

# Mouse blastocyst previtrification interventions and DNA integrity

Amr Kader, M.D.,<sup>a,b,c</sup> Rakesh K. Sharma, Ph.D.,<sup>a</sup> Tommaso Falcone, M.D.,<sup>a,b</sup> and Ashok Agarwal, Ph.D., HCLD.<sup>a</sup>

<sup>a</sup> Center for Reproductive Medicine, Glickman Urological and Kidney Institute, and Obstetrics and Gynecology and Women's Health Institute, Cleveland Clinic, Cleveland, Ohio; <sup>b</sup> Department of Obstetrics and Gynecology, Center of Surgical Innovation, Technology and Education, Cleveland Clinic, Cleveland, Ohio; and <sup>c</sup> Department of Obstetrics and Gynecology, Alexandria University, Alexandria, Egypt

**Objective:** To assess the effect of implementing different previtrification interventions on the postwarming DNA integrity of vitrified blastocysts.

**Design:** Prospective in vitro study.

**Setting:** Center for Reproductive Medicine laboratory in a tertiary hospital setting.

**Animals:** A total of 70 expanded and 46 nonexpanded mouse blastocysts were used for the study.

**Intervention(s):** [1] Twenty-two expanded blastocysts were blastocele aspirated and immediately vitrified. [2] Twelve expanded blastocysts were spontaneously hatched before vitrification. [3] Twenty-two expanded blastocysts were vitrified without intervention. [4] Sixteen nonexpanded blastocysts underwent assisted hatching using acidified tyrode's solution. [5] Seventeen nonexpanded blastocysts were vitrified without intervention. [6] Thirteen nonexpanded blastocysts and 14 expanded were used as fresh controls. Vitrification was done using a cryotip loading device.

**Main Outcome Measure(s):** DNA integrity index using terminal deoxynucleotide transferase [TdT]-mediated dUTP-digoxigenin nick-end labeling staining and confocal imaging.

**Result(s):** [1] Intervention by blastocele aspiration for expanded blastocysts or assisted hatching of nonexpanded blastocysts significantly improved the postwarming results in each blastocyst stage. [2] Allowing spontaneous hatching before vitrification is a noninvasive technique that can improve the postwarming integrity of expanded blastocysts similar to blastocele aspiration.

**Conclusion(s):** Blastocele aspiration or spontaneous hatching of expanded blastocysts and assisted hatching of nonexpanded blastocysts before vitrification helps minimize blastomere DNA damage. (Fertil Steril® 2009; ■ : ■–■. ©2009 by American Society for Reproductive Medicine.)

**Key Words:** Blastocyst, vitrification, cryopreservation, hatching, blastocele aspiration, DNA integrity index, TUNEL, apoptotic index

Blastocysts represent more mature, naturally selected embryos that exhibit higher potential for development and implantation after IVF or intracytoplasmic sperm injection (ICSI) than early stage embryos. Blastocyst transfer allows better selection of transferred embryos to patients who suffered from previous implantation failure (1). However, an adequate number of embryos need to be available to allow natural selection by extended culture.

Many reports have recorded high pregnancy rates from the transfer of vitrified blastocysts in animals (2, 3) and humans (4–10). Nevertheless, the inherent criteria of blastocysts make their cryopreservation rather challenging. First, the cryoprotectant is relatively slow to permeate the blastocyst

because of its relatively large multicellular structure. Second, the zona pellucida (ZP) presents a physiologic barrier to the cryoprotectants that prevents them from adequately permeating all blastomeres. Third, the blastocele may be insufficiently dehydrated during vitrification. As a result of any or all of the above challenges, the possibility of damage as a result of ice crystal formation still exists. The degree of blastocyst expansion has been reported to be inversely correlated with pregnancy rates resulting from vitrified blastocysts (11, 12).

To overcome these obstacles and improve both survival and clinical outcomes of blastocyst vitrification, various previtrification techniques have been improvised and implemented. These can be classified into two main categories: [1] hatching of the ZP performed by either laser, acidified Tyrode's solution, repeated micropipetting, or spontaneously by extending the culture of expanding blastocysts (12–16), and [2] blastocele collapse, passively by puncture or actively by microsuction (11, 17–19). Clinical trials adopting these techniques have shown significant improvement in terms of survival, fertilization, and pregnancy rates (15, 17–19).

