statistical significance with $p<0.05$

We next assessed the effects of vitrification on the formation of the meiotic spindles. Analysis of the configuration of the spindle apparatus in control and vitrified GV-stage oocytes showed fewer oocytes with normal spindle assembly in the vitrified compared to control group after 4 h (10.0 ± 5.8 % vs 27.5 ± 6.8 %, respectively, $p=0.045$) and 8 h (41.4 ± 5.6 % vs 70.0 ± 4.1 %, respectively, $p=0.0007$) of oocyte maturation. In contrast, the percent of oocytes with normal spindle assembly was similar between the groups at 12 h (Fig. 2).

As Aurora kinases have been indicated in several aspects of oocyte maturation, the effects of vitrification on the expression of these kinases was investigated. Quantitative-PCR was performed on RNA collected from 25 vitrified and 25 control oocytes after 0, 4, and 8 h of IVM. There was no statistically significant difference in relative levels of Aurora A, B, & C in vitrified compared to time-matched control oocytes at any of the time points (Fig. 3). Compared to the control oocytes, 51.0 % $\pm$ 7.5 % reduction was observed in Aurora A protein levels in vitrified oocytes at 0 h ($p=0.008$). No statistically significant differences were observed at 4 and 8 h (Fig. 4) of culture. Aurora B and C proteins were not consistently detected under these experimental conditions (data not shown).

Discussion

Vitrification of GV-stage oocytes is associated with impaired development compared to non-vitrified GV-stage oocytes in terms of spindle assembly, meiotic division, fertilization rate, and embryonic development [14, 15, 10]. Contributing to these differences, this research demonstrates that one of the earliest observable differences in vitrified GV-stage oocytes is a delay in both the rate of GVBD and normal spindle assembly. To this end, we observed that progression through meiosis was significantly slower in vitrified oocytes during the first 8 h of IVM. Notably, similar numbers of eggs underwent GVBD by 12 h of culture, indicating that vitrified GV-stage oocytes retain meiotic competence. These observations correspond with the time period during which the Aurora kinases are activated and involved in GVBD and meiosis I spindle function, as described below. This study demonstrates that during the first 12 h of oocyte maturation Aurora A protein levels are reduced immediately post-thaw. This may suggest that vitrification-induced changes in meiotic progression are in part due to lower levels of Aurora A.

The Aurora kinase family is known to serve an important role in normal oocyte maturation. Aurora A has been studied most extensively, and is the most highly expressed member of this family during early oocyte maturation [19, 22, 25]. As oocytes gain meiotic competence, Aurora A mRNA and protein levels both increase, and remain elevated during the process of maturation to a metaphase II oocyte [16, 19]. Aurora A is important in accurate chromosome segregation

Fig. 2 Impact of vitrification of germinal vesicle (GV) oocytes on spindle assembly during in vitro maturation (IVM). Panel **a** Representative fluorescent micrographs of tubulin (green) and chromatin (blue) staining of control and vitrified oocytes after 0, 4, and 8 h (hr) of IVM).

Panel **b** Respective percent of oocytes with normal spindle formation in control (white bars) and vitrified (black bars) GV oocytes with a normal spindle apparatus compared to control oocytes during the first 8 h of IVM. ** Indicates statistical significance with $p < 0.05$

by regulating the formation of microtubule organizing centers (MTOCs, the oocyte analogue of centrosomes), resumption of meiosis, and proper organization and function of the meiotic spindle [16, 18, 19, 21]. Aurora B and C have a high degree of homology, suggesting the possibility of functional redundancy [26]. These proteins have an important role in the regulation of chromosome condensation and alignment, and homologue separation during meiosis [23–25, 28].

Previous studies have demonstrated significant increases in the mRNA levels of all three forms of Aurora kinase during oocyte acquisition of meiotic competence, with levels remaining elevated through the maturation process. Aurora A mRNA is the predominant form, relative to Aurora B and C, which have comparable levels of increase [19, 22, 25]. In accordance to these observations, we also detected the expression of all Aurora kinases in GV-stage oocytes. Vitrification was not associated with statistically significant changes in Aurora A, B, and C mRNA levels during the first 8 h of IVM. Despite the absence of changes in mRNA levels, vitrified oocytes had reduced Aurora A protein levels immediately post-thaw, with

recovery to control protein levels by 4 h of IVM. These data may suggest degradation of Aurora A with vitrification and subsequent post-thaw repletion. Since Aurora A mRNA levels are relatively stable in vitrified oocytes, the observed post-thaw recovery of Aurora A protein levels may reflect the increased activation and utilization of the total pool of mRNA present in the thawed eggs. Since mRNA levels reflect a pool of maternal RNA and alone do not necessarily provide an accurate representation of the cellular environment, additional investigation of both transcript and protein levels in maturing eggs is necessary.

