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The effect of vitrification on maturation and viability capacities of immature human oocytes

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Abstract
Background 15 % of oocytes collected from Assisted Reproductive Technology (ART) cycles are immature. These oocytes may be matured following in vitro maturation (IVM) program. It is possible to cryopreserve the immature oocytes for further use in ART after application of IVM.
Objective The aim was to determine the maturation rate and viability of human oocytes that were matured in vitro after vitrification program.
Materials and methods 63 women (19–43 years old) who underwent controlled ovarian stimulation for ART were included in this study. 53 immature oocytes were used for fresh group (fIVM) and 50 immature oocytes for vitrification group (vIVM). The maturation medium was Ham's F_{10} supplemented with 0.75 IU FSH, 0.75 IU LH and 40 % human follicular fluid (HFF). After 36 h, maturation and morphology of all oocytes were assessed. Also, the oocyte viability was assessed using PI/Hoechst immunostaining technique.
Results The maturation rates were reduced in vIVM group (56.0 %) in comparison to fIVM group (88.7 %; $P < 0.001$). Oocyte viability rate were also reduced in vIVM group (56.0 %) in comparison to fIVM (86.8 %, $P < 0.007$).
Conclusions Cryopreservation via vitrification reduced both the maturation capacity and viability of human oocytes in IVM technology. It is, therefore, recommended to apply IVM on fresh immature oocytes, instead.

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Introduction

After ovarian stimulation, approximately 15 % of oocytes are immature at the germinal vesicle (GV) and germinal vesicle breakdown (MI) stages. In vitro maturation (IVM) and cryopreservation of immature oocytes have been proposed as an alternative for conventional in vitro fertilization (IVF) treatment. One efficient method for cryopreservation of human oocytes is vitrification technology [1, 2]. During recent years, studies have shown that vitrification offers new interesting perspectives in the field of oocyte cryopreservation, demonstrating reduced cryoinjury than other cryotechnologies [3, 4]. Female gamete cryopreservation could benefit patients in specific situation such as chemotherapy or radiation [5].

IVM has several advantages of reducing the costs, avoidance of the side effects of life threatening ovarian hyperstimulation syndrome (OHSS) and simplified treatment for certain infertile couples [1, 6]. In 1991, the first human birth resulting from IVM was reported by Cha and associates [7]. In 1994, Trounson et al. [8] reported the first birth from untreated polycystic ovarian (PCO) patient. Theoretically, given their microstructure, immature germinal vesicle (GV) oocytes should be more resistant to the damage caused by cooling and circumvent the risk of polyploidy and aneuploidies, since the chromatins are diffused and surrounded by a nuclear membrane [9, 10]. Several factors affect the survival and viability of human oocytes, such as exposure time of cells to different cryoprotectant solutions, their different concentrations and rate of ice-crystal formation [1]. The main objective of this

prospective study was investigating the success of cryopreservation of immature human oocytes using vitrification. This is followed by comparing oocytes maturation rates, as well as viability assessments with immunocytochemistry technique.

Materials and methods

Patients

A total of 63 infertile women were included in this cross-sectional study. The investigation took place over a period of 5 months in 2011 at Yazd institute for reproductive sciences. This study was approved by Ethics Committee of our institution. The patients were divided into two groups of ≥35 years and <35 years old. Oocytes were allocated in two groups of fresh-IVM (fIVM; $n = 53$) and vitrified-IVM (vIVM; $n = 50$).

Oocyte collection

The oocyte collection was performed 36 h after 10,000 IU of hCG (IBSA Co, Switzerland) injection. Transvaginal ultrasound was used for oocytes collection with a single lumen aspiration needle (Wallace, Smiths Medical International, UK) with a reduced pressure of 100 mmhg. The collected oocytes were assessed for nuclear maturity under the stereo microscope (Olympus Co, Japan). After denudation with 80 IU hyaluronidase (Sigma Co, USA) and mechanical pipetting, the oocytes were assessed for maturity. The oocytes that extruded the first polar body were considered mature (MII) and were inseminated using intracytoplasmic sperm injection (ICSI) technique.

