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ORIGINAL ARTICLE

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Effect on clinical and neonatal outcomes of blastocelic microsuction prior to vitrification

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Abstract

Purpose: Microsuction (MS) is a technique for mechanically emptying fluid from blastocele using a microneedle. In this study, we evaluated the improvement in clinical and neonatal outcomes of vitrified blastocyst transfer programs when MS of blastocelic fluid was used before vitrification.

Methods: This was a retrospective study based on data collected between March 2014 and August 2016. A total of 317 blastocysts obtained from 211 patients were analyzed. The blastocelic fluid of expanded blastocysts was aspirated completely, and blastocysts were collapsed prior to vitrification. Clinical and neonatal outcomes of warmed blastocysts were compared.

Results: The survival rate of the MS blastocyst was significantly higher compared with the nontreatment control (98.7% vs 89.3%, OR: 9.34, 95% CI: 2.35-36.8, $P < 0.01$). The rates of implantation and live birth were higher in the MS group than in the control group, but the differences were not significant. There were no differences in gestational age, birthweight, proportion of male babies, rates of cesarean section, and congenital abnormalities.

Conclusion: The MS procedure improved blastocyst survival and had little effect on further embryo development after warming.

KEYWORDS
artificial shrinkage, blastocyst, clinical and neonatal outcomes, microsuction, vitrification

1 | INTRODUCTION

Blastocyst transfer, combined with vitrification, can maximize the cumulative pregnancy rate per oocyte retrieval cycle.[1] In contrast to early-stage embryos, blastocysts represent more mature, naturally selected embryos with a higher potential for development and implantation after in vitro fertilization (IVF).[2] From the viewpoint of vitrified blastocyst transfer programs, assisted reproductive technology requires that blastocysts be completely vitrified to enhance the IVF success rate.[3,4] However, Vanderzwalmen et al[5] found that the survival rate after vitrification/warming depended on the stage of blastocyst development, with expanded blastocysts exhibiting lower survival rate than morulae or early cavitating blastocysts. Expanded blastocysts are prone to forming ice crystals during vitrification due to large amounts of water-based fluid in the blastocele cavity. This may cause ultrastructural damage and interfere with the exchange of cryoprotectants presented in vitrification media. Although cryoprotectant agents (CPAs)

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usually cause the blastocyst to collapse during vitrification,[6] it varies considerably depending on the intensity of shrinkage during the previtrification phase.

Some investigators found that artificial shrinkage (AS) of the blastocyst was effective for avoiding intracellular ice formation during vitrification. AS was aimed at dehydrating and collapsing the blastocele cavity before blastocyst vitrification. It can be performed in a variety of ways, including laser pulse shooting,[7] repeated pipetting of the blastocyst,[8] exposure to hyperosmotic sucrose solution,[9] microneedle puncture,[5,10] and mechanical microsuction (MS) of the blastocele contents.[11] Several authors have reported that previtrification AS for the blastocyst improved the survival rate and clinical outcomes of blastocyst cryopreservation programs.[7,10,12] Blastocysts were observed to be completely collapsed when MS was applied. However, research has not been frequently published that included information about live birth of a healthy infant after MS treatment.

This study retrospectively evaluated the clinical and neonatal outcomes of blastocelic MS

2 | MATERIALS AND METHODS

2.1 | Experimental data

This was a retrospective, observational study based on data collected between March 2014 and August 2016. Data were collected from 211 patients whose causes of infertility were male factor, female factor (such as ovulatory disorders and endometriosis), and/or unexplained infertility. A total of 317 blastocysts were warmed, and each was transferred to the uterus of a patient. Participation of patients in this study was obtained through an opt-out methodology. The Kurashiki Medical Center and Ethics Committee approved the project.

2.2 | Blastocyst preparations

In the oocyte retrieval cycle, ovarian stimulation was achieved using standard gonadotropin-releasing hormone agonist/follicle-stimulating hormone (FSH) protocols or an antagonist/FSH protocol. Vaginal ultrasound-guided follicle puncture was conducted at 36 hours after the human chorionic gonadotropin (Mochida) injection. The retrieved oocytes were inseminated by conventional IVF or intracytoplasmic sperm injection (ICSI) in accordance with a previously reported method.[13] The oocytes with two pronuclei and a second polar body were defined as normally fertilized at 17-19 hours after insemination. They were cultured for five days in medium (global®; Life Global) supplemented with recombinant human albumin (G-MM; Vitrolife) at 37°C in 6% CO_2, 5% O_2, and 89% N_2. On day 5, the blastocysts were evaluated for expansion. A morphological grade was given according to the Istanbul Consensus criteria.[14] Only blastocysts with diameter of 170 μm or wider were included in this study.

