

ORIGINAL ARTICLE

CRYOPRESERVATION OF OOCYTES

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Background: Various approaches have been utilized in attempting to cryopreserve oocytes, beginning with slow cooling and more recently the advent of technique of vitrification. Now it seems that oocyte cryopreservation is no longer an experimental technique and it is being increasingly utilized in clinics around the world. As successful outcome in oocyte cryopreservation can be assessed by survival through the freeze-thaw process, potential for fertilization, embryo development and dynamics of meiotic spindles. This study aimed to analyse these features in context of vitrification and slow freezing. **Methods:** In this laboratory based study, mature MII mouse oocytes from F1(C57BL/6J X CBA) mice (n=43) were divided randomly into two groups of equal numbers and were cryopreserved by slow freezing and by vitrification. Upon re-warming these oocytes were assessed for survival and for fertilization potential. Oocytes were fixed and stained to compare the effect of both protocols on spindle reassembly and chromosome configuration 10min, 1h and 3h after warming. Unfrozen oocytes were used as controls. **Results:** A greater number of vitrified oocytes survived cryopreservation than slow frozen oocytes (70.3% vs. 12.5%, $p=0.024$). After insemination, fertilization rates were higher for vitrified oocytes as compared to slow frozen oocytes (15.86% vs. 4.6%, $p=0.046$). Morphology of the meiotic spindle was found to be in a disorganized configuration in slow frozen oocytes at all-time points (10 mins, 1 h and 3h), whereas in vitrified oocytes the spindles were found to be aligned at all-time points. Chromosomes were seen to be displaced from equatorial region in both groups. **Conclusion:** Cryopreservation of mouse oocytes was conducted with greater success using vitrification, compared to slow freezing, with survival, fertilization, and spindle assembly more favourable to a successful outcome in this model.

Keywords: Oocyte cryopreservation, Cryoprotectant, Vitrification, polarization microscopy (POLSCOPE), immuno-staining, fluorescent conjugated antibodies, slow freezing

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INTRODUCTION

Cryopreservation of embryos and gametes has found numerous applications in *in vitro* fertilization (IVF) today, and has become an important part of assisted reproduction in domestic animals, and especially in human ART (Assisted reproductive techniques). Oocyte cryopreservation can be the only option for young women who are diagnosed with cancer to preserve their fertility, as many chemo and radiotherapies are known to leave patients sterile.^{1,2} Cryopreservation is also routinely used for women who want to postpone childbearing due to various personal, social and financial reasons.³ Recent improvements in oocyte cryopreservation has also led to the establishment of egg banks, providing donor eggs to prospective patients after health checks and a period of quarantine.

In Cryopreservation, cells are rapidly cooled to sub-zero temperatures in order to halt their cellular activity. One of the key mechanisms instrumental in most forms of cryopreservation is submersion of the cells in Cryoprotectants.⁴ Cryoprotectants role in cryopreservation is to displace water within the cell, as the presence of water can lead to crystallization and this can rupture the cell membrane, negatively impacting on its ability to 'reanimate'. Successful

cryopreservation through 'slow-cooling' requires the cell to undergo a series of steps where it is osmotically equilibrated with the Cryoprotectants, then a controlled-rate cooling process to subzero temperatures, at the end it can be placed in liquid nitrogen tanks (at -196°C).⁵ Membrane kinetics, presence of intracellular water, macromolecular concentration and surface to volume ratio all play a significant role in cryopreservation.⁶ Besides having decreased surface to volume ratio, the mammalian oocyte at MII stage has a plasma membrane with a low permeability coefficient, which makes movement of Cryoprotectants and water, much slower.⁷ The surrounding zona acts as an additional layer slowing the transport process further.