Follicular fluid preparation

Preparation of human follicular fluid (HFF) was performed according to the method described previously [11]. HFF was collected from women who underwent follicular puncture. HFF was centrifuged at 3,500 rpm for 10 min. Blood and granulosa cells were settled, and pure HFF was transferred to a new tube. The HFF was then inactivated in water bath at 56 °C for 30 min. At last, HFF was filtered with 0.22 μm filters, then aliquoted and stored at −20 °C before use.

In vitro maturation of oocytes

Immature oocytes were denuded of cumulus oocyte complexes (COCs), and then washed in three drops of IVM medium. They were cultured in IVM medium containing Ham's F_{10} (Biochrom Co, Germany) supplemented with 0.75 IU LH, 0.75 IU FSH (Ferring Co, Germany) with 40 % HFF at 37 °C in an incubator with 5 % CO_2 and 95 % air with high humidity. Oocytes were observed under an inverted microscope (Nikon Co, Japan) after 36 h to determine maturity.

Vitrification

Immature oocytes were frozen using a modified vitrification method [1]. Initially, immature oocytes were placed in an equilibration solution containing 7.5 % ethylene glycol (EG) (Merck Co, Germany), 7.5 % dimethyl sulphoxide (DMSO) (Merck Co, Germany) in Ham's F_{10} media supplemented with 20 % human serum albumin (HSA) (Plasbumin Co, USA) for 5–15 min at room temperature (RT). Then, oocytes were removed and placed into vitrification solution containing 15 % EG, 15 % DMSO and 0.5 M sucrose (Sigma co, USA) in Ham's F_{10} medium supplemented with 20 % HSA for 50–60 s at RT. After this stage, the oocytes were loaded into cryotops that were quickly plunged into liquid nitrogen. Next, the cap was placed on the cryotop and put into the cane. Finally, the samples were transferred to the liquid nitrogen storage tank for 1 month.

Thawing of the oocytes was performed by placing the cryotop in thawing solution in five stages: thawing solution (Ham's F_{10} supplemented with 20 % HSA and 1 M sucrose) for 50–60 s, dilution solution 1 (Ham's F_{10} supplemented with 20 % HSA and 0.5 M sucrose) for 3 min, dilution solution 2 (Ham's F_{10} supplemented with 20 % HSA and 0.25 M sucrose) for 3 min, washing solution 1 and 2 (Ham's F_{10} supplemented with 20 % HSA) each for 3–5 min. After this stage, the oocytes were placed in IVM medium for 36 h in incubator (Fig. 1).

Maturity and viability evaluations

After the culture period, all oocytes were assessed for maturity under an inverted microscope (Research instruments Ltd Co, UK). Then, oocytes were assessed for viability based on oolemma integrity with Hoechst/Propidium iodide (H/PI) nuclear staining, according to method described by Zhang et al. [12]. Supravital immunostaining was prepared with 20 μg/ml of Hoechst 33342 (Sigma, USA), and 20 μg/ml of PI (Sigma, USA) was added to Ham's F10 medium supplemented with 10 % HSA.

For viability evaluation, a few oocytes were kept in a dish with a few drops of stain. They were incubated for 15 min in the H/PI blended stain. After that, the oocytes were washed and observed under a fluorescence microscope (Olympus; Japan). The dead cells showed red fluorescence stain (PI-positive) for disruption of cellular membrane. However, the viable cells showed blue

Fig. 1 Morphological markers characterizing the maturity status of human oocytes. **a** Immature germinal vesicle (GV) oocyte. **b** Immature germinal vesicle breakdown (GVBD) oocyte (MI). **c** Mature oocyte (MII)