2.3 | Artificial shrinkage treatment

Artificial shrinkage treatment was performed following the method described in Chen et al.[11] The blastocyst was held with a micropipette, and the inner cell mass was located at the 12 o'clock position. The ICSI injection pipette (5 mm tip to elbow length and 4 μm inner diameter; ICSI Injection Pipette; Sunlight Medical) punctured the zona, broke the membrane with negative pressure, and completely aspirated the blastocelic fluid. The blastocysts were individually collapsed, which usually took 1 minute per embryo (Figure 1).

2.4 | Blastocyst vitrification and warming

The collapsed blastocysts were vitrified and warmed (Kitazato Cryotop® method; Kitazato Company).[15] During vitrification, blastocysts were loaded to the equilibration solution for 5-15 minutes and sequentially transferred to the vitrification solution for 60 seconds. The blastocysts were immediately placed on the strip (Cryotop®) with a minimum amount of vitrification solution and quickly immersed into liquid nitrogen (LN_2). After storage for several months in LN_2, blastocysts were warmed in the thawing solution for 2 minutes and sequentially transferred to the dilution solution for 3 minutes. Then, blastocysts were washed in the washing solution for 5 minutes. Assisted hatching procedures were never performed in any embryos.

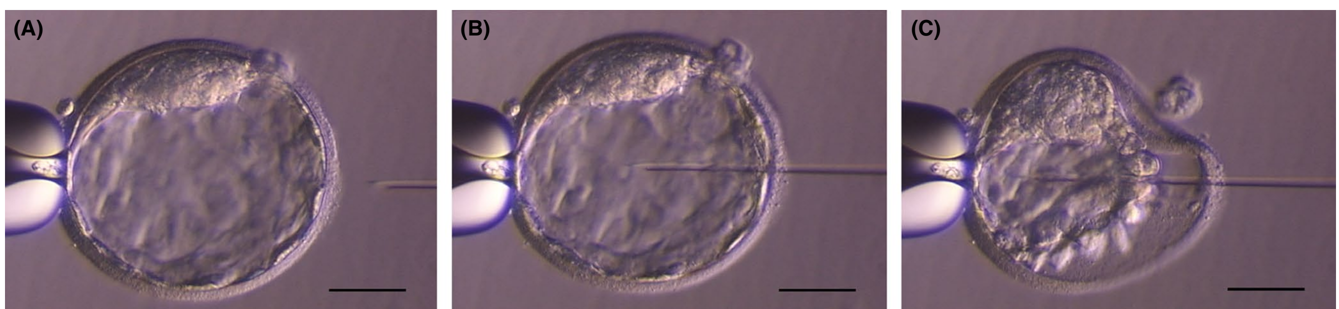

FIGURE 1 Microsuction of blastocelic fluid prior to vitrification. A, An expanded blastocyst was immobilized with a holding micropipette. The inner cell mass was located at the 12 o'clock position and the thinnest area of trophectoderm was at the 3 o'clock position. B, The aspirating needle, the same as the needle for intracytoplasmic sperm injection, was positioned at the junction of two trophectoderm cells and slowly inserted into the blastocele. C, The blastocelic fluid was aspirated completely, resulting in collapse of the blastocele. Scale bar = 50μm

TABLE 1 Demographic of patients

Characteristics	MS	NMS	*P*-value	Test statistics
Women (N)	111	100	—	—
Age (mean ± SD)[a]	35.4 ± 3.8	35.2 ± 4.2	0.80	Mann-Whitney
OPU trials (mean ± SD)	1.5 ± 0.8	1.5 ± 1.1	0.50	Mann-Whitney
Vitrified blastocysts (N)	423	482	—	—

Note: No significant difference.
Abbreviations: MS, microsuction; NMS, non-microsuction; OPU, oocyte pickup.
[a]Women age when own embryo was vitrified.

