ORIGINAL ARTICLE

Evaluation of the Clinical Efficacy of Vitrification, Warming and Blastocyst Transplantation in Assisted Reproductive Treatment

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ABSTRACT

Background and Objective: Advances in cell media have led to embryo transfer from cleavage to blastocyst. The extension of embryo culture to blastocyst stage provides some theoretical advantages and disadvantages that have been controversial. The objective of this study was to evaluate the clinical efficacy of vitrified warm cutting and blastocyst transplantation in Assisted Reproductive Technique (ART) therapy.

Methods: The study was performed on 2740 women undergoing frozen embryo thawing transfer. Patients' basic clinical information, status of frozen embryo transfer cycle, clinical pregnancy rate, early abortion rate, sex ratio of birth and birth weight were retrospectively analyzed. The main clinical outcomes of the recovery of frozen embryos at cleavage and blastocyst stages were compared. In addition, the clinical outcomes of blastocyst cryopreservation on the 5th, 6th or 7th day after oocyte retrieval according to the date of blastocyst expansion were recorded.

Results: The implantation ratio of cleavage stage embryos was 21.62% compared with 43.52% on D5 ($P < 0.05$). The D5, D6, and D7 implanting rates were statistically different. The pregnancy rates were 57.56%, 51.76% and 35.95% versus 37.79%, and the early abortion was 23.08%, 15.42% and 22.35% versus 34.55% respectively for embryos cryopreserved on D5, D6, D7 and D3. There were significant differences between D5 and D3 rates of ectopic pregnancy and early abortion. The sex ratio, the birth weight and birth defect were not statistically different among four groups.

Conclusion: Blastocyst transfer achieved a higher implantation rate than vitrified cleavage stage embryo and decreased ectopic pregnancy rate. With increased incubation days before expansion blastocyst formed, the implantation rate is reduced and the early abortion rate increases.

KEYWORDS: Vitrified-warmed, Cleavage stage, Blastocyst, Clinical outcome, Assisted Reproductive Technique.

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INTRODUCTION

In recent years, in most domestic reproductive centers of China, embryo transfer is given priority to D3 embryos, the rest of embryos at the cleavage stage are cryopreserved in good quality or cultured to blastocysts for frozen. Currently, with the rapid

development of vitrification technology and blastocyst culture program, blastocyst transplantation has become more and more common and worldwide in clinical practice of ART.[1,2] However, the optimal time for embryo transfer remains controversial.

Many studies by Cochrane Review have demonstrated the theoretical advantages of blastocyst transplantation: increased implantation potential better correlation between morphology and aneuploidy, better synchronization with endometrium.[3] But blastocyst transplantation also has some defects, like the lack of co-culture with endometrial cells for one day (the 4th day embryo enters the uterine cavity); Some embryos are more likely not to develop into blastocysts in vitro, leading to the abolition of embryo transfer; The decrease of embryo freezing rate is related to the decrease of endometrial cell co-culture rate, Technical differences in the freezing/thawing process of this expanded embryos.

Therefore, the contradictory results recently reported by Cochrane meta-analysis suggest that there is no evidence of differences in pregnancy outcomes between 2-3th and 5-6th after embryo transfer.[4]

A recent Cochrane meta-analysis found that the difference in live birth or pregnancy outcomes between 2-3th and 5-6th transfers of embryos was not significant.[5] In addition, the increase of non-transfer and the decrease of embryo freezing rate in any cycle will affect embryo transfer. ART cycle provides a further possibility of success in embryo cryopreservation and transfer except for those through fresh embryo transfer.[6,7] Therefore, we can conclude that a more accurate measure of clinical outcomes may be the cumulative pregnancy rate after fresh and additional vitrified embryos per oocyte examination cycle, rather than just the pregnancy rate per embryo transfer cycle.

The current study retrospectively analyzed the clinical pregnancy, outcome, follow-up analysis and perinatal outcome after transfer of vitrified-warmed D3 embryos, D5 blastocysts, D6 blastocysts and D7 blastocysts in Chinese women undergoing ART treatments.

