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Comparison of mechanical artificial shrinkage methods in mouse blastocyst vitrification

J.K. Joo[1], J.E. Jeong[1], S.C. Kim[1], C.W. Kim[3], G.R. Ko[2], K.S. Lee[1]

[1] Department of Obstetrics and Gynecology, Medical Research Institute, Pusan National University, School of Medicine, Busan
[2] Infertility clinic, Pusan National University Hospital, Busan
[3] Department of Obstetrics & Gynecology, School of Medicine, Sungkyunkwan University School of Medicine, Samsung Changwon Hospital, Changwon (Korea)

Summary

Purpose of investigation: This study was designed to determine which mechanical artificial shrinkage (AS) method, conducted by puncture, pipetting, or aspiration, was effective in increasing the re-expansion rate of mouse blastocysts. *Materials and Methods:* In each group, 30 mouse blastocysts were used. Before vitrification, the blastocoelic cavity was collapsed by puncture with a micro-needle, pipetting with a micro-glass pipette, and direct aspiration with an ICSI pipette. After thawing, the re-expansion rate of blastocysts was examined for each AS method. Re-expansion rate was checked at three, five, and seven hours after thawing. *Results:* The number of re-expanded mouse blastocysts at five hours after thawing was 12 in the puncture with a micro-needle group, 11 in the pipetting with a micro-glass pipette group, and 24 in the direct aspiration with an ICSI pipette group. The cumulative number of re-expanded mouse blastocysts at seven hours after thawing was 20 in the puncture with a micro-needle group, 20 in the pipetting with a micro-glass pipette group, and 28 in the direct aspiration with an ICSI pipette group. There were statistically significant differences in the cumulative number of re-expanded mouse blastocysts between five and seven hours after thawing ($p = 0.001$ and 0.021, respectively). *Conclusions:* Direct aspiration with an ICSI pipette resulted in a higher re-expansion rate than the puncture and pipetting methods. It can be considered that the direct aspiration method is more convenient and simpler than the other two methods.

Key words: Artificial shrinkage; Blastocyst; Assisted hatching.

Introduction

Due to the transfer of frozen-thawed embryos, the outcomes of IVF programs have improved [1, 2]. Frozen-thawed blastocyst transfer is performed more widely because of the increase in surplus embryos due to development of culture system and cryopreservation techniques [3].

During the vitrification process, the intracellular water is converted into ice crystals, and this is a major cause of embryo damage and lower survival rate [4]. Because the amount of intracellular water in the blastocyst is greater than that in the cleavage stage embryos, dehydration of intracellular water and influx of cryoprotectant take more time in the blastocyst. During the vitrification process, this factor could cause damage to blastocysts.

To overcome this problem, artificial shrinkage (AS) was introduced. Several studies reported the results of artificial shrinkage of blastocysts using various methods like glass micro-needle, a 29-gauge needle, micro-pipetting with a hand-drawn Pasteur pipette, laser pulse, and osmotic shock [5-8].

There are some reports suggesting that laser pulse is better than the other methods, but other studies reported opposite results. Also, among the mechanical methods, the best AS method is still unclear [5].

In this article, the authors compared survival and re-expansion rate of mouse blastocysts after artificial shrinkage and vitrification with three different mechanical artificial shrinkage methods, which were punctured with a micro-needle, pipetting with a hand-drawn glass pipette, and direct aspiration with an ICSI pipette.

Materials and Methods

Preparation of mouse blastocysts

Mouse two-cell embryos were collected from hyperstimulated female mice and cultured in G1.1 and G2.2 to blastocysts stage.

Preparation of equilibration and vitrification solutions

The solutions for equilibration, vitrification, and thawing were prepared using Dulbecco's phosphate-buffered saline (PB1) plus 20% synthetic serum substitute (SSS). Equilibration solution consisted of EBS1 (G10) and EBS2 (G10E20). EBS1 and EBS2 contained 10% glycerol and 10% glycerol + 20% ethylene glycol, respectively. Vitrification solution (VS) was composed of 25% glycerol + 25% ethylene glycol in 20% SSS + PBS.

