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GYNAECOLOGY

Research Article

Cryotop Vitrification Affects Oocyte Quality and Embryo Developmental Potential

Ling Jia*, Bo Xu*§, Yu-sheng Liu and Xian-hong Tong§

Center for Reproductive Medicine, Anhui Provincial Hospital Affiliated to Anhui Medical University, China

§Corresponding Author: Bo Xu and Xian-hong Tong, Center for Reproductive Medicine, Anhui Provincial Hospital Affiliated to Anhui Medical University, Hefei 230001, China.

*These authors contributed equally to this work.

Received: February 05, 2015; **Published:** March 18, 2015

Abstract

The vitrification technique is widely used for oocyte cryopreservation. This study aims to explore the feasibility of clinical application of oocyte vitrification. A total of 45 women whose 466 oocytes were vitrified and warmed using Cryotop method were enrolled as study group. And 104 homochromous patients with 1075 fresh oocytes undergoing intra cytoplasmic sperm injection treatment with infertility caused only by male factor were included as the control group. A retrospective analysis of clinical outcomes, including the rates of fertilization, cleavage, good quality embryo, pregnancy, implantation, abortion, and live birth, in two groups were performed. Compared to control group, the rates of cleavage ($P < 0.05$) and good quality embryo ($P < 0.05$) were significantly decreased in study group with vitrified and warmed oocytes. However, there was no difference between these two groups in fertilization rate ($p > 0.05$), clinical pregnancy rate ($p > 0.05$), implantation rate ($p > 0.05$), live birth rate ($p > 0.05$), abortion rate ($p > 0.05$). These results suggested that cryotop vitrification of human oocytes resulted in the clinical outcomes similar to fresh oocytes, however, cryopreservation of oocytes led to the decrease in oocyte quality and later embryo developmental potential. Therefore, the method for vitrification in oocyte cryopreservation program still needs further improvement.

Keywords: *Human oocyte cryopreservation; Vitrification; Survival; Embryo development*

Introduction

Since the first live birth with oocyte cryopreservation was reported at 1986 using slow freezing, more scholars have devoted themselves into this field of development and applications for oocyte cryopreservation techniques [1]. However, the number of live births using oocyte cryopreservation was small, even the slow-freezing methods for oocytes have been changed and improved over time, due to the outcomes based on slow-freezing were variable and difficult to reproduce [2-4].

Recently, the cryopreservation technique of vitrification (an ultra-rapid cooling technique) offered new interesting perspectives in the field of oocyte cryopreservation, and the conclusive evidence that has been accumulated regarding the huge potential importance of vitrification has been reviewed [5-7]. Since the first pregnancy achieved with a vitrified/warmed human oocyte and the first birth of a healthy baby, different protocols in the vitrification procedure have been reported for the human oocyte [6, 8-13]. Until now, the Cryotop based method developed by Kuwayama *et al*. is the most widely used method for vitrification of oocytes [14-17]. Several studies reported that the Cryotop based method could provide the outcomes similar to those achieved with fresh oocytes [15-17], however, it is still not clear whether the manipulations (temperature change, osmotic stress, cryoprotectant toxicity, and/or phase transitions) will cause negative effects on later embryo (vitrification freezing oocytes) developmental potential and subsequent clinical outcomes.

Therefore, a systematic evaluation is necessary to ensure the efficacy and efficiency of oocyte vitrification. In this study, we have used the Cryotop method for vitrification in oocyte cryopreservation program, and thus evaluating fertilization, cleavage, and embryo development of both vitrified/warming oocytes and fresh oocyte counterparts.

Methods and Materials

This is a retrospective study. The study was conducted in compliance with the Declaration of Helsinki and Ethics Committees on Human Research of Anhui Provincial Hospital, an affiliation of the Anhui Medical University (2010 Ethics 5th). All the subjects were patients received IVF/ICSI treatment at Anhui Province Hospital (Hefei, China) between October 2010 and May 2013. Through communication with all patients, they agreed to participate in the study and signed an informed consent form before enrolment.

The inclusion and exclusion criteria of Patients

Tee infertile couples who sought help because of infertility due to male factor, the woman's age ≤ 38 years old, physical and gynaecological examinations were normal for all subjects, and there was no family history of hereditary or chromosomal diseases. All participants had a normal karyotype, and tested negative in a screening for sexually transmitted diseases. According to the above criteria, 45 women who vitrified and warmed their oocytes between October 2010 and May 2013 at the Department of Anhui Provincial Hospital Reproductive Center were enrolled in this study. The reasons for vitrification freezing oocytes of these 45 couples were failure of sperm collection on the day of oocyte retrieval and prevention of fertilization failure. One hundred and four patients who were undergoing intra cytoplasmic sperm injection (ICSI) treatment for male factor from 104 cycles were collected as control group between October 2010 and May 2013. The basic data of patients in two groups were listed in Table 1.

