

Comparing the viability and in vitro maturation of cumulus germinal vesicle break down (GVBD) oocyte complexes using two vitrification techniques in mice

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Received: 7 October 2007; accepted: 27 December 2007

Abstract

Background: Vitrification is assumed to be a promising method to cryopreserve human oocytes but still needs optimizing.

Objective: The aim of this study was to improve the single step and step-wise vitrification effects on maturing mouse GVBD oocytes by ethylene glycol (EG) in conventional straws.

Materials and Methods: Oocytes with compact cumulus cells were cultured for 3hr in TCM199 supplemented with 10% fetal bovine serum (FBS) in 5% CO₂ in air. GVBD oocytes were randomly allocated into three groups. (1) Control (non-vitrified group), (2) exposed to single-step vitrification (contained of EG 20%+0.5M sucrose), (3) exposed to step-wise vitrification (2%, 5%, 10%, 20%EG +0.5M sucrose). In vitrification groups, oocytes were thawed and underwent additional 21 hr maturation. Viability of oocytes and maturation to MII stage were analyzed using inverted microscope and additionally by staining of propidium iodide and Hoechst 33342.

Results: All non-vitrified oocytes were viable after 24 hr; however, viability of vitrified samples in single-step group was significantly lower than that of the step-wise and control Groups. Also, the maturation rate in the step-wise group was significantly higher ($p < 0.05$) compared to single-step.

Conclusion: These results suggest that step-wise vitrification of GVBD oocytes as compared to single step vitrification was better in the rate of survival and in vitro maturation of oocytes.

Key words: *Vitrification, Viability, In vitro maturation, GVBD, Oocyte, Mouse.*

Introduction

Cryopreservation of oocytes has important roles in the preservation and management of genetic resources, genetic engineering and nuclear transfer procedure. It has the potential to become an

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important technique for preserving gametes for female whose fertility may become compromised by medical treatment (such as chemotherapy and Radiotherapy). Oocyte preservation has been successfully applied to livestock including Cattle, Goats, Sheep, and other animals, but it is still an open challenge in most mammalian species, due to the extreme sensitivity of gametes to chilling injuries (1, 2).

Oocyte cryopreservation accompanied with in vitro maturation is important in women either are at risk of ovarian hyper stimulation, or fail to hormonal stimulation response and finally in those

patients with polycystic ovarian syndrome (3). Over one decade after the first pregnancy was achieved using frozen and thawed oocyte (4), Fabbri et al (2001) reported a modified slow freezing and rapid thawing method to freeze human oocytes that gave higher rates of oocyte survival and embryo development (5) than before (6,7). The results obtained using this method revealed that frozen- thawed oocytes had significantly lower cleavage rate than non- frozen controls (8-10). Vitrification can be used to freeze mammalian oocytes and embryos (11-13). It is an alternative to traditional freezing methods (slow freezing) to avoid chilling injury and ice crystal formation (14, 15). This method is simple and rapid compared with slow freezing; however, it still needs more studies, including animal research (16). Since the major concerns about spindle damage in oocyte freezing are all linked to the use of mature oocytes (MII) (17,18), several attempts were made to freeze immature oocytes (GV), in which the meiotic spindle is not yet formed. Unfortunately not the nucleus, but the cytoplasm appears to be the main problem in immature oocytes in cryopreservation procedure (19-21). Therefore, choosing an intermediate stage, such as germinal vesicle breakdown (GVBD), may circumvent some of the problems associated with the cryopreservation of GV and MII oocytes. With regard to this fact that the oocytes have distinct properties in each species, our research has been done on GVBD oocytes of mouse. Since 1998, there have done limited appropriate researches on GVBD oocyte stage; on the other hand some researches have only been done on bovine GVBD oocyte (20-23). Therefore, the aim of the present study was to evaluate the effects of step-wise and single step vitrification on survival and maturation rates on GVBD oocytes.