Artificial shrinkage of expanding blastocysts

Before starting the vitrification procedure, AS of expanding blastocysts was performed in two equilibration solutions. In this study, we used three different AS methods.

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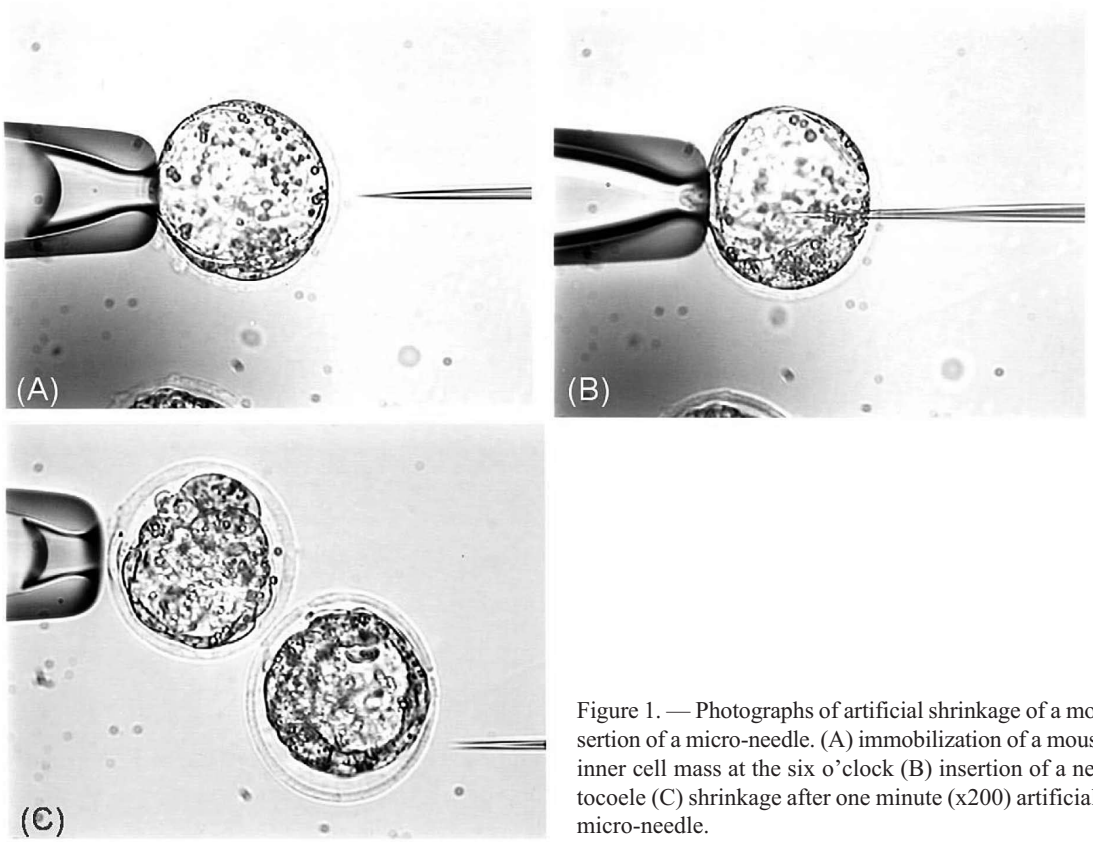

Figure 1. — Photographs of artificial shrinkage of a mouse blastocyst by insertion of a micro-needle. (A) immobilization of a mouse blastocyst with the inner cell mass at the six o'clock (B) insertion of a needle inside the blastocoele (C) shrinkage after one minute (x200) artificial shrinkage using the micro-needle.