	Vitrification Group	**Fresh Group**	***p* value**
Patients (n)	45	104	/
Mean female age (years)	29.22 ± 4.37	29.00 ± 3.60	0.207
Mean female duration of infertility (years)	4.44 ± 2.74	4.26 ± 2.57	0.755
Body mass index (kg/ m^2)	21.2 ± 3.6	22.3 ± 4.3	0.213
Days of ovarian stimulation	11.4 ± 3.0	12.2 ± 2.14	0.691
E2 levels on HCG day (pg/ml)	28045.22 ± 1337.23	3049.49 ± 1437.25	0.871
Endometrial thickness on HCG day (mm)	10.7 ± 2.2	10.6 ± 2.3	0.132

Table 1: *Comparison on basic data of the patients in two groups ($\bar{x}\pm s$).*

$p < 0.05$ was considered to be statistically significant.

Stimulated Protocol

All patients were stimulated with the long gonadotrophin-releasing hormone agonist (GnRHa, Diphereline; Ipsen Pharma. Biotech, Signes, France) protocol combined with recombinant FSH (Gonal-f, Merk Serono SA, Geneva, Switzerland). Oocytes were retrieved by transvaginal ultrasound 36h after injection of 10 000 IU human chorionic gonadotrophin (hCG, LiZhu Pharma, ZhuHai, China). The collected oocytes were cultured in IVF media (G-line products, Vitrolife, Sweden) and placed in an incubator (6% CO_2; 5% O2; 37°C) for 1-2h. Cumulus cells were removed enzymatically with 80 IU/ml hyaluronidase (SAGE In Vitro Fertilization, Inc. Trumbull, USA) and mechanically by glass pipettes.

Oocytes vitrification freezing and warming

The Cryotop Vitrification Kit (Kitazato BioPharma, Shizuoka, Japan) were used for oocyte vitrification. Briefly, the oocytes were treated for 5 min in equilibration solution [7.5% ethylene glycol (EG) and 7.5% dimethylsulfoxide (DMSO)], then transferred in a minimal volume of equilibration solution into a drop (100μL) of the vitrification solution (15% ethylene glycol plus 15% dimethysulfoxide plus

0.5 M sucrose). The oocytes were briefly rinsed three times (< 30 s every time) in three drops of vitrification solution, and then loaded onto the CryoTop. After loading, most of the solution was removed so that only a thin layer covered the oocytes. Once the oocytes were transferred into the vitrification solution, they were immediately submerged into filtered liquid nitrogen within 90s.

For the warming process, the CryoTop with vitrified oocytes, was removed from the liquid nitrogen and warmed by plunging it into 1.5 ml of thawing solution 1 (TS1, 1 M sucrose) at 37°C for 1 min. The oocytes were quickly transferred into diluent solution (DS1, 0.5 M sucrose) and washing solution 1 (0 M sucrose) for 2 min in each solution at room temperature (RT), and then transferred into washing solution 2 (0 M sucrose) for 5 min. Finally, the oocytes were kept in the fertilization medium (G-line products, Vitrolife, Sweden), under mineral oil. Subsequently, they were cultured at 37°C and 6% CO_2 in the incubator for 3h.

Post-warming survival rate was assessed using morphological criteria, indicated by the absence of overt cell degeneration, elongated shape, thick or distorted zona, expanded perivitelline space (PVS) and dark cytoplasm [15,18]. Only MII oocytes with an extruded first polar body were included in this study.

Fertilization, embryo quality and clinical outcome

After 3h incubation in fertilization medium, ICSI was performed on all surviving MII oocytes. The oocyte was considered fertilized if the second polar body was extruded or if pro-nuclei were seen 16 h after insemination. Those with two pronuclei (2PN) and a second polar body were identified as normally fertilized. The normally fertilized oocytes were transferred into drops of cleavage medium (G-line products, Vitrolife, Sweden) under mineral oil in 35-mm Petri dishes (Falcon 1008; Becton & Dickinson, Lincoln Park, NJ) until day 3. Two or three good quality embryos were selected for clinical transfer. The embryo quality was scored according to previous report [19]. Day 3 embryos that had 7-10 non-multinucleated blastomeres and < 20% fragmentation were classified as good quality embryos. Pregnancy was diagnosed by a positive blood test for hCG at 14 days after embryo transfer (ET). A clinical pregnancy was confirmed by observation on transvaginal scanning of a gestational sac with fetal heart beat~4 weeks after the positive pregnancy test. The implantation rate was defined as the number of gestational sacs visible on ultrasound divided by the total number of replaced embryos, and expressed as a percentage. All the pregnancies were followed up to the time of delivery.