METHODS

This study was performed in accordance with the Declaration of in Reproductive Hospital Affiliated to Shandong University, China. The ethical approval was obtained from the Ethic Committee of provincial hospital, and all participants had signed a written informed consent form. This study was performed on 2740 women undergoing frozen embryo thawing transfer who visited the Clinic in Reproductive Hospital Affiliated to Shandong University from Jan. to Dec. 2011. A total of 157 cycles were eliminated because of thawing D3, D5, D6 or D7 embryos in the same cycle. And 171 cycles were eliminated for embryo death. Totally, 2412 FET cycles were enrolled, of which 172 cycles transferred frozen embryos on D3, 721 cycles on D5, 1366 cycles on D6 and 153 cycles on D7. Patients' basic situation, status of frozen embryo transfer cycle, clinical pregnancy rate, early abortion rate, sex ratio of birth and birth weight are shown in Tables-1-3.

The precise synchronization of endometrial maturation and embryo development is a key factor in embryo transfer. The three methods used in endometrial preparation are the natural cycle after spontaneous ovulation. Cycles were kicked out if the endometrial thickness was thinner than 0.8 cm measured by ultrasound.

Blastocysts were vitrified by pull-cut method and cut by 0.25 ml plastic sterile straw (Bicef). Blastocysts were balanced in 7.5% ethylene glycol (EG) and 7.5% dimethyl sulfoxide (DMSO) for 20-25 minutes, exposed to phosphate buffer saline (PBS) in 15% EG and 15% DMSO, and added 0.5 M sucrose in 1 minute. Then they were immediately put into liquid nitrogen.

Before recovery, 1 ml solutions containing 0.33M sucrose, 0.2M sucrose and 0M sucrose for four orifice plates were prepared on the basis of three-hole culture and kept them at 37℃ for at least 30 min. The recovery blastula was removed and the carrier outer casing and placed the carrier terminal into the prepared 0.33M sucrose within 2 min. The embryos were then transferred into 0.2M sucrose within 3 min, finally placed the blastula into the BS within 5 min. Recovery of good blastocysts was achieved using laser assisted hatching. A punch far away from the transparent cell mass was taken as a hole the size of the cells

within an appropriate hatch is best. Then, blastula was put into blastocyst culture inside G2 (Vitrolife, Sweden) at 37° and 6% CO^2 and cultivated it for 5 h in saturated humidity to prepare for transplantation.

Progesterone was administered by injections after ET (40 – 80 mg) or orally (20 to 40 mg) daily. Serum β-hCG tests were performed two weeks after ET in all groups. The clinical pregnancy was confirmed by transvaginal ultrasound (US) three weeks later.

STATISTICAL ANALYSIS

Statistical package of social sciences (SPSS), Version 22.0 (Chicago, IL, USA) was used for statistical analysis. The continuous variables were shown as mean (x ± s). T test or Wilcoxon rank sum test and Kruskal Wallis test were used for comparison between the two groups; One-way ANOVA and factorial design ANOVA were used for comparison for more than every groups. Correlation was performed by Pearson correlation analysis. The test level alpha = 0.05; $P \leq 0.05$ was statistically significant.

RESULTS

A total of 2583 warming cycles were included in this study, there were 157 transfers were excluded from this study for women received embryos transfer from D5, D6 or D7 in the same cycles. 2412 cryo-transfers were performed, which resulted in an ET cancellation rate of 6.62%. Patients undergoing

Table-1: Frozen blastocyst transplant cycle.

