Retrospective clinical analysis of two artificial shrinkage methods applied prior to blastocyst vitrification on the outcome of frozen embryo transfer

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Abstract

Purpose Vitrification significantly improves the rates of blastocyst survival and clinical pregnancy following frozen embryo transfer (FET). However, ice crystal formation during the freezing process reduces the blastocyst survival rate. Artificial shrinkage (AS) prior to blastocyst vitrification decreases the formation of ice crystals, increasing the blastocyst survival rate. The aim of this study was to identify an efficient AS method to improve blastocyst survival rates following vitrification.

Method Use of the 29-gauge needle AS and Laser pulse AS methods prior to vitrification was compared in terms of the impacts on the rates of blastocyst survival in FET cycles, blastocyst hatching, clinical pregnancy after transfer, embryo implantation, abortion, gestational duration and birth weight. **Result** In total, 438 blastocysts in 219 cycles were thawed, resulting in survival of 407 (92.9 %). Of these, 213 cycles were transferred, resulting in 129 clinical pregnancies (60.6 %) and 140 successful births. There were no differences between the two methods in the rates of blastocyst survival, clinical pregnancy, embryo implantation and abortion. However, the 29-gauge needle AS group was associated with a significantly lower blastocyst hatching rate (83.6 % vs.

91.2 %), shorter average gestational duration (37.36 ± 2.34 vs. 38.06 ± 1.76), and higher premature birth rate (40.00 % vs. 21.15 %) compared with Laser pulse AS group.

Conclusion No significant differences in the effectiveness of the two methods applied prior to blastocyst vitrification were observed before birth, while after birth, a significantly improved clinical outcome was obtained with laser pulse AS indicating that this is a more effective pre-processing method for blastocyst vitrification.

Keywords Laser pulse AS · 29-gauge needle AS · Vitrification · Blastocyst · Outcome

Introduction

Since the technique of vitrification was shown to be successful in blastocyst freezing [1], it has been widely applied [2, 3], and has been gradually replacing the conventional slow-freezing method. However, this technique is associated with the risk of blastocoel cavity expansion due to the uptake of large amounts of fluid during vitrification, causing ice crystal formation during freezing, which impacts on blastocyst viability. Vitrification studies in mice have indicated that artificial shrinkage (AS) prior to vitrification can reduced these detrimental effects and increase the rates of survival and blastocyst hatching [4, 5]. It has also been demonstrated that the application of AS prior to vitrification improves embryo viability and the subsequent clinical outcome [6].

Several AS methods have been described. Micro-needle AS involves the use of a holding pipette to stabilize the blastocyst. A glass micro-needle is used to pierce the blastocyst and make a small hole on the trophectoderm, allowing outflow of the fluid in the blastocoel cavity and resulting in complete the shrinkage [5]; For 29-gauge needle AS, two 29-

Capsule Laser pulse AS is a more effective pre-processing method for blastocyst vitrification than 29-gauge needle AS since our results show that laser pulse AS group was associated with higher blastocyst hatching rate, longer average gestational duration and lower premature birth rate compared with 29-gauge needle AS group.

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gauge needles are used; one for stabilization and the other to pierce the blastocyst away from the inner cell mass (ICM), resulting in rapid shrinkage of the blastocyst (30 s to 1 min) [7]. Micropipetting AS involves the use of a Pasteur Pipette with a diameter slightly smaller than that of a blastocyst to expand and contract the blastocyst 2–3 times causing blastocyst shrinkage in 3–18 min under the influence of the external force [8]. In Laser pulse AS, the blastocyst is subjected to a brief ultra-high temperature pulse emitted from the laser, which causes momentary damage the trophectoderm and blastocyst shrinkage in 1 to 2 min [9]. In Sucrose AS, the blastocyst is exposed to a sucrose solution (0.125–0.25 M), resulting in release of fluid from the cavity due to isotonic exchange of fluids [6].

Previously reported studies on blastocyst freezing have focused on the effect of AS on embryo viability and implantation after the blastocyst has been frozen and thawed. However, the main priority of couples receiving this treatment is the birth of a healthy infant. Therefore, in this study, we investigated the difference between two AS methods, 29-gauge needle AS and Laser pulse AS, applied prior to blastocyst vitrification by retrospective analysis of the rates of post-warmed blastocyst survival and hatching, post-transfer clinical pregnancy and spontaneous abortion, as well as premature birth and low weight following Frozen Embryo Transfer (FET) of vitrified-warmed blastocysts in our center from March 2008 to May 2011.

