

Effect of varying equilibration time in a two-step vitrification method on the post-warming DNA integrity of mouse blastocysts

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Objective: To assess the effect of equilibration time on the DNA integrity of vitrified-warmed mouse blastocysts.

Design: Prospective in vitro study.

Setting: Embryology research laboratory.

Intervention(s): Mouse nonexpanded blastocysts (NEB) (n = 54) and expanded blastocysts (EB) (n = 56) were vitrified using cryotips. Blastocysts were vitrified using a two-step media protocol, with a first (equilibration) step of 4 minutes [NEB (n = 20), EB (n = 24)], 8 minutes [NEB (n = 17), EB (n = 16)], or 15 minutes: [NEB (n = 17), EB (n = 16)]. Control samples were fresh NEB (n = 17) and EB (n = 18) blastocysts.

Main Outcome Measure(s): DNA integrity index (DII) using terminal deoxynucleotide transferase-mediated dUTP nick-end labeling, confocal imaging, and viability.

Result(s): 1) The DII of the vitrified warmed NEB and EB improved significantly with 8-minute equilibration protocol compared with 4 minutes. 2) The DII of the EB significantly decreased with 15-minute equilibration protocol compared with 4 or 8 minutes. 3) The DII in the NEB significantly improved with 8- and 15-minute equilibration protocols compared with 4 minutes, with no significant difference between 8 or 15 minutes.

Conclusion(s): Vitrification with an 8-minute equilibration step improved DII of the NEB and EB after warming (Fertil Steril® 2009; ■: ■–■. ©2009 by American Society for Reproductive Medicine.)

Key Words: Blastocyst, vitrification, cryopreservation, media protocol, TUNEL, apoptotic index, DNA integrity index

The blastocyst characteristics (multicellularity, presence of blastocoele with high water content) make it difficult to accomplish the required level of dehydration and high viscosity evenly in all blastomeres and blastocoele as needed for proper vitrification (1). This may result in postwarming increase in apoptosis (2).

The optimum dehydration and high viscosity levels can be achieved by exposing the blastocysts to high concentrations of permeating cryoprotectants, usually in addition to an extracellular dehydrating agent such as sucrose. This could be done in one or multiple steps. One-step protocol was used in bovine blastocyst vitrification using 1–2 minutes' exposure to different cryoprotectants at high concentration (3). The inclusion of an equilibration step improved survival rates (4). Therefore, the current blastocyst vitrification protocols usually consist of 2 steps.

The first step typically entails the use of relatively low concentrations of cryoprotectants—7.5% dimethylsulfoxide (DMSO) and 7.5% ethylene glycol (EG)—for 2–5 minutes, followed by an ultra-

short (30–90 seconds') exposure to a second medium that contains a higher cryoprotectant concentration (usually double the initial concentration) as well as a dehydrating agent (commonly, a disaccharide, such as sucrose) (5–8). The objective of this stepwise exposure is to avoid toxicity and osmotic shock.

Different modifications to the two-step vitrification protocol would aim to strike a critical balance between achieving high level of dehydration and high viscosity while avoiding toxicity. These changes would include changes in types of cryoprotectants, concentrations, or exposure time at any step. Prolongation of the equilibration time in the first step of a two-step protocol using current standard concentrations of cryoprotectant for up to 7 minutes has shown adequate blastocyst survival and pregnancy rates, denoting nontoxicity (5). However, increasing the concentrations of cryoprotectants in the first step to >7.5% DMSO and EG with exposure time of 10 minutes resulted in reduced survival and hatching rates, suggesting the possibility of an increasing toxicity threshold (4, 9). Therefore, we hypothesized that prolongation of the contact time only in the first step of a standard two-step media vitrification protocol may be a safe approach to maximize delivery of the cryoprotectants to the blastocyst, thereby raising the likelihood of proper vitrification of all blastomeres and reducing the likelihood of damage as a result of ice crystal formation.

The objective of the present study was to determine whether prolongation of the first equilibration step from 4 minute' contact time to 8 or 15 minutes can improve the postwarming quality of expanded and nonexpanded blastocysts, based on the DNA integrity of warmed blastocysts.