The cryopreservation process can lead to premature discharge of cortical granules,⁸ which makes sperm penetration difficult thus employing ICSI (Intracytoplasmic sperm injection) as a rescue measure after cryopreservation^{9,10}. High lipid content in oocyte cytoplasm increases its susceptibility to chilling injury.⁷ The cytoskeletal architecture of oocyte with few actin microtubules present below the membrane make it particularly sensitive to the process of cryopreservation.¹¹ Combined, these differences in physiology have led to difficulty in

optimizing a protocol for cryopreservation in these cells.¹²

Various approaches have been utilized in attempting to cryopreserve oocytes, beginning with slow freezing and more recently shifting to the technique of vitrification.¹³ Vitrification is derived from a Latin word (vitrum) meaning glass.¹⁴ Thus, vitrification requires the cytosol to be converted into an amorphous glass like solid through intracellular dehydration. This is achieved using short exposure times and permeating Cryoprotectants in a higher concentration of (>4 mol) and non-permeating in a concentration of (>0.5 mol), in concert with extremely rapid cooling rates of ~108 °C/minute¹⁴ and relatively small volume of liquid¹⁵.

When comparing these techniques it is seen that oocytes undergo more structural modifications during slow freezing as compared to vitrification.¹⁶ Low survival rates and fertilization rates are attributed to factors like zona hardening, premature release of cortical granules.^{17,18} Studies on the mouse oocyte showed that on exposure to low temperature, spindles acquired abnormal configuration which corrected on returning to 37°C.¹⁸ Slow freezing and vitrification on oocyte's meiotic spindles, have been compared in many studies, some using computer assisted polarization microscopy (POLSCOPE) and some using immuno-staining and other methods, such as fluorescent conjugated antibodies¹⁹ and some concluded that spindle recovery is faster in vitrification as compared to slow freezing²⁰ however some found comparable recovery in the two groups²¹. The purpose and aim of this project was to compare vitrification and slow freezing in mouse oocytes, analyzing the effect of each technique on meiotic spindle formation and fertilization potential. We hypothesized that slow cooling of mouse oocytes has a detectable negative effect on, spindle integrity and fertilization potential as compared to vitrification.

MATERIAL AND METHODS

This was a laboratory based study. KSOM embryo handling medium was used for handling the oviduct, oocyte handling, and as a base medium for Cryoprotectants solutions. Sperm were dispersed in bicarbonate-buffered (K-SIFM-100; Sydney IVF, Sydney, Australia) supplemented with 5% BSA (Bovine Serum Albumin). Oocytes were denuded in 100IU/ml hyaluronidase in KSOM. *In vitro* fertilization and subsequent culture were performed in cleavage culture medium (CLM-50: Sydney IVF, Sydney, Australia) and blastocyst culture media (BLM-50: Sydney IVF, Sydney, Australia) supplemented with 5% BSA. Before use, 30µl drops of the culture medium overlaid with mineral oil

(Sigma:Aldrich) were equilibrated overnight under a humidified atmosphere of 5% CO₂ in incubator.

Oocytes were obtained from 4–6-week- old, virgin F1 hybrid mice (C57BL6/J X CBA) (n=43) which were superovulated via 5 IU intraperitoneal injection of pregnant mare serum gonadotropin (PMSG) (Folligon, Intervet, Bendigo) followed by human chorionic gonadotrophin 5 IU (hCG) (Chorulon, Intervet, Bendigo), ~48 hrs apart. The female mice were killed by means of cervical dislocation 13–15 h after hCG injection and then their oviducts were collected. Ovulated oocytes were released into KSOM handling medium by incising the ampulla of each oviduct under a stereomicroscope (Nikon SMZ-2B, Melville, NY). Cumulus cells were then removed after briefly exposing them (1 min) to 100 IU/mL bovine testis hyaluronidase (Sigma) in KSOM. Immediately after dispersion of the cumulus cells, the oocytes were collected and washed three times in KSOM, and those with extrusion of first polar body (MII) and with normal morphology were selected and allocated evenly to the vitrification or the slow freezing group.

For oocytes cryopreservation we prepared freezing and vitrification solutions in the lab using in house made KSOM handling media, and propanediol (PROH)(Sigma), sucrose(sigma), ethylene glycol(fluka), DMSO (Dimethyl sulfoxide) (sigma) in different concentrations for both the solutions.