Statistical Analysis

A comparison between fresh and vitrified/warmed groups in terms of fertilization, cleavage, pregnancy and abortion rates was performed. Statistical analysis was performed using SPSS software (SPSS Inc., version17, USA); compared with the rate of chi-square, mean compared with *t*- test. p vaule < 0.05 was considered statistically significant.

Results

Basic clinical information

No significant differences were found between the two groups in basic patient information (Age, duration of infertility), body mass index, days of ovarian stimulation, doses of used gonadotropins, values of E2 and endometrial thickness on the day of HCG (Table 1).

Outcome from vitrified/warmed and fresh oocytes

A total of 466 oocytes were vitrified and warmed, and thus 404 (86.69%) of these oocytes were survived. Of these, sixty oocytes were arrested either in metaphase I (MI) or germinal vesicle stage (GV) and therefore were not considered further (Table 2). Thus, 344 surviving mature oocytes were inseminated by ICSI and 274 (79.65%, 274/344) were normal fertilization, of which 261 (95.25%, 261/274) fertilized oocytes developed into the cleavage stage (Table 2). Therefore, 124 (47.50%, 124/261) good quality embryo were obtained, and 104 (out of 124) embryos were transferred into 45 patients (Table 2). At last, 27 (out of 104) embryos were implanted, corresponding to the implantation rate of 25.96% (Table 2). The clinical pregnancy rate was 51.11% (23 of 45) (Table 2). Four of the twenty-three patients with clinical pregnancies had a miscarriage during the first trimester (Table 2). The remaining 19 patients delivered 22 healthy

newborns (16 singletons, 3 twins), corresponding to the live birth rate of 42.22% (Table 2). At present, all infants after inspection, chromosome and development were normal.

From a total of 1075 collected fresh oocytes, 890 mature oocytes were inseminated using ICSI (Table 2). The fertilization and cleavage rates were 76.51% (681/890) and 99.11% (675/681) (Table 2). Out of the original 104 patients, a total of 222 embryo were transferred, with an average of 33.78% embryos implanted (Table 2). There were 55 clinical pregnancies of which 7 aborted and 48 delivered (Table 2).

Compared to control group, the rates of cleavage (95.65% vs. 99.11%, $p < 0.05$) and good quality embryo (47.50% vs. 57.92%, $p < 0.05$) were significantly decreased in study group of vitrified and warmed oocytes. However, there was no difference between these two groups in fertilization rate (79.65% vs. 76.51%, $p > 0.05$), clinical pregnancy rate (51.11% vs. 52.88%, $p > 0.05$), implantation rate (25.96% vs. 33.78%, $p > 0.05$), live birth rate (42.22% vs. 46.15%, $p > 0.05$), abortion rate (17.39% vs. 12.72%, $p > 0.05$, Table 2).

	Vitrification Group	Fresh Group	*p* value
Patients (n)	45	104	/
No. of cycles	45	104	/
No. of recovered oocytes	639	1075	/
No. of vitrified oocytes	466	/	/
No. of warmed oocytes	466	/	/
No. of surviving oocytes	404	/	/
No. of injected oocytes	344	890	/
Normal fertilization rate (%)	79.65 (274/344)	76.51 (681/890)	0.2379
Cleavage rate (%)	95.25 (261/274)	99.11 (675/681)	0.00011
Good quality embryo rate (%)	47.50 (124/261)	57.92 (391/675)	0.00407
No. of transfers	45	104	/
No. of transferred embryos	104	222	/
Pregnancy rate (%)	51.11 (23/45)	52.88 (55/104)	0.8422
Implantation rate (%)	25.96 (27/104)	33.78 (75/222)	0.1556
Abortion rate (%)	17.39 (4/23)	12.72 (7/55)	0.5894
Live birth rate (%)	42.22 (19/45)	46.15 (48/104)	0.6578

Table 2: *Comparison of pregnant outcomes after ICSI-ET in two groups.*
$p < 0.05$ was considered to be statistically significant.

Discussion

Although embryo cryopreservation is the most reliable effect of fertility preservation [20], oocyte cryopreservation represents a necessary complement to assisted reproductive technologies. Indications include preserving fertility in ovarian cancer patients, avoiding severe ovarian hyper stimulation syndrome and coping with failure to obtain sperm [21-25]. This approach is remarkably useful for ovum donation programs because no synchronization is needed between the donor and recipients, which makes it more adjustable, easier, and safer as a procedure because a quarantine period can be used before oocyte donation. Also, it does not require the presence of a male partner [26], therefore having great impact on assisted reproduction technology.