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A.K. has nothing to disclose. A.C. has nothing to disclose. R.S. has nothing to disclose. T.F. has nothing to disclose. A.A. has nothing to disclose. Reprint requests: Ashok Agarwal, Ph.D., H.C.L.D., Professor and Director, 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, Ohio 44195 (FAX: 216-445-6049; E-mail: Agarwaa@ccf.org).

MATERIALS AND METHODS

Embryos

Commercially available cryopreserved four-cell mouse embryos (Embryotech Laboratories, Wilmington, MA) were thawed and incubated as previously described (2). Embryo development was monitored after 24, 48, and 72 hours of incubation. Embryos showing signs of fragmentation and/or delayed or accelerated development were discarded (2).

Nonexpanded blastocysts (NEB) were defined as embryos with blastocoele volume <50% of the total volume of the blastocyst, and expanded blastocysts (EB) were defined as embryos with blastocoele volume >50% of the total volume (2). In total, 145 blastocysts were included in the study. Three vitrification protocols were used, with a fixed second step of 90 seconds' exposure and a first step (equilibration) exposure time of either 4, 8, or 15 minutes. Fresh NEB and EB were used as controls. The blastocysts were distributed as follows:

1. NEB, fresh (n = 17).
2. NEB, 4-minute equilibration vitrification protocol (n = 20).
3. NEB, 8-minute equilibration vitrification protocol (n = 17).
4. NEB 15-minute equilibration vitrification protocol (n = 17).
5. EB, fresh (n = 18).
6. EB, 4-minute equilibration vitrification protocol (n = 24).
7. EB, 8-minute equilibration vitrification protocol (n = 16).
8. EB 15-minute vitrification protocol (n = 16).

Vitrification and Warming

Vitrification was performed using the Irvine vitrification kit with a cryotip loading device (Irvine Scientific, Santa Ana, CA). Blastocysts were placed in a 20- μ L droplet of equilibration solution (7.5% DMSO, 7.5% EG, and 20% serum substitute supplement [SSS]) for either 4, 8, or 15 minutes. After equilibration, the blastocysts were propagated into three subsequent 20- μ L droplets of vitrification solution (15% DMSO, 15% EG, and 20% SSS) for 5 seconds, 5 seconds, and 60 seconds in the first, second and third drops, respectively. The blastocysts were loaded in 1 μ L vitrification solution in the cryotips. The devices were heat sealed and immediately plunged into liquid nitrogen.

Two to three blastocysts were loaded per cryotip. A total of 43 cryotips were used in the study.

After 24 hours, the cryotips were removed from the liquid nitrogen and placed in a 37°C water bath for 3 seconds. The cryotip was cut at one end, and the embryos were placed in thawing medium (HEPES-buffered Medium 199, gentamicin sulfate 35 μ g/mL, 1 mol/L sucrose, and 20% SSS) for 1 minute. This was followed by 4 minutes in an incubation dilution media (HEPES-buffered Medium 199, gentamicin sulfate 35 μ g/mL, 0.5 mol/L sucrose, and 20% SSS) and 9 minutes in a washing solution (HEPES-buffered Medium 199, gentamicin sulfate 35 μ g/mL, and 20% SSS). The embryos were finally incubated in human tubal fluid medium in 5% CO₂ for 4 hours before any further processing. Blastocyst survival was measured by the partial or complete reexpansion of the blastocyst after warming and incubation. (2).

DNA Damage by TUNEL Staining

Blastocysts were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4) at room temperature for 1 hour, followed by three washings in PBS with 0.3% polyvinylpyrrolidone (PVP). They were permeabilized with 0.5% Triton X-100 for 2 minutes on ice. After three washes in the PBS/PVP solution, the blastocysts were incubated in terminal deoxynucleotide transferase-mediated dUTP nick-end labeling (TUNEL) reaction solution (Roche Diagnostics Corporation, Indianapolis, IN) at 37°C for 1 hour in the dark, washed in PBS, and mounted in 4',6-diamidino-2-phenylindole (DAPI) containing Vectashield (Vector Labs, Burlingame, CA). The blastocysts in the mounting drops were covered with a cover slip. The edges were sealed with clear nail polish and stored at -20°C in the dark for confocal imaging (2).

