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Experimental Research

Anatomy Section

# The Nuclear Maturation and Embryo Development of Mice Germinal Vesicle Oocytes with and without Cumulus Cell after Vitrification

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## ABSTRACT

**Background:** Cryobiology is an essential tool in assisted reproductive technology. Research in this area focuses on the possibility of restoring fertility in women with reproductive problems or after cancer treatments.

**Aim**: The purpose of this study was to evaluate viability of oocytes, In vitro maturation and embryo development in vitrified germinal vesicle oocytes with and without cumulus cell after single and stepwise vitrification procedure.

**Materials and Methods**: Germinal vesicle oocytes with or without cumulus cells were obtained from 4 weeks old female mice 48h after intraperitoneal injection of 7.5 IU pregnant mare serum gonadotropin (PMSG). For vitrification collected oocytes vitrification were exposed to cryoprotectant, 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 exposure to cryoprotectant and immerged in liquid nitrogen, the oocytes were thawed and washed in medium TCM199 two times. Then the oocytes transferred to IVM medium for maturation and embryo development to blastocyst.

**Results**: The oocytes survival rates after vitrifying-warming, maturation rate, the capacity of fertilization and embryonic development to blastocyst were examined in vitro. The oocytes survival, maturation to MII, fertilization developmental rate in the step-wise exposure and with cumulus cell was significantly higher ($p<0.05$) as compared with corresponding rate in the single step procedure without cumulus cell.

**Conclusion**: The results of present study indicated that oocytes vitrified with cumulus cells and stepwise procedure had positive effect on maturation and developmental rate to blastocyst than oocytes without cumulus cell and single step procedure.


**Keywords:** Cryoprotectant, Ethylene glycol, Immature oocytes, Stepwise vitrification

## INTRODUCTION

An important factor for the advances in reproductive biology and infertility treatment is the storage of oocytes [1]. Oocyte cryopreservation is a successful alternative for storing the excess oocytes during the assisted reproductive technology therapies [2]. Cryopreservation is widely applied in reproductive biology to preserve the reproductive potential of excess embryos and, therefore, to reduce the need for repeated gonadotropin-stimulation protocols [1]. The clinical use of germinal vesicle (GV) stage oocytes to be matured for in vitro fertilization (IVF) may be preferable to traditional IVF treatment as the risk of ovarian hyperstimulation syndrome is reduced [3-4]. High rates of nuclear maturation have been reported following cryopreservation and in vitro maturation (IVM) of murine GV-stage oocytes using a variety of cryopreservation techniques [5-7]. In some cases, the rate of fertilization was reported to be similar for thawed and fresh control oocytes [7]. It has been established that coupling of somatic cumulus cells with the GV-stage oocyte is vital to the progression of oocyte maturation and subsequent embryo development [8,9]. 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 [10,11]. Cumulus cells play an important role in oocyte maturation since they provide and transfer several known and unknown factors that are essential for normal nuclear and cytoplasmic maturation of oocytes and subsequent embryonic development after fertilization [12,13].

In the present study mouse oocytes were used as an easily available source. The aim of the study was to evaluate the influence of a stepwise and single step vitrification method on embryo development of oocytes with and without cumulus cells. The GV oocytes were evaluated by post thawing survival, maturation, fertilization and developmental capacity to blastocyst stage.

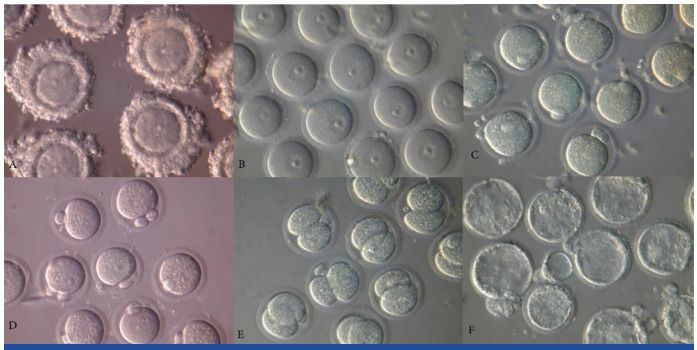
## MATERIALS AND METHODS

**Collection of GV oocytes:** In this study 3-4 week old ICR strain female mice have been used for oocytes collection. The mice were kept under standard protocol. The mice were stimulated by an intraperitoneal injection of 7.5 IU pregnant mare serum gonadotropin. The animals were killed after 48h. A 28G micro-injection needle under a stereomicroscope was used puncturing ovarian antral follicles to release the GV-stage oocytes. The collected GV oocytes with and without cumulus cell were randomly assigned to control and experimental groups.