Materials and methods

All chemicals were obtained from sigma (St.Louis, MO) unless indicated otherwise.

Experimental design

Two experimental and one control groups were used in the present study. Experimental groups were vitrified immediately after getting to GVBD in step-wise or single step methods. While non-vitrified GV oocytes in control group were opposed to maturation protocols only (for 24 hr).

Animals and collection of GV oocytes

Female NMRI virgin mice aged 3 to 4 weeks old were prepared from pasture institute (Iran) and maintained for adaptation in anatomical laboratory of Tehran Medical Sciences University for 2 weeks. The animals were injected intra-peritoneal (IP) with 10 IU of pregnant mare serum gonadotropin (PMSG) for ovulation induction. The animals were killed by cervical dislocation 48 hr after PMSG administration, and the ovaries were removed and transferred into a holding medium, which consisted of TCM199 (Gibco, UK) supplemented with 10% fetal bovine serum. GV oocytes were obtained as cumulus–oocyte complexes (COCs) by puncturing antral follicles in the ovaries with a 29-G needle in the holding medium. In all experiments, only full-grown COCs, surrounded by at least two layers of cumulus cells, were selected, whereas partly or completely naked oocytes were discarded.

In vitro maturation of GV oocytes

Fresh GV oocytes were cultured in 100- μ l droplets of TCM-199 supplemented with 10% fetal bovine serum (FBS), 0.23 mM sodium pyruvate , 10 ng/mL epidermal growth factor , 100 mIU FSH (GONAL- F serono), 75 μ g/ml of penicillin G-K salts , and 50 μ g/ml of streptomycin sulfate (Sigma) under liquid paraffin oil at 37°C in an atmosphere of 5% CO₂ in humidified air, as described by Albarracin et al. (23), with some modifications. In experimental groups the maturation duration for getting to GVBD oocytes before vitrification was 3 to 4 hr.

Oocytes vitrification and thawing

Vitrification: The GVBD oocytes were vitrified by solid surface vitrification, as described by Aono et al. (10), with some modifications. The holding medium used for handling oocytes during vitrification was TCM199 containing 10% FBS. All vitrification solutions were prepared using this holding medium. Manipulation of oocytes and vitrification process were performed at room temperature (25°C). In the single step group, oocytes were exposed to %20 ethylene glycol (EG) +0.5 M sucrose in a holding medium for 40-60 sec. Oocyte in the stepwise group was exposed to vitrification solution of %2 EG for 5 min (step one), %5 EG for 3 min (step two), %10 EG for 2 min (step three) and %20 EG +0.5 M sucrose for 40-60 sec (step four). Oocytes were loaded into straws by aspiration and the straws were plunged

directly into liquid nitrogen. The straws were stored for 7 days in liquid nitrogen.

Thawing: The straws were thawed in air for 10 sec, and immediately plunged into a water bath at 37°C for 10 sec.

Thawing was carried out in four steps using sucrose solution in a holding medium containing %10 FBS at 37°C, after which the oocytes were exposed to decreasing concentrations of sucrose (0.5, 0.2, 0.1 and 0.05 M) for 1 minute. GVBD oocytes were washed three times at 37°C in maturation medium before being transferred for maturation protocol. After thawing, GVBD oocytes were matured for additional 21 hr to fulfill the 24 hr maturation requirement.

Assessment of oocyte viability

Oocyte survival was evaluated morphologically based on the integrity of the oolema and zona pellucida; Oocytes were also assessed for viability based on oolema integrity by propidium iodide (PI) and Hoechst.

For this purpose oocytes were stained with PI (10µg/ml) and Hoechst 33342 (10µg/ml) for 10 minutes, washed, and then observed under a fluorescence microscope. The dead cells showed red fluorescence (PI-positive) for disruption of cellular membrane. The viable cells showed blue fluorescence without red fluorescence (PI-negative) for the intact cell membrane (24).