Confocal Imaging

We used a Leica TCS-SP2 laser scanning spectral confocal microscope (Leica Microsystems, Heidelberg, Germany) with HCX Plan Apo 63X, 1.32 NA, oil immersion objective to visualize the DNA damage. The DAPI and TUNEL stains were visualized at excitation wavelengths of 364 nm and 488 nm, respectively. Sequential images were collected along the z-axis at 1–2- μ m intervals. Leica software was used to collect the stack of images generated by the confocal scanning. Figure 1 shows the snapshots of the collected stacks of one embryo in two- or three-dimensional reconstructions, with separate and merged blue and green fluorescent channels (2).

DNA Integrity Index

Velocity 64 software (Improvision, Lexington, MA) was used to analyze and count all blastomeres in each blastocyst. The software uses the stack of images created by the Leica software to reconstruct the blastocyst. With this software, the blastocysts can be viewed layer by layer. Going back and forth in between layers allows an accurate counting of the blastomeres, thereby avoiding any repetition and excluding any artifacts from being counted. Individual blastomeres were tagged as TUNEL⁺ (green) or TUNEL⁻ (blue) and counted using the software. The DNA integrity index (DII) was finally calculated for each individual blastocyst as the percentage of TUNEL⁺/total number of blastomeres (2). Figure 2 describes the counting technique using the software.

Statistical Analysis

Analysis of the DII in all groups was performed using Wilcoxon signed rank test with *P* values from the paired *t* test. R statistical software version 2.3.1 was used (R Development Core Team, 2006) (2). *P*<.05 was considered to be statistically significant.

RESULTS

Table 1 summarizes the DII results in the different blastocyst groups. Figure 3 shows a red line connecting the median DIIs in the NEB groups and a blue line connecting the median DIIs in the EB groups to demonstrate changes with increasing equilibration time before vitrification.

Vitrification of the Nonexpanded Blastocysts

DNA integrity index In the 4-minute equilibration vitrification protocol, the DII of the NEB was significantly lower than the DII in the fresh control samples (*P*=.013). When the equilibration time was prolonged from 4 to 8 minutes, there was a significant improvement in the DII (*P*=.043). Further extension of the exposure time from 8 to 15 minutes did not significantly improve the DII (*P*=.5).

The DII of the vitrified NEB was similar to that of the fresh control samples at 8 and 15 minutes' equilibration times (*P*=.56 and *P*=.82, respectively). The DII was significantly higher in the NEB after 15 minutes of equilibration than after 4 minutes (*P*=.001).