Materials and methods

Patient and FET data

Data were collected on all the patients that had received two thawed embryos at the Department of Reproduction, Nanjing Maternity and Child Health Care Hospital Affiliated to Nanjing Medical University, Nanjing, China from March 2008 to May 2011. All the patients have undergone physical examination before FET and were all proved to be healthy. In total, 219 cycles/438 blastocysts analyzed. Prior to freezing, 115 cycles/230 embryos were subjected to 29-gauge needle AS and 104 cycles/208 embryos were subjected to laser pulse AS. The mean age of patients was 29.26 ± 3.76 years (range, 22–41 years). Of 438 warmed embryos, 407 survived, 213 cycles were transferred, and 129 clinical cycles developed. Three cases were lost to follow-up, and detailed medical records of pregnancy and birth were kept for the other 126 cycles. A total of 140 babies were born (33 twins and 47 singletons) to 107 couples.

Embryo generation and transfer

Day-3 transfer of 2–3 embryos was performed for all patients; the remaining embryos were cultured to Day-5 until blastocyst formation, followed by vitrification. Ten minutes before the freezing, the blastocyst was subjected to AS. Blastocyst freezing was performed according to the Gardner criteria [10], and the blastocysts were graded at least B for either trophectoderm or ICM when the blastocysts developed to the third or fourth period. Only the blastocysts in third or fourth period were chosen to freezing. And all the post-thaw blastocysts were cultured 16 h before ET. The procedures used for ovulation induction, embryo culture, vitrification and thawing, and embryo transfer have been described in detail previously [11, 12].

Artificial shrinkage

The following AS methods were investigated for the capacity to avoid ICM damage:

29-gauge needle AS: The inclined cross-section of a 29-gauge needle was used to hold the blastocyst (with the ICM at the edge of the blastocyst in direct view) in a droplet with a mesh bottom. The tip of a second needle was used to rapidly enter the center of the blastocyst, causing shrinkage within 1 min.

Laser pulse AS: The ICM was positioned at a distance from the focus of the laser beam (ZILOS-tk system, Hamilton Thorne Instruments Biosciences, Beverly, MA, USA) before being subjected to a 200 ms laser pulse to generate a small hole in the trophectoderm and resulting in the release of fluid from the blastocoel cavity. Blastocyst shrinkage occurred within 1–2 min.

All the procedures were performed by one operator.

Clinical and birth outcomes

After thawing, blastocysts were cultured in vitro for a further 16 h prior to being implanted into the patient's uterine cavity the next day. At 30 days post-transfer, the fetal heartbeat was monitored by ultrasound to confirm the establishment of clinical pregnancy. At week 20, details of the patient's ongoing pregnancy were recorded in a follow-up phone call. Delivery before week 37 was defined as premature birth and birth weight lower than 2,500 g was defined as low birth weight.

Statistical analysis

Statistical analysis was completed with the SPSS 13.0 package (SPSS, Chicago, IL, USA). Groups of data were compared with the Student's *t*-test or Mann–Whitney *U* test.

Proportional data were compared in χ^2 analysis. $P<0.05$ was considered statistically significant.

Results

Like most similar studies, the major focus of our investigations was placed on the viability and development of embryos after blastocyst warming. As shown in Table 1, a total of 438 blastocysts were thawed, of which, 407 survived, indicating an embryo survival rate of 92.9 %. No difference in this rate was detected for the 29-gauge needle AS group and Laser pulse AS group (93.0 % vs. 92.8 %, respectively).

Our other consideration was the development potential after warming, which is referred to as Blastocyst Hatching Rate. The blastocyst hatching rate was significantly lower in the 29-gauge needle AS group than that in the Laser pulse AS group (83.6 % vs. 91.2 %; $P<0.05$). And we compared the good embryo rate before freezing (the fourth period blastocysts/all the blastocysts) and good embryo rate after thawing (the sixth period blastocysts/all the blastocysts) in the two group, we found that good embryo rate has no difference between the two group before freezing (70.87 % vs. 74.52 %; $P=0.392$) and has significant difference after thawing (45.33 % vs. 75.19 %; $P<0.001$).

As shown in Table 2, 67 and 62 cycles were completed in the 29-gauge needle AS group and laser pulse AS group, respectively (a total of 129 cycles), among which 47 cycles resulted in twins, 81 in singletons, one ectopic pregnancy and no cases of triplets. The clinical pregnancy rates for each group were similar (60.36 % vs. 60.78 % respectively). The twin/singleton and embryo implantation rates in the laser pulse AS group were slightly higher than those in the 29-gauge needle AS group, although these differences were not statistically significant.

