Evaluation of post-thaw DNA integrity of mouse blastocysts after ultrarapid and slow freezing

Amr Kader, M.D., Ashok Agarwal, Ph.D., H.C.L.D., Hussein Abdelrazik, M.D., Rakesh K. Sharma, Ph.D., Ali Ahmad, Ph.D., H.C.L.D., and Tommaso Falcone, M.D.

Objective: To evaluate the effect of vitrification and two other methods of slow cryopreservation on DNA integrity in expanded and nonexpanded blastocysts.

Design: Prospective in vitro study.

Setting: Tertiary care academic hospital.

Intervention(s): 1) Twenty-two expanded blastocysts (EB) and 17 nonexpanded blastocysts (NEB) vitrified in cryotips; 2) 15 EB and 16 NEB by slow freezing using propanediol; 3) 11 EB and 16 NEB by slow cryopreservation using glycerol; and 4) 14 EB and 13 NEB as fresh control samples.

Main Outcome Measure(s): DNA fragmentation by TUNEL and confocal imaging.

Result(s): Blastocysts slowly cryopreserved with glycerol showed DNA integrity of 94.76 ± 4.70% and 90.87 ± 6.16% for NEB and EB, respectively. Propanediol cryopreservation showed values of 72.63 ± 13.44% and 56.19 ± 25.49% and vitrification 84.36 ± 7.6% and 77.61 ± 16.65%, respectively, for the same groups. The NEB showed less DNA fragmentation than EB in all cryopreservation techniques, but this was significant only with slow freezing using propanediol.

Conclusion(s): All cryopreservation techniques induce DNA damage to blastocysts. Damage is maximal with propanediol and minimal with slow freezing using glycerol. The more expanded the blastocyst, the greater is the susceptibility to DNA damage during cryopreservation.

Key Words: Blastocysts, cryopreservation, vitrification, ultra-rapid freezing, apoptotic index, DNA integrity index, TUNEL, cryotip, confocal imaging

Surplus embryos resulting from stimulated ICSI or IVF cycles can now be cryopreserved, thereby avoiding the risks of multiple pregnancies (1). Embryo cryopreservation is a cost-effective and convenient method for future embryo transfer in the event of an unsuccessful initial transfer or a couple’s desire for more children. It minimizes the risks of ovarian hyperstimulation and allows cryopreservation of embryos for later transfer or for screening them for genetic diseases (2, 3).

With the improvements in extended embryo culture, transfer of blastocysts is now possible. Blastocysts invariably represent an embryo that has exhibited its developmental potentials in vitro. This natural selection is by far more reliable than selection based on morphology of earlier-stage embryos (4). This makes transfer of blastocysts a better option, especially for patients with previous implantation failure (5).

With the improvements in cryopreservation protocols, cryopreservation of blastocysts has become a readily available and reliable tool to preserve the embryos that have shown the best in vitro developmental potential (6). Glycerol has been the most widely used cryoprotectant for slow cryopreservation of blastocysts (7). Propanediol has been the main cryoprotectant used in most protocols of slow cryopreservation of early embryo stages in animals and humans for the last 2 decades (6). There are few reports on the use of propanediol as a cryoprotectant for blastocysts in humans or in animal models (8–13). Cryopreservation of blastocysts is challenging because of its inherent characteristics, such as: 1) slow permeation of the cryoprotectant into the blastocyst largely due to the multicellular shape; 2) zona pellucida (ZP) acting as a physiologic barrier to the permeation of the cryopreservation media; and 3) presence of the blastocele which might be inadequately dehydrated during cryopreservation. Therefore, there is an increased likelihood of damage by ice crystals from inadequate cryoprotection (14).

Vitrification prevents ice crystal formation with the ultra-rapid transformation from vegetative to solid state. This requires ultrarapid heat transfer rates. It also requires the cells to be adequately dehydrated and permeated with a high concentration of cryoprotectant (15). The degree of success of
vitrification is therefore expected to correlate with the fulfillment of the previously mentioned conditions. Ideally, vitrification can therefore be considered to be a more efficient alternative to slow cryopreservation of blastocysts (15). Clinical success after transfer of vitrified blastocysts has been reported (16). Vitrification allows flexibility and practicality, can be performed quickly (a few minutes), and eliminates the need for expensive/high maintenance equipments (15). Vitrification success is very sensitive to time and technique, however, and therefore it requires proper training and standardization to obtain reliable and reproducible results (17).

Apoptosis is the ultimate cellular response to suboptimal conditions and different kinds of stress that an embryo might encounter during its freeze-thaw cycle. Embryo viability and developmental potential are related to the ratio of relative number of intact to damaged blastomeres, known as apoptotic index. Lower-grade embryos show higher apoptotic index. The evaluation of DNA fragmentation by terminal deoxynucleotidyl transferase [TdT]–mediated dUTP nick-end labeling (TUNEL) has been used as a reliable method for detection of apoptosis in embryos (18).