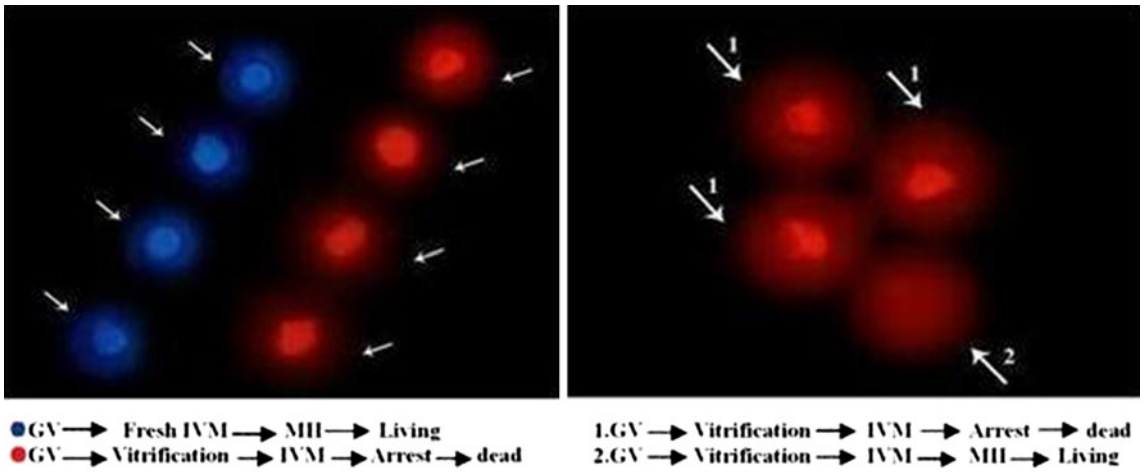

Fig. 2 GV oocyte after staining with Hoechst/PI. Viable oocyte (*blue fluorescence*), dead cell (*red fluorescence*)

fluorescence (PI-negative) for the intact cell membrane. Cells in intact conditions are non-penetrable to PI, but penetrable to H stain (Fig. 2).

Statistical analysis

Statistical analyses were performed using SPSS (version 16). The data were analysed using the Chi square test. P value of <0.05 was considered statistically significant.

Results

There were no significant differences in oocyte maturation rates in regards to maternal age (Table 1). As shown in Table 2, the most pronounced maturation of immature oocytes was in the group with endometriosis infertility. The findings also showed that the least rates of maturation were related to the group with unexplained infertility. Table 3 shows the differences in rates of oocyte viability in regards to the etiology of infertility.

Table 1 Frequency of maturation of immature oocytes according to patient's age

Group	Maturation rate	
	Age < 35	Age > 35
Fresh-IVM	61 (72.6 %)	23 (27.4 %)
Vitrified-IVM	14 (93.3 %)	1 (6.7 %)

As presented in Table 4, there were no differences between maturation rates of GV and MI oocytes in fIVM group ($P = 0.65$). However, in vIVM group, the percentile of MI maturation was significantly higher than GV. The rate of GV maturation in fIVM group was significantly higher than vIVM group. Table 5 shows that in fIVM oocytes, there was no difference between viability of GV and MI ($P = 0.54$). But, in vIVM group, the percentile of MI viability was significantly higher than GV. The data also confirmed that the rate of GV viability in fIVM was significantly higher than vIVM groups. Although, the viability rates in MI oocytes were similar in two groups.

Table 2 Frequency and percentile of maturity in immature oocytes according to the etiology of infertility

Group	Oocyte number	Percent
Unexplained		
Maturation	16	59.3
Oocyte arrest	5	18.5
Degeneration	4	14.8
Parthenogenesis	1	3.7
Maturation and degeneration	1	3.7
Male factor		
Maturation	35	72.9
Oocyte arrest	7	14.6
Degeneration	4	8.3
Parthenogenesis	1	2.1
Maturation and parthenogenesis	1	2.1
Tubal factor		
Maturation	14	87.5
Maturation and degeneration	1	6.3
Maturation and parthenogenesis	1	6.3
Polycystic ovary (PCO)		
Maturation	3	60
Oocyte arrest	2	40
Endometriosis		
Maturation	7	100