The freezing solutions were prepared using KSOM handling media supplemented with 0.1M sucrose and 1.5 M PROH (Propanediol). For adding Cryoprotectants the oocytes were shifted first to freezing solution containing PROH for 10 minutes and then to second freezing solution containing KSOM+0.1 M sucrose for another 10 minute interval, during which time the oocytes were loaded onto pre-labelled plastic straws with minimum of 3 MII oocytes per straw and sealed using PVA. All these procedures were performed at room temperature.

After adding Cryoprotectants the straws were loaded on to pre-cooled Slow Cooling Machine (CryoLogic CL-863) at 20 °C, which was filled with liquid nitrogen. When the temperature reached -6°C at the rate of 3°C/min, seeding was done manually by touching straws with forceps chilled in liquid nitrogen, and machine was allowed to run for 45 mins during which time the temperature dropped to -40°C at the rate of 0.3°C/min. After complete run the straws were shifted to goblets placed in liquid nitrogen and eventually into the liquid nitrogen tanks.

For thawing straws were removed from the goblets and held in vapour phase for 10 seconds, then in air for another 30 seconds and finally in water bath at 30°C for 20 seconds. After these contents were squeezed into well of an empty Nunc dish after

cutting open the straws at the PVA end. The oocytes were then shifted into thawing solutions, which contained decreasing concentration of sucrose in KSOM handling medium to remove the permeating cryoprotectants for 10 minutes each before being shifted into handling media for 20 minutes in which last 10 minutes the temperature was increased up to 37°C. The oocytes used in IVF were finally put in culture for 3 hours at 37°C.

For vitrification of MII oocytes, the protocol using 16.5% v/v DMSO and Ethylene glycol was used. The MII oocytes were first transferred to pre-warmed KSOM handling medium and were then kept at room temperature for 10 minutes. And then in equilibrium solution containing permeating Cryoprotectants DMSO and Ethylene glycol for ~4 minutes and from there the oocytes were shifted to vitrification solution containing sucrose along with DMSO and Ethylene glycol for efficient dehydration. After about 45 seconds oocytes were loaded onto pre-labelled Fibre plug Prototype R2-Flat Tip (CryoLogic®, Mulgrave, Victoria, Australia) with 1.5ul of Vitrification solution with maximum of two oocytes per fibre plug. For 2 seconds the fibre plugs were touched on metal alloy block placed in liquid nitrogen. The fibre plugs were placed in pre-labelled goblets placed in liquid nitrogen bath and finally shifted to liquid nitrogen tanks.

For warming, the fibre plugs containing goblets were removed from LN2 tanks and put into an esky with LN2 and the fibre plugs were removed from the storage straw by keeping it in LN2 vapours. After this the hooks were submerged into one well of the pre-prepared and pre-warmed Nunc dish containing warming solution comprising of (KSOM+1 M sucrose) and then immediately shifted to the second well of same media for about 5 minutes and then shifted to another well containing about (KSOM+0.5 M sucrose) again for 5 minutes before being put in handling media.

For *in vitro* fertilization oocytes were inseminated with F1 hybrid mice sperm (C57BL/6/J X CBA) and then culture was performed under a humidified atmosphere of 5% CO₂ in air, at 37°C. For IVF, sperm were obtained from the cauda epididymides of mature (4–6-month-old) male mice. We used a total of 5 male mice for 5 repeats of the experiments. Sperm were released into the medium by gently puncturing the epididymides (5–7 times) with a hypodermic needle and were allowed to disperse for 30 minutes at 37°C. The sperm concentration was determined using a MAKLER counting chamber kit (Irvine Scientific). Insemination volume was calculated as concentration of sperm/2,500,000 and then on

dividing 50 ul by this value volume of the sperm was obtained. The oocytes, after 6 hours of incubation with the sperm were shifted to separate culture media and assessed next day for 2 cell formation. The media was changed over to blastocyst media after 48h and the rate of formation of morula and blastocyst were assessed.