Few reports are available on the changes in the apoptotic index after cryopreservation or vitrification of blastocysts (19). Therefore, the purpose of the present study was to compare the effect of vitrification and slow freezing with either glycerol or propanediol and study the alterations in blastocyst apoptotic index after thawing.

**MATERIALS AND METHODS**

**Embryos**

Cryopreserved 2-cell mouse embryos were purchased from Embryotech Laboratories (Wilmington, MA). Thawing was done by removing the straw from the liquid nitrogen and exposing to room temperature for 2 min. Each straw was bisected between the lower heat seal and the column of medium. By using the stylet, the contents of the straw were flushed as a single drop into a sterile culture dish containing prewarmed Quinn’s media (Sage In-Vitro Fertilization, Trumbull, CT).

After rinsing the embryos in the first dish, they were transferred to human tubal medium with 10% albumin (HTF) and incubated in 5% CO₂ at 37°C. Embryos were examined after 24, 48, and 72 h for development monitoring and embryo grading. Embryos showing signs of fragmentation or delayed development were excluded from the study.

**Embryo Grouping**

After assuring the quality of the embryos, they were stratified according to the degree of the blastocele expansion and age into 2 groups: 1) nonexpanded day 5 blastocysts: blastocele volume less than one-half of the total volume of the blastocyst; and 2) expanded day 6 blastocysts: blastocele volume more than one-half of the total volume of the blastocyst. Embryos of each group were randomly distributed into the different experimental groups or the control group.

**Vitrification**

Vitrification was done using vitrification medium (Irvine Scientific, Santa Ana, CA) and cryotip loading device according to the manufacturer’s recommended protocol. Embryos were placed in 20-μL droplets of the equilibration medium (7.5% dimethyl sulfoxide [DMSO] and ethylene glycol and 20% serum substitute supplement) for 5 min and transferred into 3 droplets of 20 μL vitrification medium (15% DMSO and ethylene glycol and 20% serum substitute supplement) for 5 s in the first and second droplet and for 90 s in the last droplet. Embryos were then loaded in the cryotip loading device with 1 μL vitrification medium. The cryotip was sealed from both sides with heat pulse sealer and immediately transferred to liquid nitrogen.

Thawing was carried out after at least 24 h by removing the cryotip from the liquid nitrogen and immediately placing it in a 37°C water bath for 3 s. The cryotip was cut at its lower end, and the contents were brought down near to the thawing medium in the pre-prepared thawing Petri dish. Thawing dish contains 1 droplet of 20 μL of thawing medium, 2 droplets of 20 μL of dilution medium and 3 droplets of 20 μL of washing medium. The embryos retrieved after thawing were placed in thawing media droplets for 1 minute, in each of the dilution medium droplets for 2 minutes and finally in each of the washing medium droplets for 3 minutes. Embryos were finally incubated in HTF medium in 5% CO₂ for 4 h before any further processing.

**Slow Freezing**

Slow cryopreservation was done using blastocyst freeze media (protocol 1, glycerol based) and embryo freeze media (protocol 2, propanediol based) according to the manufacturer’s instructions (Irvine Scientific). In protocol 1, embryos were immersed in 20-μL droplets for 10 min in media containing 5% glycerol and transferred to a 40-μL droplet containing 9% glycerol with 0.2 mol/L sucrose for another 10 min before loading into conventional cryopreservation straws. In protocol 2, embryos were immersed in 20-μL droplets for 10 min in media containing 1.5 mol/L propanediol and transferred to a 40-μL droplet containing 1.5 mol/L propanediol with 0.1 mol/L sucrose for another 5 min before loading into conventional cryopreservation straws. All media were presupplemented with 12 mg/mL human serum albumin.

After loading, the straws were transferred to Cryopanner device (Kryo 360 1.7; TS Scientific, Perkasie, PA), seeding was done at −7°C, and the temperature was cooled from 4°C to −7°C at a rate of 3°C/min, holding for 10 min at −7°C, then at a rate of −0.3°C/min to −40°C, and finally from −40°C to −140°C at 10°C/min followed by immediate transfer to liquid nitrogen.

**Embryo DNA Fragmentation**

Embryos were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4) for 1 h at room temperature.
After fixation, embryos were washed at least three times in PBS containing 0.3% polyvinylpyrrolidone (PVP) and permeabilized in 0.5% Triton X-100 on ice for 2 min. The embryos were then washed three times in PBS/PVP and incubated in TUNEL reaction cocktail (In-situ cell death detection system; Roche Diagnostic Corp., Indianapolis, IN) at 37°C for 1 h in the dark. Positive and negative control samples were included in each analysis. Blastocysts exposed to DNase I for 15 min at room temperature served as positive control. Blastocysts that were not exposed to the TdT enzyme served as negative control. Embryos were extensively washed in PBS and mounted in 4',6-diamidino-2-phenylindole (DAPI) containing Vectashield antibleaching solution (Vector Labs, Burlingame, CA). Drops of mounting medium were covered with cover slide, and the edges were sealed with clear nail polish and stored at −20°C in the dark for analysis by confocal microscope.