Embryo viability and developmental potentials are related to the relative number of intact versus damaged blastomeres.

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Reprint requests: Ashok Agarwal, Ph.D., HCLD., Center for Reproductive Medicine, Glickman Urological & Kidney Institute and Obstetrics and Gynecology and Women's Health Institute, Cleveland Clinic, 9500 Euclid Avenue, Desk A19.1, Cleveland, OH 44195 (FAX: 216-445-6049; E-mail: Agarwaa@ccf.org).

Embryos with more blastomeres showing DNA damage and undergoing apoptosis are considered to be of lower grade (2). Our objective was to examine different previtrification interventions and evaluate the extent of DNA damage with each.

In the present study, we have categorized the previtrification interventions according to the degree of embryo expansion. For the nonexpanded blastocele, we hypothesized that assisted hatching as a minimally invasive procedure may be adequate to provide better exposure to the cryoprotectant as well as proper dehydration. As the expanded blastocysts carry a higher risk of damage during vitrification and thawing because of the large blastocele fluid volume, larger size, and number of cells, a more invasive technique (blastocele aspiration) was adopted for this developmental stage. We also studied spontaneous hatching as a simple, noninvasive alternative that may be applied when appropriate, for expanded blastocysts to improve their permeation with cryoprotectants and their dehydration. We used mouse embryos in this study as a suitable model for studying pathophysiologic events associated with the embryos (20, 21).

## MATERIALS AND METHODS

### Embryos

Cryopreserved two-cell mouse embryos were purchased from Embryotech (Embryotech Laboratories, Inc., Wilmington, MA). Embryo straws were thawed according to the manufacturer recommendations then incubated in human tubal fluid (HTF) medium supplemented with 10% albumin in an incubator with 5% CO<sub>2</sub> at 37°C. Embryos were examined after 24 hours, 48 hours, and 72 hours for development monitoring and embryo grading. Embryos showing signs of fragmentation, delayed or accelerated development were excluded from the study (22).

### Embryo Grouping

After assuring embryo quality, the blastocysts were classified initially according to the degree of blastocele expansion and integrity of the ZP into the following: [1] nonexpanded blastocysts with blastocelic volume less than half the total volume of the blastocyst, [2] expanded blastocysts with blastocelic volume less than half the total volume of the blastocyst; [3] spontaneously hatched blastocysts identified as having a discontinuation of the ZP, generally associated with some herniation of the trophoblast.

After classification, embryos were randomly assigned to the experimental groups, with fresh controls, vitrification with or without active intervention as follows: [1] 22 expanded blastocysts were blastocele aspirated then vitrified; [2] 12 expanded blastocysts were spontaneously hatched before vitrification; [3] 22 expanded blastocysts were vitrified without intervention; [4] 16 nonexpanded blastocysts underwent assisted hatching using acidified Tyrode's solution; [5] 17 nonexpanded blastocysts were vitrified without intervention; [6] 13 nonexpanded and 14 expanded blastocysts were used as fresh controls.

### Assisted Hatching

Assisted hatching was performed with the aid of two hydraulic micromanipulators (Narishige, Tokyo, Japan) and microsyringes (IM-6, Narishige) mounted on an inverted microscope (Olympus, Tokyo, Japan). Each blastocyst was kept in place with a holding micropipette, and the inner cell mass was oriented to the 12 o'clock position. Assisted hatching was performed using acidified Tyrode's solution (Irvine Scientific, Santa Ana, CA).

### Spontaneous Hatching

Expanded embryos were allowed to spontaneously hatch by prolonged incubation (day 6). Hatching was verified under the dissecting microscope by the presence of a discontinuous zona with or without herniation of the trophoblast.

### Blastocele Aspiration

Microsuction was performed with the aid of two hydraulic micromanipulators (Narishige, Tokyo, Japan) and microsyringes (IM-6; Narishige) mounted on an inverted microscope (Leica Microsystems GmbH, Wetzlar, Germany). The blastocyst was held in place with a holding micropipette, and the inner cell mass was oriented to the 12 o'clock position. An ICSI injection needle (Swemed, Sweden; Vitrolife, Englewood, CO) was used to penetrate the zona and penetrate the blastocelic cavity with a slight negative pressure. The needle was gradually withdrawn as the blastocelic cavity was collapsing.