All chemicals used for fixation and labelling were purchased from Sigma unless stated otherwise. Simultaneous fixation and extraction of oocytes were performed in 4% PFA in PBS well for 30 minutes. Fixed oocytes were then transferred into 0.1% Triton-X 100 in PBS for another 10 minutes. After this the oocytes were washed in PBS before being stored in a Nunc dish at 4°C. As fluctuations in temperature and pH before fixation could result in rapid depolymerization or polymerization of microtubules and were avoided.

After treatment with the fixative at 37°C, the oocytes were washed in a blocking solution, i.e., PBS containing 0.1% BSA, 0.01% Tween-20, 0.02% NaN₃ for 10 minutes and were incubated for 2h in primary antibody (anti-β Tubulin (ab134185) in 1:50 dilution in blocking solution). The oocytes were washed through 4 drops of blocking solution for 30 minutes before being incubated for 1 hour in secondary antibody (1:1000 goat anti rabbit Alexa-488 conjugated secondary in blocking solution). After secondary antibody incubation oocytes were incubated in 5ug/mL Hoechst 33342 in PBS. From this point onwards the exposure to light was minimized. After washing in blocking solution for 30 minutes, the oocytes were mounted in vector shield mounting medium (Vector Laboratories) containing DNA labelling agent (DAPI).

Nikon ECLIPSE Ci-E microscope, with fluorescein, rhodamine, and Hoechst selective filter sets was used for differential interference contrast and fluorescence microscopy of specimens and acquired images were processed using Adobe Photoshop 3.0.

After a freeze-thaw, and vitrified-warmed cycle the oocytes were assessed for survival by morphologic criteria. The oocytes were classified as morphologically viable if they displayed homogeneous cytoplasm, an intact plasma membrane, and the zona pellucida (ZP). Oocytes showing one polar body (MII) were included in the study. In this series of experiments, cryopreserved metaphase II oocytes were randomly divided into two experimental groups after cryopreservation, one is slow frozen other vitrified.

For IVF the oocytes were put in culture for ~3 hrs. For control fresh MII oocytes were

taken from female mice. There were 5 repeats of the whole experiment for IVF. There was only round of spindle fixing and staining. Three time frames were used for assessing spindle morphology after cryopreservation. Oocytes to be used at 10 minutes were immediately fixed. For 1 and 3 hour group the oocytes were immediately transferred to culture dish (washed through one drop and left in one drop for 1 hour, one drop for 3 hour group). Fresh oocytes were also fixed and stained as control.

Ethical approval was obtained from MMC (Monash Medical Centre) Animal Ethics Committee 'A' under approval no. MMCA 2011/84.

Statistical analysis was performed using SPSS (SPSS Inc. an IBM company, Chicago). Comparisons were made using paired *t*-tests. Differences between groups were considered statistically significant at $p \leq 0.05$. Five replicates were conducted and results were calculated as total of all repeats.

RESULTS

Of 297 frozen MII oocytes, there were 152 in slow frozen group and 145 in the Vitrification group. Of the slow frozen group, 19 (12.5%) survived the freeze thaw process, compared to the vitrified oocytes, where 102 (70.3%) survived the process and exhibited normal morphology. When IVF was performed on slow frozen unfertilized MII oocytes (152) the developmental rate to 2-cell embryos of the oocytes after insemination was 4.6% (7/152). In the vitrified group the developmental rate to 2 cell embryos was 15.86% (23/145).

From the 2 cell embryos formed in the slow frozen group⁷, 1 progressed to morula stage which later on developed into a blastocyst (0.65%). Whereas for the Vitrified group out of 23 2-cell embryos, 5 progressed to morula (3.44%) and 2 developed into blastocysts (1.38%). Fresh Oocytes were taken as control (119) and after insemination 59 (49.6%) developed into 2-cell embryos, out of which 36 reached morula (61%) and 35 developed into blastocysts (59.3%).

