Vitrification of in vitro matured oocytes diminishes embryo development potential before but not after embryo genomic activation

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Abstract

Purpose The aim of this study is to evaluate the impact of oocyte vitrification on embryo development potential and to assess the chromosome abnormalities of blastocysts derived from fresh/vitrified-warmed oocytes to assure the safety of the oocyte cryopreservation technique.

Methods In vitro matured oocytes derived from immature oocytes were retrieved from small follicles during IVF/intracytoplasmic sperm injection (ICSI) cycles were randomly divided into a fresh and vitrified-warmed groups. After intracytoplasmic sperm injection, the fertilization rate, embryo quality, and developmental status were compared between the two groups. Blastocysts derived from both groups were analyzed using the copy number variation (CNV)-seq technique to evaluate DNA abnormalities.

Results The fertilization rate with ICSI and the cleavage rate were similar between the two groups. Among the vitrified-warmed group, there was a lower incidence of usable embryos on day 3 (16.42 vs. 28.57 %; $P < 0.05$) and a lower incidence of blastocysts (7.46 vs. 17.86 %; $P < 0.05$). However, the proportions of embryos that developed to blastocysts from the day 3 available embryos were similar between the two groups (62.5 vs. 45.45 %; $P > 0.05$). In the day 3 embryos, the

proportion of >5 cell embryos in the fresh group was markedly higher than in the vitrified-warmed group (41.67 vs. 21.64 %; $P < 0.05$), and the proportion of embryos with ≥ 50 % fragments was not significantly different between the two groups (39.29 vs. 43.28 %; $P > 0.05$). The result of CNV-seq demonstrated that there was no difference in chromosomal abnormalities between the two groups (20 vs. 20 %).

Conclusions Oocyte vitrification and the warming procedure diminished the embryo development potential before day 3, when embryo genomic activation started. The day 3 usable embryos derived from vitrified-warmed oocytes had the same potential for developing into blastocysts. Vitrification and the warming procedure did not increase the chromosome abnormalities of the blastocysts. Oocyte vitrification is a safe technique for those patients who have no other options, although the oocyte efficiency may be diminished after the vitrified-warmed procedure.

Keywords Oocyte vitrification · ICSI · Blastocyst · CNV-seq

Introduction

Oocyte cryopreservation is a widely used approach to preserve female fertility and provides a number of irreplaceable advantages. First, it prevents ethical and legal issues associated with embryo preservation. Second, it provides the opportunity for women undergoing potentially sterilizing medical and surgical treatments to preserve fertility and have children later in life [1]. Third, an egg bank also makes the donor oocyte programme more convenient and efficient [2]. Finally, it also provides a remedial measure for patients undergoing in vitro fertilization cycles who have no available sperm to use. Initial progress on the mature oocyte cryopreservation technique was very slow due to the metaphase-II (MII) oocyte's

Capsule Oocyte vitrification diminishes embryo development potential but does not increase chromosomal abnormalities of blastocysts. It is a safe technique for those patients who have no other options.

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large size, water content, and the fragile meiotic spindle structure [3]. Only with the recent advances in the vitrification technique has the efficacy of oocyte cryopreservation undergone important improvement in terms of survival and pregnancy rates. Many published articles have provided good evidence that fertilization and pregnancy rates are similar to IVF/intracytoplasmic sperm injection (ICSI) with fresh oocytes when vitrified/warmed oocytes are used as part of IVF/ICSI in young patients [4–6]. The American Society for Reproductive Medicine (ASRM) has announced that oocyte vitrification and warming should no longer be considered experimental but has also recommended that more data are needed before this technology should be used routinely in lieu of embryo cryopreservation [7]. Further research should be carried out to investigate the efficiency and safety of the oocyte vitrification technique. In this study, we focused on whether the technique affects the utility efficiency of the oocyte and increases chromosomal damage.

Copy number variation (CNV) sequencing has been proved to be highly sensitive and specific for the detection of whole and partial chromosome aneuploidies, submicroscopic deletions and duplications <1 Mb, and ring chromosomes. In addition, CNV-seq is capable of reproducibly detecting both heterozygous and homozygous deletions as small as 0.1 Mb in size [8]. CNV-seq may serve as a useful tool to assess the full spectrum of chromosomal aberrations that arise in human embryos at the research and clinical level [8–10]. CNV-seq also has demonstrated high level of consistency with an established methodology, such as array-CGH and became a robust high-throughout methodology ready for clinical application with potential advantages of reduced costs and enhanced precision. Clinical results also reported high pregnancy outcomes following transfer of screened embryos using this method [11, 12].

