JOURNAL OF CLINICAL AND DIAGNOSTIC RESEARCH

How to cite this article:

MAHMOUDI R ,RAGARDI KASHANI I ,ABBASI M ,AMIDI F, SOBHANI A ,ABOLHASANI F SAADIPOUR KH,AMIRI I.IN VITRO MATURATION AND FERTILIZATION CAPACITY OF MOUSE GV-STAGE OOCYTE FOLLOWING STEPWISE VITRIFICATION. Journal of Clinical and Diagnostic Research [serial online] 2008 December [cited: 2008 December 1]; 2:1234-1239.
Available from
http://www.jcdr.net/back_issues.asp?issn=0973-709x&year=2008&month=December&volume=2&issue=6&page=1234-1239&id=259

ORIGINAL ARTICLE

In Vitro Maturation And Fertilization Capacity Of Mouse GV-Stage Oocyte Following Stepwise Vitrification

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ABSTRACT

Background: The storage of oocytes is important for advances in reproductive biology and infertility treatment. Although successful procedures for cryopreservation of human metaphase II oocytes have been reported, their results have proven unsatisfactory, and appear to require further progress. The microtubular spindle of MII oocytes is sensitive to temperature changes. Germinal vesicle (GV) stage oocytes do not have microtubular spindle, so its cryopreservation may be an alternative approach to the storage of gametes.

Aims: examining the viability and subsequent developmental ability of murine GV oocytes after vitrification.

Setting: Embryology research center at an academic institution.

Design: Original Article

Methods and Materials: Germinal vesicle oocytes with cumulus cells were transferred to vitrification solution, which was composed of 30% (v/v) ethylene glycol, 18% (w/v) Ficoll-70, and 0.3 M sucrose either by single step or in a step-wise way. After vitrification and storage in liquid nitrogen, the oocytes were thawed and washed two times in medium TCM199 and then subjected to in vitro maturation, fertilization and culture.

Results: The oocyte survival, maturation to MII, fertilization and development rates in step-wise exposure were found to be significantly higher ($P<0.05$) when compared with corresponding rates in the single step procedure. Collected data was analyzed by one way ANOVA test.

Conclusion: The results of present study indicate that GV stage oocytes have better morphology and viability after vitrification in stepwise method; and their rates of maturation and development to 2-cell stage are also higher as compared to oocytes cryopreserved via single step procedure.

Key Words: Oocytes Cryopreservation, Germinal Vesicle Oocyte, Vitrification, Microtubule.

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Introduction

Cryopreservation of human gametes and embryos has become an integral part of assisted reproduction. The major developments in cryopreservation technology have mirrored the rapid expansion of reproductive technology over the past 2 decades. It is now possible to cryopreserve sperm, oocytes and embryos at their various stages of development [1]. There are two methods of cell cryopreservation, slow cryopreservation and vitrification. Many cell types can be successfully cryopreserved by slow cryopreservation but some cell types can not be cryopreserved by slow equilibrium methods, or yield poor survivals.

The common reasons for failure are high sensitivity to extreme temperatures, inability to maintain an intracellular supercooled state, and disruption of complex cell-cell interactions by extracellular ice. One important cell type that has been found difficult to cryopreserve by slow freezing, is the mammalian oocyte. Generalized high sensitivity to extremes of temperature is one reason. Another, important reason is that mature oocytes are locked in the metaphase of Meiosis II; i.e., the chromosomes are arrayed on the meiotic spindle. The spindle is composed of microtubulin material, and like microtubules in general, it becomes disaggregated by being cooled to temperatures around 0°C[2],[3],[4],[5].

In case of cells and tissue types where equilibrium slow freezing yields inferior results, increasing efforts are being made to achieve cryopreservation by vitrification procedures. Vitrification has been used successfully for storage of mature (metaphase II stage) oocytes [6],[7],[8],[9],[10]. But data about vitrification of germinal vesicle (GV)-stage oocytes are limited, although this mode of cryopreservation can be promising [11], [12].Up till the stage of Germinal vesicle (GV) oocytes have not formed spindles, which poses significant problems in successful cryopreservation of oocytes. At this stage, the chromosomes are decondensed and enclosed within nuclear membrane, potentially making them more resilient to cooling.

