Original Article

Comparison of Different Vitrification Procedures on Developmental Competence of Mouse Germinal Vesicle Oocytes in the Presence or Absence of Cumulus Cells

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Abstract

Background: An evaluation of the developmental competence of vitrified mouse germinal vesicle (GV) oocytes with various equilibration and vitrification times; in the presence or absence of cumulus cells and by comparison between the cryotop method and straws was performed.
Materials and Methods: Mouse GV oocytes were considered in cumulus-denuded oocytes (CDOs) and cumulus-oocyte complexes (COCs) groups. Their survival and developmental rates were studied in the following experiments: (I) exposure to different equilibration times (0, 3 and 5 minutes) and vitrification (1, 3 and 5 minutes) without plunging in LN2 as toxicity tests, (II) oocytes were vitrified using straws followed by exposure to equilibration solution for 0, 3 and 5 minutes and vitrification solution for 1 and 3 minutes, and (III) oocytes were vitrified by cryotop following exposure to equilibration for 5 minutes and vitrification for 1 minute, respectively.
Results: Maturation and developmental rates of the COCs were higher than CDOs in the non-vitrified group ($p<0.05$). The survival and maturation rates were low in all oocytes exposed to vitrification solution for 5 minutes ($p <0.05$). In vitrified CDOs and COCs using straws, the survival rates ranged from 56.9% to 85.4% and 44.0% to 84.5%, and the maturation rates from 35.3% to 56.8% and 25.8% to 56.2%, respectively; which were lower than non-vitrified samples ($p <0.05$). Cryotop vitrified oocytes showed higher survival, maturation and fertilization rates when compared to straw in both CDOs and COCs ($p <0.05$).
Conclusion: The presence of cumulus cells improves developmental competence of GV oocytes in control groups but it did not affect the vitrified group. Vitrification of mouse GV oocytes using cryotop was more effective than straws, however both vitrification techniques did not improve the cleavage rate.

Keywords: Oocytes, Cryopreservation, Cumulus Cells, *In vitro*

Introduction

Oocyte cryopreservation has an important role in preserving the fertility potential of different mammalian species (1). In spite of certain advantages in cryopreservation of oocytes rather than embryos; it is more difficult to cryopreserve oocytes successfully due to their size, plasma membrane properties, cytoskeleton and meiotic characteristics (2, 3).
It has been shown that the different meiotic stages of oocytes from germinal vesicle (GV) to metaphase II (MII) has some effects on their viability after vitrification and thawing (4, 5). Another issue that could influence oocytes survival and maturation after cryopreservation is the presence or absence of cumulus cells. Controversial results have been obtained in this regard (6-12). The presence of cumulus cells is essential for acquisition of developmental competence by fresh oocytes (6, 7). Cumulus cells may protect oocytes from the adverse effects of chilling injury and may enhance fertilization rates via impeding premature zona reaction after oocyte cryopreservation (8). Conversely, other studies have reported that partially denuded oocytes (9) and denuded oocytes (10, 11) exhibited better fertilization rates after cryopreservation. Also, Zhang et al. (12) have shown no differences in developmental competence between oocytes with or without cumulus cells after vitrification.
Some attempts have been undertaken to improve the vitrification protocol for oocyte cryopreserva-

Received: 14 Jun 2009, Accepted: 19 Sep 2009
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Royan Institue
International Journal of Fertility and Sterility
Vol 3, No 3, Nov-Dec 2009, Pages: 111-118

tion by evaluating different cryoprotectant agents (CPAs) (13, 14) undergoing equilibration and vitrification times in a stepwise manner, (15, 16) and using different carrier systems (17, 18).
It appears that ethylene glycol (EG), as a permeable cryoprotectant, is a good candidate for vitrification because ethylene glycol has a low toxicity effect and rapid cell permeation (5, 19). Ethylene glycol, ficoll and sucrose solution (EGFS) is a vitrification solution which contains not only EG, but also sucrose and ficoll as nonpermeating agents (20). High survival rates have been obtained by vitrification of embryos (21, 22) with EGFS, however, there is little information about the effectiveness of EGFS on the survival and development of GV oocytes following vitrification.
The toxic effects of CPAs on cells are time dependent (10, 16, 23) and differences in exposure time for different cell types may impact their outcomes. It has been demonstrated that the viability rates of mouse and bovine oocytes and embryos could be affected by exposure time to both equilibration and vitrification solution (16, 23).
Increasing the cooling rate could facilitate vitrification and increasing the thawing rate could prevent de-vitrification. Minimum volume methods, such as the open cryotop, (24) are other approaches to increase cooling and thawing rates. There is limited data on the effect of effect of vitrification methods by EGFS in a stepwise manner using straw or cryotops on the survival maturation and development of GV oocytes.
Thus, the aims of this study by using the mouse model, were to evaluate the effect of several parameters, including: 1. determination of the presence or absence of cumulus cells before vitrification, 2. dehydration and equilibration of samples in a stepwise manner, 3. analyzing conventional straw and cryotop as carrier systems on the survival, maturation and fertilization rates and their subsequent embryo development.