In the present study, we investigated the difference in development potential between fresh and vitrified-warmed oocytes using the oocytes matured in vitro from the immature oocytes that were retrieved from small follicles during IVF/ICSI cycles. The chromosome abnormalities of blastocysts derived from fresh/vitrified-warmed oocytes were also evaluated using the CNV-seq technique.

Material and methods

Source of oocytes

The study group comprised of 341 women who each underwent an IVF or ICSI cycle ($n=467$) with written consent between June 2012 and March 2014 in the Ruijin Hospital of Shanghai Jiaotong University of Medicine in Shanghai. Written informed consent was also obtained from each infertile couple before the use of their immature oocytes. This study was approved by the ethics committee of our hospital.

Inclusive criteria were age <38 years [1], basal FSH <10 mIU/ml [2], the couples each have normal chromosome types [3], and infertility was only due to male infertility and/or oviduct factors [4]. Patients with more than two preceding failure transfer cycles were excluded. The oocytes retrieved from follicles smaller than 10 mm in diameter and possessed one to five layers of overlying corona cells were defined as immature oocytes [13]. For each cycle, at least one immature oocyte was retrieved.

Oocyte recovery procedures and in vitro maturation

The patients were given standard controlled ovarian stimulation with a long GnRH agonist or antagonist protocol as previously described [14]. Human chorionic gonadotropin (hCG, Lizhu, China) was administered to trigger egg maturation when two or more follicles reached the size of ≥ 20 mm in diameter, and transvaginal oocyte retrieval was performed 36 h later. The immature oocyte-cumulus complexes were cultured in fertilization medium (HTF medium, Irvine Scientific, USA) supplemented with 20 % autologous follicle fluid with final concentrations of 0.075 IU/mL for both FSH and LH at 37 °C in an atmosphere of 5 % CO₂ and 95 % air with high humidity for maturation in culture. The maturity of oocytes was assessed 24 h after IVM culture, and the oocytes with a polar body after the denudation of cumulus cells were regarded as mature. The oocytes were evaluated microscopically based on morphology to identify normal oocytes with an appropriate size, normal zona pellucida, first polar body, and cytoplasm. Oocytes with abnormal morphology were excluded.

Randomization

The randomization was on a 1:2 basis. Random numbers from 0–3 were produced using the random number function in Microsoft Excel. The numbers were allocated to mature oocytes in sequence. The oocytes with number ≤ 1 were performed ICSI immediately and the oocytes with a number range from 1–3 were vitrified.

Vitrification and thawing procedure

The vitrification procedure was performed at room temperature by first equilibrating in equilibration solution (ES) containing 7.5 % ethylene glycol (EG) and 7.5 % dimethylsulfoxide (DMSO) [15]. The equilibration was performed gradually, and then, the oocytes remained in ES for 8–10 min until the oocytes returned to their original size from the dehydration caused by the cryoprotectants. The oocytes were then transferred into vitrification solution (VS) containing 15 % EG, 15 % DMSO, and 0.5 mol/L sucrose for 45–60 s, at which point they were rapidly loaded onto a Cryotop strip and quickly submerged into liquid nitrogen (LN2). The Cryotop was capped

with a protective cover while submerged in LN2 and stored in an LN2 tank at -196°C .

For warming, the Cryotop was removed from the nitrogen, immersed into a solution (containing 1 mol/L sucrose) at 37°C , and warmed for 60 s. The oocytes were then transferred to a dilution solution (containing 0.5 mol/L sucrose) for 3 min, followed by a dilution solution (containing 0.25 mol/L sucrose) for 3 min and then a washing solution without sucrose for 1 min. The warmed oocytes were placed in fertilization medium for 2 h before intracytoplasmic sperm injection (ICSI).

Fertilization and embryo culture

Fresh oocytes and oocytes cultured for 2 h after vitrification and the warming procedure were inseminated by ICSI according to the routine of our reproductive centre. ICSI was performed with cryopreserved sperm donated by fertile men. Oocytes were checked for the presence of pronuclei at 16–18 h after ICSI. The zygotes were transferred to culture medium (CSC medium, Irvine Scientific, USA) for the following 48 h. The quality of the embryos was evaluated 48 h later (day 3) based on the number of blastomeres, the degree of fragmentation, and the equality of blastomeres, according to the criteria previously described [14]. Embryos with more than five blastomeres, $<50\%$ fragments, and a total score of more than 5 on day 3 were considered available. The further development of all embryos was recorded at intervals of 24 h for up to 3 more days (day 6). The quality of the blastocysts was evaluated according to the Gardner grade [16].