**Vitrification:** The GV cumulus oocytes complexes (GV-COC) [Table/Fig-1A] and GV denuded oocytes (GV-DO) [Table/Fig-1B] were randomly divided into either a stepwise group or a single-step group. In the stepwise group, the oocytes were exposed first for 5min to 200-ml droplets of solution A, then for 2 min to 200- µl droplets of solution B and finally for 1 min to 200- µl droplets of solution C. The solution A consisted of 10%(v/v) ethylene glycol, 4.5% (W/V) Ficoll-70, and 0.075 M sucrose, the solution B, consisted of 20% (V/V) ethylene glycol, 9.0% (W/V) Ficoll-70, and 0.15 M sucrose, and the solution C, consisted of 30% (V/V) ethylene glycol, 18% (W/V) Ficoll-70, and 0.3 M sucrose in 4-well dishes. In the single step group, the GV-stag was exposed for 1 min only to 200- µl drop of solution C.

The vitrified GV oocytes were loaded into a 0.25 ml plastic straw (IVM, I Aigle, France). The straw then was submerged into liquid nitrogen, The cryoprotectant was removed from the oocytes during warming. The oocytes transferred into 400 µl drop with sequentially solutions of 0.5, 0.25, and 0.125 M sucrose by keeping for 90s in each thowing solution. The thawing oocytes washed for 3 min in TCM 199 medium contained 20% fetal bovin serum. All procedures were done at room temperature of 22-24ºC.

**Maturation of GV oocytes:** The vitrified-thawed GV oocytes or fresh GV oocytes (control group) were cultured in 100 µl drop of IVM



**[Table/Fig-1]:** . A) GV oocytes with t cumulus cell. B) GV oocytes without cumulus cell. C) Maturation to MIIoocytes. D) Fertilization oocyte (2PN). E) 2-cell stage. F) blastocysts stage

| Groups | No. of GV oocyte examination | Oocyte Survival% | Final stage of oocytes maturation | | |
|---|---|---|---|---|---|
| | | | No GV% | N. of GVBD% | Maturation MII% |
| Control COC | 252 | 252(100)a | 0 | 24 (9.47) | 226(89.41)a |
| Control DO | 221 | 221(100)a | 28(12.66 ) | 23(10.40 ) | 169 (77.47 )a |
| Stepwise COC | 150 | 134(88.96)b | 2(1.34) | 34(25.37) | 98(73.23)b |
| StepwiseDO | 169 | 144(85.20) | 30( 17.75) | 30( 17.75) | 86(50.88) |
| Single-stepCOC | 168 | 116(70.6) | 9(6.89) | 35(30.67) | 72(62.42) |
| Single-stepDO | 156 | 103 (66/02) | 29(18.58 ) | 17(10.89) | 54 (34.61 ) |

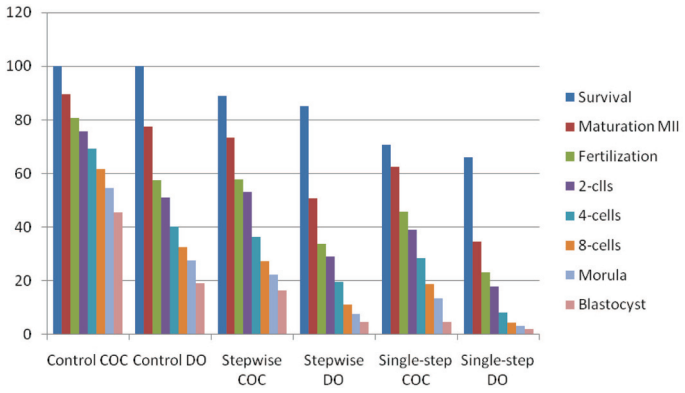
**[Table/Fig-2]:** Fertilization, and cleavage rates and embryo development to blastocysts of vitrified mouse GV oocytes with and without cumulus cell *compared to all groups p<0.05; b compared to Stepwise DO and Single-step DO p<0.05; c compared to Stepwise DO, Single-step COC and Single-step DO p<0.05; d compared to Single-step DO p<0.05.*

medium (α-MEM). 24h after the culture, the GV oocytes with first polar body were defined as mature MII oocytes [Table/Fig-1C].