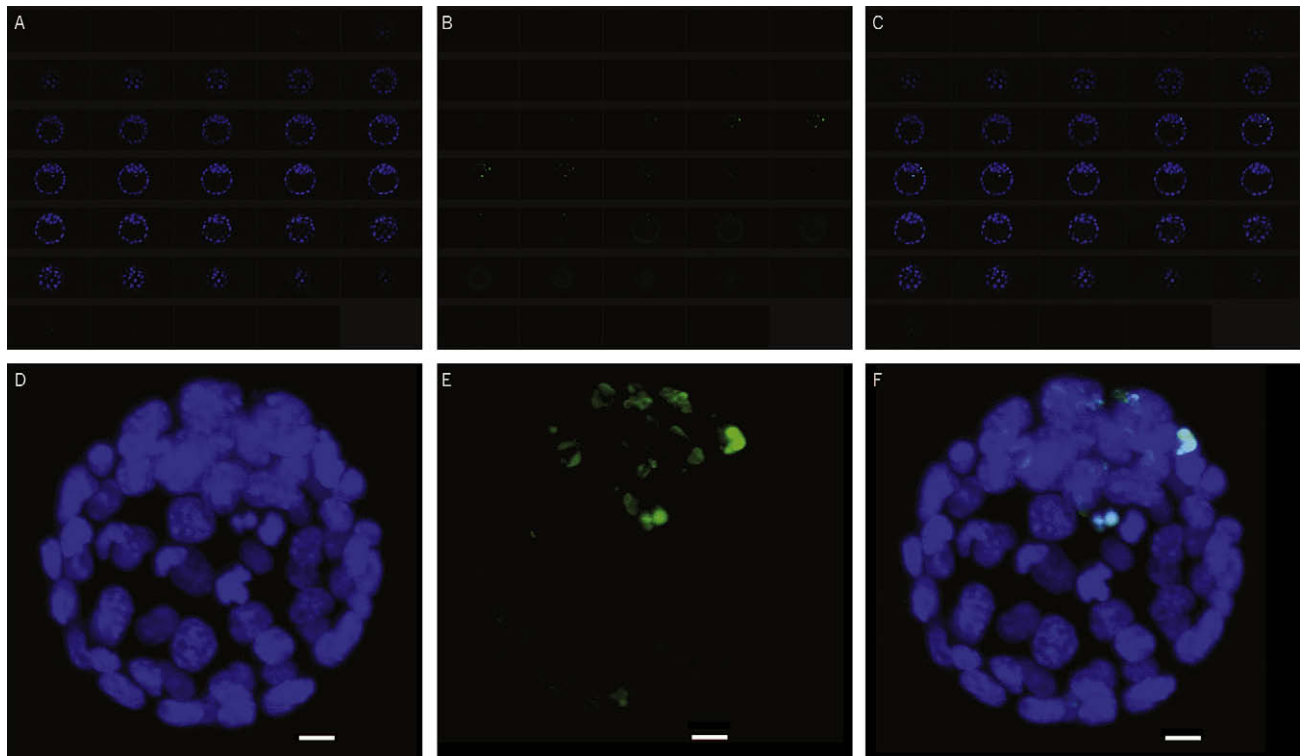
Viability The survival and reexpansion rates were 100% for all of the NEB vitrification protocols.

Vitrification of Expanded Blastocysts

DNA integrity index In the 4-minute equilibration protocol, the DII of the EB was significantly lower than the DII in the fresh control samples (*P*<.001). Similarly to the NEB, when the equilibration time was prolonged from 4 to 8 minutes, there was a significant improvement in the DII (*P*<.001). However, further extension of the exposure time from 8 to 15 minutes resulted in a significant deterioration of the DII of the EB (*P*<.001). The DII of the EB after 8 minutes was similar to that of the fresh control samples (*P*=.2). However, the EB with 15 minutes' equilibration time had

FIGURE 1

Imaging with confocal Leica software. (A) Different sections of the blastocyst in the blue fluorescence channel. (B) Different sections of the blastocyst in the green fluorescence channel. (C) Different sections of the blastocyst in both the blue and the green fluorescence channels merged. (D) Overlay image of the blastocysts in the blue fluorescence channel. (E) Overlay image of the blastocysts in the green fluorescence channel. (F) Overlay image of the blastocysts in both the blue and the green fluorescence channels merged. Scale bar = 10 μm .



Kader. Effect of equilibration time on blastocyst vitrification. *Fertil Steril* 2009.

a significantly lower DII than the fresh control samples. The DII of the EB was significantly lower after 15 minutes' equilibration than after 4 minutes ($P=.042$).

Viability The survival and reexpansion rates were 91.6% at 4 minutes, 100% at 8 minutes, and 93.7% at 15 minutes of equilibration.

DISCUSSION

The main objective in this study was to determine whether prolongation of the first equilibration step in the vitrification of mouse blastocysts from 4-minute contact time to 8 or 15 minutes can improve the postwarming quality of EB and NEB, based on their DII. Overall, we found that extending the equilibration time from 4 to 8 minutes significantly improved the DII of the NEB. Further extension of the exposure time to 15 minutes failed to provide any significant additional improvement. For the EB, extension of the equilibration media from 4 to 8 minutes also significantly improved the DII whereas further extension of the equilibration time to 15 minutes resulted in a significant deterioration of the DII compared with the 4-minute protocol.