2.5 | Transfer of postwarmed blastocysts

Blastocyst survival was defined as partially intact after warming and re-expansion after culture in vitro before transfer. Each surviving blastocyst was transferred to a patient's uterus. Common modalities for blastocyst transfer were natural cycles or hormonal replacement cycles for endometrial preparation. Blastocyst transfer was performed under ultrasound guidance using embryo transfer catheter.

2.6 | Follow-up and evaluation index

To confirm the establishment of a clinical pregnancy, an ultrasound check was performed to visualize a gestational sac and fetal heartbeat. The loss of fetus with a gestational age of <20 weeks was considered a spontaneous abortion. A live birth rate was defined as live birth delivery cycles divided by transfer cycles. Preterm birth was defined as <37 gestational weeks. Birthweight <2500 g was defined as low birthweight. The duration of pregnancy, mode of delivery, and weight and sex of the child were recorded as neonatal outcomes. Cumulative implantation rate and cumulative live birth rate were the proportion of women that had at least one implantation and live birth, whether from the first transfer attempt or subsequent transfers of vitrified supernumerary blastocysts after the oocyte retrieval. Once a woman obtained a pregnancy from vitrified embryo transfer, she did not contribute any more to the cumulative rate.

2.7 | Statistical analysis

Analyses were done using the Statcel 2 program (OMS Publishing). Continuous variables were represented as mean ± SD and investigated to determine whether they were normally distributed. All continuous variables were not normally distributed, and the Mann-Whitney *U* test was used to compare the MS and non-MS groups (NMS). Categorical variables were described as frequency and percentage, with between-group difference tested by the chi-squared test and Fisher's exact test when the expected frequencies were <5. Odds ratio (OR) with 95% confidence interval was analyzed using an online calculator. $P < 0.05$ was considered statistically significant.

3 | RESULTS

The patient characteristics are summarized in Table 1. The two groups (MS and NMS) were comparable for the mean age of patients (35.4 ± 3.8 years vs 35.2 ± 4.2 years) and number of oocytes pickup trial cycles (1.5 ± 0.8 vs 1.5 ± 1.1), respectively. Overall in all cycles, 317 blastocysts were warmed, of which 298 survived (survival rate was 94.0%).

In all cycles, single embryo transfer was completed. In the MS group, 158 vitrified blastocysts were warmed for transfer and 156 survived. The survival rate was 98.7%. In two cycles, no blastocysts survived, and embryo transfer was cancelled. In the NMS group, 159 vitrified blastocysts were warmed for transfer and 142 survived. The survival rate was 89.3%. In 17 cycles, no blastocysts survived, and embryo transfer was cancelled. The survival rate was significantly higher in the MS group than in the NMS group (OR: 9.34, 95% CI: 2.35-36.8, $P < 0.01$). The rates of implantation (31.0% vs 24.5%) and live birth (20.3% vs 18.9%) were higher in the MS group, but not different between the groups. The cumulative implantation rate (44.1% vs 39.0%) and cumulative birth rate (28.8% vs 30.0%) were comparable in both groups. The details are summarized in Table 2.

The neonatal outcomes of 62 children born after transfer are shown in Table 3. Thirty-two babies were born in the MS group, and 30 babies were born in the NMS group. There were no differences between the MS and NMS groups with respect to the average gestational weeks (37.8 ± 3.4 weeks vs 38.2 ± 1.5 weeks), preterm birth rate (9.4% vs 13.3%), mean birthweight (2810.3 ± 707.9 g vs 3076.8 ± 409.1 g), and low birthweight rate (12.5% vs 3.3%). The cesarean section (37.5% vs 43.3%), proportion of male babies (37.5% vs 50.0%), and congenital abnormality rates (3.1% vs 0%) also had no differences. One baby with cleft lip was born in the MS group.

4 | DISCUSSION

The MS blastocyst survival was significantly higher than the NMS blastocyst survival. Furthermore, MS had little effect on further embryo development after warming.