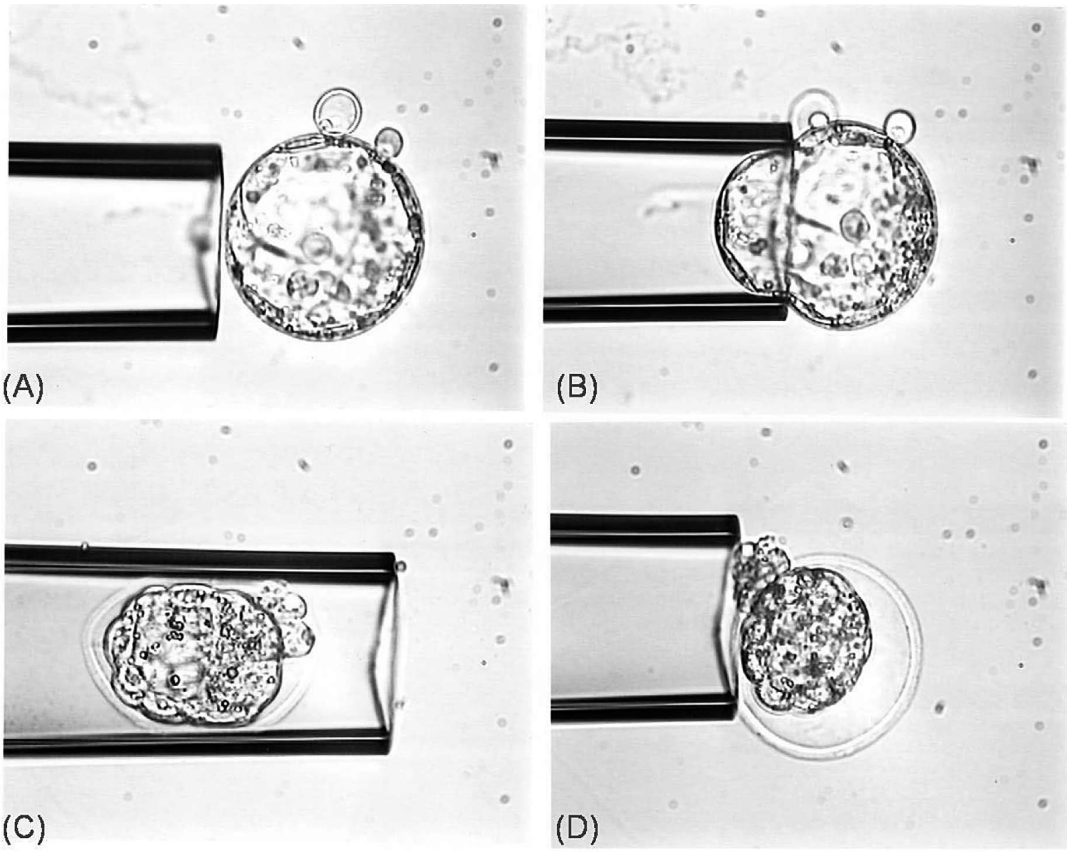

Figure 2. — Photographs of artificial shrinkage of a mouse blastocyst by pipetting with 100 um (O.D) glass pipette connected to the injector of a micromanipulator. (A) Before artificial shrinkage. (B) Start of pipetting. (C) A completely pipetted mouse blastocyst. (D) Shrunken mouse blastocyst (x200).

