

Original Article

Transfer of vitrified-warmed day 7 blastocysts following in vitro fertilization/intracytoplasmic sperm injection to Chinese women resulted in the successful delivery of nineteen healthy babies

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Abstract: This retrospective study aimed to investigate whether the transfer of human vitrified-warmed day 7 blastocyst has the potential to be clinically important. It included 94 patients aged from 22 to 45 years old who had failed to get ideal embryos after in vitro fertilization (IVF) cycles at day 3, day 5 and day 6, or whose embryos had slow development. Blastocysts were collected on day 7 and were biopsied and screened for aneuploidy. A total of 154 day 7 blastocysts were vitrified by using cryotop with 153 blastocysts surviving after warming (rate of survival was 99.4%). A total of 151 day 7 blastocysts were transferred to 94 patients. Results showed an implantation rate of 17.9% and a clinical pregnancy rate of 16.6% (25 patients had clinical pregnancies). From these, 17 patients successfully delivered healthy babies (68%), while eight patients had miscarriages (32%). Our clinical data has shown that vitrified day 7 blastocysts can be transferred and successfully implant to give rise to viable pregnancies and subsequent delivery. Therefore, the transfer of day 7 vitrified-warmed blastocysts has the potential to be clinically important.

Keywords: Vitrified/cryopreservation, day 7, blastocyst, pregnancy, IVF

Introduction

Vitrification of human embryos has been extensively applied to assisted reproductive technology (ART) since it was first reported by Mukaida et al. in 1998 [1]. Compared with conventional slow cryopreservation, the vitrification technique has increased the post-warm survival rate, transfer rate and pregnancy rate, thus greatly increasing the clinical efficacy of surplus embryos [2, 3]. Therefore, vitrification has become an important cryopreservation technique in ART due to easy operation and technical reliability.

With the development of science and technology, researchers are making every effort to find biomarkers that can predict embryo viability. Attempts have been made to evaluate embryo quality by analyzing embryo culture media [4-7], eggs and even granule cells [8, 9].

However, none of these methods can be applied in clinical practice at present. Blastocyst transfer is an alternative method for selecting embryos, as those embryos which reach the blastocyst stage at days 5-7 have demonstrated their developmental potential. Furthermore, the transfer of blastocysts provides better synchronicity between the embryo and the endometrium [10]. With growing advances in embryo culture systems, the in vitro culture and cryopreservation of blastocysts has become an effective compensatory means of ART. It greatly increases overall pregnancy rate per egg pick-up, and reduces the risk of multiple pregnancies in IVF cycles and the occurrence of severe ovarian hyperstimulation syndrome (OHSS) [11-13]. Additionally, the effective preservation of leftover blastocysts can reduce the medical cost and pain borne by patients who would otherwise have to undergo further rounds of egg pick-ups.

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Blastocyst culture and transfer has been gradually accepted and widely applied to clinical practices. However, a subset of embryos develops slowly and do not mature on days 5 or 6, only forming blastocysts on day 7. Unfortunately, many centers culture embryos to day 6 and then discard any retarded embryos that have not yet developed to the blastocyst stage. Thus, the majority of studies focus on the transfer of day 5 or day 6 rather than day 7 blastocysts [14].

The success of embryo transplantation relies on embryo quality, endometrial receptivity, and embryo-endometrial synchrony [15]. The best available evidence suggests that differences in transferring day 5, day 6 and day 7 blastocyst are only present in fresh embryo transfer, but not in frozen embryo transfer (FET) cycles. This may be related to the endometrial receptivity [16]. Recently, Kovalevsky et al. reported the pregnancy outcomes from using vitrified day 5, day 6 and day 7 blastocyst after warming. The result showed that the rate of clinical pregnancy and ongoing pregnancy of day 5 blastocysts was higher than that of day 6 blastocysts, but there was no significant difference between day 5 and day 7 blastocysts or between day 6 and day 7 blastocysts [17]. Conventional wisdom may suggest that the transfer of day 7 blastocyst would have higher miscarries than day 5 and day 6 blastocysts. However, with the development of preimplantation genetic screening (PGS) and its extensive application in the clinic, the assessment of embryo quality has become more sophisticated and no longer relies only on morphology for the selection embryos with good developmental potential. Additionally, not all day 3 embryos have the potential to develop to blastocysts. As such, the extended period of culture applies a selective pressure which prevents the transfer of these low quality embryos.