There is a fundamental problem that AS for blastocysts prior to vitrification is successful in some laboratories but not in others. It is challenging to explain the reason for this discrepancy, because many factors may affect the survival rate of human blastocyst vitrification, such as the method of AS, protocols of vitrification, types of carrier used for vitrification, previtrification handling of blastocysts, quality and size of blastocysts, and criteria for survival of warmed blastocysts. In our experience, the survival rate declines in completely expanded blastocysts after warming without AS prior to vitrification. This leads to performing AS prior to vitrification when blastocyst size is large. We tried various AS methods with expanded blastocysts, including repeated pipetting of the blastocyst,[8] exposure to hyperosmotic sucrose solution,[9] and laser pulse shooting.[7] Nevertheless, we

TABLE 2 Clinical outcomes with MS and NMS groups

Warming cycle results	MS	NMS	Odds ratio (95% CI)	*P*-value	Test statistics
Warming cycles (N)	158	159	—	—	—
Warmed blastocysts (N)	158	159	—	—	—
Survived blastocysts (N, % per warmed blastocysts)	156 (98.7)	142 (89.3)	9.34 (2.35-36.8)	<0.01	Chi-squared
Implantation (N, % per warmed blastocysts)[a]	49 (31.0)	39 (24.5)	1.38 (0.85-2.26)	0.20	Chi-squared
Cumulative implantation (N, % per women)	49 (44.1)	39 (39.0)	1.24 (0.72-2.14)	0.45	Chi-squared
Live birth (N, % per warmed blastocysts)	32 (20.3)	30 (18.9)	1.09 (0.63-1.90)	0.76	Chi-squared
Cumulative live birth (N, % per women)	32 (28.8)	30 (30.0)	0.95 (0.52-1.70)	0.85	Chi-squared
Multiple birth (N, % per live birth)	0 (0)	0 (0)	—	—	—

Note: Significant at $P < 0.05$.
Abbreviation: CI, confidence interval; MS, microsuction; NMS, non-microsuction.
[a]Defined as a gestational sac identified with ultrasound.

could not reproduce the satisfactory results described in those papers. We then tried the MS method, and a high survival rate was obtained after vitrification of large-sized blastocysts, as demonstrated in this study. These results are similar to previous reports.[11,16,17]

Among various methods of AS, laser puncturing of blastocysts before vitrification is the most popular. We tried laser pulse shrinkage of expanded blastocysts following Mukaida's method.[7] One to three laser pulses (0.2-0.8 ms; RI Saturn 5 active laser system [6-47-500; Research Instruments]) were targeted at the cellular junction of the trophectoderm cells to induce blastocelic cavity collapse. After 5-10 minutes, the collapsed blastocysts were immediately vitrified (Kitazato Cryotop® method). Embryo viability and developmental potentials were related to the relative number of intact vs damaged blastomeres. Embryos with more blastomeres showing DNA damage and undergoing apoptosis were of lower grade.[18] Desai et al[19] reported that AS before vitrification could reduce DNA damage in expanding blastocysts. So, we evaluated the extent of DNA damage using the TUNEL (terminal deoxynucleotidyl transferase-mediated d-UTP nick end labeling) technique. The DNA damage after warming was higher in laser group (32.3%) compared to the MS (13.7%) and NMS (21.2%) groups (not published).

TABLE 3 Neonatal outcomes with MS and NMS groups

Delivered baby data	MS	NMS	Odds ratio (95% CI)	*P*-value	Test statistics
Babies (N)	32	30	—	—	—
Gestational age (weeks; mean ± SD)	37.8 ± 3.4	38.2 ± 1.5	—	0.62	Mann-Whitney
Preterm birth (N, %)	3 (9.4)	4 (13.3)	0.67 (0.15-2.97)	0.46	Fisher's exact
Birthweight (g; mean ± SD)	2810.3 ± 707.9	3076.8 ± 409.1	—	0.12	Mann-Whitney
Low birthweight (N, %)	4 (12.5)	1 (3.3)	4.14 (0.57-28.9)	0.20	Fisher's exact
Cesarean section (N, %)	12 (37.5)	13 (43.3)	0.79 (0.29-2.14)	0.64	Chi-squared
Proportion of male babies (N, %)	12 (37.5)	15 (50.0)	0.60 (0.22-1.63)	0.32	Chi-squared
Congenital abnormalities (N, %)	1 (3.1)[a]	0 (0)	Inf (0.24-Inf)	0.52	Fisher's exact

Abbreviation: CI, confidence interval; MS, microsuction; NMS, non-microsuction.
[a]One baby with cleft lip.