The rates of abortion, gestational duration and birth weight are shown in Table 3. There were no differences with respect to spontaneous abortion rate, sex and twin/singleton birth rates and average birth weight between the two groups. The patients in the 29-gauge needle AS group are younger than in the laser pulse AS group (29.14 ± 2.91 vs. 30.73 ± 3.80 ; $P=0.017$). However, compared to the laser pulse AS group, the 29-

gauge needle AS group had a higher premature birth rate (40.00 % vs. 21.15 %, $P<0.05$). And it seemed that the 29-gauge needle AS group had a higher low birth weight rate than the laser pulse AS group (15.07 % vs. 8.96 %).

Discussion

This study involved a retrospective analysis of the detailed medical records of pregnancy until birth resulting from 219 freeze-thaw cycles performed in this Reproduction Center from 2008 to 2011. This study is the largest analysis of the influence of AS methods on blastocyst vitrification reported to date. Since the APGAR score is relatively objective and the 107 mothers included in the study were not all delivered in the same one hospital, this data was not statistically analyzed here. The birth outcome was examined by focusing on two sets of objective data; the gestational duration and birth weight. However, due to insufficient samples, data on birth defects was not collected. The aim of this study was to compare the effects of the 29-gauge needle AS method and the laser pulse AS method applied prior to blastocyst vitrification on the in vitro and in vivo development of embryos.

Artificial shrinkage causes relative little damage to embryos, and more importantly, it can improve embryo viability after blastocyst thawing and the pregnancy rate after transfer. Vanderzwalmen et al. [5] showed that, compared with no AS treatment, AS treatment of blastocysts was associated with higher rates of implantation and pregnancy. Furthermore, Mukaida et al. [9] demonstrated that AS resulted in significantly higher embryo viability after thawing and pregnancy rates than those observed in a control group. It was also found that AS pre-processing can be applied not only prior to vitrification, but also prior to the fresh blastocyst transfer and that this approach also increased the pregnancy rate after transfer [13]. These reports demonstrate, not only that AS does not cause major damage to blastocysts, but also that this approach improves the results of blastocyst transfer.

A number of AS methods are widely used, although only two studies have been reported focusing on the comparison of different AS methods prior to vitrification [6, 9]. Moreover,

Table 1 Blastocyst viability and subsequent development after thawing

	29-gauge needle AS	Laser pulse AS	<i>P</i>
Thawed cycles	115	104	/
No. of blastocysts thawed	230	208	/
Good embryo rate before freezing (%)	70.87 % (163/230)	74.52 % (155/208)	$P=0.392$
Blastocyst survival rate	93.0 % (214/230)	92.8 % (193/208)	$P>0.05$
Blastocyst hatching rate	83.6 % (179/214)	91.2 % (176/193)	$P=0.023$
Good embryo rate after thawing (%)	45.33 % (97/214)	75.19 % (133/193)	$P<0.001$

Table 2 Clinical pregnancy outcome after frozen embryo transfer

	29-gauge needle AS	Laser pulse AS	<i>P</i>
Transferred cycles	111	102	/
Mean age of patients	28.97±3.76	29.59±3.75	<i>P</i> >0.05
No. of transferred embryos	213	193	/
Mean no. of transferred embryos	1.92±0.73	1.89±0.51	<i>P</i> >0.05
Clinical pregnancy rate	60.36 % (67/111)	60.78 % (62/102)	<i>P</i> >0.05
Twin/singleton	22/45	25/36	<i>P</i> >0.05
Ectopic pregnancy	0	1	/
Embryo implantation rate	41.8 % (89/213)	44.6 % (86/193)	<i>P</i> >0.05

the outcome analysis covered no more than pregnancy outcome, without data on birth outcome. Mukaida et al. [9] compared the effect of glass micro-needle AS and Laser pulse AS and found that the two groups had similar effects on the rates of blastocyst survival, clinical pregnancy, implantation and spontaneous abortion. Iwayama et al. [6] also stated that sucrose treatment AS and Laser pulse AS had similar effects on the rates of clinical pregnancy and implantation following transfer.