Statistical analysis

Collected data were analyzed by chi-square test. The differences in the values of survival and maturation rates, were considered significant when p<0.05.

A total of 422 GV oocytes with cumulus cells were obtained from 20 ovaries that were used for 2 vitrified and 1 non-vitrified (control) groups. The number of oocytes collected averaged 21.1 per mouse ovary.

Morphology of GV oocytes

Morphological figures of vitrified GVBD oocytes using non-vitrified (control) and either single step or step-wise method is shown in figure 1 (1A, 1B and 1C). Cumulus-oocyte complexes in the single step method show dispersed cumulus cells (Figure 1C). In step-wise method (Figure 1B) cumulus-oocyte complexes were almost similar to those in the control group (Fig. 1A). Also, viability assessment of oocytes based on oolema integrity by Hoechst and PI showed red fluorescence (Figure 2A) for dead cells for disruption of cellular membrane (PI-positive) and blue fluorescence (Figure 2B) for viable cells for the intact cell membrane (PI-negative).

Viability and in vitro maturation of vitrified GV oocytes

The survival and maturation rates of GV oocytes after different treatments including exposure to 2 type of vitrification and non-vitrified oocytes shown in Table I. The survival and maturation rate in the step-wise group (81.97% and 68.43% respectively) were significantly (p<0.05) higher than single step group (58.04% and 57.12% respectively). But maturation rate in control group was significantly higher than single step and stepwise vitrified groups (p<0.05). The vitrifying/warming process in two experimental groups were greatly reduced the developmental capacity of GVBD oocytes compared to control as assessed by in vitro maturation (p<0.05).

Results

Table I. Viability and in vitro maturation of vitrified mouse GVBD oocyte

Treatment groups	No. of GV oocytes	No. of GVBD oocytes	No. of survived oocytes (after vitrification)	No. MII oocytes
Control (non vitrified)	120 (100%)	109 (90.83%)	-	92 (84.40%)*
Step-wise	160 (100%)	148 (92.50%)	121 (81.97%)*	83 (68.43%)
Single step	142 (100%)	133(93.66%)	78 (58.04%)*	44 (57.12%)*

GV= Germinal vesicle, GVBD = GV breakdown. *= $p < 0.05$

Discussion

In this study, we showed that the cryopreservation of mouse GVBD oocytes by step-wise and single step vitrification enabled oocytes to survive and mature. Successful cryopreservation of GVBD oocytes has been reported only in bovine and calf (20,22

,23), but there were low rates of survival capacity after cryopreservation. As some of researcher reported, oocytes at different maturation stages respond to cryopreservation differently (19, 25). In fact, several authors have reported that GV oocytes are just as sensitive to chilling injury (26) or more sensitive to chilling or cryopreservation than MII oocytes (27,28).Bovine

oocytes at the GVBD stage have been described as more resistant to cooling than GV or MII oocytes. However, when cryopreservation was attempted, Men et al (2002) observed that a significantly higher proportion of cleaved bovine embryos from vitrified MII oocytes developed into blastocysts than those derived from vitrified GVBD oocytes (20).

In addition to GVBD oocytes, attempts have also been made on vitrifying oocytes at other maturation stages. Thus when bovine oocytes were vitrified 0, 6, 12 or 24 hr after the onset of maturation, Hochi et al (1998) found the best stage for vitrification was that of oocytes matured for 12 hr (22). The reason for the high sensitive nature of bovine oocytes at GVBD oocytes to cryopreservation is unknown. In bovine and other mammalian oocytes, as Hunter and Moor (1987) reported, active transcription and translation occur at GVBD and later stages of meiotic maturation (28,30). Therefore, in addition to the detrimental effect on cytology, the biochemical process with the oocytes may also be affected by cryopreservation. Consequently, the impaired biochemical process will negatively influence the cytoplasmic maturation of oocytes. In addition to meiotic stages, the protocol of vitrification is important in survival, in vitro maturation and cleavage rate of oocytes after thawing. In the present study, we used step-wise and single-step vitrification methods to cryopreserve GVBD oocyte of mice. We found high rates of survival and IVM rates when the oocytes were vitrified after step-wise method. The survival and in vitro maturation in single step was lower in comparison to step-wise. Our results in step-wise group in survival rate are consistent with Men et al (20) that reported vitrification of bovine GVBD