	D3 Leavage Stage Embryo	*D5 Blastocyst*	*D6 Blastocyst*	*D7 Blastocyst*
Thaw cycles	187	736	1477	183
Give up cycles (rate)	15 (8.02%)a	15 (2.04%)b	111 (7.52%)a	30 (16.39%)c
Transplantation cycles	172	721	1366	153
Average age (y)	33.0 ± 4.26a	30.72 ± 4.41b	31.40 ± 4.63c	32.88 ± 5.02a
Average number of embryos	2.45 ± 0.62a	1.58 ± 0.49b	1.63 ± 0.49c	1.49 ± 0.51d
Lining thickness (cm)	1.01 ± 0.18	1.01 ± 0.16	1.02 ± 0.16	1.02 ± 0.15

a.b.c.d.: Statistically different between different peer letters ($P < 0.05$).

Table-2: Different culture times of frozen blastocysts compared to clinical outcomes after recovery.

Item	*D3 Cleavage-Stage Embryo*	*D5 Blastocyst*	*D6 Blastocyst*	*D7 Blastocyst*
cycles	172	721	1366	153
Embryo transfer number	421	1142	2233	228
7week clinical pregnancy	91	569	937	62
Implantation rate	21.62%a	49.82%b	41.96%c	27.19%a
Clinical pregnancy(rate)	65 (37.79%)a	415(57.56%)b	707 (51.76%)b	55 (35.95%)a
Ectopic pregnancy(rate)	3 (4.62%)ab	6 (1.45%)a	7 (0.99%)ac	1 (1.82%)a
Early abortion cycles (rate)	15 (23.08%)a	64 (15.42%)ab	158 (22.35%)ac	19 (34.55%)ad

a.b.c.: statistically difference between different letters in the peer ($P < 0.05$).

Table-3: Different incubation time of blastocyst refrigerant recovery after follow-up result comparison.

Item	*D3 Cleavage-Stage Embryo*	*D5 Blastocyst*	*D6 Blastocyst*	*D7 Blastocyst*
Cycles	172	721	1366	153
Clinical pregnancy	65	415	707	55
Live birth cycle	47	345	542	35
Preterm delivery cycles (rate)	8 (17.02%)	65 (18.84%)	89 (16.73%)	5 (14.29%)
Twins (rate)	15 (23.08%)a	142 (34.22%)a	211 (29.84%)a	5 (9.09%)b
Multiple births (rate)	5 (7.70%)a	6 (1.45%)b	9 (1.27%)b	1 (1.82%)ab
Gestational age	38 ± 2 weeks 0 day	38 weeks 1day ± 2 weeks 2 day	38 weeks 3 day ± 2 weeks 1 day	38 weeks 4 day ± 1 weeks 6 day
Birth sex ratio	103: 100	126: 100	119: 100	105: 100
Male baby birth weight	3.03 ± 0.99a	2.98 ± 0.82ab	3.21 ± 0.70ac	3.33 ± 0.77a
Female baby birth weight	2.82 ± 0.71a	2.96 ± 0.62ab	3.15 ± 0.58ac	3.00 ± 0.68a
Live birth	59 (30,29)	447 (250,197)	665 (361,304)	41 (21,20)
Tiny deformity		4	5	0
Severe deformity	2#	6*	6	1
Total birth defects (rate)	2 (3.39%)	10 (2.24%)	10 (1.50%)	1 (2.44%)

a.b.c.: statistically difference between different letters in the peer ($P < 0.05$).
Double egg twins：1 Cleft lip and 1 cleft palate
* With double egg twins of adrenal cortex hyperplasia
Tiny deformities include：Hernia, congenital laryngeal stridor, skin hemangioma
Severe deformities include：Congenital heart disease, nervous system abnormalities, cleft lip and palate, adrenal cortex hyperplasia, bladder fistula, Downs Syndrome.

blastocyst-stage ET were younger than those undergoing cleavage-stage embryo transfers ($P < 0.05$). The proportion of ETs was statistically lower when embryos were vitrified on day 3 ($P < 0.05$). A comparison of these patients' basic status was in Table-1.