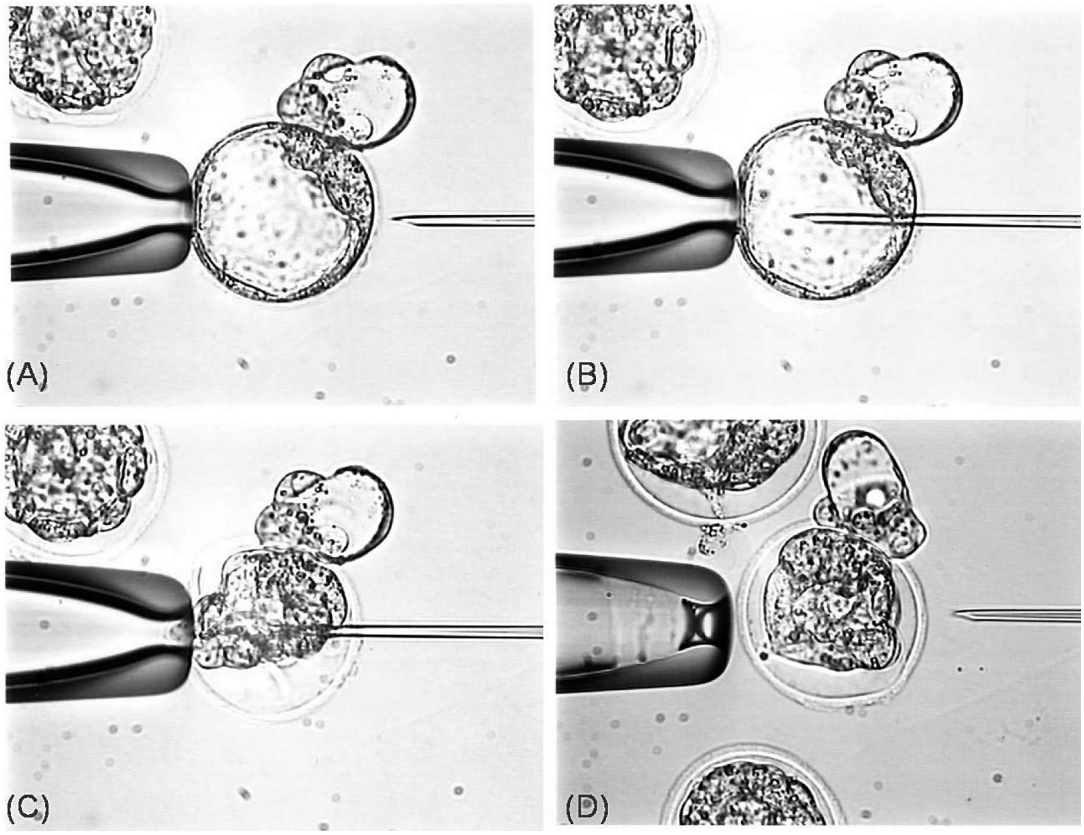

Figure 3. — Photographs of artificial shrinkage of a mouse blastocyst with the ICSI pipette. (A) Holding mouse blastocyst with a holding pipette. (B) Insertion of the ICSI pipette inside the blastocoele cavity. (C) Aspiration of the blastocoelic fluid. (D) Removal of the ICSI pipette (x200).

1) Puncture with a micro-needle (group A)

The puncture needle was prepared by using a puller. It was inserted into the blastocyst and it created two holes in the blastocyst. The technique of shrinkage using micro-needle puncture has been described previously [5]. The expanding blastocyst was held with a holding pipette and the inner cell mass (ICM) was placed at the six or 12 o'clock position, and a glass micro-needle was pushed through the cellular junction of the trophoectoderm into the blastocoelic cavity until it shrank (Figure 1). After removing the micro-needle, the authors observed contraction of the blastocoele within a few minutes.

2) Pipetting with a hand-drawn glass pipette (group B)

Pipetting of the expanding blastocyst was conducted using a glass pipette with slightly smaller in diameter than the expanding blastocyst. The pipettes were manually made from a Borosilicate micro-glass pipette hand-drawn using a microforge. The inner diameter of the pipette was 100 um. The pipette was connected with the pipette holder of a micromanipulator. Pipetting was repeated until the blastocoele collapsed completely (Figure 2).

3) Direct aspiration of the blastocoelic fluid with an ICSI pipette (group C)

In the same manner as in the ICSI procedure, the blastocyst was fixed with a holding pipette after the ICM part in the expanding mouse blastocyst turning to a six or 12 o'clock. Then, the ICSI pipette was inserted into the blastocoelic cavity and the blastocoelic fluid was aspirated (Figure 3).