As a result, MS was used as a primary AS method in our laboratory and used in this study design. We analyzed small numbers of blastocysts in unpublished data, and it was difficult to conclude whether laser or MS was better. In our experience, poor-quality blastocysts often degenerated after laser puncturing, although it should be interpreted with caution due to the small sample size. Mukaida et al[7] suggested that a single laser pulse created an instant heat effect that caused the cellular degeneration of the trophectoderm. Conversely, the MS method is a technique for mechanically emptying the fluid from a blastocele via microneedle puncture. It is possible to completely collapse a blastocele within 1 minute. The key to the success of expanded blastocyst vitrification may be to make a hole in the trophectoderm layer and cause a collapse of the blastocele cavity.

Microsuction is one of the methods for AS using ICSI injection and holding micropipettes. In animal models, Chen et al[11] reported significant improvement in survival rate (59%-89%) of blastocysts treated with MS prior to vitrification. Kader et al[16] also demonstrated that MS treatment significantly improved the DNA integrity index.

We performed additional analyses to more clearly distinguish this method. In the MS group, 32 babies were born, including one baby with cleft lip. In the control group, 30 babies were born. There were no differences between the two groups in birthweight, gestational age, rate of cesarean section, proportion of male babies, and congenital abnormalities. This result should be interpreted with caution due to the small study size and high risk of bias. In our findings, there were no monozygotic twins derived from a mechanically punctured blastocyst. Wang et al[20] demonstrated that there was an increased risk of producing monozygotic twins after AS by laser pulse. They speculated that this could be caused by damage of the zona pellucida, creating a hole larger than the microneedle puncture. Monozygotic twins are associated with a range of well-documented risks linked to the health of the mother and fetus.

Artificial shrinkage treatment may shorten the equilibration period for embryo vitrification. This may reduce the harmful effects from CPAs contained in vitrification media. However, few studies and reviews have demonstrated any concern about side effects of equilibration periods for human embryos prior to vitrification. Xiong et al[21] reported that shortening the exposure of the embryo in equilibrium solution was associated with low implantation rate and clinical outcomes, so that they concluded that appropriately prolonged equilibrium time would be needed to improve the clinical outcomes. To our knowledge, dimethyl sulfoxide has been especially well-proven as a toxic CPA for human embryonic stem cells,[22] but the impact is not clear for the early embryo stage. The Kitazato Cryotop® method that evolved over a long period of time has optimized the CPAs types, concentrations, and procedures. Many healthy babies have been born after using the Kitazato Cryotop® method, and there are few reports that prolongation of the equilibration time affected the future health of babies. Kitazato also recommended the AS treatment (exposure in hyperosmotic solution) for expanded blastocysts to achieve high survival rates of embryos after vitrification, but they did not mention whether the equilibration period could be shortened. In our laboratory, four embryos are vitrified in 15 minutes. The embryos are exposed to equilibration medium at least 5 minutes, and then, each is sequentially vitrified every 2 minutes. In other words, we start vitrifying the first embryo at 5 minutes after exposure in equilibration medium and the final embryo at 11 minutes or later. It may be enough for blastocysts to expose in equilibration medium at least 5 minutes before vitrification, and initial equilibration periods up to 15 minutes may have little effect on human embryos and further development.

In conclusion, this study shows that the MS method reduces blastocelic fluid prior to vitrification and may enhance the survival and implantation rates after warming. The MS method group also tends to have better clinical outcomes than nontreatment group. Because total available numbers of surviving MS blastocyst will be increasing, the cumulative clinical outcomes will be expected to be high in further large-scale studies. Further prospective randomized studies will be needed to confirm the findings of this study.

DISCLOSURES

Conflict of interest: Shingo Mitsuhata, Yoshitaka Fujii, Yuji Endo, Momoko Hayashi, and Hiroaki Motoyama declare that they have no conflict of interest. *Human rights statement and informed consent*: All the procedures were followed in accordance with the ethical standards of the institutional ethical committee and with the Helsinki Declaration of 1964 and its later amendments. All the study's participants provided informed consent, and the study design was approved by the appropriate ethics committee of Kurashiki Medical Clinic, Okayama, Japan. *Animal studies*: This article does not contain any studies with animal participants performed by any of the authors.

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