On comparing slow freezing with vitrification regarding the survival rates, we found a statistically significant difference (p -value=0.024). Similarly the difference between fertilization rates for two groups was again statistically significant (p -value =0.046).

Whereas there was no statistically significant difference between the two groups on comparing the rates of morula and blastocyst formation.

To evaluate the effect of cryop-reservation on spindle morphology, we carried out qualitative

assessment. The oocytes were divided into 3 time points 10 minutes, 1 hour and 3 hours incubation in both slow freezing and vitrification groups and they were then compared to the fresh controls. Using fluorescence microscopy spindle morphology was assessed and it showed variable dynamics of spindle organization upon thawing.

The fresh control oocytes displayed bipolar spindles with chromosomes aligned along the metaphase plate as expected. The spindle appears to be barrel shaped, bipolar with organized microtubules. The chromosomes appear to be aligned at the equatorial region.

SLOW FREEZING GROUP:

a) Slow frozen–Thaw at 10 minutes: Following slow freezing, the oocytes were first assessed at 10 minutes and they showed the spindles to be highly disorganized not confining to the normal barrel shape. Also the chromosomes were not seen at the equatorial region rather they were more concentrated at the poles. This pointed towards the effect of slow freezing protocol on spindle configuration.

b) Slow frozen–Thaw at 1 hour: When oocytes were assessed after 1 hour of incubation in the slow freeze thaw group, it was seen that the spindles had not yet regained their normal configuration and were still quite scattered. Regarding the alignment of chromosomes, they were still away from the equatorial region and were more aggregated towards the poles.

c) Slow frozen –Thaw at 3 hour: Finally analysing the slow frozen thawed oocytes after 3 hours of incubation, it was hard to see spindle morphology as well as the chromosomes. The spindles appeared as a disorganized group with absent chromosomes indicating our limitation in clearly pointing out the recovery of spindle morphology after 3 hours of culture as was expected.

VITRIFICATION GROUP:

a) Vitrified–Warmed at 10 minutes: In vitrified warm group, the oocytes were first seen after 10 minutes and it showed the spindles to be barrel shaped with organized microtubules and chromosomes aligned along the metaphase plate.

b) Vitrified–Warmed at 1 hour: In the 1 hour group, it was apparent that although the spindle morphology seemed to be normal, but the chromosomes started to lose their equatorial alignment.

b) Vitrified –Warmed at 3 hours: After 3 hours of incubation although the spindle maintained their normal organization, but the chromosomes appeared to be misaligned compared to the other groups.

Figure-1 depicts the spindle morphology and chromosomes alignment in all experimental group.