Nevertheless, the influence of cryopreservation on oocyte quality and clinical outcome was still unclear and often doubted. Several reports suggested that the clinical pregnancy rate and live birth rates from vitrified/warmed oocytes were similar with those from fresh oocytes [15-17]. In this study, we also found that oocyte cryopreservation did not affect the clinical pregnancy rate and live birth rate.

However, the cleavage rate and good quality embryo rate were decreased in oocytes after cryopreservation. This data was in agreement with ultra structural analysis of vitrified oocytes. Previous studies suggested that the spindle structure and mitochondria were impaired in vitrified-warmed oocytes based on ultra structural analysis [27,28]. Moreover, oocyte cryopreservation may result in an adverse impact on the later development of the embryo [29]. These suggested that the oocyte cryopreservation technology still needed to be improved. Since the vitrification freezing technology was used for embryo and oocyte cryopreservation, the composition of cryoprotectant agents (CPA) has been in continuous improvement. Although human oocytes have been successfully vitrified, the degree and exact effect of CPA toxicity is poorly understood. Certain combinations of CPAs, such as EG and DMSO, resulted in the best morphology, they were the most detrimental in terms of total mitochondrial and tubulin retrieval after vitrification, indicating that the oocytes' morphological appearance alone is not an efficient measure of the quality of vitrified oocytes and can be misleading [29]. We need further investigate which CPA or combination of CPAs could result in the minimal damage and the most viable oocytes.

In addition to the CPA, there were several other factors which may have an effect on embryo development and clinical outcomes, including the intrinsic quality of the gametes and the impact of any ex vivo manipulations. Female age remains the most critical factor that could affect clinical outcomes after oocyte vitrification [30], which given the well known decline in female fertility beyond the age of 30 years [31,32]. Assisted reproduction is unable to fully overcome the effect of age on fertility loss after the age of 35 years. Additionally, The impact of increasing female age is not only associated with a statistically significant decrease in human ovarian follicle number, but also is related to increasing adverse reproductive outcomes and a higher proportion of maternal and/or fetal morbidity and mortality. Therefore, in order to achieve higher success rates with IVF, the age of application of oocyte vitrification should be at an age younger than 35 years.

At the same time, the mature oocytes must be fertilized at the right time. Otherwise, oocytes will undergo apoptosis [33]. Dozortsev *et al*. observed that the optimal time of ICSI for human oocytes was from 37 to 41 hours after administration of hCG to prevent the oocyte aging. Those fertilized oocytes could achieve the best development potential [34]. It has been well documented that various un physiological alterations occur in the oocyte during the course of cryopreservation, one of the most prominent is the transient disappearance of the meiotic spindle. The meiotic spindle plays a critical role in process of meiosis and further development after fertilization. Fortunately, the meiotic spindle does regenerate when culturing oocytes for 1 to 3 hours after cryopreservation [27,35]. The degree and speed of recovery of the oocyte spindle depend on time intervals after thawing and on the freezing method. Chen *et al*. found that the rates of fertilization and blastocyst formation of vitrified mouse oocytes inseminated immediately or at 1 hour of incubation were significantly lower than controls, but they were improved when inseminated at 2 or 3 hours of incubation [13]. Therefore, considering both aspects of spindle recovery and oocyte aging, our center set the inseminated time at 3 hours after thawing of oocytes which may achieve higher success rates in IVF cycles.

What is noteworthy is that before human MII oocytes are vitrified they are usually denuded from their cumulus cells. However, our previous studies have found that fertilization rates were improved after IVF if the corona radiata was left intact in vitrified-warmed human oocytes. This indicates that human oocytes with an intact corona radiata do not develop hardening of the zona pellucida after vitrification with the CryoTop method, and that poor penetration of the zona pellucida after vitrification has probably been due to the removal of the cumulus cells [36]. The ICSI technique has been widely used in vitrified-thawed oocytes to overcome the potentially low fertilization rates with conventional insemination, IVF. Our findings suggested that there is no need for ICSI, which not only decreases the workload in the laboratory but also decrease potential genetic risks in the offspring through bypassing the natural selection and penetration of high-quality spermatozoa into the cytoplasm of the oocyte [36].

In our center, oocyte vitrification did not affect the clinical pregnancy and live birth rates; however, the rates of cleavage and good quality embryo were decreased comparing with those of fresh oocytes. These results suggested that the oocyte cryopreservation may result in an adverse impact on the oocyte quality and embryo development potential. Cryotop is, at present, the most efficient vitrification method available, but still need needs further improvement.

Acknowledgments

This research was supported by the Natural Science Foundation of Anhui Provincial of China (1308085QH131), Natural Science Research Project of Anhui Provincial Education Department (KJ2013Z134), and the National Natural Science Foundation of China (81000237).

Competing Interests

The authors declare that they have no competing interests.

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Volume 1 Issue 2 March 2015
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