There are few studies that have reported the culture of day 7 blastocyst and the outcome of pregnancy after transfer of vitrified-warmed day 7 blastocyst. Shoukir et al. reported 16 cases of vitrified day 5 or day 8 blastocyst transfer, in which pregnancy was only successfully achieved in one case [18]. Bielanska et al. found that non-euploid embryos developed more slowly [19]. However other studies found that the rate of cell division was not associated with the state of chromosomes [20]. Richter et

al. reported a clinical pregnancy rate of 15% for the transfer of day 7 blastocysts. This finding proved that embryos at day 7 are still viable for use in clinical treatment [21]. As such, it may be unwise to discard day 7 blastocysts as it represents a potential even a loss of opportunity and resources. Additional studies have also argued that day 7 blastocysts have potential for development and can give rise to successful pregnancies [22-24].

The present study included a relatively larger sample size of vitrified day 7 blastocyst transfers, although day 7 blastocyst cryopreservation is not a routine practice in most IVF centers and there have been few reports about it. Cultivation of blastocysts to day 7 has become a routine practice in our ART Center at the Ninth People's Hospital of Shanghai Jiaotong University School of Medicine. We tried to culture embryos to day 7 when we were unable to harvest mature and high-quality embryos on day 3 and day 5, or day 6. This study reports the pregnancy outcomes of 94 cycles of 154 vitrified day 7 blastocyst for ART between January 2007 and May 2012 in our center.

Materials and methods

Patients

This study was a retrospective analysis from January 2007 to May 2012, conducted at the Department of Assisted Reproduction of the Ninth People's Hospital of Shanghai Jiaotong University School of Medicine. A total of 95 patients with a mean age of 31.4 ± 3.8 years (range 22-45 years) who received IVF/intracytoplasmic sperm injection (ICSI) treatment regimens were included in the study. The pregnancy outcomes of 95 cycles of 154 vitrified day 7 blastocyst for ART were analyzed. All blastocysts were warmed after vitrified cryopreservation and the inclusion criteria are presented in **Figure 1**.

Ethical approval

The study protocol was approved by the Ethics Committee of the Ninth People's Hospital of Shanghai. The trial was conducted according to the Declaration of Helsinki for medical research. All participants provided informed consent after counseling for infertility treatments and routine IVF procedures.

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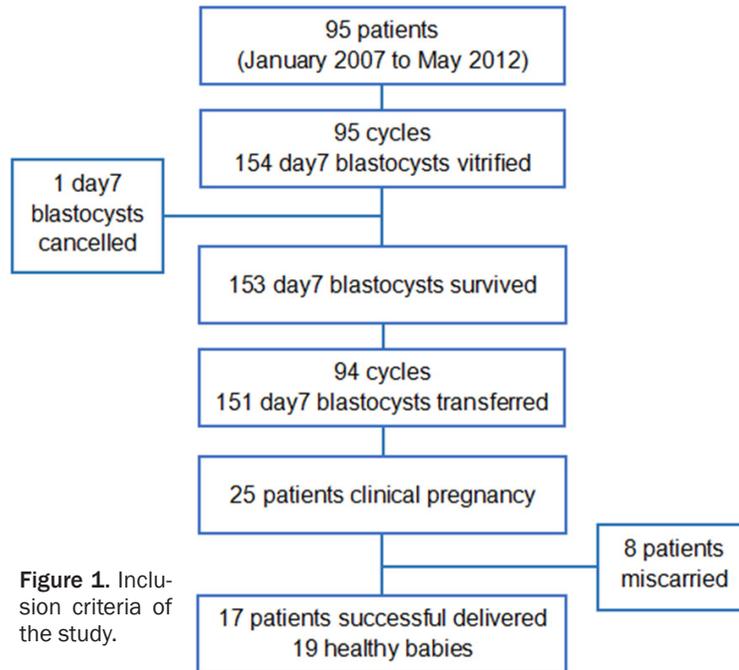


Figure 1. Inclusion criteria of the study.