The clinical pregnancy rate per transfer was 51.49%, and miscarriage rate was 20.61%. A total of 421 cleavage-embryos were transferred (mean: 2.457 ± 0.62) in 172 cycles; among them 91 were able to implant (overall implantation 21.62%). And 3603 blastocysts were transferred (mean: 1.61 ± 0.49) in 2240 cycles; among them 1568 implanted (overall implantation 43.52%). Clinical pregnancy rate, ectopic pregnancy rate, and miscarriage rate were differed among embryos vitrified on D3, D5, D6, D7 (data shown in Table-2).

In this study, 1242 pregnancy led to the birth of 970 live born (78.10%), of which 576 were singletons (59.39%), 373 were twins (38.45%) and 21 were triples (2.16%). Mean gestational age, birth weight, sex ratio and birth defects for different groups are listed in Table-3. The mean gestational age, sex ration and birth defects were comparable among different groups.

DISCUSSION

Cleavage stage frozen embryos transfer cycles were less because of a small number of good qualities D3 embryos and decreased opportunity of the poor quality of cleavage embryos developing into blastocysts.

Traditionally, cleavage-stage embryos were transferred or cryopreserved on day 3, but over the past decades there had been a tendency to blastocysts stage embryos transfer.[8] With the development of efficient culture systems, it is becoming more reliable to obtain blastocysts in vitro. Blastocysts are preimplantation embryos that have successfully passed the critical step of genomic activation and have a high developmental potential.[9] In addition, blastocyst transferring is considered to be more physiologically appropriate as it more closely mimics the time of natural implantation and may improve synchrony between endometrium and embryo development.[10,11] In our study, the implantation and clinical pregnancy rates in D3 group were statistically lower than blastocyst groups (21.62% vs. 43.52%, 37.79% vs. 52.54%).

Vitrification technology was applied in this study for embryos and blastocysts cryopreserving, and blastocysts transfer on day 5 have the statistical lower cancellation rate due to the higher survival rate. The presence of the mitotic apparatus in cleavage embryos might be disturbed during vitrification procedures. Another explanation might be that D3 embryos vitrification might carried out when a patient has achieved relatively few high morphologic quality embryos, which was considered as another explanation, it also explained why patients undergoing early cleavage-stage vitrification were older than those undergoing D5 blastocyst vitrification.[12,13]

Although blastocyst transfer has many advantages, there are still some disadvantages, such as the poor culture conditions, resulting in a slight delay of embryo development with the general situation of in vitro culture. The average age of in vivo mature blastocysts collected by uterine lavage is reported to be 4.5 days after ovulation, but mature blastocysts usually need to develop to the fifth day in vitro. [14] Both differentially developed blastocysts (day 5 vs. day 6 vs. day 7) and cleavage embryos (day 3) in a whole year period were involved in present study. The proportion of day 6 blastocyst vitrifying-warming cycles were higher than day 5 and day 7. According to the time of appearance of Pinot protein, histological characteristics and steroid receptor down-regulation, the receptive window moves earlier than the natural cycle under stimulation,[15] thus increasing the potential for later developed blastocysts to miss the implantation window. Nevertheless, it was controversial whether the delayed development in vitro would influence the clinical and perinatal results in warming cycles.[16] As showed in present study, expanded blastocyst on day 5 after warming achieved statistical higher implantation rate, clinical pregnancy rate and lower miscarriage rate among three differentially developed blastocysts groups. Also, the D7 blastocyst cryotransfer resulted in an implantation rate of 27.19%, and the miscarriage rate was 34.55%, which is significantly higher than D5 and D6. There is little information on the maturation of blastocysts 7 days after transfer,[17] and such embryos are usually discarded because slow-growing embryos are generally not considered feasible. Blastocysts that mature on day

