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Vitrification of human oocyte using cryoloop

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Abstract

Background: The cryopreservation of human oocyte would make a significant contribution to infertility treatment, such as using it for oocyte donation and for patients a bout to lose ovarian function due to surgery or chemotherapy. Despite of using standard freezing straws and cryovials or even open pulled straws, only a few successful pregnancies have been arisen from cryopreserved human oocytes. This situation has been primarily attributed to poor survival, fertilization and development of cryopreserved oocytes.

Objective: The aim of this study was to evaluate the novel cryoloop vitrification method for cryopreservation of human oocytes.

Materials and Methods: Nine infertile couples participated in this study. In all women proper regulation and desensitization was done using GnRH agonist during luteal phase. Mature oocytes allocated into two groups randomly. In group I, 34 oocytes were vitrified in conventional straws, while in group II, 33 oocytes were vitrified in cryoloop. After a store time of 1-6 months the oocytes were thawed, incubated for 2 hours and subsequently the ICSI was done on survived oocytes. To verify normal fertilization of vitrified oocytes the number of pronuclei in the cytoplasm was counted 16-18 hours after ICSI and good morphological quality embryos were transferred on day 2 or 3 after sperm injection. Pregnancy was identified by the serum ß HCG level, checked 14 days after embryo transfer.

Results: The present study shows that the rate of survival of vitrified human oocytes in two groups has no significant difference (52.94% in group I versus 63.63% in group II) but the fertilization rate of vitrified oocytes by cryoloop was greater than vitrified oocytes by conventional straws (73.7% versus 55.55% respectively). One of the embryo transfers achieved clinical pregnancy and resulted in the delivery of healthy baby.

Conclusion: Vitrification by using cryoloop can improved the fertilization rate and developmental capacity of vitrified thawed oocyte.

***Key words**: Vitrification, Human oocyte, Survival rate, Fertilization.*

Introduction

Cryopreservation of oocyte is one of the most promising options of the future in human female gamete preservation and donation.

Technology for the long term preservation of mammalian gametes and zygotes has improved greatly over the past 25 years (1, 2) and currently is used for supporting various assisted reproductive technologies in human reproductive medicine.

Successful freezing of human oocytes has several advantages (3-5) such as allowing the establishment of oocyte banks. It also provides an alternative to embryo preservation for the avoidance of ethical Problems.

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Vitrification is a process by which liquid turns into solid without the formation of ice crystal (6). Vitrification was achieved simply by plunging the sample into liquid nitrogen at -196ºC (7). In this method, heat transfer from the sample into the LN2 leads to the evaporation of LN2 around the sample, resulting in the formation of a nitrogen gas layer, which acts as an insulator. This insulation reduces the heat transfer and makes it impossible to achieved uniform and rapid cooling rates (8). However, the post thaw and subsequent development of frozen oocytes has been extremely poor in published studies (9-12) and further research to enhance the viability of cryopreserved oocytes is required. The cryoloop, used as a vessel in vitrification, is a thin nylon loop used to suspend a film of cryoprotectant containing the oocytes and directly immerse them in liquid nitrogen. Vitrification of oocytes using the cryoloop has advantages over conventional vitrification procedures in that the open system lacking any thermo insulating layer, coupled with the small volume of <1µl, results in both rapid and uniform heat exchange during cooling.

So far the cryoloop has been used on human, mouse (13) and monkey (14) blastocysts with excellent survival rates. Mavrides et al. (15) demonstrated that the new cryoloop vitrification technique followed by ICSI produce good embryo formation results compared with slow freezing and could hold the future for effective bovine oocyte cryopreservation. Lieberman and Tucker (16) demonstrated that using the cryoloop or the hemi straw system which enabled the achievement of ultra rapid cooling rates, in combination with the mixture of ethylene glycol and DMSO as cryoprotectant agents for vitrification can effectively improve survival rates of human oocytes after warming. Cai et al. (17) recently showed that the cryoloop vitrification method in combination with 20% ethylene glycol and 20 % dimethylsulphoxide as cryoprotectant had the minimal adverse effects on the spindle configuration of rabbit oocytes and embryo development.

Until now, direct comparison of different carrier of conventional straw and the cryoloop for human oocytes has not been reported. The goal of this study was to evaluate the novel cryoloop vitrification method for the cryopreservation of human oocytes followed by ICSI.