Regimes for collection of fully-grown GV-stage human oocytes have been devised that involve modification of mature oocyte collection techniques [13] and reduction in hormonal stimulation. Studies have shown that GV-stage oocytes which are stripped of cumulus cells have a reduced developmental capacity compared with that of cumulus-enclosed GV-stage oocytes [14]. Cumulus cells play an important role on oocyte maturation, since they provide and transfer several known and unknown factors that are essential in regulation of meiotic progression, normal nuclear and cytoplasmic maturation of oocytes and subsequent embryonic development after fertilization [15],[16],[17],[18]. The aim of this study was to compare the effects of stepwise and single step exposure to cryoprotectant on the developmental ability of vitrified mouse oocyte in ethylene glycol-sucrose in presence of cumulus cells.

Methods and Materials

All chemicals were purchased from Sigma Chemical Co., St. Louis, MO, except for the ones specifically described.

Collection Of GV Oocytes

Oocytes were obtained from 3-4 week old ICR strain female mice, the animal were kept under controlled condition (14h light: 10h dark) and fed water and pellets ab

libitum. Mice were stimulated by an i.p. injection of 7.5 IU pregnant mare serum gonadotropin (PMSG). 46h later the animals were killed by cervical dislocation and the ovaries were removed in Hepes-buffered human tubal fluid medium (HTF) (sigma) supplemented with 5mg/ml BSA. The GV-stage oocytes of ovarian antral follicles were released by puncturing with a 28G micro-injection needle under a stereomicroscope. The “GV cumulus oocyte complexes” were randomly allocated to three groups:control without treatment, stepwise preservation and single-step vitrification group.

Preparation Of Vitrification And Thawing Solutions

The solutions for vitrification and dilution were prepared using PB1 plus 20% fetal bovine serum. The stepwise vitrification solution consisted of solution A, which was composed of 10%(v/v) ethylene glycol, 4.5%(W/V) Ficoll-70, and 0.075M sucrose, and solution B which was composed of 20% (V/V) ethylene glycol, 9.0% (W/V) Ficoll-70, and 0.15 M sucrose, and solution C, which was composed of 30% (V/V) ethylene glycol, 18% (W/V) Ficoll-70, and 0.3 M sucrose. Single-step vitrification solution consisted only of 30% (V/V) ethylene glycol, 18% (W/V) Ficoll-70, and 0.3 M sucrose. The solutions for thawing were made of 0.5, 0.25 and 0.125 M sucrose.

Vitrification And Thawing

The GV-COC were randomly divided into either stepwise group or single-step group. In stepwise group, the GV-stage oocytes were exposed first for 5 min to 200-ml droplets of solution A, then for 2 min to 200- µl drop of solution B and finally for 1 min to 200- µl drop of solution C in 4-well plates. In single step group, the GV-stage oocytes were exposed for 1 min to 200- µl drop of solution C.

The procedures were performed at room temperature of 22-24ºC. After obtaining equilibrium, 10-15 GV oocytes were loaded into a 0.25 ml plastic straw (IVM, I Aigle, France). The straw was filled with 1 cm of vitrification medium, 0.5 cm of air, 2 cm of Vitrification medium containing oocytes, 0.5 cm of air and 3.5 cm of vitrification medium. The straw was heat sealed and plunged into liquid nitrogen. After storage for 1-5 days, straw was taken out of liquid nitrogen, held in air for 10 s, and then plunged into water of 37ºC for 10 s. Straw was then removed from water and wiped dry. It was cut with scissors and the contents containing oocytes were expelled into 400-µl drop with a sequential series of 0.5, 0.25, and 0.125 M sucrose by keeping for 90 s in each solution, and washed for 6 min in α-MEM medium supplemented with 20% FBS.