oocytes after a two-step vitrification (81.97% versus 79.59%). Four-step vitrification in our study might be responsible for higher survival rate than the study of Men et al, in where they used two-step vitrification. Other reasons could explain such differences between their results and ours: the different size of oocyte in bovine and mouse and the different cryoprotectant agents (CPAs) in it.

Mahmmoudi et al (2005) reported that intact mouse oocytes had a higher developmental competence than denuded oocytes (31) and damage to connection between oocyte and cumulus cells after exposure to cryopreservation has adverse effect on in vitro maturation after thawing. Hurt et al (2000) reported that vitrification with EG would lead to expansion of cumulus cells probably due to damage of the gap junction between cumulus and oocytes (32). In this study, as Cetin and Bastan stated (33), contrary to Hurt et al. (32) in step-wise vitrification with EG, distribution of cumulus cells did not occur after dissolution.

Finally, as reported by other investigators, the cryodamage of the oocytes is the major determinant to the significant low maturation rate in cryopreserved oocytes. Therefore, we do not regard present vitrification protocol as a definitive protocol. Anyway, further research will help clarify the cellular and molecular mechanisms of cryopreservation-induced injury.

Conclusion

Our results suggest that step-wise vitrification of GVBD oocytes as compared to single step vitrification was better in rate of survival and in vitro maturation of oocytes.

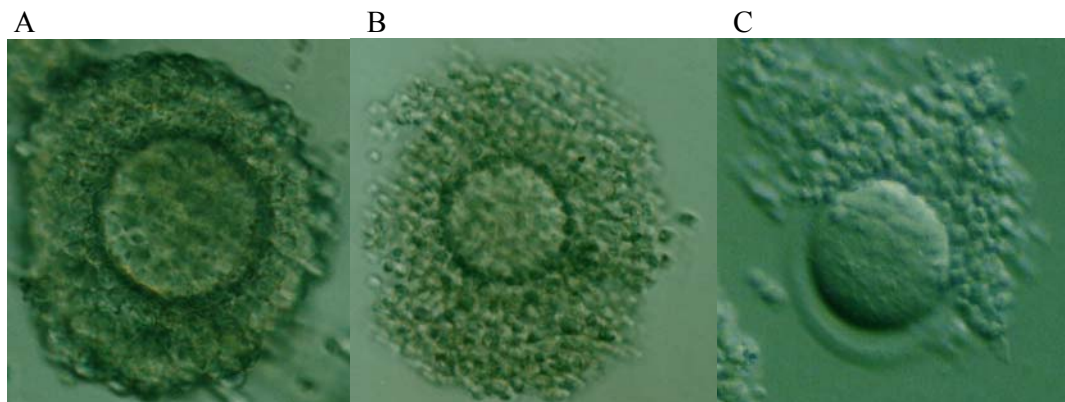


Figure 1. GVBD A, before vitrification. B, after step wise vitrification C, after single step vitrification