Adequate intracellular delivery of cryoprotectants and sufficient dehydration are the main objectives for vitrification media protocols. Researchers have suggested modifying these protocols to improve the outcome. One strategy was to increase the concentration of cryoprotectant in a one-step vitrification protocol to increase intracellular delivery. However, omission of the equilibration step

by Zhu et al. (10) appeared to be a key factor in poor survival of bovine blastocysts. Inclusion of an equilibration step improves survival rates most likely by reducing osmotic shock and toxicity (11).

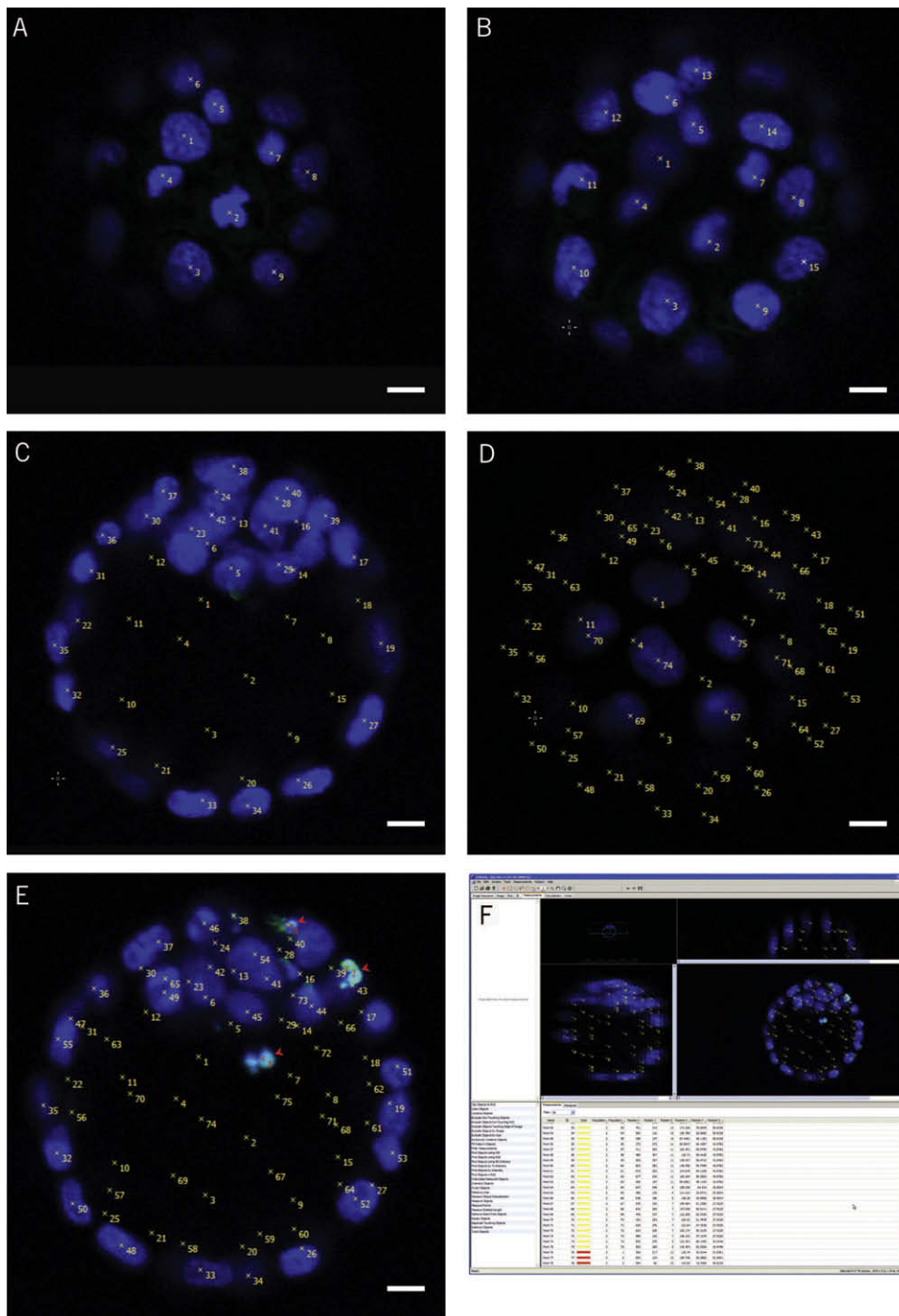
To investigate the effects of using more concentrated vitrification solutions in two-step vitrification media protocols, Cuello et al. (12) vitrified expanded porcine blastocysts in final vitrification solutions up to 17% EG + 17% DMSO. The highest survival rate was associated with the highest concentration of vitrification solution. This result seemed to imply that the use of more concentrated vitrification solutions increases intracellular cryoprotectant concentration for proper vitrification without breaching toxicity thresholds. However, much higher concentrations used by Cseh et al. (4) (47.4% glycerol + 6% propylene glycol) resulted in poor murine blastocyst survival, denoting a possible toxicity. The EB suffered more damage than the NEB, possibly owing to increased vulnerability at this developmental stage to the toxic effects of high media concentrations.