Of the three members of the Aurora kinase family, protein levels were only detectable for Aurora A when using groups of 50 oocytes. This supports previous studies showing that Aurora A is the most abundant member of the family during this period of oocyte development. Interestingly, the immediate post-thaw (0 h) decrease in Aurora A protein levels in vitrified relative to control GV-stage oocytes resolved by 4 h of culture. During the time period of reduced Aurora A protein levels, the vitrified GV-stage eggs also lag behind in GVBD

Fig. 3 Aurora kinase A, B, and C mRNA transcript expression during in vitro maturation (IVM). This graphical representation demonstrates respective mRNA expression (real-time PCR) in each member of the Aurora kinase family in control (white bars) and vitrified (black bars)

oocytes after 0, 4, and 8 h (hr) of IVM ($n=25$ oocytes/group). Fold change in mRNA transcripts of vitrified oocytes were calculated relative to the control group of the same time point. No statistically significant differences were seen between the groups

Fig. 4 Aurora A protein expression during GV oocyte in vitro maturation (IVM). Panel **a** Representative western blot of Aurora A protein levels in control (C) and vitrified (V) oocytes after 0, 4, and 8 h (hr) of (IVM), with 50 oocytes per lane. Panel **b** Vitrified GV oocytes (black bars)

demonstrate decreased expression of Aurora A protein compared to control oocytes (white bars) after 0 h of IVM. Fold change in protein levels of vitrified oocytes were calculated relative to the control group of the same time point. ** Indicates statistical significance ($p=0.008$)

and formation of meiotic spindle. Since these differences can no longer be detected by 12 h of maturation, we speculate that a threshold level for Aurora A protein expression may be required for oocyte to proceed with normal meiotic maturation.

The observation that vitrification is associated with decreased Aurora A protein levels, which was associated with a delay in both GVBD and normal spindle assembly, is consistent with prior functional studies. Direct cytoplasmic injection of Aurora A antibodies has been shown to decrease the rate of GVBD and produces a distorted metaphase I spindle [18]. Additionally, the reduction of Aurora A using RNAi results in disruption of meiotic resumption and spindle assembly [16, 21]. Our findings support these earlier observations and provide further evidence that decreased Aurora A protein levels result in abnormal oocyte maturation.

The inability to measure Aurora B or C by Western blot is most likely the result of insufficient protein for detection. Indeed, only Aurora A and C, but not Aurora B, have previously been shown to be detectable by Western blot [16, 27]. Yang et al. showed detection of Aurora C, but not Aurora B, after 8 h of in vitro maturation using groups of 500 GV oocytes [27]. Unfortunately, input of 500 oocytes was not feasible within the experimental design of this study. To this end, future investigation is necessary to delineate the role(s) of these Aurora kinases in vitrification-induced changes in IVM.

It is unclear if vitrification causes a targeted reduction in Aurora A protein levels, or if it has a global detrimental impact on oocyte proteins and cellular function. Prior studies have demonstrated significant cytoplasmic and nuclear changes following vitrification beyond those on the spindle apparatus and chromosomes, such as mitochondrial alterations,

suggesting potential widespread effects of vitrification on oocytes [10, 30–32]. Future studies are necessary to delineate the degree to which vitrification affects the oocyte maturation pathways, as well as global cellular function.

Limitations of this study include the potential effect of the experimental protocol on oocytes. Although oocytes were maintained at 37 °C for the majority of time during preparation for the vitrification process, irreversible disruption of the meiotic spindle has been seen with cooling of an oocyte to room temperature [22]. It is possible that the findings noted in this study that have been attributed to the vitrification process may be due in part to cooling to room temperature during manipulation. It is also possible that results may vary according to different vitrification protocols. Another consideration is that for the 0 h oocyte groups, control oocytes in vitro matured for 10–15 min at 37 °C while vitrified oocytes were carried through the freeze/thaw process. This could potentially impact mRNA and protein levels, contributing to the differences seen at this time points. Albeit, it may be expected to have a small to negligible impact over such a short timeframe. An additional limitation is the inability to detect Aurora B and C protein levels by Western blot under these experimental conditions, precluding any conclusions of how vitrification impacts their role in the maturation process. Finally, cryoprotectants are known to be toxic to oocytes, and the absence of a cryoprotectant control is a limitation of this study. A previous study showed no difference in the rate of spindle abnormalities or abnormal chromosome separation between oocytes exposed or not exposed to 1 M concentrations of the cryoprotectant propanediol [15]. However, there are no studies to indicate if these results can be extrapolated to the impact of cryoprotectant on Aurora kinases, or to the impact of

alternative cryoprotectants (in the case of these experiments, 7.5 % (v/v) of DMSO and ethylene glycol).