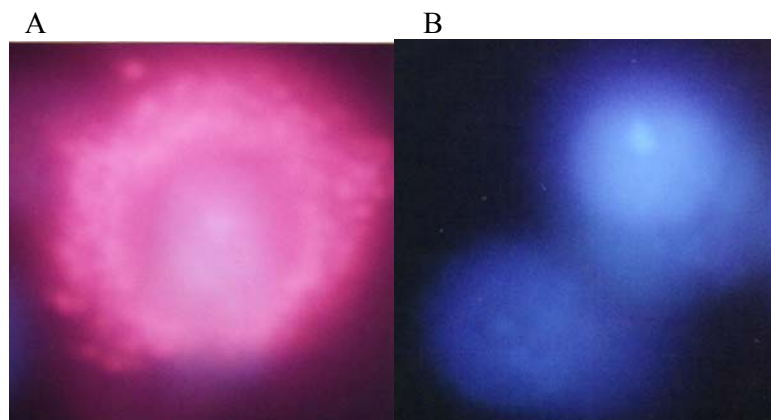


Figure 2. GVBD stained by propidium iodide and Hoechst. A, Dead B, Viable

Acknowledgements

This project financially supported by research deputy of Tehran University of Medical Sciences and Health services grant No. 85-04-30-4669. So, we would like to thank for their helps.

References

1. Kuleshova LL, Lopata A. Vitrification can be more favorable than slow cooling. *Fertil Steril* 2002; 78: 449-454.
2. Van der Elst J. Oocyte freezing: here to stay? *Hum Reprod Update* 2003; 9:463-470.
3. Cooper A, Paynter SJ, Fuller BJ, Shaw RW. Differential effects of cryopreservation on nuclear or cytoplasmic maturation in vitro in immature mouse oocytes from stimulated ovaries. *Hum Reprod* 1998; 13: 971-978.
4. Chen C. Pregnancy after human oocyte cryopreservation. *Lancet* 1986; 1: 884-886.
5. Fabbri R, Porcu E, Marsella T, Rocchetta G, Venturoli S, Flamigni C. Human oocyte cryopreservation: new perspectives regarding oocyte survival. *Hum Reprod* 2001; 16:411-416.
6. Porcu E, Ciotti PM, Fabbri R, Magrini O, Seracchioli R, Flamigni C. Birth of a healthy female after intracytoplasmic sperm injection of cryopreserved human oocytes. *Fertil Steril* 1997; 68:724-726.
7. Tucker MJ, Morton PC, Wright G, Sweitzer CL, Massey JB. Clinical application of human egg cryopreservation. *Hum Reprod* 1998; 13:3156-3159.
8. Otoi T, Yamamoto K, Koyama N, Tachikawa S, Suzuki T. Cryopreservation of mature bovine oocytes by vitrification in straws. *Cryobiology* 1998; 37:77-85.
9. Lane M, Gardner DK. Vitrification of mouse oocytes using a nylon loop. *Mol Reprod Dev* 2001; 58:342-347.
10. Aono N, Naganuma T, Abe Y, Hara K, Sasada H, Sato E, Yoshida H. Successful production of blastocysts following ultra rapid vitrification with step-wise equilibration of germinal vesicle-stage mouse oocytes. *J Reprod Dev* 2003; 49:501-506.
11. Hong SW, Chung HM, Lim JM, Ko JJ, Joon TK, Yee B, et al. Improved human oocyte development after vitrification: a comparison of thawing methods. *Fertil Steril* 1999; 72:142-146.
12. Lane M, Bavister BD, Lyons EA, Forest KT. Containerless vitrification of mammalian oocytes and embryos. *Nat Biotechnol* 1999; 17: 1234-1236.
13. Yoon TK, Chung HM, Lim JM, Han SY, Ko JJ, Cha KY. Pregnancy and delivery of healthy infants developed from vitrified oocytes in a stimulated in vitro fertilization-embryo transfer program. *Fertil Steril* 2000; 74: 180-181.
14. Rall WF, Fahy GM. Ice-free cryopreservation of mouse embryos at -196°C by vitrification. *Nature* 1985; 313: 573-575.
15. Gupta MK, Uhm SJ, Lee HT. Cryopreservation of immature and in vitro matured porcine oocytes by solid surface vitrification. *Theriogenology* 2007; 67: 238-248.
16. Cai XY, Chen GA, Lian Y, Zheng XY, Peng HM. Cryoloop vitrification of rabbit oocytes. *Human Reprod* 2005; 20: 1969-1974.
17. Mandelbaum J, Anastasiou O, Lévy R, Guérin JF, de Larouzière V, Antoine JM. Effects of cryopreservation on the meiotic spindle of human oocytes. *Eur J Obstet Gynecol Reprod Biol* 2004; 113:17-23.
18. Hyttel P, Vajta G, Callesen H. Vitrification of bovine oocytes with the open pulled straw method: Ultra structural consequences. *Mol Reprod Dev* 2000; 56: 80-88.
19. Otoi T, Yamamoto K, Koyama N, Suzuki T. In vitro fertilization and development of immature and mature bovine oocytes cryopreserved by ethylene glycol with sucrose. *Cryobiology* 1995; 32: 455-460.
20. Men H, Monson RL, Rutledge JJ. Effect of meiotic stages and maturation protocols on bovine oocyte's resistance to cryopreservation. *Theriogenology* 2002; 57:1095-1103.
21. Ambrosini G, Andrisani A, Porcu E, Rebellato E, Revelli A, Caserta D, et al. Oocyte's cryopreservation: state of art. *Reprod Toxicol* 2006; 22: 250-262.
22. Hochi S, Ito K, Hirabayashi M, Ueda M, Kimura K, Hanada A. Effect of nuclear stages during IVM on the survival of vitrified-warmed bovine oocytes. *Theriogenology*. 1998; 49:787-796.
23. Albarracin JL, Morato R, Izquierdo D, Mogas T. Vitrification of calf oocytes: effects of maturation stage and prematuration treatment on the nuclear and cytoskeletal components of oocytes and their subsequent development. *Mol Reprod Dev* 2005; 72:239-249.