Confocal Imaging

Images were collected with a Leica TCS-SP2 laser scanning spectral confocal microscope (Leica Microsystems, Heidelberg) using an HCX Plan Apo ×63, 1.32 NA, oil immersion objective at zoom 2. The specimen was excited at 364 nm (UV) for DAPI and 488 nm for fluorescein isothiocyanate-conjugated TUNEL. The emitted fluorescence from each of the two labels was detected with two separate photomultiplier detectors whose spectrophotometer slits were set for 400–490 and 500–550, respectively. Images were collected sequentially at each level of the specimen to prevent cross-talk of the fluorophores and were then collected along the z-axis of the sample with a step size of 1–3 μm. A projection of the three-dimensional (3D) stack of images was then created using Leica software. The original stack of embryo images was transferred to the Velocity 64 software (Improvision, Lexington, MA), which reconstructs a 3D image of the blastocysts. On the 3D image, individual blastomeres are labeled as either intact (TUNEL−ve, blue stain) or fragmented DNA (TUNEL+ve, green stain) and counted. The apoptotic index was calculated as the ratio of TUNEL+ve/total number of blastomeres, and DNA integrity index was calculated as TUNEL−ve/total number of blastomeres.

Statistical Analysis

The total number of blastomeres and DNA integrity indices were summarized within study groups numerically using mean and standard deviation. The DNA integrity indices within groups were also displayed along with boxplots showing 10th and 90th percentiles, 25th and 75th percentiles, and median. Comparisons of groups were performed using Wilcoxon signed rank test, with P values reported from pairwise group tests. Analyses and graphing were performed using R version 2.3.1 (www.R-project.org).

RESULTS

After counting of individual blastomeres, DNA integrity and apoptotic indices were calculated for each blastocyst. Results for NEB and EB are shown in Table 1. The DNA integrity index was used for better visualization of the results (Fig. 1). An example of the blastocysts images using the confocal microscopy in different groups is illustrated in Fig. 2. We observed an increase in DNA fragmentation index with vitrification and slow cryopreservation using propanediol and glycerol (DNA fragmentation index of all the cryopreserved blastocysts vs. the fresh control embryos). This increase was not significant for slow cryopreservation with glycerol (P = .3), and it was significant with vitrification and slow cryopreservation with propanediol (P < .001; Table 1).

Comparing the different methods of cryopreservation, conventional blastocyst cryopreservation with glycerol showed significantly better DNA integrity (lower DNA fragmentation index) after thawing compared with vitrification, for both NEB (P < .001) and EB (P = .002). It also showed significantly better results compared with slow cryopreservation with propanediol for all NEB (P < .001) and EB blastocysts (P < .001; Table 1).
The increase in DNA fragmentation with the cryopreservation using propanediol was significantly higher compared with vitrification for NEB ($P = .002$) and EB ($P < .001$). Table 1 summarizes the results from comparison of different cryopreservation techniques with the fresh control samples for NEB and EB.

To evaluate the effect of the blastocyst expansion on the postwarming or thawing results, NEB were compared with EB in all of the cryopreservation methods. Expanded blastocysts showed an increase in their apoptotic index over NEB for all cryopreservation and vitrification methods. However,
this increase only reached statistical significance in slow cryopreservation with propanediol, though it was a constant trend in all of the methods of cryopreservation. The effect of blastocyst expansion on the DNA integrity index after different cryopreservation techniques is shown in Table 2.

**DISCUSSION**

In this study, we examined the effect of different methods of cryopreservation and vitrification on inducing apoptosis in mouse blastocysts. Slow freezing is based on equilibration between the embryos and low concentration of the cryoprotectant. This would result in low toxicity and osmotic stress, but still carries a high risk of ice crystal formation. Glycerol is a natural metabolite and is considered to be a safe option for the metabolically and genetically active blastocysts (6). Clinical trials using glycerol have reported implantation rates of 16%, pregnancy rates of 25% to 28%, and a take-home baby rate of 10% (7, 19).

Propanediol as a cryoprotectant is characterized by its low toxicity with high penetrating power and less tendency to crystallize compared with glycerol or DMSO (20). Its use has been mostly restricted to earlier embryo stages: from pronuclei to morula stages. Few reports have described propanediol as a cryoprotectant in slow cryopreservation of mouse or bovine blastocysts (8, 11–13).