In summary, these findings demonstrate that vitrification delays both GVBD and normal spindle assembly during the first 8 h of vitrified GV-stage oocyte IVM, and is associated with decreased Aurora A protein expression during the initial post-thaw period. Taken together, these results suggest that Aurora A may play a role in GV-stage oocyte maturation abnormalities following vitrification. These findings expand our understanding of the effect of vitrification on the enzymatic and cellular processes vital to oocyte maturation and development. These findings also provide a foundation for future investigation of the effects of vitrification, and may facilitate modifications which improve clinical outcomes with oocyte cryopreservation and assisted reproductive technology.

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Conflict of Interest The authors of this manuscript have no conflict of interest to declare.

References

- Pickering SJ, Braude PR, Johnson MH, Cant A, Currie J. Transient cooling to room temperature can cause irreversible disruption of the meiotic spindle in the human oocyte. *Fertil Steril*. 1990;54(1):102–8.
- Coticchio G, Bromfield JJ, Sciajno R, Gambardella A, Scaravelli G, Borini A, et al. Vitrification may increase the rate of chromosome misalignment in the metaphase II spindle of human mature oocytes. *Reprod BioMed Online*. 2009;19 Suppl 3:29–34.
- Eroglu A, Toner M, Leykin L, Toth TL. Cytoskeleton and polyploidy after maturation and fertilization of cryopreserved germinal vesicle-stage mouse oocytes. *J Assist Reprod Genet*. 1998;15(7):447–54.
- Bromfield JJ, Coticchio G, Hutt K, Sciajno R, Borini A, Albertini DF. Meiotic spindle dynamics in human oocytes following slow-cooling cryopreservation. *Hum Reprod*. 2009;24(9):2114–23. doi:10.1093/humrep/dep182.
- Tamura AN, Huang TT, Marikawa Y. Impact of vitrification on the meiotic spindle and components of the microtubule-organizing center in mouse mature oocytes. *Biol Reprod*. 2013;89(5):112. doi:10.1095/biolreprod.113.108167.
- Trounson A, Wood C, Kausche A. In vitro maturation and the fertilization and developmental competence of oocytes recovered from untreated polycystic ovarian patients. *Fertil Steril*. 1994;62(2):353–62.
- Chian RC, Buckett WM, Tulandi T, Tan SL. Prospective randomized study of human chorionic gonadotrophin priming before immature oocyte retrieval from unstimulated women with polycystic ovarian syndrome. *Hum Reprod*. 2000;15(1):165–70.
- Lee HJ, Jee BC, Suh CS, Kim SH, Moon SY. Oocyte maturity in relation to woman's age in in vitro fertilization cycles stimulated by single regimen. *Yonsei Med J*. 2012;53(1):181–5. doi:10.3349/ymj.2012.53.1.181.
- Cha KY, Chian RC. Maturation in vitro of immature human oocytes for clinical use. *Hum Reprod Update*. 1998;4(2):103–20.
- Van Blerkom J, Davis PW. Cytogenetic, cellular, and developmental consequences of cryopreservation of immature and mature mouse and human oocytes. *Microsc Res Tech*. 1994;27(2):165–93. doi:10.1002/jemt.1070270209.
- Agca Y. Cryopreservation of oocyte and ovarian tissue. *ILAR J / Nat Res Counc, Inst Lab Anim Resour*. 2000;41(4):207–20.
- Lee JA, Sekhon L, Grunfeld L, Copperman AB. In-vitro maturation of germinal vesicle and metaphase I eggs prior to cryopreservation optimizes reproductive potential in patients undergoing fertility preservation. *Curr Opin Obstet Gynecol*. 2014;26(3):168–73. doi:10.1097/GCO.0000000000000062.
- Lei T, Guo N, Liu JQ, Tan MH, Li YF. Vitrification of in vitro matured oocytes: effects on meiotic spindle configuration and mitochondrial function. *Int J Clin Exp Pathol*. 2014;7(3):1159–65.
- Jee BC, Chen HY, Chian RC, Suh CS, Kim SH, Moon SY. Vitrification of immature mouse oocyte using stepwise equilibration before or after in vitro maturation. *Fertil Steril*. 2009;92(3):1153–7. doi:10.1016/j.fertnstert.2009.02.030.
- Park SE, Son WY, Lee SH, Lee KA, Ko JJ, Cha KY. Chromosome and spindle configurations of human oocytes matured in vitro after cryopreservation at the germinal vesicle stage. *Fertil Steril*. 1997;68(5):920–6.
- Saskova A, Solc P, Baran V, Kubelka M, Schultz RM, Motlik J. Aurora kinase A controls meiosis I progression in mouse oocytes. *Cell Cycle*. 2008;7(15):2368–76.
- Andresson T, Ruderman JV. The kinase Eg2 is a component of the *Xenopus* oocyte progesterone-activated signaling pathway. *EMBO J*. 1998;17(19):5627–37. doi:10.1093/emboj/17.19.5627.
- Yao LJ, Zhong ZS, Zhang LS, Chen DY, Schatten H, Sun QY. Aurora-A is a critical regulator of microtubule assembly and nuclear activity in mouse oocytes, fertilized eggs, and early embryos. *Biol Reprod*. 2004;70(5):1392–9. doi:10.1095/biolreprod.103.025155.
- Swain JE, Ding J, Wu J, Smith GD. Regulation of spindle and chromatin dynamics during early and late stages of oocyte maturation by aurora kinases. *Mol Hum Reprod*. 2008;14(5):291–9. doi:10.1093/molehr/gan015.
- Ma C, Cummings C, Liu XJ. Biphasic activation of Aurora-A kinase during the meiosis I–meiosis II transition in *Xenopus* oocytes. *Mol Cell Biol*. 2003;23(5):1703–16.
- Ding J, Swain JE, Smith GD. Aurora kinase-A regulates microtubule organizing center (MTOC) localization, chromosome dynamics, and histone-H3 phosphorylation in mouse oocytes. *Mol Reprod Dev*. 2011;78(2):80–90. doi:10.1002/mrd.21272.
- Uzbekova S, Arlot-Bonnemains Y, Dupont J, Dalbès-Tran R, Papillier P, Penetier S, et al. Spatio-temporal expression patterns of aurora kinases a, B, and C and cytoplasmic polyadenylation-element-binding protein in bovine oocytes during meiotic maturation. *Biol Reprod*. 2008;78(2):218–33. doi:10.1095/biolreprod.107.061036.
- Vogt E, Kipp A, Eichenlaub-Ritter U. Aurora kinase B, epigenetic state of centromeric heterochromatin and chiasma resolution in oocytes. *Reprod BioMed Online*. 2009;19(3):352–68.
- Ruchaud S, Carmena M, Eamshaw WC. Chromosomal passengers: conducting cell division. *Nat Rev Mol Cell Biol*. 2007;8(10):798–812. doi:10.1038/nrm2257.
- Shuda K, Schindler K, Ma J, Schultz RM, Donovan PJ. Aurora kinase B modulates chromosome alignment in mouse oocytes. *Mol Reprod Dev*. 2009;76(11):1094–105. doi:10.1002/mrd.21075.
- Tseng TC, Chen SH, Hsu YP, Tang TK. Protein kinase profile of sperm and eggs: cloning and characterization of two novel testis-specific protein kinases (AIE1, AIE2) related to yeast and fly chromosome segregation regulators. *DNA Cell Biol*. 1998;17(10):823–33.