Materials and Methods

Animals

Female (4-6 week old) and male (8-12 week old) NMRI mice were cared for and used following the guidelines of laboratory animals at Tarbiat Modares University. Animals were provided with water and chow without restriction and maintained under a 12 hour light:12 hour dark regimen (light on at 7:00 am), at a temperature of 23 ± 3°C and relative humidity of 44 ± 2% for at least one week.

Isolation of GV oocytes from mice

All chemicals and media were purchased from UK Sigma Chemical Company, unless otherwise stated and all media were made with Mili-Q water. Female mice were primed with an intraperitoneal injection of 7.5 IU pregnant mare's serum gonadotropin (Folligon, Intervet, Australia) then sacrificed 48 hours later by cervical dislocation. Their ovaries were immediately removed and placed in HEPES-buffered TCM199 medium (Gibco-BRL) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco-BRL), 0.23 mM sodium pyruvate, 50 µg/ml penicillin and 75 µg/ml streptomycin. Under the view of a stereomicroscope, GV oocytes were released from large antral follicles by repeated puncturing with a 30 gauge sterile needle. Cumulus-oocyte complex (COC) with several layers of granulose cells, homogenous cytoplasm and uniform size was selected for experiments. The cumulus-denuded oocytes (CDO) were obtained by repeated pipetting and flushing a portion of the COCs through a small fine bore mouth-controlled pipette. After washing the oocytes in fresh HEPES - buffered TCM199 medium; they were used for the following experiments.

Equilibration, vitrification and thawing solutions

The solutions for equilibration, vitrification and thawing were prepared using PB1 (modified phosphate-buffered saline) as a basic isotonic solution that contained phosphate-buffered saline supplemented with 0.33 mM sodium pyruvate, 5.56 mM glucose, 3 mg/ml bovine serum albumin (BSA), 50µg/ml penicillin and 75 µg/ml streptomycin. The equilibration solutions ES were composed of 20% (v/v) ethylene glycol, 5 mg/ml BSA and 80% (v/v) ficoll-sucrose (FS) solution; the components of the FS solution were 30% (w/v) ficoll 70 and 0.5 M sucrose in PB1 medium. The vitrification solution (VS) was composed of 40% (v/v) ethylene glycol and 60% (v/v) FS solution and the thawing solutions consisted of PB1 medium containing 1, 0.5 and 0.25 M sucrose (20).

Experiment I: Evaluation of different equilibration and vitrification times on the survival and maturation rates of mice GV oocytes without plunging in LN_2 (toxicity tests)

COCs and CDOs were exposed to one droplet of equilibration solution (200 µL) for different equilibration times of 0, 3 or 5. Then, oocytes were transferred in a droplet of VS (200 µL) for different vitrification times of 1, 3 or 5 minutes, and, without plunging in liquid nitrogen, they were transferred into decreasing concentrations of 1.0,

0.5, 0.25, and 0.125 M sucrose at an interval of 1.5 minutes. The oocytes, after washing three times in HEPES-buffered TCM199, were subjected to *in vitro* maturation. After a 24 hour culture period, survival and maturation rates were microscopically assessed and compared with their respective control groups which were directly cultured in HEPES-buffered TCM 199.
The times that led to no significant differences in the survival rate in groups comparing to the respective control were selected for subsequent experiments. Each experiment was repeated at least four times.