### Vitrification and Warming

Vitrification was performed using the Irvine vitrification kit (Irvine Scientific, Santa Ana, CA) with the cryotip loading device according to the manufacturer's recommended protocol. As previously described, the blastocysts were placed in 20  $\mu$ L droplet of the equilibration medium (7.5% of dimethyl sulfoxide [DMSO] and ethylene glycol and 20% serum substitute supplement) for 5 minutes then transferred quickly to three droplets of 20  $\mu$ L of the vitrification medium (15% of DMSO and ethylene glycol and 20% serum substitute supplement) for 5 seconds in the first and second droplet. Within 90 seconds of being in the last droplet, the blastocysts were loaded in the cryotip with 1  $\mu$ L of the vitrification medium, sealed from both ends with heat pulse sealer, and immediately transferred to liquid nitrogen.

After 24 hours, thawing was performed by removing a cryotip from the liquid nitrogen and immediately placing it in a 37°C water bath for 3 seconds. The cryotip was cut at its lower end, and the contents were brought down near to the thawing medium. The embryos retrieved after thawing were placed in one droplet of thawing medium containing 1 M sucrose for 1 minute. Blastocysts were further propagated into two droplets of dilution medium containing 0.5 M sucrose, remaining for 2 minutes in each droplet. They were finally washed in a series of three droplets of washing medium (containing only buffered HEPES and serum

substitute supplement and no sucrose) for 3 minutes in each droplet. Embryos were finally incubated in HTF medium in 5% CO<sub>2</sub> for 4 hours before any further processing. Blastocysts' survival was judged by the partial or complete reexpansion of the blastocysts after warming and incubation.

### Staining for DNA Damage

As previously described, embryos were fixed in 4% paraformaldehyde in PBS (pH 7.4) for 1 hour at room temperature. After fixation, embryos were washed at least three times in phosphate-buffered saline (PBS) containing 0.3% polyvinylpyrrolidone (PBS/PVP) and permeabilized in 0.5% Triton X-100 on ice for 2 minutes. The embryos were then washed X3 in PBS/PVP and incubated in the TUNEL (Terminal deoxynucleotide transferase [TdT]-mediated dUTP nick-end labeling) reaction cocktail (In situ Cell Death Detection System; Roche Diagnostic Corporation, Indianapolis, IN), at 37°C for 1 hour in the dark. Positive (exposed to DNase I) and negative controls (no DNase I exposure) were included in each analysis. After an extensive wash in PBS, blastocysts were mounted in 4', 6-diamidino-2-phenylindole (DAPI) containing Vectashield antibleaching solution (Vector Labs, Burlingame, CA). The mounting medium drops were covered with a cover slide, and the edges were sealed with clear nail polish and stored at -20°C in the dark for analysis by confocal microscope (22).

### Confocal imaging and DNA Integrity Index

Specimens were excited at 364 nm (UV) for DAPI and 488 nm for fluorescein isothiocyanate-conjugated TUNEL, and images were collected with a Leica TCS-SP2 laser scanning spectral confocal microscope (Leica Microsystems, Heidelberg, GmbH) using an HCX Plan Apo 63X, 1.32 NA, oil immersion objective at zoom 2. The emitted fluorescence from each of the two labels was detected with two separate photomultiplier detectors with the spectrophotometer slits set for 400 to 490 and 500 to 550, respectively. Sequential images were collected at each level of the specimen along the z-axis of the sample with a step size of 1 to 3 μm. A projection of the three-dimensional stack of collected images was then created using Leica software. Volocity 64 software (Improvision, Lexington, MA) was used to reconstruct the 3D image of the blastocysts, and individual blastomeres were labeled as TUNEL<sup>+ve</sup> or TUNEL<sup>-ve</sup> and counted. The DNA integrity index was calculated for each individual blastocyst as the percentage of TUNEL<sup>-ve</sup>/total number of blastomeres (22). Figure 1 shows overlay images taken by confocal microscopy of blastocysts in the different experimental groups.