CNV sequencing and data analysis

Blastocysts were washed three times in PBS, carefully transferred by micropipette into 2.5 μL of PBS placed in the bottom of a 200- μL PCR tube, and then frozen at -20°C . Blastocysts, as well as PBS blank controls, were subjected to whole genome amplification (WGA). The WGA products were

fragmented to an average size of 300 bp, and sequencing libraries were prepared as previously described [17]. The libraries were subjected to sequencing on the HiSeq 2500 Illumina platform to generate approximately five million 36-bp single-end reads, representing 0.1-fold genome coverage. All of the sequences were collated and aligned to the unmasked genome using the Burrows-Wheeler algorithm.

Statistical analysis

The data are reported as the mean $\pm$ SD and were analysed using the SPSS 16.0. Chi-squared or Fisher's Exact test whenever there were small numbers ($n < 5$) involved and Student's *t* test analysis were also performed. A two-sided *P* value of <0.05 was considered as statistically significant.

Results

A total of 506 immature oocytes were collected from 467 oocyte retrieval cycles. After in vitro maturation, 361 oocytes were matured (71.34 %). And 313 MII oocytes from 297 oocyte retrieval cycles were evaluated microscopically as morphologically normal. Of these normal oocytes, 106 were directly fertilized by ICSI and 207 underwent the procedure of vitrification-warming, with 175 (84.54 %) of these surviving the warming procedure. There were no differences between the patient demographics of the two groups, as shown in Table 1.

The fertilization rate and cleavage rate were similar in the two groups. However, there were significant differences between the two groups when comparing the subsequent embryo development. The available embryos rate on day 3 was much lower in the vitrified-warmed group than in the fresh group (16.42 vs. 28.57 %; $P < 0.05$). The proportion of >5 cell embryos in the fresh group was markedly higher than in the vitrified-warmed group (41.67 vs. 21.64 %; $P < 0.05$). However, the proportion of embryos with $\geq 50\%$ fragments was

Table 1 Patient demographics of the fresh and vitrified-warmed groups

	Fresh group	Vitrified-warmed group	<i>P</i>
No. of cycle	98	199	
Female age (mean years $\pm$ SD)	30.12 $\pm$ 3.81	30.31 $\pm$ 3.64	NS
Baseline FSH (mean mIU/mL $\pm$ SD)	5.93 $\pm$ 1.92	5.86 $\pm$ 1.91	NS
Previous IVF cycles (mean $\pm$ SD)	0.74 $\pm$ 0.85	0.81 $\pm$ 0.87	NS
GnRH-agonist long protocol (%)	65 (66.3)	134 (67.3)	NS
Antagonist protocol (%)	33 (33.7)	65 (32.7)	NS
Days of stimulation (mean $\pm$ SD)	10.85 $\pm$ 1.12	10.69 $\pm$ 1.07	NS
Total gonadotropin amount IU (mean $\pm$ SD)	2020.78 $\pm$ 450.66	1969.55 $\pm$ 502.43	NS
Peak E2 (pg/mL $\pm$ SD)	3674 $\pm$ 1323	3589 $\pm$ 1190	NS

NS not significant

Table 2 Comparison of embryos derived from fresh oocytes and vitrified-warmed oocytes

	Fresh group	Vitrified-warmed group	<i>P</i>
MII oocytes (%)	106	207	
No. of oocytes survived after warming	–	175 (84.54)	–
Total fertilization (%)	88 (83.02)	143 (81.71)	NS
2PN (%)	83 (94.32)	135 (94.41)	NS
1PN or 3PN (%)	5 (5.68)	8 (5.59)	NS
Embryo cleavage (%)	84 (95.45)	134 (93.71)	NS
Day 3 available embryos (%)	24 (28.57)	22 (16.42)	<0.05
Day 3 no. of >5 cell embryos (%)	35 (41.67)	29 (21.64)	<0.05
No. of fragment $\geq$ 50 % embryos	33 (39.29)	58 (43.28)	NS
No. of blastocytes (%)	15 (17.86)	10 (7.46)	<0.05
Blastocytes/day 3 available embryos (%)	15/24 (62.5)	10/22 (45.45)	NS
No. of blastocysts of abnormal CNV-seq result (%)	3 (20)	2 (20)	NS

NS not significant

not significantly different between the two groups (39.29 vs. 43.28 %; $P>0.05$). The day 3 unavailable embryos all failed to develop to blastocysts. We can conclude that the diminishment of the embryo development potential before day 3 was mostly due to the slower developing speed. Because of the lower available embryos rate on day 3, the blastocysts rate on day 5 was much lower in the vitrified-warmed group than in the fresh group (7.46 vs. 17.86 %; $P<0.05$). However, the proportions of embryos that developed to blastocysts from the day 3 available embryos were similar between the two groups (62.5 vs. 45.45 %; $P>0.05$), which meant that day 3 available embryos in the two groups had the same potential for developing into blastocysts. The results of CNV-seq demonstrated that there were no differences in chromosomal abnormalities between the two groups (20 vs. 20 %) (Table 2).