Experiment II: Evaluation of different vitrification procedures with conventional straw on the survival and developmental rates of GV oocytes
Groups of 10-15 COCs or CDOs were exposed to one droplet of equilibration solution (200µL) for different periods of 0, 3 or 5 minutes. Oocytes were then transferred in a droplet of vitrification solution (200 µL) for 1 or 3 minutes and loaded into a 0.25ml straw (I.M.V., L'Aigle, France) (19). The straw was sealed and plunged directly into LN2. For thawing, the straw was taken out and held in the air for 5 seconds and then immersed into 37ºC water for 30 seconds. The straw end was cut and its contents were expelled into a drop of 1 M sucrose (500 µL). The oocytes were transferred into droplets of 0.5, 0.25, and 0.125 M sucrose at an interval of 1.5 minutes and placed in HEPES-buffered TCM 199 solution for washing and maturation. The viability of the oocytes was morphologically assessed using an inverted microscope. Oocytes with a clear, bright homogenous cytoplasm and intact zona pellucida were classified as normal, while oocytes with a dark or granular cytoplasm, cracked zona pellucida or misshapen were recorded as abnormal. After 24 hours, the MII oocytes were inseminated and their developmental rates were assessed. The equilibration and vitrification condition which had a high survival and maturation rate was selected for experiment III.

Experiment III: The evaluation of vitrification procedures with cryotop on the survival and developmental rates of GV oocytes
Based on the results of experiment II, COCs or CDOs were equilibrated for 5 minutes in EFS20 and then transferred to EFS40 for 1 minute. Then, oocytes were loaded onto a cryotop (Kitazato Supplies, Japan) as reported by Kuwayama (25) and immediately plunged into IN_2. For thawing, the cryotop cap was removed while immersed in IN_2. The strip was submerged directly into a drop of 1M sucrose (500 µL) and oocytes were transferred in the descending concentrations of sucrose as described in experiment II. After thawing, the viability and developmental competence of vitrified GV oocytes were evaluated as described previously for experiment II.

In vitro maturation of GV oocytes
GV oocytes were collected separately from experiments I-III, cultured and matured in HEPES-buffered TCM 199 medium which was supplemented with 50 µg/ml penicillin, 75 µg/ml streptomycin, 0.23 mM sodium pyruvate, 10% FBS, 75 mIU/ml recombinant human follicle stimulating hormone (rFSH) and 10 IU/ml human chorionic gonadotropin (hCG). COCs and CDOs were cultured in 5-10 µL drops of maturation medium under mineral oil at 37ºC, 100% humidity in 5% CO_2 for 24 hours. The oocytes at MII stages were collected and used for *in vitro* fertilization.

In vitro fertilization and embryo culture
Spermatozoa were extracted from the cauda epididymis of 7 to 8 week-old male NMRI mice and capacitated for 1.5 hours in T6 medium supplemented with 5 mg/mL bovine serum albumin (BSA). The collected MII oocytes from experiments II and III were separately transferred to T6 medium containing capacitated spermatozoa and supplemented with 15 mg/mL BSA. The oocytes were washed three times in T6 medium with 5 mg/mL BSA 4-6 hours after insemination and cultured for 120 hours. The rates of fertilization, two-cell embryos, morula, and hatching blastocyst embryos were assessed 10, 24, 72, and 120 hours later (26).

Blastocyst staining
Embryos at the expanded stage were chosen and stained by incubation in 250 µl droplets of T6 containing 0.1% toluidine blue for 60 seconds. The stained blastocysts were mounted on glass microscope slides, and cell counting was performed under a light microscope in 2 or 3 planes.

Statistical analysis
The percentage of survived, MII and cleaved oocytes were analyzed by two-way ANOVA and Tukey's HSD was used as post hoc test. Data were analyzed after arcsine of sqtr transformation using SPSS (version 16) software. P value less than 0.05 was considered as significant.

Results

Experiment I: The survival and maturation rates of mouse GV oocytes exposed to various equilibration and vitrification times without plunging in IN_2 (toxicity tests)

The survival and maturation rates of freshly collected COCs and CDOs after exposure to equilibration and vitrification solutions at various times in a stepwise manner followed by removal of cryoprotectant without plunging in liquid nitrogen are shown in Table 1.

The survival and maturation rates of CDOs and COCs in all experimental groups were comparable with their respective control groups, with the exception of treatment nos. 3, 6, and 9 (5 minute vitrification time) that showed lower survival and maturation rates ($p<0.05$). After 24 hours IVM, there were no significant differences in the survival rates between COCs and CDOs groups in the control and their experimental groups ($p< 0.05$). The percentage of oocytes that reached MII were significantly higher in COCs when compared to CDOs in treatments 1,2,4,5,7 and 8 and the control groups ($p< 0.05$).