### Statistical Analysis

Total number of blastomeres and DNA integrity indices were summarized within study groups numerically using mean and standard deviation. DNA integrity indices within groups also were displayed along with box plots showing 10th and 90th percentiles, 25th and 75th percentiles and medians. Compar-

isons of groups with respect to the mean and standard deviation of the DNA integrity indices in each group were performed using the Wilcoxon signed rank test, with *P* values reported from pairwise group tests. Analyses and graphing were performed using R version ([www.R-project.org](http://www.R-project.org)) (22).

## RESULTS

### Experiment 1: Effect of Assisted Hatching of Nonexpanded Blastocysts on Postwarming DNA Integrity

We evaluated the effect of vitrification of nonexpanded blastocysts on DNA integrity of individual blastomeres, as well as the effect of assisted hatching before vitrification. Vitrification of nonexpanded blastocysts resulted in DNA damage with a decreased DNA integrity index compared with fresh control (*P*=.006). Assisted hatching of nonexpanded blastocysts significantly improved the postwarming DNA integrity of blastomeres (*P*<.001). Assisted hatching of nonexpanded blastocysts yielded blastocysts that were almost similar to the fresh control (*P*=.98) (Table 1 and Fig. 2A).

### Experiment 2: Effect of Blastocle Aspiration of Expanded Blastocysts on Postwarming DNA Integrity

This experiment evaluated the effect of vitrification of expanded blastocysts on the blastocysts' DNA integrity index, as well as the effect of blastocle aspiration before vitrification. Vitrification of expanded blastocysts decreased the integrity index of blastocysts compared with fresh control (*P*<.001). Blastocle aspiration of expanded blastocysts significantly improved the postwarming integrity index (*P*=.001) compared with nonintervention vitrification. However, even after blastocle aspiration, significant decrease in the integrity index was seen after vitrification when compared with fresh control (*P*=.003) (Table 2 and Fig. 2B).

Blastocysts with blastocle aspirated before vitrification all partially or totally reexpanded within 4 hours of incubation.

### Experiment 3: Effect of Spontaneous Hatching on Postwarming DNA Integrity

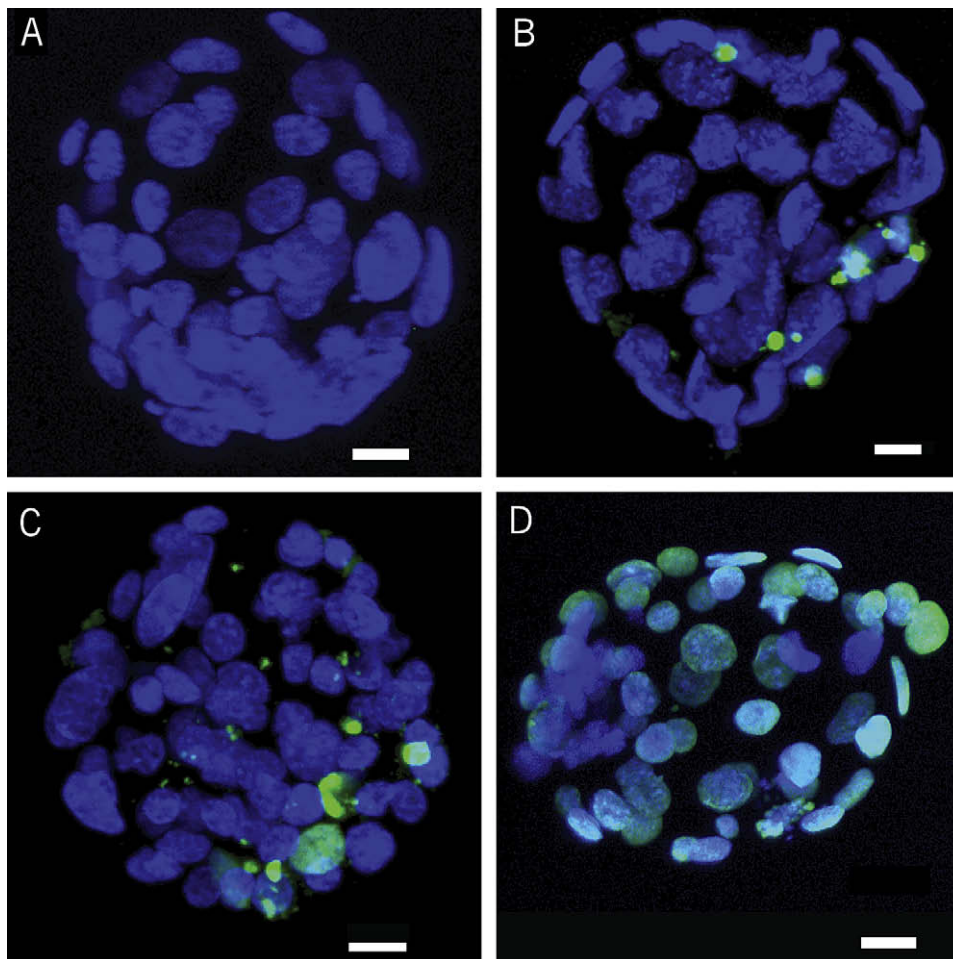
This experiment evaluated the effect of spontaneous hatching before vitrification on the blastocyst DNA integrity index. Spontaneous hatching of expanded blastocysts significantly improved the postwarming DNA integrity index (*P*=.012) compared with vitrified expanded blastocysts with intact zona. Spontaneously hatched blastocysts still exhibit lower DNA integrity index following vitrification compared with fresh control (*P*=.001) (Table 2 and Fig. 2B).