Gardner's grade of the blastocysts that developed in the two groups and the CNV-seq results of each blastocyst are shown in Table 3.

Discussion

The present study demonstrated that the oocyte vitrification and warming procedure diminished embryo development potential. This result was in agreement with our unpublished clinical data and prior study [18]. Although several articles have demonstrated that vitrified-warmed oocytes had an equivalent clinical outcome [4–6], most of these focused on the outcome of the transferred embryos which would not reflect the overall developed potential of the oocytes and special patient populations, such as oocyte donors or infertile couples with supernumerary oocytes [19].

In our study, no significant difference in the fertilization rate was observed between the fresh and vitrified-warmed oocytes, which was different from the result of prior study [18]. The conclusion that no significant effect on spindles

Table 3 The quality of the blastocysts derived from vitrified-warmed oocytes and the results of the CNV-SEQ

	Gardner's grade of blastocysts		CNV-seq result
	EH stage	ICM and TE grades	
Blastocysts derived from Vitrified-warmed oocytes			
No. 1	4	BC	Euploid
No. 2	4	AB	Euploid
No. 3	4	BC	Euploid
No. 4	3	CB	Euploid
No. 5	4	BB	Euploid
No. 6	4	BB	Euploid
No. 7	3	CC	−19
No. 8	4	BB	Euploid
No. 9	3	BC	Euploid
No. 10	4	BC	del (15q25.1qter, 22.44 Mb)
Blastocysts derived from fresh oocytes			
No. 1	4	AB	Euploid
No. 2	4	BA	Euploid
No. 3	3	BC	Euploid
No. 4	4	CC	Euploid
No. 5	2	–	+21
No. 6	5	BA	Euploid
No. 7	4	CB	Euploid
No. 8	3	CB	Euploid
No. 9	2	–	Euploid
No. 10	3	CC	Euploid
No. 11	4	BB	+14
No. 12	4	CB	Euploid
No. 13	3	CB	−(9q34.11qter, 10.08 Mb)
No. 14	4	AC	Euploid
No. 15	3	BB	Euploid

and chromosomes was observed after the vitrification-warming procedure [20] may partly explain our result. Although the vitrification procedure may bring change to the zona pellucida of the oocytes, we could obtain the same fertilization rate using the ICSI technique. We found that the diminishment of embryo development potential before day 3 was mostly due to the slower developing speed. However, the day 3 available embryos in the two groups had the same potential to develop to blastocysts. Day 3 coincides with the time when embryo genomic activation starts [21].

The preimplantation development consists of two main transitions: from a metaphase II oocyte to a four-cell embryo where mainly the maternal genes are expressed and from an eight-cell embryo to a blastocyst with downregulation of the maternal genes and upregulation of the embryonic genes [22]. During the first cell cycles, the embryo relies on the reserves of mRNA and proteins stored in the oocyte cytoplasm, and it is only later in the preimplantation development that embryo genomic activation (EGA) occurs, marking the beginning of self-sustained cellular biology. The translation and degradation of maternally inherited mRNAs stored in the oocyte cytoplasm prior to ovulation is both concomitant with and required for the successful completion of EGA [23]. The widespread cytoplasmic catabolism of oocyte-inherited proteins is required for the correct initiation of EGA [24]. The relatively poor developmental competence of primate oocytes is likely caused in part by failure in the timely onset of embryonic genome activation resulting from incomplete cytoplasmic maturation, which frequently results in developmental arrest [25]. Thus, we infer that the diminishment of developing potential of embryos before day 3 was due to the damage of the oocyte cytoplasm. The embryos that started embryo genomic activation normally could recover their developing potential. This inference is consistent with the data of our other experiments, which demonstrated that the reactive oxygen species (ROS) was higher and that certain mRNA levels were lower in the vitrified-warmed oocytes (data unpublished).

The data of this study demonstrated that blastocysts created from vitrified-warmed oocytes had no more risk of aneuploidy than those derived from fresh oocytes. This conclusion was in agreement with previous study [18]. We used the CNV-seq technique, which was able to detect chromosome abnormalities in more detail. However, we did not detect the arrested embryos that did not reach the blastocyst stage, as those embryos had no implantation potential. Although our work was in in vitro matured oocytes and in vivo matured oocytes vitrified at the MII stage might have difference, our data to a certain degree demonstrated that oocyte vitrification is a safe technique for those patients who have no other options, although the oocyte utility efficiency may be diminished after the vitrified-warmed procedure. Further studies are also required to investigate the specific influence of vitrification on the oocyte cytoplasm to guide the improvement of the oocyte

cryopreservation technique to increase the utility efficiency of the oocyte.

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