Experiment II: The survival and developmental rates of vitrified mouse GV oocytes using conventional straw in different equilibration and vitrification times

The survival and maturation rates of vitrified and non-vitrified mouse GV oocytes in both CDOs and COCs groups are summarized in Table 2.

The survival and maturation rates were lower in both vitrified COCs and CDOs than non-vitrified samples ($p<0.05$).

In different vitrified CDOs and COCs groups the survival rates ranged from 56.9% to 85.4% and 44.0% to 84.5%, and the maturation rates from 35.3% to 56.8% and 25.8% to 56.2%, respectively.

Higher survival and maturation rates were observed in treatment 5 (the exposure time to ES and VS for 5 and 1 minutes, respectively) than other vitrified groups ($p< 0.05$).

Data for the fertilization and developmental rates of vitrified and non-vitrified GV oocytes in both CDOs and COCs groups are summarized in Table 3.

Table 1: The survival and maturation rates of mouse GV oocytes exposed to equilibration and vitrification solutions at different times (toxicity tests)

			After 24 h *in vitro* culture					
			CDO			COC		
Treatment	**ET**	**VT**	**NO**	**(NO) Survival % ± SD**	**(NO) MII % ± SD**	**NO**	**(NO) Survival % ± SD**	**(NO) MII % ± SD**
Control	0 min	0 min	197	(183) 93.2 ± 2.2	(135) 65.7 ± 2.9	205	(195) 94.8 ± 2.7	(178) 86.6 ± 3.7[b]
1	0 min	1 min	183	(168) 91.8 ± 3.1	(119) 65.1 ± 3.8	170	(159) 93.5 ± 3.3	(145) 84.4 ± 2.1[b]
2	0 min	3 min	198	(173) 87.3 ± 3.3	(121) 61.1 ± 1.9	188	(168) 90.2 ± 5.2	(147) 78.9 ± 4.5[b]
3	0 min	5 min	231	(134) 58.6 ± 6.7[a]	(83) 36.0 ± 6.1[a]	230	(136) 60.5 ± 7.2[a]	(99) 44.3 ± 7.5[a]
4	3 min	1 min	198	(181) 91.3 ± 3.1	(126) 63.6 ± 4.3	214	(195) 90.0 ± 4.0	(176) 81.9 ± 4.1[b]
5	3 min	3 min	206	(187) 90.6 ± 3.4	(128) 64.2 ± 2.2	197	(181) 91.7 ± 2.8	(160) 80.9 ± 2.5[b]
6	3 min	5 min	192	(116) 60.7 ± 6.3[a]	(78) 41.2 ± 5.6[a]	223	(126) 56.9 ± 3.7[a]	(105) 47.1 ± 2.8[a]
7	5 min	1 min	227	(212) 92.7 ± 3.3	(141) 61.6 ± 4.3	176	(161) 91.5 ± 3.4	(143) 81.3 ± 3.0[b]
8	5 min	3 min	233	(210) 89.9 ± 4.1	(144) 61.9 ± 3.5	251	(228) 90.6 ± 3.9	(203) 80.7 ± 3.4[b]
9	5 min	5 min	222	(109) 48.0 ± 6.2[a]	(62) 27.7 ± 3.8[a]	231	(109) 47.5 ± 6.0[a]	(74) 32.6 ± 3.9[a]

Four experimental replicates were performed for each group.
ET: Equilibration time; VT: Vitrification time; MII: Metaphase II oocyte; COC: Cumulus oocyte complex; CDO: Cumulus denuded oocyte
The survival and maturation rates were based on the total number of GV oocytes.
a: Indicates significant difference compared with the control group in the same columns ($p<0.05$).
b: Indicates significance differences for COC vs. CDO in the same rows ($p<0.05$).

In non-vitrified samples, there were significant differences ($p< 0.05$) between CDOs and COCs groups in fertilization rates (60% vs. 87%), development two-cell (46% vs. 78%) and blastocyst stages (12% vs. 36%). Among all vitrified groups using conventional straw, the fertilization and cleavage rates in CDOs ranged from 16.6% to 38.6% and 6.5% to 27.5%. In COCs, they ranged from 14.7% to 40.4% and 2.8% to 28.3%, respectively. None of the vitrified GV oocytes reached the blastocyst stage (Table 3). Treatment 5 which had the highest survival, maturation, fertilization and cleavage rates ($p< 0.05$) among all vitrified groups (Tables 2 and 3) were selected for experiment III.