### Evaluation of Spontaneous Hatching versus Blastocle Aspiration of Expanded Blastocysts before Vitrification

We compared the effect of active previtrification intervention (blastocle aspiration) versus passive, noninvasive intervention (spontaneous hatching) on the DNA integrity index of expanded blastocysts. Vitrification, after both active and passive previtrification interventions still resulted in significant

## FIGURE 1

Confocal images of different blastocysts, stained with DAPI and TUNEL for DNA integrity showing: (A) Control nonexpanded blastocyst with no DNA damage. (B) Expanded spontaneously hatched vitrified blastocyst showing slight DNA damage. (C) Expanded vitrified blastocyst showing more DNA damage. (D) Positive control showing extensive DNA damage. All blastomeres stained blue with DAPI, whereas those showing DNA fragmentation showed a superimposed green staining. Scale bar = 10  $\mu\text{m}$ .



Kader. Blastocyst previtrification interventions. *Fertil Steril* 2009.

decrease in the DNA integrity index with values of  $P=.003$  and  $.001$ , respectively, compared with fresh control. However, the two interventions significantly decreased the DNA damage resulting from vitrification with significant improvement in the DNA integrity index ( $P<.001$ ) for blastocle aspiration and a value of  $P=.012$  when compared with vitrification without intervention. The two intervention techniques were comparable with no statistical difference ( $P=.35$ ) with regard to the DNA integrity index (Table 2 and Fig. 2B).

#### Vitrification of Expanded versus Nonexpanded Blastocysts

Nonexpanded vitrified blastocysts resulted in a better DNA integrity index ( $84.36 \pm 8.76$ ) compared with expanded ( $77.61 \pm 16.65$ ) blastocysts without any interventions. However, these differences were not statistically significant.

#### DISCUSSION

Our study showed that assisted hatching of nonexpanded blastocysts as well as spontaneous hatching of expanded blastocysts before vitrification both significantly improve the DNA integrity of the blastocysts when compared with vitrification of similar blastocysts. This demonstrates that the breach in the integrity of the zona would minimize the effect of the zona being a barrier for proper cryoprotectant exposure and dehydration of the blastocyst.

In the only study that attempted to clinically evaluate this concept, Zech et al. (12) have studied the effect of previtrification assisted hatching and spontaneous hatching of human blastocysts for their impact on the outcome of transfers to patients who previously failed at least one cycle of assisted reproduction. They demonstrated a nonsignificant

**TABLE 1****Effect of assisted hatching of nonexpanded blastocysts on postwarming DNA integrity.**

Parameter	Nonexpanded blastocysts		
	Fresh	Vitrified	
	Control (n = 13)	Nonhatched (n = 17)	Assistedly hatched (n = 16)
Total blastomeres (mean ± SD)	33.15 ± 14.09	39.24 ± 11.33	39.00 ± 12.67
Survival	100%	100%	100%
DNA integrity Index (%) (mean ± SD)	93.94 ± 6.64	84.36 ± 8.76 ( $P = .006$ ) <sup>a</sup>	94.63 ± 5.10 ( $P = .98$ ) <sup>a</sup> ( $P < .001$ ) <sup>b</sup>

Note:  $P < .05$  was considered to be significant using Wilcoxon signed rank test.