**In vitro fertilization and development:** We used ICR male mice of 12 week old obtained spermatozoa. The caudal epididymis was punctured into TYH medium contained with 4 mg/ml bovine serum albumin. The released sperms were transferred in 200 µl droplets of the IVF medium. The droplets were covered with mineral oil and incubated for 1-2 h at a 37ºC in a humidified atmosphere with 5% $CO_2$ for sperm capacitation. About 15-20 mature MII oocytes were added to 200 µl droplets of IVF medium. Sperm suspension at a concentration of 10-20µl was added to each droplet to obtain a concentration of 1-2 × $10^6$ motile sperms and then incubated for 5 h at 37ºC. The oocytes were pipetted to remove the sperms and washed in fresh TYM medium and transfered in $CO_2$ incubator at 37ºC. At 6-8 h post-insemination, embryos with two distinct pronuclei and a second polar body were classified as PN stage [Table/Fig-1D] observed under a phase-contrast inverted microscope. The fertilized oocytes (2pn) were transferred to 100 µl of potassium simplex optimized medium for more development to 2-cell [Table/Fig-1E] and blastocyst stage [Table/Fig-1F]. A total of 1116 oocytes were obtained from 60 ovaries and they were used for in vitro maturation. The average number of collected oocytes was almost 19 per ovary.

## STATISTICAL ANALYSIS

Data were analyzed by SPSS version 17. We calculated the proportion of survival and maturation rates of GV oocytes to MII and embryo development from MII to the blastocyst stage and then compared the proportion rate of each stage in different groups in a 2x2 tables and then summarized the results. Chi-Square test was used to compare the groups in term of maturation, fertilization and developmental rate. The differences in the proportions were considered significant when p<0.05.




**[Table/Fig-3]:** Survival, Maturation, Fertilization and Embryo Development to Blastocyst rates of Vitrified mouse GV oocytes., *COC= cumulus-oocytes complex, DO= Denuded oocytes*

| Groups | Fertilization (%) | 2cell (%) | 4cell (%) | 8cell (%) | Morula (%) | Blastocyst (%) |
|---|---|---|---|---|---|---|
| Control COC | 204(80.76)a | 192(75.58) a | 177(69.38) a | 157(61.58) a | 139(54.65) a | 117(45.62) a |
| Control DO | 127(57.46)b | 113(51.13) b | 89 (40.27)b | 72 (32.57) b | 61(27.60)b | 42 (19.04)b |
| Stepwise COC | 77(57.80)c | 70(53.19 )c | 50(36.50 )c | 39(27.35 )c | 32(22.20)c | 22(16.41)c |
| Stepwise DO | 57(33.72) | 49 (28.99) | 33(19.52) | 19(11.24) | 13(7.69) | 8(4.73) |
| Single-step COC | 53(45.68)d | 44(38.95 )d | 32(28.45 )d | 22(18.76 )d | 16(13.47 ) | 6(4.84) |
| Single-step DO | 36(23.07) | 28(17.94) | 13 (8.33) | 7 (4.48) | 5(3.20) | 3(1.92) |

**[Table/Fig-4]:** Fertilization, and cleavage rates and embryo development to blastocysts of vitrified mouse GV oocytes with and without cumulus cell, *a compared to all groups p<0.05; b compared to Stepwise DO and Single-step DO p<0.05; c compared to Stepwise DO, Single-step COC and Single-step DO p<0.05; d compared to Single-step DO p<0.05*

## RESULTS

**Survival and in vitro maturation of vitrified GV oocytes:** The survival and maturation rates of GV oocytes after different treatments including vitrification and non-vitrified oocytes are shown in [Table/Fig-2,3]. The survival and maturation rates in the stepwise COC group were significantly higher than those for the single-step DO group, but the maturation rate in the control group was significantly higher than that in the single step and stepwise vitrified group (p<0.05).

**In vitro fertilization and embryo developmental of vitrified GV oocyte:** The in vitro fertilization and development of GV oocytes after in vitro maturation is shown in [Table/Fig-4,3]. The rates of fertilization, cleavage and embryonic development to blastocyst stage in the control group were significantly higher than those for all of the vitrified groups. Among the vitrified groups, the fertilization and developmental rates in the stepwise group were significantly higher than those for the single step group (p <0.05).