Maturation Of GV Oocytes

The vitrified-thawed GV oocytes or fresh GV oocytes (control group) were cultured in 100 µl drop of IVM medium composed of α-MEM supplemented with 0.23 mM sodium pyruvate, 1 mg/ml of fetuin, antibiotic and antimycotic solution (comprising 100 U of penicillin, 100 µg of streptomycin, and 0.25 µg of amphotericin B), 10 ng/ml of mouse epithelial growth factor of culture-grade (EGF, Upstate Biotechnology Inc, Lake placid, NY), 75 mU/ml of follicle-stimulating hormone (fertinome, serono, Geneva, Switzerland), and 3 mg/ml of bovine serum albumin (BSA, Fraction V, Sigma) under mineral oil of embryo-tested grade (sigma), and incubated at 37ºC in an atmosphere of 5% CO_2 in humidified air. 16-18 h after culture, the GV-COC oocytes with first polar body were defined as mature MII oocytes.

In Vitro Fertilization And Development

Spermatozoa from male ICR mice of 12 weeks age were released by cauda Epididymis puncture in to IVF medium TYH medium supplemented with 4 mg/ml BSA. The sperms were suspended in 200 µl droplet of the IVF medium, were covered with mineral oil and incubated at 37ºC for 1-2 hrs in humidified atmosphere of 5% CO_2 for capacitation. Mature oocytes (n=15-20) were placed in separate 200 µl droplet of

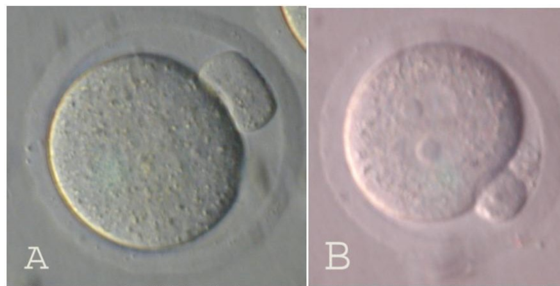
IVF Medium under mineral oil. Sperm mixture (10-20 µl) was added to each droplet to obtain a concentration of 1-2 × 10^6 motile sperm/ml. after co-incubation for 5 h at 37ºC, the oocytes were then removed and washed in fresh TYH medium and placed in a 5% CO2 incubator at 37ºC. At 6-8 h post-insemination, embryos with two distinct pronuclei and a second polar body were classified as PN stage, observed under a phase-contrast inverted microscope. The pronuclei stages were then transferred to 100 µl of KSOM (potassium Simplex optimized medium) for 2-cell stage development.

Statistical Analysis

Collected data was analyzed by one way ANOVA test. The difference in values of Survival, Maturation, Fertilization and Developmental rate, were considered significant when p value was<0.05.

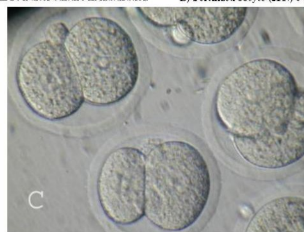
Result

A total of 570 GV oocytes with cumulus cells were obtained from 26 ovaries that were used for vitrified and non-vitrified (control) group.



A) Maturation to metaphase II 24 h after culture in maturation medium.

B) Fertilized oocyte (2PN) 6-8 h after insemination.



C) Development to 2-cell stage 24 h after insemination.

(Table/Fig 3) Maturation, fertilization and development to 2-cell stage 0f GV oocytes after vitrification.

All pictures taken under inverted microscope at ×400 original magnification

Morphological figures of nonvitrified (controls) and vitrified GV oocytes using either single step or stepwise method are shown in [Table/Fig 3]. Partly dispersed cumulus cells and large subzonal space are noticeable. In stepwise method COC were similar to those in the control group

Survival and in vitro maturation of vitrified GV oocytes

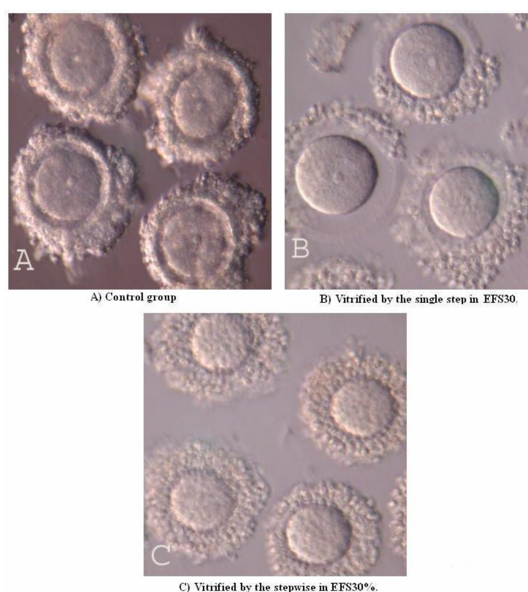
The survival and maturation rates of GV oocytes after different treatments including exposure to cryoprotectant, vitrification and for non-vitrified oocytes are shown in [Table/Fig 1]. The survival and maturation rate in the stepwise group was significantly higher than single step group (p<0.05). But maturation rate in control group was significantly higher than both single step and stepwise vitrified groups(P<0.05).