Table 3 Frequency and percentile of immature oocytes viability according to the cause of infertility

Group	Oocyte number	Percent
Unexplained		
Alive	16	59.3
Dead	11	40.7
Male factor		
Alive	34	70.8
Dead	14	29.2
Tube factor		
Alive	14	87.5
Dead	2	12.5
Polycystic ovary (PCO)		
Alive	3	60
Dead	2	40
Endometriosis		
Alive	7	100
Dead	–	–

Discussion

Nowadays, maturation of immature oocytes in vitro is one of the approaches, which has attracted the attention of many researchers in ART. Technological developments for preserving gametes as well as cryopreservation have contributed to subsequent fertility in women who undergo surgery, radiation or chemotherapy for cancer treatment. Efforts have led to successful cryopreservation with the use of different protocols. Rall and Fahy [13] reported an extremely rapid method called vitrification, in which embryos suspended in a high concentration of cryoprotectant solution placed into liquid nitrogen. They used dimethylsulfoxide (DMSO), acetamide and propylene glycol as the cryoprotectant. Ethylene glycol (EG) has been reported to be less toxic to oocytes. Generally, oocyte cryopreservation is carried out through the following steps: exposing cryoprotectant agents (CPAs); cooling below 0 °C, storing, thawing, removing CPAs and returning to the physiological mode [14]. It is proved that oocyte cryopreservation process during any of the above steps causes damage to oocytes. Permeable CPAs are necessary for intracellular dehydration, while they decrease the freezing point in cells, such as oocytes. CPAs which are used ordinarily for oocyte cryopreservation include EG [15] and DMSO [16]. They are toxic in high concentrations, although their toxicity reduces with lower temperatures and short durations [17]. Quality of oocytes after thawing, viability, percentage of degeneration, and oocyte arrest after vitrification and their comparison with oocytes from non-cryopreserved group cultured in identical conditions determines the success of the protocol. The survival rates of cryopreserved oocytes depend upon several mechanisms related to cell injury, such as the chemical toxicity of the cryoprotectant, intracellular ice formation, fracture damage, and osmotic swelling during the removal of the cryoprotectant [18].

In the present study, human immature oocytes occurring in the phase of GV and MI underwent IVM by the use of two approaches. Then, viability in both groups was studied to determine whether vitrification process has any effects on oocyte viability compared to the fresh in vitro maturated group. Is it probable for chemical and physical impacts of cryopreservation to produce any unwanted variation in the viability of oocytes? Can we use the method of vitrification for immature oocytes as an efficient approach in ART? Therefore, the patients underwent ovarian stimulation and immature oocytes were studied in two groups after aspiration from ovary. In the first group, GV and MI oocytes were maturated in vitro by using laboratorial technology (fIVM). However, the immature oocytes in the second group, were cryopreserved by vitrification, and then maturated after thawing (vIVM). Then, in both groups, the spindle and chromosome viability were analyzed using immunofluorescent technique, after IVM, and used Hoechst and Propidium iodide staining. Hoechst stains are part of a family of blue fluorescent dyes used to stain DNA that bound to the minor groove of DNA. Therefore, these stains are often

Table 4 Frequency of maturation according to the stage of immature oocytes in two groups

Group	Fresh-IVM ($n = 53$)		Vitrified-IVM ($n = 50$)	
	GV = 33	MI = 20	GV = 19	MI = 31
Maturation	29 (87.9 %)	18 (90 %)	6 (31.6 %)	22 (71 %)
Oocyte arrest	1 (3 %)	1 (0 %)	8 (42.1 %)	4 (12.9 %)
Degeneration	1 (3 %)	0 (0 %)	5 (26.3 %)	2 (6.5 %)
Parthenogenesis	1 (3 %)	1 (5 %)	–	–
Maturation and degeneration	1 (3 %)	0 (0 %)	0 (0 %)	1 (3.2 %)
Maturation and parthenogenesis	–	–	0 (0 %)	2 (6.5 %)
P value	0.646		0.002	