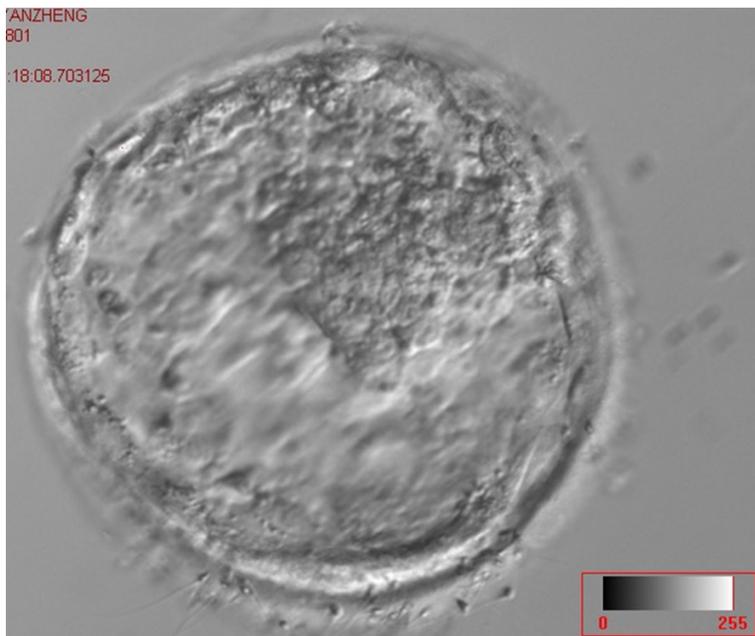


Figure 2. A hatched blastocyst developed from vitrified cleaved embryos and empty zona pellucida observed on day 7 after oocyte retrieval: a before vitrification of blastocysts.

Blastocyst grading system

The morphological evaluation of blastocysts was performed according to the Gardner and Schoolcraft grading system and included three different parameters: expansion and hatching

(EH) stage, Inner cell mass (ICM) grade and trophoblast (TE) grade. The EH stage was assessed as one of the following; Grading of blastocyst development: grade 1: the blastocoel cavity was smaller than 50% of the total volume of the embryo; grade 2: the blastocoel cavity was larger than 50% of the total volume of the embryo; grade 3: the blastocoel cavity occupied the total volume of the embryo, and the total volume of the embryo became larger; grade 4: the blastocoel cavity was the full volume of the embryo, and the embryo became larger, thinner and more transparent; grade 5: part of the blastocyst escaped from the zona pellucida; grade 6: the blastocyst had escaped from the zona pellucida completely. The ICM was assessed as one of the following; grade A: there were numerous cells in close arrangements; grade B: there were some cells in loose arrangements; grade C: there were minimal cells. The TE was assessed as one of the following; grade A: the trophoblast cell layer was composed of numerous closely structured cells; grade B: the trophoblast cell layer was composed of some loosely structured cells; grade C: the trophoblast cell layer was composed of sparse cells (**Figure 2**).

Blastocyst vitrification

The vitrification of blastocysts was performed according to the Cryotop method previously described by Kuwayama et al. [25]. Before cryopreservation, the Equilibration Solution (ES) and Vitrification Solution (VS) were pre-warmed at room temperature (20-25°C) for 30 min. Then, the petri dish containing the blastocysts to be vitrified

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was removed from the incubator, and the blastocysts were moved with caution to the ES (7.5% EG+7.5% DMSO-20% SSS mHTF) surface, letting them fall freely to the bottom of the dish by gently turning dish three times. The equilibration time of the blastocysts was 8-10 min. If artificial shrinkage (AS) was needed, the equilibrium time was reduced to 5 min. Three 50 μ L drops of VS (VS: 15% EG+15% DMSO+0.5 M sucrose-20% SSS mHTF) were prepared on the central well dish cover. Below, these are referred to as VS1, VS2 and VS3. The equilibrated blastocysts were moved in sequence to VS1 for 5 seconds, then VS2 for 10 seconds, and VS3 for 20 seconds, and then loaded onto the Cryotop with caution, before plunging it directly into liquid nitrogen for vitrification.

Warming of blastocysts

Before warming, warming fluid 1 (1 M sucrose-20% SSS mHTF) was inverted gently three times to ensure the thorough mixing of the fluid. Then, 1.0 ml was added to the central hole of the central well dish and pre-warmed in the 37°C incubator. Warming fluids 2, 3 and 4 were pre-warmed at room temperature for at least 30 min before use. The blastocyst to be warmed was quickly moved from the liquid nitrogen cylinder to a foam box containing an appropriate amount of liquid nitrogen. The foam box was then placed in proximity to the warming microscope. 0.5 ml of warming fluids 3 and 4 were added at the periphery of another central well dish, and 0.5 ml of warming fluid 2 was added to the central well. Warming fluid 1 was quickly moved from the incubator to the platform of the microscope, and the cryotop containing the blastocyst to be warmed was quickly inserted into warming fluid 1 and kept there for 1 min. The blastocyst was then moved to warming liquid 2 for 3 min by turning the position gently three times. The embryo was then gently moved to the warming liquids 3 and 4 for 6 min by turning their positions gently three times. The embryo was then moved to equilibrated culture drops for continuous washing for 5-10 drops and cultured continuously for 2-3 h before transfer.