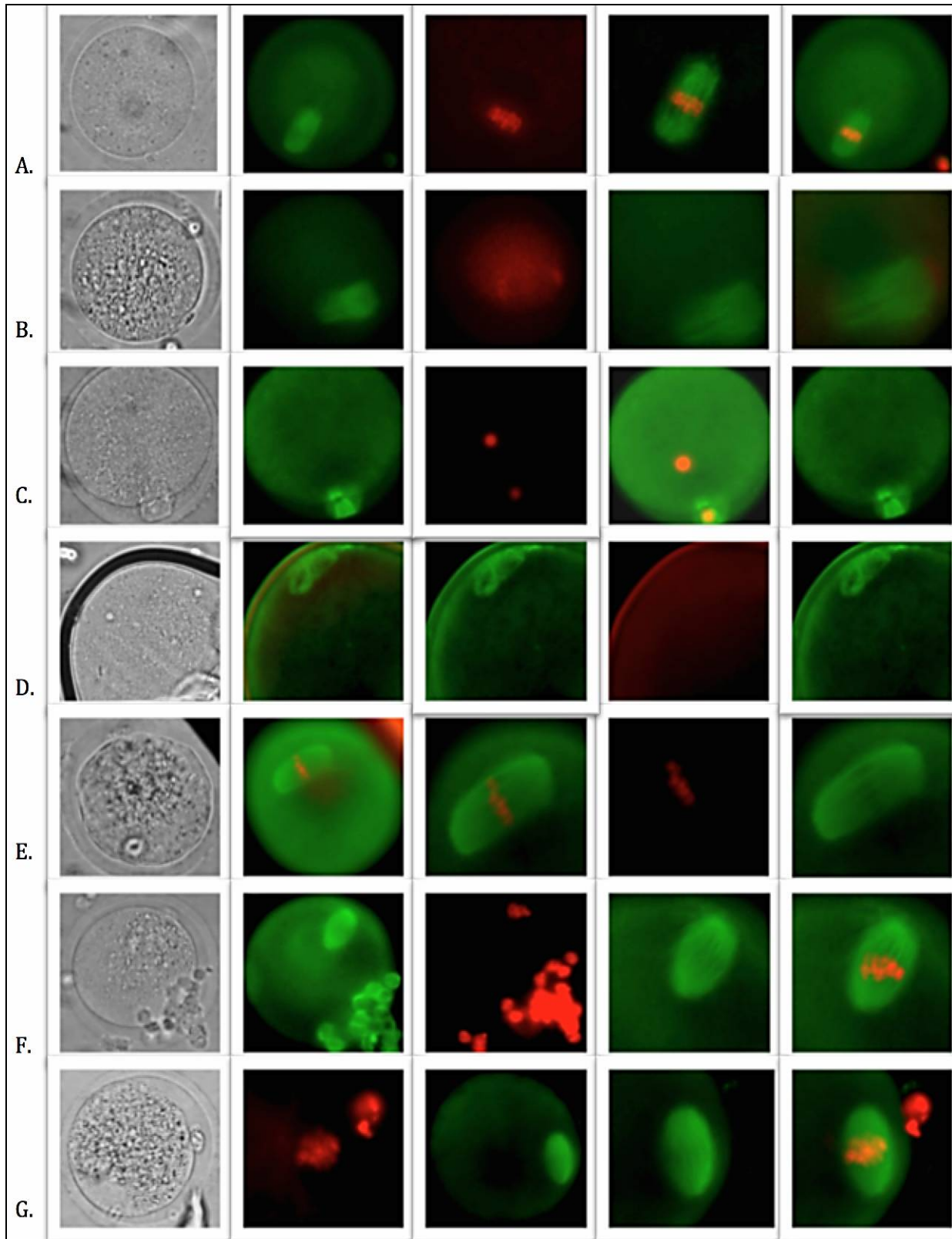


Figure-1: Bright field and high magnification fluorescence image showing spindles (green) configuration with location of chromosomes (red) in, A) Fresh oocytes, B, C, D) Slow frozen oocytes at 10 mins, 1 hour and 3 hours respectively. E, F, G) Vitrified oocytes at 10 minutes, 1 hour and 3 hours, respectively.

DISCUSSION

As mentioned previously various factors are to be taken into consideration for developing a successful cryopreservation protocol. The oocyte has proved a challenge in this regard, not only due to a reduced surface to volume ratio but also due to its altered membrane kinetics and permeability to water and Cryoprotectants.¹⁴ Various studies have investigated the effect of cryopreservation on oocyte survival and compared vitrification to slow cooling in context to oocyte survival, fertilization potential and spindle integrity, both in animal models and humans.²² In our study we tried to reassert the previous findings and tried to see which protocol works best for cryopreserving oocyte. Beginning with slow freezing, we used 1.5 M PROH as a penetrating Cryoprotectants along with 0.1M sucrose.

The survival rates for the slow frozen group was about 12.5%, which is quite better than one of the initial studies using the same protocol, where survival rates for unfertilized M11 oocytes were about 4%.¹¹ PROH has been shown to be less toxic as compared to DMSO²³ for cryopreservation in both mouse and human oocytes. The fertilization rate for slow frozen oocytes in our study was about 4.6% which again showed the detrimental effect of slow freeze thaw process on developmental competence of oocytes. Out of the oocytes which fertilized in our slow freeze thaw group only one was able to progress to morula stage and eventually developed into blastocyst.