## DISCUSSION

In this study, we showed that after stepwise and single step exposure to cryoprotectants, the mouse GV oocytes with and without cumulus cells were able to survive, mature, fertilize, cleavage and develop to blastocyst stage through vitrification. The injury to the cells in the process of cryopreservation can be due to osmotic effects accompanying saturation with permeable cryoprotectants [14]. The reduction in subsequent developmental competence caused by exposure to cryoprotectant solutions has been shown to be more severe in the GV oocytes than MII oocytes [15-16].

Whittingham [17] reported that survival rate of mouse oocyte after freezing-thawing is not different between oocytes with and without cumulus cells prior to freezing although beneficial effects of cumulus cells on oocyte survival after freezing-thawing have been reported by

some investigators [18-19]. The beneficial effects of cumulus cells on oocytes survival rate after freezing-thawing have been reported when a slow freezing method was used [18, 20-21]. Johnson and Packering [18] have suggested that the presence of the cumulus cells can reduce the adverse effects of DMSO on the oocytes. It has been suggested that the presence of cumulus cells can minimize the release of cortical granules and premature zona reaction for zona hardening resulting in low fertilization rates [22]. Chian, et al., [23] reported that oocytes survival rates following vitrification are not affected by the presence or absence of cumulus cells.

Fuku, et al., [24] and Kasai [25] proposed that the supplementation of saccharides such as sucrose in the vitrification medium could reduce toxicity to the embryos by reducing the extracellular concentration of the cryoprotectant. In the present study, the GV oocytes vitrified by stepwise exposure to cryoprotectant showed significantly higher IVM and IVF and cleavage rates and lower degeneration rate than the corresponding rates vitrified by the single step. In the single-step method, as in the previous report [26] treatments of EFS30 showed that the GV-COCs after vitrification had partly dispersed cumulus cells and oocytes with shrunken cytoplasm. The connection between cumulus cells and the oocyte in the GV-COCs is shown to be important for completion of normal oocyte maturation in vitro [27]. Hochi, et al., [28] vitrified immature bovine oocytes in straws by using a mixture of 40% EG, ficoll and sucrose as a vitrification medium. They reported 47.5% fertilization rate from the vitrified bovine oocytes. In another study in which immature bovine oocytes were vitrified using a mixture 2.5 M EG, ficoll and sucrose in open pulled straws [29], a successful maturation rate of 60% was recorded. Cetin, et al., [30] vitrified immature bovine oocytes and found that 34.1% of oocytes reached the MII stage in EG group. In the present study, we found the decrease of IVM rate in the single step with EFS30 indicating that, during vitrification of mouse GV-COCs, the disconnection between cumulus cells and the oocyte may be caused by the same phenomenon explained in the reports mentioned above. In addition, in the present study, the time for exposing oocytes to the final cryoprotectant was only 1 min, which seems to provide insufficient penetration of the cryoprotectant into the COCs in the single-step group, although the prolongation of treated time resulted in a lower viability by toxic effect of cryoprotectant [31]. Vitrification is a non-equilibrium cryopreservation method that needs relatively high concentrations of cryoprotectants, and a stepwise addition of cryoprotectants may reduce the toxic effect of cryoprotectants and be considered to minimize damage due to extreme cell-volume expansion [32,33]. Ohboshi, et al., [34] reported that vitrification of bovine blastocysts, and with two-step exposure to the cryoprotectants showed less damage compared with the single-step procedure. Abe, et al., [16] reported survival, fertilization, maturation and developmental rates to blastocyst of bovine GV-COCs, using Nylon-Mesh and expoxer with stepwise cryoprotctant, were significantly higher compared with the single-step vitrification. Aono et al., [34-35] reported higher survival, maturation and blastocysts rates, using ultra rapid vitrification accompanied by step-wise equilibration in mouse GV oocytes than single step vitrified group [35-36]. We showed in our previous study that using step-wise vitrification method increased the rates of maturation, fertilization and blastocyste formation in GV oocytes. Since vitrification is a nonequilibrium cryopreservation method that needs a relatively high concentration of cryoprotectants, a step-wise addition of cryoprotectants may reduce the toxic effects of cryoprotectants and be considered to minimize damage due to extreme cell-volume expansion [13,37].

## CONCLUSION

GV oocytes with and without cumulus can be vitrified successfully with single step and stepwise methods. Better survival, maturation and developmental rates to blastocysts can be obtained when GV oocytes are vitrified by stepwise procedure using conventional straws (0.25 ml). It can be concluded that more successful results could be achieved by using containers that obtain a higher cooling-warming ratio (Cryotop) or minimum drop size.

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