Blastocyst survival

The warmed blastocysts were cultured for 1-3 h to observe expansion of the cavity. Full or par-

tial expansion of the blastocoel cavity was considered to indicate survival and that the blastocyst could be used for transfer.

Endometrial preparation and clinical outcomes

Natural or artificial endometrial preparation was selected according to the ovulatory function of the patient. B-ultrasound was used to monitor the follicular development in clients having natural cycles with luteal support being administered after ovulation. For luteal support in patients with artificial cycles, oral estradiol valerate (Progynova, Schering, Germany) was administered in the early stage of menorrhagia, and luteosterone was injected intramuscularly when B-ultrasound showed that the endometrium had grown thicker than 8 mm. The surviving blastocysts were transferred under abdominal ultrasound guidance. Serum e-human chorionic gonadotrophin (ehCG) was detected two weeks after blastocyst transfer, and those who were positive for ehCG underwent B-ultrasound examination. The presence of a gestational sac was used to diagnose a clinical pregnancy, and termination of fetal development or discharge of the gestational sac within 12 weeks of pregnancy was considered to indicate an early abortion.

Statistical analysis

All statistical calculations were performed using the SPSS (Statistical Package for the Social Science; SPSS Inc., Chicago, IL) statistical program for Microsoft Windows and GraphPad Prism 5. All variables were checked by a normality test. The normality of continuous variables was tested using the Shapiro-Wilk test. A random effects logistic regression model was performed. Results are expressed as the mean \pm SD. Categorical data are presented as performance rate (%).

Results

The pregnancy outcomes of 95 cycles of 154 vitrified-warmed day 7 blastocysts were analyzed. These included embryos that were unable to develop to grade 4, grade 5 or higher blastocysts by day 5 or day 6 and therefore required further culture to day 7. Cultivation of those embryos to day 7 has become a routine practice in our ART Center. There were 95 cycles of vitrified day 7 blastocysts in patients

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Table 1. General data and laboratory of the patients with vitrified cryopreservation of day 7 blastocysts

Parameter	Value
Patient age (year) \pm SD	31.4 \pm 3.8 (22-45)
Infertility years \pm SD	4.6 \pm 3.0 (1-16)
Mean number of prior embryo transfer failures \pm SD	1.8 \pm 1.8 (0-9)
Number of warmed blastocysts	154
Number of surviving blastocysts (%)	153 (99.4)
Number of cancelled cycles	1
Number of transferred cycles	94
Number of transferred blastocysts	151
Mean number of transferred blastocysts \pm SD	1.6 \pm 0.6

Plus-minus values represent the mean \pm SD.

Table 2. Clinical pregnancy outcomes of vitrified day 7 blastocyst

Parameter	Value
Number of HCG-positive pregnancies (% per transferred cycle)	34 (36.2)
Number of clinical pregnancies (% per transferred cycle)	25 (16.6)
Number of implant embryos (% per transferred blastocyst)	27 (17.9)
Number of miscarriages (% per clinical pregnancy)	8 (32.0)
Number of ectopic pregnancies (% per clinical pregnancy)	0

Plus-minus values represent the mean \pm SD.

Table 3. Results of vitrified human day 7 blastocyst transfer

Parameter	Value
Number of deliveries (% per clinical pregnancy)	17 (68.0)
Number of living births	19
Number of singletons (% per living delivery)	15 (88.2)
Number of twins (% per living delivery)	2 (11.8)
Number of males (%)	13 (68.4)
Number of females (%)	6 (31.6)
Mean gestational age (wk) \pm SD	38.5 \pm 0.8
Preterm (<37 wk) (%)	0
Mean birth weight (g) \pm SD	3157 \pm 392.4

Plus-minus values represent the mean \pm SD.

with a mean age of 31.4 \pm 3.8 (22-45) years, an infertility duration of 4.6 \pm 3.0 (1-16) years, and 1.8 \pm 1.8 (0-9) transfer failures. They had no high-quality embryos on day 3 and no embryos developed to grade four or higher blastocysts on day 5 or day 6. Of the 154 warmed blastocysts, 153 survived, with a survival rate of 99.4%. The number of transfer cycles was 94, of which one was canceled. The number of transferred blastocysts was 151. The mean number of blastocysts transferred per cycle was 1.6 \pm 0.6. General data and the laboratory values of the included patients are presented in the **Table 1**.

Overall, 94 transfer cycles were performed with day 7 blastocysts. From these hCG was positive in 34 patients and the biochemical pregnancy rate was 36.2%. Twenty-five patients achieved clinical pregnancy, with a clinical pregnancy rate of 16.6%. From there 17 patients have successful implantation-achieving an implantation rate of 17.9%. Ultimately there were eight abortions, however no ectopic pregnancies occurred in any patients. The clinical pregnancy outcomes of vitrified blastocysts are presented in **Table 2**.