As it is known that fertilization and embryo development are the two key functions which can indicate the cryo-survival of the oocyte, so reduced fertilization rate indicates that different mechanisms in slow freezing thawing process affects critical organelles in oocyte required for successful fertilization process. When comparing vitrification we saw that using DMSO and EG (Ethylene glycol) along with sucrose in our study, the survival rates were about 70.3% whereas fertilization rate was about 15.83%. The survival rate is comparable to other studies which show survival rate of 60–70% using a similar protocol.²⁴ But fertilization rate are pretty low in our study as compared to other studies (62%). In another study comparing slow freezing using choline based sodium depleted freezing method, the survival rates for vitrification were considerably greater than slow freezing (88.9 5.8% vs. 69.4 8.7%, p -value<0.05 in 10 replicates.²⁵ In vitrified oocytes after insemination the cleavage and blastocyst rates were also better than those of slow frozen oocytes (63.6% vs.39.9% and 30.5% vs. 19.1% respectively, p -value<0.05). The low fertilization rate can be attributed to various factors

like zona hardening which is more pronounced in slow freezing but cannot be completely ruled out in vitrification. So ICSI has been routinely applied for fertilization to overcome zona hardening. So ICSI has been routinely applied for fertilization to overcome zona hardening. However some scientists reported equal fertilization rate after insemination and ICSI.²⁶ For control, we can see the fertilization rates for fresh oocytes is about (49.6%), whereas the rate of morula and blastocyst formation is about 61% and 59.1%, respectively.

As it is known that the mammalian M11 oocyte is a mature secondary oocyte with chromosomes attached to the spindles uncovered by nuclear membrane making them rather unstable. We tried to study the effect of slow freezing and vitrification on the meiotic spindle apparatus, but due to few number of oocytes we were unable to commute the percentage of oocytes, effected in two groups as well as the rate of recovery But on studying the available stained oocytes we saw that in vitrified group even after 10 minutes the spindles were aligned with chromosomes arranged at the equatorial plate. In the 1 hour group the spindles maintained their configuration with attached chromosomes but after 3 hours the chromosomes were seen to be losing their equatorial alignment and were moving towards the pole although spindle were still barrel shaped. When compared with other studies it is evident that vitrified -warmed mouse oocytes tend to recover their spindle configuration after a period 1–3 hours.¹⁹ Our results show that after 3 hours, although the spindles maintain their shape, but the chromosomes were scattered away from the metaphysical plate. As it is known that concentration of Cryoprotectants influence the meiotic spindle assembly,²⁷ so the difference in our study can be because of use of a different protocol, but in our suggestion the optimum time for insemination or ICSI should be between 1–3 hours and not more than that to avoid oocyte aging as well as formation of aneuploid embryos. Looking at the slow freeze group, the oocytes have disorganized spindles, 10 minutes post thaw which is a finding consistent with other studies.²⁸ But most of the other studies reported recovery of spindles after incubation 1–3 hrs. post thaw.²⁹ In our study we failed to see recovery of spindle configuration after interval of 1–3 hours. Although it is seen that 1.5 M PROH protects the meiotic spindles from low temperature injury,²⁷ but in our study the spindles in slow freeze process were badly affected. The recovery of spindle configuration is important as it can lead to formation of aneuploid embryos and for this incubation time after thawing and warming is important.²⁸

CONCLUSION

As it is apparent that current protocols for cryopreservation have evolved over long period of time and the modifications kept on increasing the cryo-survival rate. Our study is an effort to validate the efficacy of these protocols and see how cryopreservation techniques effect fertilization potential and spindle integrity of oocytes. Despite limitations we were able to see that vitrification works better than slow freezing as far as survival, fertilization and configuration of meiotic spindles of mouse oocytes are concerned. However further studies are needed to study these phenomena in details and deciding optimum time for insemination after thawing or warming can be a crucial factor in deciding the successful outcome of these procedures.

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