In another approach, Kaidi et al. (9) vitrified expanded bovine blastocysts using a three-step protocol. The blastocysts demonstrated a low hatching rate after warming, possibly owing to the high toxicity of the final cryoprotectant concentrations and prolonged exposure in the second step with high concentrations of the cryoprotectants.

We therefore opted to keep a two-step vitrification media protocol using the current standards of cryoprotectants and investigate the effect of prolonging the first step. In our experiment, we investigated

FIGURE 2

Calculating the DNA integrity index by counting the blastomeres using “Volocity 64” software. TUNEL⁻ blastomeres are blue; TUNEL⁺ blastomeres are green. (A) Start of counting at the superficial layer of the blastocyst. (B) Continuous counting at a deeper cross-section. (C) Continuous counting at an intermediate cross-section. (D) Counting at a bottom layer of the blastocyst. (E) Counting of the TUNEL⁺ cells, which are marked with a red arrow for better visualization. (F) Counting of TUNEL⁺ TUNEL⁻ blastomeres all gathered in the “Volocity 64” software with information on each blastomere location. Scale bar = 10 μ m.



Kader. Effect of equilibration time on blastocyst vitrification. Fertil Steril 2009.

TABLE 1

Comparison of the DNA integrity index of nonexpanded and expanded blastocysts following 4, 8, and 15 minutes' equilibration vitrification protocols.

Parameter	Nonexpanded blastocysts (n = 71)			
	Fresh (n = 17)	4-min equilibration (n = 20)	8-min equilibration (n = 17)	15-min equilibration (n = 17)
Total blastomeres (mean ± SD)	32.00 ± 12.71	38.75 ± 11.41	44.18 ± 10.10	37.53 ± 9.73
Survival, %	NA	100	100	100
DNA integrity index, % (mean ± SD)	94.13 ± 6.36	86.37 ± 9.51 <i>P</i> = .013*	93.03 ± 6.81 NS* <i>P</i> = .043**	95.40 ± 3.91 NS* NS***
Parameter	Expanded blastocysts (n = 74)			
	Fresh (n = 18)	4 min equilibration (n = 24)	8 min equilibration (n = 16)	15 min equilibration (n = 16)
Total blastomeres (mean ± SD)	46.33 ± 14.17	46.42 ± 14.49	59.69 ± 6.61	44.44 ± 14.89
Survival, %	NA	91.66	100	93.75%
DNA integrity index, %	94.61 ± 4.75	78.76 ± 16.38 <i>P</i> < .001*	96.40 ± 3.82 NS* <i>P</i> < .001**	73.87 ± 10.88 <i>P</i> < .001* <i>P</i> = .042** <i>P</i> < .001***

Note: *P* < .05 was considered to be significant by Wilcoxon signed rank test.

* Compared with fresh.