Experiment III: The comparison of vitrification technique using cryotop and conventional straw on the developmental competence of GV oocytes in the presence or absence of cumulus cells

The GV oocytes which were vitrified in a cryotop carrier showed significantly higher ($p< 0.05$) survival rates when compared to the conventional straw in both the CDOs (97% vs. 85.4%) and COCs (96.5% vs. 84.5%) groups, respectively. There was no significant difference in the survival rates of oocytes between the non-vitrified and vitrified groups using cryotop (Table 2). Maturation rates of the oocytes were also significantly higher in the vitrified groups which used the cryotop than conventional straw for both CDOs (63.9% vs. 56.8%) and COCs (64.5% vs. 56.2%) groups, respectively ($p< 0.05$).

However in the COCs groups, this rate was significantly lower ($p< 0.05$) in the cryotop carrier group (64%) when compared to the respective non-vitrified group (86%).

Higher fertilization rates were observed in the vitrified group using cryotop than conventional straw (54.2% vs. 40.4% for COCs and 52.3% vs. 38.6% for CDOs, respectively). The COCs vitrified group that used cryotop exhibited a significantly lower ($p< 0.05$) fertilization rate than non-vitrified COCs (54.2% vs. 87.8%).

There was no significant difference in the fertiliation rate between vitrified CDOs in the cryotop carrier group and their respective non-vitrified samples. The cleavage rate of CDOs and COCs declined significantly in both vitrified groups using conventional straw and cryotop compared to their respective non-vitrified groups ($p< 0.05$).

The rates of blastocyst formation in COCs vitrified group using cryotop were significantly lower ($p< 0.05$) than non-vitrified COCs group (9.8% vs. 36.8%).

Table 2: The survival and maturation rates of vitrified mouse GV oocytes

			CDO			COC		
Treatment	**ET**	**VT**	**NO**	**(NO) Survival % ± SD**	**(NO) MII % ± SD**	**NO**	**(NO) Survival % ± SD**	**(NO) MII % ± SD**
Control	0 min	0 min	197	(197) 100 ± 0.0	(135) 68.6 ± 3.2^{c}	205	(197) 100 ± 0.0	(178) 86.6 ± 3.7
Straw 1	0 min	1 min	258	(157) 59.6 ± 6.7^{abc}	(65) 41.4 ± 2.9^{abc}	220	(98) 44.0 ± 3.7^{ab}	(25) 25.8 ± 3.0^{ab}
Straw 2	0 min	3 min	222	(126) 56.9 ± 2.3^{ab}	(44) 35.3 ± 4.6^{ab}	275	(153) 55.1 ± 5.2^{ab}	(56) 36.5 ± 2.2^{ab}
Straw 3	3 min	1 min	287	(215) 75.0 ± 4.1^{a}	(98) 45.5 ± 2.7^{ab}	217	(162) 74.4 ± 4.3^{ab}	(75) 46.4 ± 4.4^{ab}
Straw 4	3 min	3 min	229	(148) 64.1 ± 5.9^{ab}	(67) 44.6 ± 4.0^{ab}	234	(148) 68.8 ± 4.9^{ab}	(59) 40.9 ± 6.7^{ab}
Straw 5	5 min	1 min	281	(241) 85.4 ± 4.2^{a}	(137) 56.8 ± 3.2^{a}	273	(231) 84.5 ± 2.9^{a}	(130) 56.2 ± 2.3^{a}
Straw 6	5 min	3 min	265	(164) 61.3 ± 4.7^{ab}	(65) 5.5ab ± 38.8	216	(125) 4.0ab ± 58.6	(49) 2.8ab ± 39.2
Cryotop	5 min	1 min	226	(219) 97.0 ± 1.2^{b}	(140) 63.9 ± 4.4^{b}	214	(207) 96.5 ± 1.4^{b}	(133) 64.5 ± 3.5^{ab}

Four experimental replicates were performed for each group.
ET: Equilibration time; VT: Vitrification time; MII: Metaphase II oocyte; COC: Cumulus oocyte complex; CDO: Cumulus denuded oocyte
The survival rates were based on the total number of vitrified GV oocytes.
The maturation rates were based on the number of oocytes which survived.
a: Indicates significant differences compared to control group in the same column ($p<0.05$).
b: Indicates significant differences compared to treatment 5 in the same column ($p<0.05$).
c: Indicates significant differences for COC vs. CDO in the same rows ($p<0.05$).