<sup>a</sup> Comparing fresh controls with vitrified nonhatched and assistedly hatched blastocysts.

<sup>b</sup> Comparing vitrified nonhatched and assistedly hatched blastocysts.

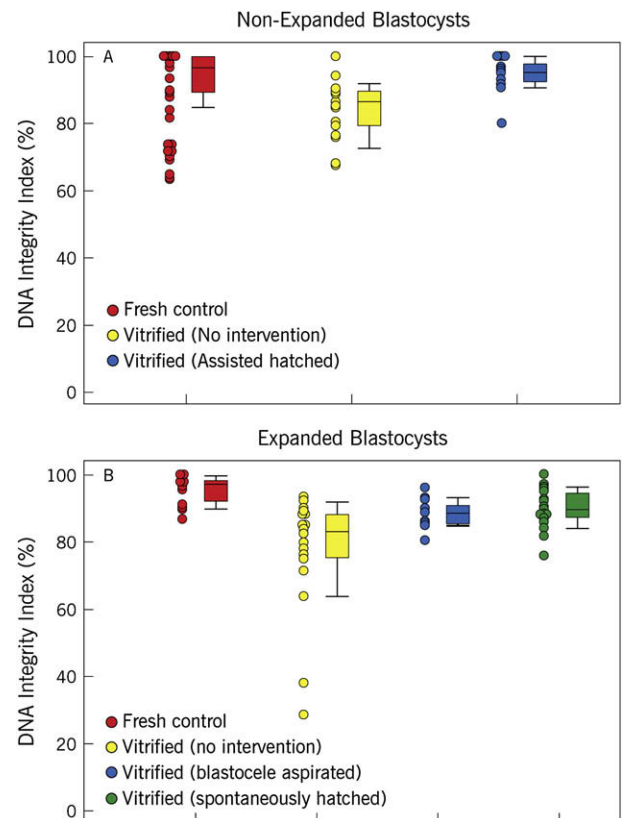
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increase in the embryo survival rate in the nonexpanded blastocysts from 73% to 78%. Survival increased from 55% to 81% in the expanded blastocysts. Nonexpanded blastocysts survival was low in their study; however, it was much lower in the expanded blastocysts. We have demonstrated higher survival rates (100% nonexpanded hatched or nonhatched and expanded hatched, 90.9% in expanded intact blastocysts). This may be because of: [1] different vitrification protocols; [2] the fact that mice blastocysts are smaller in size, have less number of cells, can better tolerate freezing injury; [3] the limited sample size. However, our findings on the DNA integrity index confirmed a significant beneficial effect of opening the zona in the expanded and nonexpanded blastocysts. With the increased vulnerability of the expanded blastocysts, it is possible that in a clinical situation, the beneficial effect of performing the zonal hatching can be more significant than when applied to nonexpanded blastocysts, which are less affected by vitrification as described by Zech et al. (12). We find it interesting in our study that only the assistedly hatched vitrified nonexpanded mouse blastocysts had a DNA integrity index that was not statistically different than fresh control. Lack of similar results with collapsed or hatched expanded blastocysts may denote that the blastocoele expansion may not be the only factor that increases the vulnerability of the mouse blastocyst to vitrification. Other factors such as culture extension may have a role in the expanded blastocysts' vulnerability to vitrification.

Using mouse embryos, Zhu et al. (13) and Pribenszky et al. (2003) evaluated the vitrification of spontaneously hatched blastocysts. Both investigators concluded the possibility of vitrifying the hatched blastocysts using glycerol- (13) or ethylene glycol (EG)-based (23) vitrification solutions. Zhu et al. (13) achieved a 65% survival rate when doing a one-step vitrification method, which increased to 89% to 94% when a two-step method was applied. The use of two-step

**FIGURE 2**

DNA integrity index in the nonexpanded (A) and expanded blastocysts (B) with different previtrification interventions compared with fresh control and vitrification with no intervention.