Continuous culture, exposure to the vitrification solution, and vitrification

First, to compare the effects of AS methods, blastocysts were cultured immediately after AS in each group and the re-expansion rate was checked. As the next step, to observe the effects of vitrification solution on artificially shrunken blastocysts, blastocysts were shrunken using each AS method and exposed to the VS solution. Immediately after exposure to the VS solution, blastocysts were cultured for seven hours.

The authors also checked the re-expansion rate of frozen-warmed blastocysts. After blastocoele contraction, the blastocysts were equilibrated in the equilibration solution for another two minutes before exposure to the vitrification solution. The blastocysts were then incubated in the vitrification solution and loaded on the tip of the capped-pulled straw within 30 seconds. Then, the straw was immediately plunged into liquid nitrogen.

Thawing of blastocysts and observation of re-expansion rate

Re-expansion was defined as the full expanding state of the blastocyst. The proportion of re-expanding blastocysts at three, five, and seven hours after thawing was observed in each group.

Statistical analysis

The data obtained were examined for differences using Student's t-test and Fisher's exact probability test as appropriate. Results are expressed as mean ± SD.

Results

The procedures used in this study are described in Figures 1, 2, and 3. The conduction time was less than three minutes for all three methods, respectively. For adequate shrinkage, puncture was performed at least two times and pipetting was performed three times. Direct aspiration was

Table 1. — *Cumulative number of re-expanded mouse blastocysts after artificial shrinkage with three different mechanical methods.*

No. of re-expanded mouse blastocysts according to the passage of time	Methods of artificial shrinkage Group A (Micro needle, n=30)	Group B (Glass pipette, n=30)	Group C (ICSI pipette, n=30)	*p*-value
3 h (%)	3 (10.0)	4 (13.3)	6 (20.0)	0.533
5 h (%)	16 (53.3)	16 (53.3)	24 (80.0)	0.049
7 h (%)	23 (76.7)	24 (80.0)	30 (100)	0.21

Table 2. — *Cumulative number of re-expanded mouse blastocysts exposed to the vitrification solution after artificial shrinkage with three different mechanical methods.*

No. of re-expanded mouse blastocysts according to the passage of time	Methods of artificial shrinkage Group A (Micro needle, n=30)	Group B (Glass pipette, n=30)	Group C (ICSI pipette, n=30)	*p*-value
3 h (%)	2 (6.7)	5 (16.7)	6 (20.0)	0.533
5 h (%)	14 (46.7)	16 (53.3)	27 (90.0)	0.000
7 h (%)	21 (70.0)	23 (76.7)	29 (96.7)	0.013

Table 3. — *Cumulative number of re-expanded mouse blastocysts after vitrification and warming following artificial shrinkage with three different mechanical methods.*

No. of re-expanded mouse blastocysts according to the passage of time	Methods of artificial shrinkage Group A (Micro needle, n=30)	Group B (Glass pipette, n=30)	Group C (ICSI pipette, n=30)	*p*-value
3 h (%)	2 (6.7)	3 (10.0)	6 (20.0)	0.260
5 h (%)	12 (40.0)	11 (36.7)	24 (80.0)	0.001
7 h (%)	20 (66.7)	20 (66.7)	28 (93.3)	0.021

performed with only a single insertion of the ICSI pipette.

Table 1 shows the number of re-expanding mouse blastocysts after artificial shrinkage with three different mechanical methods. At five hours after AS, the cumulative number of re-expanding mouse blastocysts was 16 in group A, 16 in group B, and 24 in group C, respectively. At seven hours after AS, the cumulative number of re-expanding mouse blastocysts was 23 in group A, 24 in group B, and 30 in group C, respectively. There was a statistically significant difference in the cumulative number of re-expanding mouse blastocysts at five hours after AS ($p = 0.049$).