In this study, we conducted a comprehensive investigation of the effects of 29-gauge needle AS and Laser pulse AS applied prior to blastocyst vitrification in order to determine the most effective method. Here, 438 blastocysts were thawed resulting in implantation of 406 blastocysts and 140 births. In accordance with the two previous studies, we also confirmed that there were no differences between the two methods investigated with regard to the rates of blastocyst survival, clinical pregnancy, implantation and spontaneous abortion. However, we found that the good embryo rate is significantly higher in the laser pulse AS group than in the 29-gauge needle AS group after thawing while there is no difference before freezing. We also performed further analysis to more clearly distinguish these two methods. In total, following transfer of embryos subjected to 29-gauge needle AS and laser pulse AS,

babies were born to 55 and 52 couples, respectively. However, the premature birth rate in the laser pulse AS group is lower than in the 29-gauge needle AS group while the patients in the laser pulse AS group is older than in the 29-gauge needle AS group. There were no significant differences in other birth outcome parameters such as the twin/singleton, sex ratios, gestational duration and average birth weight between the two groups. It can be speculated that the 29-gauge needle AS could increase the probability of premature birth because the tip of the 29-gauge needle is large relative to the blastocyst diameter, thus creating a large hole and thus more damage to the trophoctoderm during AS treatment, relative to that caused by laser pulse AS treatment. The trophoctoderm forms a critical part of the placenta and umbilical cord that are developed in the embryo at the later stage. Based on the critical importance of these structures for uterine implantation and delivery of nutrition to the fetus during the whole development process, we propose that damage to the trophoctoderm may have a detrimental impact on the late development and function of the placenta and umbilical cord. This degradation is unlikely to affect early fetal development, thus accounting for the absence of any difference observed between the 29-gauge needle AS group and laser pulse AS group with regard to the spontaneous abortion rate. During the later stages, the

Table 3 Pregnancy outcome after frozen embryo transfer

	29-gauge needle AS	Laser pulse AS	<i>P</i>
Intrauterine clinical pregnancies	67	61	/
Loss to follow-up	2	1	/
Abortion rate	15.38 % (10/65)	13.33 % (8/60)	<i>P</i> >0.05
Male/female	44/29	39/28	<i>P</i> >0.05
Twin/singleton	18/37	15/37	<i>P</i> >0.05
Gestational duration (weeks)	37.36±2.34	38.06±1.76	<i>P</i> =0.036
Age	29.14±2.91	30.73±3.80	<i>P</i> =0.017
Premature birth rate	40.00 % (22/55)	21.15 % (11/52)	<i>P</i> =0.035
Caesarean/Physiological production	46/9	48/4	<i>P</i> =0.170
Average birth weight	3101.23±634.17 g	3283.28±628.31 g	<i>P</i> =0.091
Low weight rate (%)	15.07 % (11/73)	8.96 % (6/67)	<i>P</i> >0.05

demand for nutrition is increased as is the fetal weight; thus damage to the trophectoderm incurred during AS pre-processing and the associated detrimental effects on the placenta and umbilical cord impair subsequent fetal development, with an increased probability of premature birth.

It should be noted, however, that the rates of blastocyst survival and clinical pregnancy (93.0 % and 60.36 % respectively) associated with 29-gauge needle AS pre-processing are acceptable. In addition, the 29-gauge needle AS is a low-cost approach, costing no more than 10 RMB in China to purchase two 29-gauge needles, which is far cheaper than the cost of a ZILOS-tk system, which may be more than 300,000 RMB, and thus prohibitively expensive for some small reproduction centers. In terms of the micropipetting method for AS, the diameter of the Pasteur pipette used must be suitable for the blastocyst diameter; that is, a larger Pasteur pipette is not suitable for AS, while a smaller one may cause critical damage to the embryo, or even the blastocyst ICM. Therefore, the 29-gauge needle AS method is more controllable. Micro-needle AS is also easy to apply, although the micro-needle costs more than 300 RMB, which is more than ten times the cost associated with the 29-gauge needle method.

In conclusion, we did not determine a significant advantage of either AS method in terms of pre-birth parameters; however, FET of the blastocysts subjected to laser pulse AS applied prior to the vitrification showed a better outcome in terms of average gestational duration and average birth weight compared with that of the 29-gauge needle AS method. Furthermore, laser pulse AS is an easy-to-operate and highly effective AS method that can be utilized by appropriately sized reproduction centers to significantly improve rates of survival rate and pregnancy following FET, with limited damage to the blastocyst. However, the 29-gauge needle AS method remains a practical approach based on its low-cost and acceptable rates of survival and post-transfer pregnancy.

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