(Table/Fig 1) The percentage of mouse oocytes that survived after vitrification.The percentage of Matured oocyte after 24 h culture and the percentage fertilized oocyte after IVF. The percentage of 2-cell embryos developed from with fertilized oocytes.The maturation, fertilization and developmental rates in the stepwise group significantly higher than single step group (p <0.05).

Groups	No. GV oocyte	Survival rate (%)	Maturation rate after 24h culture following fertilization and cleavage rate				
			No. of GV (%)	No. of GVBD (%)	No. of MII (%)	Fertilization Rate (%)	2cell Stage (%)
Control	252	250(99.2)	0	24 (9.47)	228 (89.41)	204 (80.76)	192 (75.58)
Stepwise	150	136 (90.9)	2 (1.34)	39 (29.27)	95 (71.23)	77 (57.80)	72 (53.19)
Single step	168	116 (69.1)	9 (6.89)	35 (30.67)	72 (62.42)	53 (46.78)	44 (38.95)

In Vitro Fertilization And Development Of Vitrified GV

In vitro fertilization and development after in vitro maturation of vitrified GV oocytes is shown in [Table/Fig 2]. The rates of fertilization and development to 2-cell stage in the control group were significantly higher than the entire vitrified group. Among the vitrified groups, the fertilization and developmental rates in the stepwise group treatment with EFS10, EFS 20 and EFS 30% were significantly higher than those of the single step treatment only with EFS30%. Furthermore, in stepwise vitrified group the fertilization and developmental rates were significantly higher than those in single step group (p <0.05).



A) Control group

B) Vitrified by the single step in EFS30.

C) Vitrified by the stepwise in EFS30%.

(Table/Fig 2) Figures of the GV-COCs.

All pictures taken under inverted microscope at ×200–400 original magnification

Discussion

In the present study mouse oocytes have been used as an easily available source. We have investigated effects of stepwise and single step exposure to cryoprotectant on the developmental ability of vitrified oocyte in ethylene glycol-sucrose in presence of cumulus cells. The vitrified oocytes were evaluated by post thawing survival, in vitro maturation, in vitro fertilization and developmental capacity to 2-cell stage. We provide evidence that vitrification of immature mouse oocytes in a stepwise manner results in satisfactory maturation and fertilization rates.

Also, this study emphasizes that the connection between cumulus cells and the oocyte in the GV-COCs is important for completion of normal oocyte maturation in vitro. Partly dispersed cumulus cells in the oocytes after single step vitrification, shows that the connection between cumulus cells and oocytes may be disturbed during vitrification and result in lower in vitro maturation rates in this group. The role of cumulus cells on oocyte maturation, ovulation, and fertilization has been identified before [18].Our results show that the presence of cumulus cells is important for oocyte maturation and fertilization after vitrification. In agreement to our study, Abe et al [19] reported survival, fertilization, maturation and developmental rate of bovine GV-COCs with using Nylon-Mesh and exposure with stepwise cryoprotectant to be significantly higher ($p < 0.05$) compared with the single-step vitrification. Also, Anon et al [20],[21] reported higher survival, maturation and development rates when using ultrarapid vitrification accompanied with step-wise equilibration in mouse GV oocytes than single step vitrified group.

Conclusion

In conclusion, the introduction of vitrification and rapid freezing techniques is gaining widespread recognition and might become the procedure of choice over traditional slow freezing methods. This study shows that GV oocytes can be vitrified successfully with both single step and stepwise methods. But, better survival and maturation and developement rates can be obtained when GV oocytes are vitrified by stepwise procedure with conventional straws.

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