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TABLE 2

**Effect of blastocele aspiration and spontaneous hatching on expanded blastocysts postwarming DNA integrity.**

Parameter	Expanded blastocysts			
	Fresh	Vitrified		
	Control (n = 14)	No intervention (n = 22)	Blastocele aspirated (n = 22)	Spontaneously hatched (n = 12)
Total blastomeres mean $\pm$ SD	48.00 $\pm$ 15.66	46.91 $\pm$ 15.05	39.95 $\pm$ 12.84	62.00 $\pm$ 13.10
Survival	100%	90.9%	100%	100%
DNA integrity index mean $\pm$ SD	95.47 $\pm$ 4.25	77.61 $\pm$ 16.65 ( $P < .001$ ) <sup>a</sup>	90.08 $\pm$ 5.59 ( $P = .003$ ) <sup>a</sup> ( $P = .001$ ) <sup>b</sup>	88.45 $\pm$ 4.35 ( $P = .001$ ) <sup>a</sup> ( $P < .012$ ) <sup>c</sup> ( $P = .35$ ) <sup>d</sup>

Note:  $P < .05$  was considered to be significant using Wilcoxon signed rank test.

<sup>a</sup> Comparing fresh controls with vitrified with no intervention, blastocele aspirated, vitrified, and spontaneously hatched vitrified blastocysts.

<sup>b</sup> Comparing vitrified with no intervention and aspirated vitrified blastocysts.

<sup>c</sup> Comparing vitrified with no intervention and spontaneously hatched blastocysts.

<sup>d</sup> Comparing blastocele aspirated vitrified and spontaneously hatched vitrified blastocysts.

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vitrification on mice blastocysts explains the high survival rate in this study as well as in our study where we used different cryoprotectants and concentrations that are in contemporary clinical practice. However, none of these two studies examined any added value of vitrifying the hatched blastocysts. In our study, we found that spontaneous hatching before vitrification of expanded blastocysts significantly improved the DNA integrity compared with vitrification of the expanded nonhatched blastocysts. This could be considered as a watchful noninvasive approach to improve the outcome of expanded blastocysts vitrification.

One of the potential causes of the increased vulnerability of expanded blastocysts is the large blastocelic volume and its more difficult dehydration. We have evaluated collapsing the blastocele of expanded blastocysts by aspiration. Our results showed a significant improvement in the DNA integrity index of expanded blastocysts when the blastocele was aspirated before the vitrification procedure. All blastocysts were viable and expanding after warming and incubation for 4 hours. Different reports tested collapsing the blastocele through different approaches. Collapsing the blastocele using a microneedle puncture before vitrification was first reported by Son et al. (19). They reported a survival rate of 90% and an implantation rate of 29% after transferring human blastocysts using this technique. Hiraoka et al. (18) reduced the blastocelic volume of day 5 and day 6 human blastocysts by micropipetting before blastocysts vitrification. The reexpansion of the vitrified blastocysts was 98%, whereas implantation rate was 33%. Unfortunately, the study did not include any prospective or retrospective controls to assess exactly the added value of the technique. Nevertheless, it did emphasize the

safety and effectiveness of the technique when applied to human embryos.

Applying a similar technique to the one we adopted in our study to collapse the blastocyst, Chen et al. (11) collapsed the blastocele in mouse blastocysts by microsuction before vitrification and evaluated the postwarming survival and pregnancy rates. They noted that nonexpanded blastocysts had better postwarming survival (80%) compared with the expanded ones (59%). We noted a significant difference in the DNA integrity index between the nonexpanded and expanded groups. However, our results show higher postwarming survival of 100% for nonexpanded and 90.9% for expanded blastocysts. This may be related to the different vitrification protocol we used, the preselection of good-quality embryos for study enrolment and to the limited number of embryos in the study groups. The same group also concluded that microsuction greatly improved the postwarming survival, with nonexpanded blastocyst survival increasing to 92%, and expanded blastocyst survival increasing to 89%. Our results on the DNA integrity index are in agreement with these findings of improvement in the postwarming outcome with the blastocele collapse before vitrification.