** Compared with 4 minutes.

*** Compared with 8 minutes.

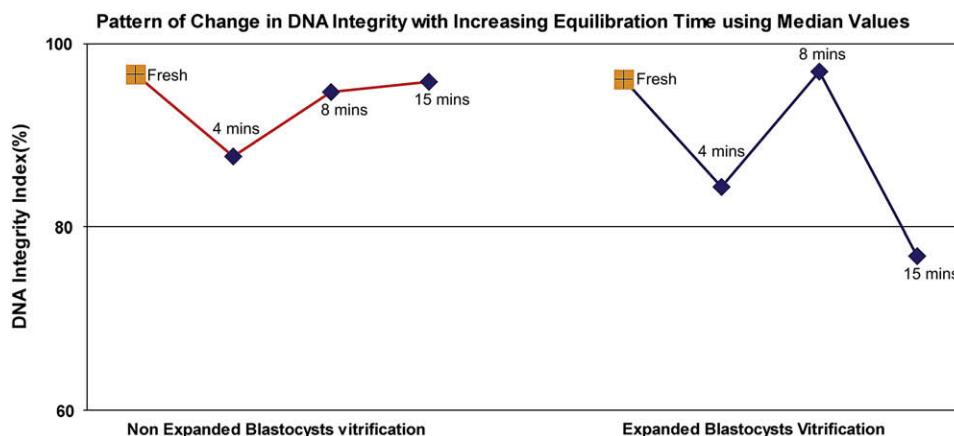
Kader. Effect of equilibration time on blastocyst vitrification. Fertil Steril 2009.

the effect of extending the equilibration time in the first step of a media protocol from 4 to 8 or 15 minutes, hypothesizing that prolonging exposure may garner outcome improvement. At the same time, we maintained the standard ultrashort second step of the vitrification media protocol to avoid the risk of inducing toxicity or osmotic damage at this critical step.

Other investigators have found superior survival, implantation, and pregnancy rates with vitrified NEB compared with EB (5, 6). Increasing the concentration of cryoprotectants in the media also previously led to poor EB survival (4). This may be related to a vulnerability to highly concentrated cryoprotectants that progresses with blastocyst development (2).

FIGURE 3

Median DNA integrity indices in the fresh and vitrified nonexpanded and expanded blastocysts after 4, 8, or 15 minutes' equilibration time. The red line connects the median DNA integrity indices of the nonexpanded blastocysts. The blue line connects the median DNA integrity indices of the expanded blastocysts.



Kader. Effect of equilibration time on blastocyst vitrification. Fertil Steril 2009.

Two opposing mechanisms seem to coexist and affect the outcome of blastocyst vitrification: the permeation of the cryoprotectants and the toxicity of these agents (2, 13). Although extending the exposure time can improve the cryoprotection, it increases the risks of toxicity. Therefore, an ideal exposure time would be the longest time that can improve the cryoprotection and still avoids toxicity.

As blastocysts develop, both their blastocoele volume and the number of cells increase, which increases the need for maximal equilibration with cryoprotectants. At the same time, the increased metabolic activity of the dividing cells potentially increases their susceptibility to the toxic effect of cryoprotectants. These two risks become exaggerated in the EB, making it particularly challenging to improve cryoprotection while avoiding toxicity.

In the present study, extending the exposure time to 8 minutes improved the vitrification outcome of both the EB and the NEB. We could therefore conclude that an 8-minute exposure to the cryoprotectants was not toxic to any of the blastocyst stages. We could also

conclude that extending the exposure time to 15 minutes may have had a more toxic than cryoprotective effect. This was more evident in the EB, which were more vulnerable to toxicity. The toxic effect was greater than any possible beneficial effect from better cryoprotection. This resulted in significant deterioration of the DII. There was no significant effect in the DII in the NEB when the exposure time was extended from 8 to 15 minutes, probably denoting a balance between the cryoprotection and toxicity.

Although mouse blastocysts have different tolerances to cryopreservation than do human embryos, the mouse embryo is a suitable model for studying pathophysiologic events associated with vitrification (14, 15). The developmental potential of an embryo is directly proportional to its DII (16). Based on the three exposure times studied in the present experiment, the 8-minute exposure to the equilibration media seems to be the most appropriate as a unified protocol for the two blastocyst stages. However, further investigations to elucidate the clinical impact and significance of our basic findings would be required.

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