Materials and Methods

Patients

Nine infertile couples participated in this prospective study. The mean (±SD) age of women was 31.5±5.7 years and the mean (±SD) duration of infertility was 6.3±2.8 years.

Reasons for the ICSI-ET were as follows: tubal factor (n=3), unexplained cause (n=2), asthenospermia (n=2), an ovulation (n=1), and endometriosis (n=1).

In all women proper regulation and desensitization was done using GnRH agonist during luteal phase. Then controlled hyperstimulation was initiated with FSH-hMG. Ovulation was triggered with 10,000 IU of HCG. When controlled ovarian hyperstimulation had resulted in development of at least two follicle>18 mm in diameter, transvaginal oocytes retrieval was done 34-36 hours after the HCG injection. A total of 99 cumulus-corona-acolyte complexes were retrieved. After removals of granulose cells with hyaluronidase 80 IU/ml and mechanical dissection, 67 oocytes were found to be at metaphase II (Mature) stage, 14 at the metaphase I (MI) stage, 10 at the germinal vesicle (GV) stage, and 5 were degenerative. Mature oocytes allocated into two groups randomly.

In group I, 34 oocytes were vitrified in conventional straws, while in group II, 33 oocytes were vitrified in cryoloop.

Pretreatment, vitrification in cryoloop and dilution solution

The selected oocytes for vitrification were exposed to PBI for few minutes at room temperature then they were placed in EFS 20% for 100 seconds, and then the oocytes were transferred to EFS 40% for 40 seconds. In this time the cryoloop placed into V2 in order to make a film. The cryoloop has been transferred under stereomicroscope and then 2-3 oocytes transferred from V2 to cryoloop by mouth pipette. Then loaded cryoloop was plunged into LN2 and screwed on the cryovial which already was immersed in LN2. After a store time of 1-6 months, the cryoloop was removed from the LN2 and placed on top of thawing medium (0.5M sucrose in PBI) and as soon as the cryoloop contents liquefied and the loop was immersed in thawing medium, the oocytes were transferred to HTF medium with 10% synthetic serum substitute and washed 3 times.

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Pretreatment, vitrification in conventional straw and dilution solution

The 34 oocyte for vitrification were exposed to PBI for few minutes, Then they were placed in EFS %20 for about 2 minute and were transferred to EFS %40 for about 1 minute. The 0.25 ml plastic straw was filled with 1 cm of vitrification medium (EFS %40), 0.5 cm of air, 2 cm of vitrification medium containing oocytes (2-3), 0.5 cm of air and 3.5 cm of vitrification medium and straw immersed in LN2. After storage time of 1-6 months, the straw was taken out and held in air for 5 seconds, and then it was plunged into water at room temperature for 10 seconds.

The vitrification solution kept transparent in the liquid nitrogen, air, and water. The content containing oocytes was expelled into a drop of 0.5M sucrose. Finally oocytes were transferred into the HTF medium with 10% synthetic serum substitute. The procedures were performed at a room temperature of 22 to 24ºC.