Table 5 Frequency of viability in immature oocytes in two groups

Group	Fresh-IVM ($n = 53$)		Vitrified-IVM ($n = 50$)	
	GV = 33	MI = 20	GV = 19	MI = 31
Alive	29 (87.9 %)	17 (85 %)	6 (31.6 %)	22 (71 %)
Dead	4 (12.1 %)	3 (15 %)	13 (68.4 %)	9 (29 %)
P value	0.536		0.007	

called supravital, which means that cells survive a treatment with these compounds. Propidium iodide (PI) fluorescent is molecule with a molecular mass of 668.4 Da that can be used to stain cells. It can be used to differentiate necrotic, apoptotic and normal cells; PI is membrane impermeant and generally excluded from viable cells. PI is commonly used for identifying dead cells in a population and as a counterstain in multicolor fluorescent techniques. Results of this research indicate that there were no significant differences in oocyte maturation rates in regards to maternal age ($P = 0.07$) since all the patients underwent ovarian stimulation protocols. The most pronounced maturation of immature oocytes was in the group with endometriosis infertility; studies proved the successful condition of these patients in case they are candidate donors. Also, the findings showed that the maturity was higher in fIVM when compared with vIVM. Also, the rates of oocyte arrest in vIVM were higher than fIVM, and the rates of oocytes degeneration in vIVM were higher than fIVM. In each group, only a few oocytes underwent degeneration or parthenogenesis after IVM. This is probably due to aging of the patients or a delay from the time of ovarian puncture to the transfer of the oocytes into culture media or freezing container. According to Kim et al. [11], almost 78.5 % of the immature oocytes retrieved in stimulated cycles were matured in vitro, and 12.3 % of the oocytes remained at the GV stage. Moreover, Russel et al. [19] showed that maturation rate was 63 % in naked and compact oocytes in unstimulated cycles. Toth et al. [20] demonstrated that 83.3 % of the oocytes were matured after thawing of cryopreserved oocytes. Recently, Cao et al. [21] reported that the maturation rates of human immature oocytes in vitrified group were 50.8 % in comparison with the control group 70.4 %; in the present study, the rates of maturation in vitrified oocytes were similar to study done by Cao et al [21]. But, rates of maturity following IVM of immature oocytes without vitrification were higher when compared to above. Although the rate of degeneration and oocyte arrest was high in vitrification group, the rate of maturation in comparison with other studies seems acceptable. The findings also showed that the viability status was higher in fIVM (86.8 %) when compared to vIVM (56.0 %). Mahmoudi et al. [22], by using mouse oocytes, showed the viability rate in the step-wise group (81.75 %) was significantly higher than that in the single-step group (58.68 %) [22]. Men et al. [23] reported that the survival rate after a two-step vitrification of bovine GVBD oocytes was 79.59 %. The process of cryopreservation can contribute to the death of oocytes, parthenogenetic activation [24], chromosomal anomaly [25, 26] and perturb the normal activity of the cells microtubules [27, 28], as a direct consequence of intracellular ice-crystal formation, rapid or massive osmotic and temperature change and the relative toxicity of cryoprotectant [29–31]. A too-long exposure time for cells to cryoprotectant will more substantially alter intracellular pH as well as the subsequent post-thawing developmental potential of the cryopreserved cell than would be the case for a shorter exposure time, whereas a too-short exposure time of oocytes to cryoprotectant may induce incomplete dehydration [29]. Koutlaki et al. [32] proposed that the result of vitrification depends on two factors: skill and technique of an individual who accomplishes vitrification and kind of container in which oocytes are placed.

In conclusion, the oocytes exposed directly to IVM (fIVM) possess more rates of maturation and viability than vitrification group (vIVM). It is proposed that immature oocytes survive better in routine IVM than cryopreservation for future use in IVM. Therefore, immature oocytes in ART cases should immediately undergo the IVM technology, though they may be cryopreserved successfully if it becomes necessary.

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Conflict of interest We declare that we have no conflict of interest.

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