The cumulative number of re-expanding mouse blastocysts exposed to the vitrification solution after AS is presented in Table 2. At five hours after exposure to the vitrification solution, the cumulative number of re-expanding mouse blastocysts was 14 in group A, 16 in group B, and 27 in group C, respectively. At seven hours after exposure to the vitrification solution, the number of re-expanding mouse blastocysts was 21 in group A, 23 in group B, and 29 in group C, respectively. There were statistically significant differences in the cumulative number of re-expanding mouse blastocysts at five and seven hours after exposure to the vitrification solution ($p = 0.000$ and 0.013, respectively).

The cumulative number of re-expanding mouse blastocysts that were vitrified-warmed after AS is presented in Table 3. At five hours after warming and vitrification, the cumulative number of re-expanding mouse blastocysts was 12 in group A, 11 in group B, and 24 in group C, respectively. At seven hours after warming and vitrification, the cumulative number of re-expanding mouse blastocysts was 20 in group A, 20 in group B, and 28 in group C, respectively. There were statistically significant differences in the cumulative number of re-expanded mouse blastocysts at five and seven hours after warming and vitrification ($p = 0.001$ and 0.021, respectively).

Discussion

In slow freezing of blastocysts, the survival rate was very low [9]. The reasons for this are damage to the trophoblast caused in the process of dehydration by the freezing solution and ice crystal formation due to insufficient dehydration [10-12].

For overcoming these problems, AS of blastocysts was introduced. Many studies have reported the beneficial effects of AS of blastocysts [5-7]. Mukaida *et al.* reported a high survival rate (97.2%) and pregnancy rate (60.2%) in warmed-vitrified blastocyst transfer cycles using AS before vitrification [4].

Several different AS methods were introduced, such as micro-needle stimuli [4, 5], 29-G needle puncture [6], laser pulse [4], and pipetting [7]. Among these methods, laser pulse is used more widely, but the laser equipment is quite expensive and is not always possible to provide this facility in all IVF clinics. AS methods using a needle or pipette however do not need any special equipment and the biologist in the IVF labs familiar with the procedure. Considering the economic aspect and accessibility, these methods are very feasible.

In this study, the re-expansion rate in the direct aspiration with an ICSI pipette group was higher than that in the other two groups. Many factors can be considered to be responsible for this result such as human error and mechanical damage. Technical errors can occur due to the complexity of methods. During all of the AS procedures, a micromanipulator was used. This made delicate handling of the blastocysts possible and the probability of occurrence of human errors was controlled well in all of the three AS methods.

Lesser mechanical damage might be the main cause for the good result in the direct aspiration group. In the puncture method, puncture was performed at least two times. Also, in the pipetting method, pipetting was performed two to three times to cause complete shrinkage. Repeat procedures might cause trophoblast damage and influence the survival rate and re-expansion rate.

Direct aspiration of the blastocoelic fluid might have some other benefits. It is a familiar method for those who conduct the ICSI procedure. A relatively short procedure time can be helpful for survival of the embryo and for the laboratory technician. It could result in less human errors.

These methods can be applied to the human embryo, and actually in the present clinics, this procedure was used several years ago [13]. However, the present authors have no comparative data in human IVF.

The results in humans could be different from those in mouse blastocysts. However, the aspiration method is relatively easy and simple, but a learning curve is needed to achieve consistent results in the human IVF program. This study showed statistically better outcomes in the aspiration group and it may be valuable for further research.

Considering the economic aspect and accessibility, mechanical AS is considered to be an efficient method for frozen-warmed blastocyst transfer. Especially, the present authors think that direct aspiration of the blastocoelic fluid with an ICSI pipette is a very simple and cost-effective method.

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Address reprint requests to:
KYU-SUP LEE, M.D.
Department of Obstetrics and Gynecology
Pusan National University, School of Medicine
Gu-Deok-Ro 305, Seo-Gu
Busan (Republic of Korea)
e-mail: kuslee@pusan.ac.kr