Table 3: The fertilization and developmental rates of vitrified mouse GV oocytes

Treatment	ET	VT	CDO (NO) Fertilized % ± SD	CDO (NO) 2Cell % ± SD	CDO (NO) Blastocyst % ± SD	CDO Cell count	COC (NO) Fertilized % ± SD	COC (NO) 2Cell % ± SD	COC (NO) Blastocyst % ± SD	COC Cell count
Control	0 min	0 min	(81) 60.5 ± 7.8^{c}	(64) 46.1 ± 6.6^{c}	(16) 12.0 ± 2.7^{c}	86.5 ± 7.8	(156) 87.8 ± 4.0	(140) 78.2 ± 6.8	(67) 36.8 ± 4.5	89.5 ± 6.6
Straw 1	0 min	1 min	(14) 17.8 ± 8.0^{abc}	(8) 10.8 ± 6.3^{ab}	(0) 0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	(4) 1 4.7 ± 10.1^{ab}	(1) 2.8 ± 5.6^{ab}	(0) 0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
Straw 2	0 min	3 min	(7) 16.6 ± 6.4^{ab}	(3) 6.5 ± 4.4^{ab}	(0) 0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	(7) 12.8 ± 2.9^{ab}	(5) 9.0 ± 1.9^{ab}	(0) 0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
Straw 3	3 min	1 min	(36) 36.7 ± 2.5^{a}	(26) 26.4 ± 2.9^{a}	(0) 0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	(28) 37.1 ± 2.2^{a}	(17) 22.9 ± 2.5^{a}	(0) 0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
Straw 4	3 min	3 min	(15) 22.1 ± 1.8^{ab}	(8) 11.2 ± 4.7^{ab}	(0) 0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	(12) 20.4 ± 4.4^{ab}	(6) 10.0 ± 2.8^{ab}	(0) 0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
Straw 5	5 min	1 min	(53) 38.6 ± 4.3^{a}	(38) 27.5 ± 2.8^{a}	(0) 0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	(52) 40.4 ± 6.0^{a}	(37) 28.3 ± 3.3^{a}	(0) 0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
Straw 6	5 min	3 min	(11) 16.7 ± 3.9^{ab}	(7) 10.5 ± 2.9^{ab}	(0) 0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	(8) 16.1 ± 4.9^{ab}	(4) 7.6 ± 5.6^{ab}	(0) 0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
Cryotop	5 min	1 min	(73) 52.3 ± 3.4^{b}	(50) 35.7 ± 2.9^{a}	(11) 7.5 ± 3.8b	80.3 ± 13.8	(73) 5 4.2 ± 4.5^{ab}	(48) 3 6.2 ± 3.3^{ab}	(13) 9.8 ± 1.6^{ab}	81.8 ± 11.7

Four experimental replicates were performed for each group.
ET: Equilibration time; VT: Vitrification time; MII: Metaphase II oocyte; COC: Cumulus oocyte complex; CDO: Cumulus denuded oocyte
The fertilization rates and developmental rates to blastocyst stage were based on the number of MII oocyte
a: Indicates significant differences compare to control group in the same column (p<0.05).
b: Indicates significant differences compare to treatment 5 in the same column (p<0.05).
c: Indicates significant differences for COC vs. CDO in the same rows (p<0.05).

However, no significant difference in blastocyst formation rate was seen between vitrified CDOs in the cryotop carrier and their respective non-vitrified groups. Also the mean cell numbers per blastocyst derived from vitrified cryotop oocytes did not show any significant difference compared to their respective non-vitrified groups for both COCs (89.5 vs. 81.8) and CDOs (86.5 vs. 80.3) groups.

Discussion

The result of the present study showed that, in toxicity tested groups, prolonged exposure to vitrification solution (up to 5 minutes) resulted in lower survival and maturation rates compared to the non-vitrified control and other experimental groups. Establishment of a balance between the duration of equilibration and vitrification time is critical to gain a successful vitrification method. Hence, exposure time to cryoprotectant agents is very critical and should be adjusted properly. In the vitrification procedures, high concentrations of cryoprotectant were used which could result in osmotic and toxic injuries. In order to decrease the damage of osmotic and toxic stress, cryoprotectant should be added in a step-wise manner (15, 27).