Of the 94 cycles that involved the transfer of vitrified day 7 blastocyst after warming, seventeen patients delivered nineteen healthy babies successfully. The rate of delivered was 68%. This included fifteen singletons (88.2%) and two twin births (11.8%) with a mean gestational age of 38.5 \pm 0.8 weeks. Of the nineteen babies, thirteen were boys (68.4%) and the remaining six were girls (31.6%) with a mean birth weight of 3157.9 \pm 392.4 g. There were no cases of malformation in any of the births (**Table 3**).

Discussion

At present, embryos are usually scored and clinically graded on day 3 after fertilization. High-quality embryos are used for transfer or vitrified cryopreservation. In addition, the time of blastocyst culture is usually limited to day 5 or day 6, and rarely extended to day 7. Even though there are day 7 blastocysts, they are mostly discarded and rarely used for transfer, mainly because of the belief that the extended delay in the development of these embryos is a sign of poor quality [26, 27]. However, for some patients embryos do not develop to blastocysts by day 3, or are not pregnant when transferred as day 5 or day 6 blastocyst. This study demonstrates that transferring human vitrified-warmed day 7 blastocyst has an important clinical potential when

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the embryos were not mature on day 3, day 5 or day 6, or when those embryos have slow development. Furthermore, these results proved that the transfer of vitrified-warmed day 7 blastocysts can increase the accumulative pregnancy rate. In addition, there was only eight miscarriages and no case of malformation in the births, which suggests that the use vitrified day 7 blastocyst which had slow development did not increase the risk of natural miscarriage and birth defects. Nonetheless, the transfer of vitrified day 7 blastocyst should be approached with caution until further data have collected, analyzed and confirmed [28].

The best available clinical evidence indicates that the transfer of blastocysts creates a higher clinical pregnancy rate than the transfer of day 3 embryos. Under normal conditions day 3 embryo are in the fallopian tube. As such if they are transferred on day three the conditions they face in the uterus will be sub-optimal and could increase the miscarriage rate. However, if embryos are cultured and then transferred on day 5 or day 6 the uterus will represent an appropriate growth environment. As such blastocyst transplantation better reflects the embryo-endometrial synchrony. Much evidences suggests that there are more high quality blastocysts on day 5 than day 6 or day 7, but implantation rates were not found to differ by day of transfer so long as the same quality of blastocyst was being transferred [29]. Therefore, it was reasonable to expect that the transfer of day 7 blastocyst would not result in high miscarriage rate.

In this article, we reported the pregnancy outcomes of 95 cycles of day 7 blastocyst transferred during the past four years in our center. Our research significantly expands the data available on the transfer of vitrified-warmed day 7 blastocysts. And the data analysis did not make any remarkable findings with respect to the age, years of infertility or the number of previous transfer failures in these infertile patients. These patients were all healthy and had no other diseases. Their only problem was an inability to harvest high-quality embryos on day 3 and day 5, or day 6, as a result of slow development. We tried to culture these embryos to day 7, and after scoring and grading, vitrified those that were worthy of cryopreservation for attempted transfer after warming.

Thereafter, PGS biopsy was conducted and it was found that day 7 blastocyst were able to tolerate vitrified cryopreservation, implant well and offer a relatively good pregnancy outcome. In addition, the sample size of the present study is large enough to assure that our conclusion is valid. Although the birth rate was not high, the clinical pregnancy rate for ART overall is only 40%, and these normally discarded embryos can give rise to successful pregnancies in patients who would otherwise have no other options than an additional cycle. There is no doubt that the outcome of the present study should bring new hope to those patients who are unable to harvest high-quality embryos on day 3 or day 5 and day 6 or have advanced maternal age with follicular reduction.

In summary, day 7 blastocyst can be used for vitrification and transfer. This has the potential to not only avoid the waste of active embryos and increase the accumulative pregnancy rate, but also is of great significance to those patients who do not have high-quality embryos on day 3, day 5, and/or day 6. There is no doubt that the quality of the embryo before cryopreservation has important impact on the clinical outcome of the post-warm transfer. Vitrified day 7 blastocyst survives at a high rate and can implant successfully and offer living deliveries. However, as the pregnancy rate of vitrified day 7 blastocyst transfer is relatively low, the health and safety of the offspring will need to be carefully studied in long-term follow-up cohorts.

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Disclosure of conflict of interest

None.

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