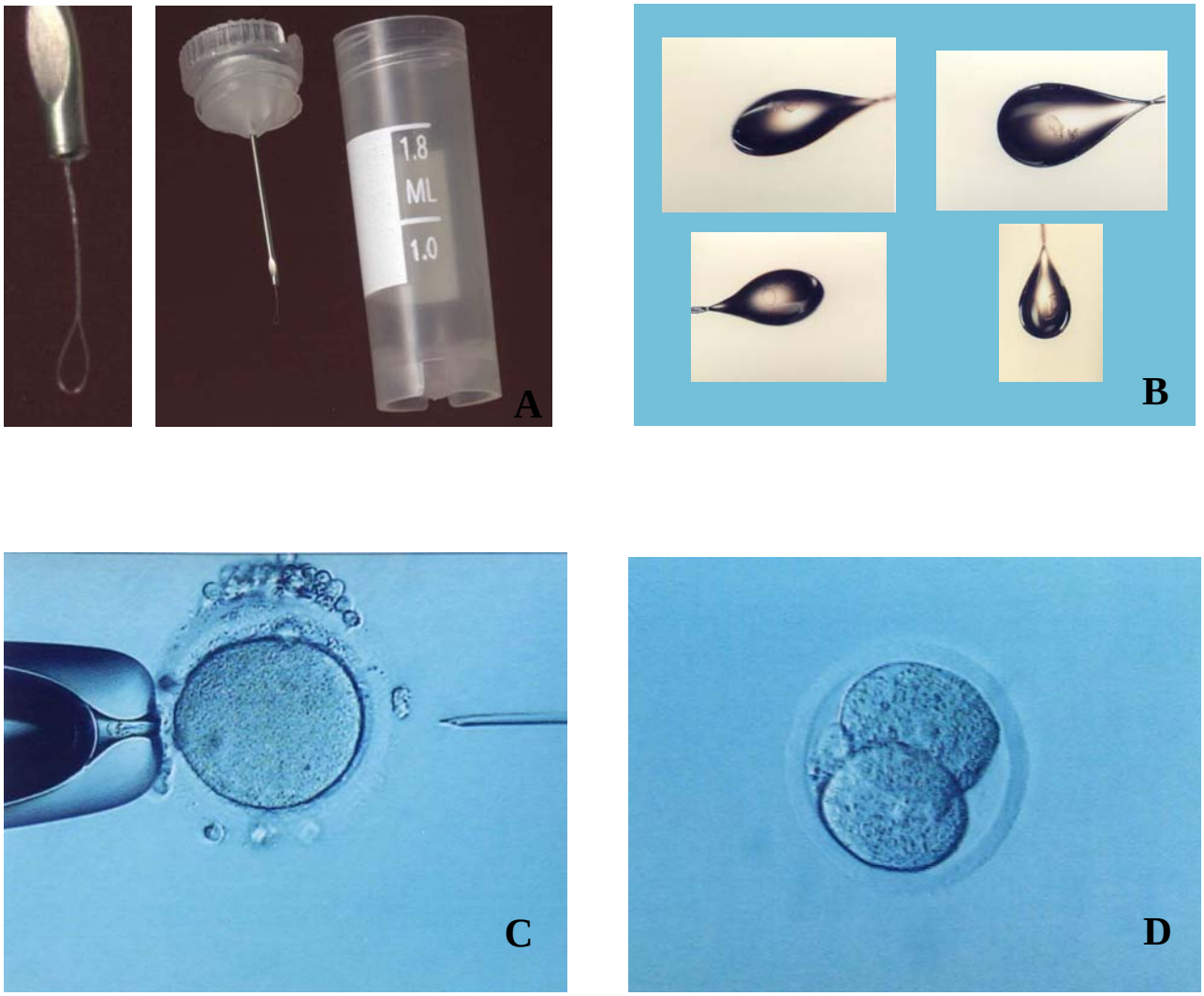

Figure I. Cryoloop and cryovial used for vitrification of oocytes(A) The loaded cryoloop(B) Injection of sperm in freez-thawed oocyte(C) 2 cell embryo 24 hours after sperm injection (D). 4 cell embryo 48 hours after sperm injection (E).

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Table I. Oocyte recovery, survival and fertilization rates, developmental capacity of vitrified oocytes, number of embryo transfer and delivery pregnancy

Group	No. of oocytes vitrified and thawed	Survival rate 2h post thawing (%)	No of 2PN* (%)	No of 6-8 cells embryo (%)	ET patients	Delivered pregnancy (%)
Straws Group I	34	18 (52.94٪)	10 (55.55٪)	4 (40%)	2	0 (0%)
Cryoloop Group II	33	21 (63.63٪)	15 (73.7٪)	9 (60%)	3	1 (33%)
p value	-	>0.05	<0.05	<0.05	-	<0.05

**:2PN=female and male pronuclei*

Intracytoplasmic sperm injection, embryo culture and embryo transfer

After washing all oocytes, they were cultured in same medium for 2 hours. Oocytes with fragmented cytoplasm, indistinct oolemma, increased perivtelline space or damaged zona pellucida were considered as non survivors. For inracytoplasmic injection, a motile spermatozoon was identified, immobilized and injected into the ooplasm using an inverted microscope with a micromanipulation system. To verify normal fertilization of vitrified oocytes the number of pronuclei in the cytoplasm was counted 16-18 hours after sperm injection using X200 magnification seeking two pronuclear and two polar bodies. The embryos from 2PN were cultured in HTF medium with 10% synthetic serum substitute and good morphological quality embryos were transferred on day 2 or 3 after sperm injection. Pregnancy was identified by the serum ß HCG level, checked 14 days after ET.