When the oocytes were exposed to equilibration solution before being subjected to vitrification media, a shorter time was required for sufficient permeation of cryoprotectant when they were placed in vitrification solution. Therefore, decreasing the exposure time to the vitrification solution resulted in a higher survival rate and developmental competence due to lesser toxicity and osmotic shock (16, 28). Long exposure to vitrification solution resulted in reduction of viability which may be due to inactivation of an enzyme needed for meiotic progression (28).

In the present study, in spite of high survival rates, the developmental competence of vitrified GV oocytes was considerably diminished (15, 29). It seems that these differences are due to a different protocol which was used for mouse GV oocyte vitrification. Also, it has been demonstrated that cryopreservation of mouse (15, 30) and bovine (27, 31, 32) GV stage oocytes showed lower survival and developmental rates than oocytes at the MII stage. In these studies the developmental competence of vitrified GV oocytes was approximately 50% lower than that of controls (15, 27, 30-32).

It appears that, the low cleavage rates obtained from our study were due to many factors, such as the susceptibility of the oocytes' sub-cellular structure to low temperature (26, 33), osmotic and ionic injuries (34, 35). These osmotic and ionic injuries may have an effect on the cytoskeleton and criti-

cal organelles of the cells such as the mitochondria (26), thus arresting their development and mitotic division; however additional studies are needed to verify this.

In the present study, it was observed that in the non-vitrified control group, the maturation rate and development to blastocyste stage in COCs were higher than CDOs. Denuding of oocytes prior to IVM resulted in a decrease in developmental competence. It seems that cumulus cells produce and secrete factors that act on oocyte development via the paracrine way (36). In agreement with our results, the role of cumulus cells in development of oocytes has been previously demonstrated (7, 36).

The results obtained from this study indicated that the presence or absence of cumulus cells could not influence the GV oocyte survival rate and developmental competence after vitrification and thawing. It does not appear that this reduction of developmental competence of COCs after vitrification is caused by exposure to cryoprotectant agents, because, oocyte exposure to equilibration and vitrification solutions in toxicity tested groups could not change the developmental competence of oocytes. It may result by interruption of gap junction communication between the oocyte and cumulus cells. The communication between cumulus cells and oocytes is very susceptible to the physical condition caused by cryopreservation (37). In agreement with our results some investigators have shown that cumulus cell projections were disturbed by vitrification (11, 12).

In this study, the effect of cryotop and conventional straw as carrier systems on the survival rates and developmental competence of mouse GV oocytes in the presence or absence of cumulus cells were compared. Results indicated that oocyte vitrification by cryotop was of more benefit on the survival rate and developmental competence after thawing in comparison to the conventional straw, but there were no significant differences between the COCs and CDOs groups in vitrification by cryotop.

To achieve a higher cooling rate by decreasing the volume of vitrification solution, the cryotop method was introduced by Kuwayama et al. (24). It seems that, when chilling injury was avoided by increasing the cooling rate in the vitrification method by using cryotop; the integrity of oocytes was better preserved in comparison to vitrification with conventional straw. Also, zona pellucida fracture damage as a consequence of cryopreservation in straw could relatively be prevented by using the cryotop method (25) .

Also our results showed that the fertilization rate in both vitrification techniques was lower than the non-vitrified group. This might be due to a cryoinjury to the zona pellucida which caused some zona hardening. In agreement with this suggestion, it has been shown that zona hardening took place during cryopreservation by premature release of cortical granules which could affect sperm penetration and fertilization rates (38, 39).

Conclusion

In conclusion, the present study demonstrated that the presence of cumulus cells improved developmental competence of GV oocytes in non-vitrified control groups but it could not improve developmental capacity of GV oocytes after vitrification and thawing. The vitrification of mouse GV oocytes using cryotop was more effective than conventional straw in respect to survival, maturation and fertilization rates, but both vitrification techniques did not result in an improvement in the cleavage rate in comparison to the non-vitrified group.

Acknowledgements

This work was conducted and funded partially by the Faculty of Medical Sciences, Tarbiat Modares University and Veterinary Medicine of Urmia University. The authors thank Mr. Mohammad Asghari for his statistical analysis assistance. There is no conflict of interest in this article.

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