In a large clinical trial, Mukaida et al. (17) evaluated reducing the blastocelic volume of human blastocysts before vitrification by piercing the blastocele using a microneedle or a laser pulse. These investigators reported a significant improvement in their results with the survival rate increasing from 85% to 97.2% and the pregnancy rate increasing from 34.1% to 60.2% in comparison to a retrospective cohort. Our results again came in harmony with those findings

emphasizing the value of blastocele evacuation before vitrification. Using a prospective design and testing the blastocele collapse by microsuction before vitrification only on expanded blastocysts that are more vulnerable to vitrification, we were able to demonstrate significant improvement in the DNA integrity index postwarming compared with vitrification of expanded blastocysts with no intervention. The survival of expanded blastocysts with no intervention in our study was 90.9% compared with 100% when the blastocele was aspirated before vitrification.

Expanded blastocysts seem to be more vulnerable to damage by vitrification and this was invariably shown in the different studies discussed as well as in our study. We have compared the noninvasive technique of watchful waiting for spontaneous hatching before vitrification to the blastocele aspiration that could be considered the most invasive technique of previtrification intervention. Both techniques significantly improved the DNA integrity of the expanded blastocysts after vitrification. However, the two techniques were not significantly different from each other, and both were still showing significantly less DNA integrity index compared with fresh control. These findings suggest that the expanded blastocysts are highly vulnerable to vitrification. This vulnerability can be lessened when the blastocele fluid is removed or the ZP is breached. However, despite the intervention, the expanded blastocyst may remain more vulnerable to vitrification, as this may be related to their more prolonged in vitro culturing.

All blastocysts that were vitrified in our study showed deterioration in their DNA integrity index, which was statistically significant when compared with fresh control with the exception of the assisted hatched nonexpanded blastocysts, which came similar to fresh control. This may suggest that vitrifying-assisted hatched blastocysts may be the best option when trying to vitrify blastocysts. Although we cannot exactly predict the clinical significance of this finding, we can anticipate it will be true when using human embryos. All clinical results previously discussed were showing nonexpanded blastocysts vitrification giving better results than expanded. They also showed that hatching whether assisted or spontaneous is improving the outcome of vitrified blastocysts.

Our study aimed to provide basic evidence of improvement with the application of various previtrification interventions to blastocysts. Interpretation of our results was primarily based on the evaluation of the DNA integrity of the blastocyst's individual blastomeres represented by the integrity index. These changes may not be morphologically evident unless the damage is extensive. Nevertheless, sublethal embryo damage might still reflect on the embryo's potential to implant, grow into a healthy pregnancy, and finally produce a live birth.

From our study, we can derive several conclusions that may provide some clinical insight: [1] vitrification, although an extremely promising technique for blastocyst cryopreservation, still induces some DNA damage to the blastomeres, both in expanded and, to a smaller extent, in nonexpanded

blastocysts; [2] assisted hatching of nonexpanded blastocysts or blastocele aspiration of expanded blastocysts are two techniques that can be adopted to significantly minimize the DNA damage induced by vitrification; [3] spontaneous hatching can be used as a noninvasive alternative to blastocele aspiration before vitrification, resulting in vitrification outcomes similar to those achieved with expanded blastocysts; and [4] assisted hatched nonexpanded blastocysts seem to be the most appropriate choice if blastocysts are to be vitrified, as they were comparable to fresh control embryos.

Our results are in agreement with previous findings on previtrification interventions in mouse as well as in human blastocysts. We investigated and analyzed for the first time the different previtrification interventions when applied on either expanded or nonexpanded blastocysts at a cellular level using the DNA integrity index. Our study, together with the few clinical trials that have assessed previtrification interventions, may provide a foundation to justify the implementation of such techniques and suggest an algorithm of action to clinical practices adopting or considering blastocyst vitrification. The mouse blastocyst model that we used is expected to be less sensitive to damage during vitrification, and therefore, we would expect that our results may be even magnified when applied on human blastocysts. Although all the techniques described have been clinically tested by other groups, the clinical implementation of our specific findings and recommendations needs to be further evaluated for their clinical value in future clinical trials.

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