27. Yang KT, Li SK, Chang CC, Tang CJ, Lin YN, Lee SC, et al. Aurora-C kinase deficiency causes cytokinesis failure in meiosis I and production of large polyploid oocytes in mice. *Mol Biol Cell*. 2010;21(14):2371–83. doi:[10.1091/mbc.E10-02-0170](https://doi.org/10.1091/mbc.E10-02-0170).
28. Schindler K, Davydenko O, Fram B, Lampson MA, Schultz RM. Maternally recruited Aurora C kinase is more stable than Aurora B to support mouse oocyte maturation and early development. *Proc Natl Acad Sci U S A*. 2012;109(33):E2215–22. doi:[10.1073/pnas.1120517109](https://doi.org/10.1073/pnas.1120517109).
29. Selesniemi K, Lee HJ, Muhlhauser A, Tilly JL. Prevention of maternal aging-associated oocyte aneuploidy and meiotic spindle defects in mice by dietary and genetic strategies. *Proc Natl Acad Sci U S A*. 2011;108(30):12319–24. doi:[10.1073/pnas.1018793108](https://doi.org/10.1073/pnas.1018793108).
30. Manipalviratn S, Tong ZB, Stegmann B, Widra E, Carter J, DeCherney A. Effect of vitrification and thawing on human oocyte ATP concentration. *Fertil Steril*. 2011;95(5):1839–41. doi:[10.1016/j.fertnstert.2010.10.040](https://doi.org/10.1016/j.fertnstert.2010.10.040).
31. Rho GJ, Kim S, Yoo JG, Balasubramanian S, Lee HJ, Choe SY. Microtubulin configuration and mitochondrial distribution after ultra-rapid cooling of bovine oocytes. *Mol Reprod Dev*. 2002;63(4):464–70. doi:[10.1002/mrd.10196](https://doi.org/10.1002/mrd.10196).
32. Yan CL, Fu XW, Zhou GB, Zhao XM, Suo L, Zhu SE. Mitochondrial behaviors in the vitrified mouse oocyte and its parthenogenetic embryo: effect of Taxol pretreatment and relationship to competence. *Fertil Steril*. 2010;93(3):959–66. doi:[10.1016/j.fertnstert.2008.12.045](https://doi.org/10.1016/j.fertnstert.2008.12.045).