Statistical analysis

The survival rates of vitrified oocytes after dilution and two hours post incubation were calculated. The fertilization rate after ICSI was calculated. The X^2 test was used for statistical comparison. The difference was considered statistically significant when $p<0.05$.

Results

The results are presented in table I. The present study showed that the survival rate of cryopreserved human oocytes vitrified in two groups have no significant difference (52.94% in group I, and 63.63% in group II) but the fertilization rate of oocytes vitrified by cryoloop was greater than oocyte vitrified by conventional straws. (73.7% versus 55.55% respectively) and this difference was significant ($p<0.05$). The number of oocytes which reached to 6-8 cells embryos were higher in cryoloop group that in the straw group (60% versus 40% respectively) and the difference was significant ($p<0.05$). In group I, four 6-8 cell embryos from vitrified oocytes were transferred to two patients, while in group II, nine 6-8 cell embryos from vitrified oocytes were transferred to three patients. There was no clinical pregnancy in the first group while, only one of three patients achieved clinical pregnancy and resulted in the delivery of a healthy baby. The rate of pregnancy between two groups (0% versus 33%) was significantly different ($p<0.05$).

Discussion

This study has shown that oocytes vitrified using cryoloop had the same chance of morphological survival with those vitrified using conventional straw. These observations were supported by Lieberman and Tucker (10) who reported that the viability of oocytes after vitrification using the hemi–straw system was slightly higher than this when using the cryoloop but this difference was not significant.

Improving the survival rate and fertilization rate remains a major challenge in cryopreservation technology. ICSI has been proposed as a solution to cryodamage involving the zona pellucida and cortical granules. Porcu et al. (1997) introduced ICSI which led to an increase in the fertilization rate (5).

In this study, we performed ICSI to achieve fertilization for the vitrified thawed oocytes.

Our result showed that the embryo formation by ICSI following cryopreservation of oocytes using the cryoloop vitrification method is more than fertilization rate of vitrified-thawed oocyte using the conventional straws. Previous study had shown that the microtubules of oocytes are vulnerable to cryoprotectants and to temperature changes (18). Damage to meiotic spindle may impair fertilization and the development of

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embryos (19). It was estimated that the cooling rate achieved by plunging a straw into liquid nitrogen is 2500ºC/min. To obtain a more rapid cooling rate, some investigators brought oocyte in a small amount of vitrification solution into direct contact with liquid nitrogen with use of an electron microscope grid or open pulled straw (20). They suggested that the developmental capacity could be improved by reducing the time to traverse the damaging temperature. Briefly our study indicated that the oocytes which held in cryoloop for vitrification achieve a faster cooling and warming rate than those in conventional straws therefore, the disruption in cryoloop method is seemed to be occurred less than the disruption of microtubule in conventional straws. More over, oocyte in small amount of vitrification solution can be directly warmed and immediately diluted into the dilution that allow reduced exposure to unsuitable temperatures and concentrated cryoprotectants. In contrast, the conventional straw is warmed in water and then cut with scissors. The oocytes in larger vitrification volume are expelled into the dilution solution and then placed into another dilution solution. This allows more time to pass through the inappropriate conditions. The above mentioned points, may explained why vitrification of oocyte using cryoloop like ops, cps or grids preserved spindles better than conventional straws. It may also partly explain the finding of others (20) that the developmental competence of vitrified bovine oocytes could be enhanced using ops or grids, compared with conventional straws.

In our study a male infant delivery at 39 weeks arose from transfer of 3 embryos to an ovulated woman where oocyte vitrified in cryoloop and post thawed was fertilized by ICSI. Previously several successful pregnancies from vitrified human acolytes using ops or grids have been reported (21, 22). Pregnancies reported here and by others indicate a burgeoning awareness of the potential benefits of oocyte vitrification, pregnancy cautious optimism for the future of this technology. The risk of contamination, that may result from the direct contact of the straw content with liquid nitrogen, can be eliminated by nitrogen filtration or freezing in concentrated nitrogen vapor (-170°C). Another method that can considerably reduce the contamination is repeated washing of thawed oocytes in several drops of the culture medium.

Conclusion

It can be concluded from the results presented here that vitrification by using cryoloop can improved the fertilization rate and developmental capacity of vitrified thawed oocytes. More studies on vitrification and thawing procedures are needed to develop more efficient and optimal vitrification methods.

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