7 may still be feasible in the case reports of fresh embryo transfer and frozen embryo transfer.[18] The data from present study indicates the day 7 blastocysts could still be considered to be cryopreserved if no blastocysts were harvested on D5 or D6. Also, the high miscarriage rate should be informed to the patients. The decrease in implantation and increase in abortion rate for later developing blastocysts in warming cycles could be explained that more viable embryos would reach expanded blastocyst earlier.[19,20] However, another important question is, whether extended *in vitro* culture itself would decrease the competence of embryos to interact with endometrium? Or whether the *in vivo* circumstance would provide an important dynamic immune and endocrine microenvironment for blastocysts to implant better and maintain ongoing pregnancy? The longer the embryo is kept in vitro, the more genetic traits and biological characteristics might be changed.[21] There is evidence that the blastocyst is involved in the regulation of endometrial chemokines during blastocyst attachment.[22] In present study, The miscarriage rate in present study of D3 embryos (23.08%) was comparable to blastocysts groups (D5, 15.42%; D6, 24.35%; D7, 34.55%), despite the latter groups were thought to be more viable. Similar results were found in other studies. Ana Cobo analyzed 3150 cases of clinical outcomes of frozen cleavage stage embryos and blastocysts. The early abortion rate on D3, D5 and D6 was 17.3%, 18.9% and 29%,[23] respectively, which was similar to the results of the current study.

In the D3 embryo group of our study the incidence of ectopic pregnancy was 4.62% after embryo transfer, while in D6 transplanted blastocysts the rate was 0.99%. Research suggests that D3 after embryo transfer into the uterus may be a "wandering" phenomenon, thus increasing the ectopic pregnancy rate.

The birth sex ratio of different cultivated days after embryo transfer was not significantly different. The birth sex ratio of boys and girls was 103:100 and 121:100 in the cleavage stage group and the blastocyst groups respectively. A follow-up research showed that the fresh blastocyst boy proportion was 58.5% compared with 50.3% for frozen blastocyst boys after transplantation.[24] Another study on fresh and frozen blastocysts showed that in frozen cycles the boy proportion was 34.4% compared with 55% for fresh transplanted blastocysts.[25] In this study, frozen surplus embryos after transplantation of D3 embryos continued to cultivate into high-quality blastocysts or all embryos were cultured to blastocysts.

The birth weight of babies in the D5 and D6 blastocysts groups was significantly different. Maybe the reason is that the multiple rates is higher in the D5 blastocyst groups, as is a higher incidence of preterm labor. As a result, the D7 blastocyst twin ratio is lower than the rest ($P < 0.05$) and the birth weight is slightly higher. The literature report found that the frozen blastocyst birth defect rate was approximately 1.0% (1/103); with the slow freezing cleavage stage embryos, the rate of birth defects was 4.1% (8/194).[23,25] In this study, the vitrification D3 frozen embryos birth defect rate was slightly higher, but there was no significant difference with or between the blastocyst groups.

Regardless, blastocyst transplantation has a higher implantation rate than using divided stage of embryo transfer and decreasing rate of ectopic pregnancy. The later developing blastocysts transfer after warming resulted in a reduced implantation and increased early abortion rate. In any case, the ultimate goal of reproductive medicine practitioners to improve antiretroviral therapy in bringing babies home has been achieved, but the best outcome of current art practice is to deliver single births rather than multiple births. Finally, with the continuous improvement of culture system in vitro, blastocyst vitrification combined with single blastocyst transplantation has become an efficient human art therapy program.

CONCLUSION

Blastocyst transfer achieved a higher implantation rate than vitrified cleavage stage embryo and decreased ectopic pregnancy rate. With increased incubation days before expansion blastocyst formed, the implantation rate is reduced and the early abortion rate increases.

LIMITATIONS OF THE STUDY

Ideally, vitrification systems must have been

validated with QC considerations to enhance procedural consistency and to minimize intra- and inter-laboratory variation. Serum hormones at peri-implantation were not determined. Patient's monitoring/screening data is not included. The effect of co-interventions (dual triggering) was not considered because of retrospective nature of the study.

CONFLICT OF INTEREST

None to declare.

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Author's Contribution

LX, SG, JJ, MS, YS, RT: Conceptualization and design of study, Acquisition of data and Drafting of manuscript.

All Authors: Approval of the final version of the manuscript to be published.