24. Zhang X, Li XH, Ma X, Wang ZH, Lu S, Guo YL. Redox-induced apoptosis of human oocytes in resting follicles in vitro. *J Soc Gynecol Investig* 2006; 13:451-458.
25. Lim JM, Ko JJ, Hwang WS, Chung HM, Niwa K. Development of in vitro matured bovine oocytes after cryopreservation with different cryoprotectants. *Theriogenology* 1999; 51:1303-1310.
26. Martino A, Pollard JW, Leibo SP. Effect of chilling bovine oocytes on their developmental competence. *Mol Reprod Dev* 1996; 45:503-512.
27. Zeron Y, Pearl M, Borochoy A, Arav A. Kinetic and temporal factors influence chilling injury to germinal vesicle and mature bovine oocytes. *Cryobiology* 1999; 38:35-42.
28. Goud A, Goud P, Qian C, Van der Elst J, Van Maele G, Dhont M. Cryopreservation of human germinal vesicle stage and in vitro matured M II oocytes: influence of cryopreservation media on the survival, fertilization, and early cleavage divisions. *Fertil Steril* 2000; 74:487-494.
29. Sirard MA, Florman HM, Leibfried-Rutledge ML, Barnes FL, Sims ML, First NL. Timing of nuclear progression and protein synthesis necessary for meiotic maturation of bovine oocytes. *Biol Reprod* 1989; 40: 1257-1263.
30. Hunter AG, Moor RM. Stage-dependent effects of inhibiting ribonucleic acids and protein synthesis on meiotic maturation of bovine oocytes in vitro. *J Dairy Sci* 1987; 70:1646-1651.
31. Mahmoudi R, Subhani A, Pasbakhsh P, Abolhasani F, Amiri I, Salehnia M, et al. The Effects of cumulus cells on in vitro maturation of mouse germinal vesicle stage oocytes. *IJRM* 2005; 3:74-78.
32. Hurt AE, Landim F, Seidel GE, Squires EL. Vitrification of immature and mature equine and bovine oocytes in an ethylene glycol, ficoll and sucrose solution using open-pulled straws. *Theriogenology* 2000; 54:119-128.
33. Cetin Y, Bastan A. Cryopreservation of immature bovine oocytes by vitrification in straws. *Anim Reprod Sci* 2006; 92:29-36.