An initial case report described the first transfer of human blastocyst cryopreserved with propanediol that resulted in birth of a healthy baby (10). In 2006, Magli et al. reported the cryopreservation of blastocysts after a cleavage-stage embryo biopsy, using propanediol on 47 blastocysts (9). This resulted in a survival rate of 53%, implantation rate of 32%, and clinical pregnancy rate of 39%. Biopsying the embryos did not have any effect on the subsequent development and implantation of the embryos. Those results are better than those generally reported with the use of glycerol for slow cryopreservation of intact embryos. The difference may be attributed to the small sample size. Also, embryo biopsying may have added an additional stress, making the development to blastocysts more selective for the best-quality embryos. Contrary to the earlier reports of successful cryopreservation of blastocysts with propanediol, we recorded the highest levels of

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**TABLE 2**

Comparison of the DNA integrity index of nonexpanded and expanded blastocysts with different cryopreservation techniques.

<table>
<thead>
<tr>
<th>Group</th>
<th>Nonexpanded blastocysts (n = 62)</th>
<th>Expanded blastocysts (n = 62)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh control</td>
<td>93.94 ± 6.64 (n = 13)</td>
<td>95.47 ± 4.25 (n = 14)</td>
<td>.49</td>
</tr>
<tr>
<td>Vitrification</td>
<td>84.36 ± 8.76 (n = 17)</td>
<td>77.61 ± 16.65 (n = 22)</td>
<td>.11</td>
</tr>
<tr>
<td>Slow glycerol</td>
<td>94.76 ± 4.70 (n = 16)</td>
<td>90.87 ± 6.16 (n = 11)</td>
<td>.09</td>
</tr>
<tr>
<td>Slow propanediol</td>
<td>72.63 ± 13.44 (n = 16)</td>
<td>56.19 ± 25.49 (n = 15)</td>
<td>.037</td>
</tr>
</tbody>
</table>

*Note: P < .05 was considered to be significant comparing the nonexpanded to expanded blastocysts by Wilcoxon signed rank test.*

apoptosis in the blastocysts cryopreserved by this method. Our study had no external effectors, such as the embryo biopsy in Magli et al.’s report, and therefore the results correlate only with the method of cryopreservation.

Although vitrification avoids ice formation, which is a serious concern in blastocyst freezing, it exposes the blastocysts to high concentrations of cryoprotectants that may be toxic if exposure time is not controlled. Previtritification interventions such as blastocoele collapse and assisted hatching have resulted in improved clinical outcomes (21–23). Pregnancy rates ranging from 15% to 60% and implantation rates of 18%–46.7% were reported with highest results being obtained after blastocoele puncture (14, 16, 22, 24, 25).

Lieberman and Tucker compared 254 vitrified blastocysts transfer cycles with a similar number of conventional glycerol rate-controlled freezing cycles (26). No overall differences were seen between the two freezing methods regarding implantation rate (42.9% vs. 46.1%, respectively) or clinical pregnancy (28.9% vs. 30.6%, respectively). Vitrification on day 5 blastocysts were reported to have significantly better implantation and clinical pregnancy rates than day 6. Implantation rates were 33.4% and 25.9%, and pregnancy rates were 48.7% and 42.8%, respectively, for day 5 and day 6 blastocysts. This difference was not seen in slow freezing, and all blastocysts were exposed to laser-assisted hatching after thawing or warming to compensate for any possible zonal hardening from cryopreservation.

Mukaida et al. have reported 207 transfer cycles with 725 day 5 or day 6 vitrified blastocysts (16). They reported survival of 87% of day 5 blastocysts versus 55% of day 6 with a statistically significant difference between the two groups. No statistically significant difference was noted regarding the implantation and pregnancy rates, and overall pregnancy rate was 37%.

Our results show an increase in the apoptotic index of EB versus NEB in all cryopreservation methods or vitrification. Although this increase did not reach statistical significance, it is in accordance with the earlier reports that show significant differences in a number of outcome parameters after vitrification of day 5 and day 6 blastocysts. Lack of significance in the present findings may be related to either the small sample size or more likely the fact that apoptosis induction, which was the focus of the present experiment, may not be the only player. The interplay of different biologic factors, such as apoptosis induction, metabolic changes, and gene expression alteration, may all contribute to the fate and outcome of embryos undergoing a freeze-thaw cycle (27–29).

Although many trials in humans and animals have evaluated the use of cryopreserved or vitrified blastocysts on their survival, implantation, and ongoing pregnancy rates, very few studies have examined the mechanism of how cryopreserved blastocysts are affected. During a freeze-thaw cycle, cells experience a variety of stressful events. The cellular response to these factors may be either by adaptation or yielding by upregulation of apoptosis (30, 31). The apoptotic index of a blastocyst is an important parameter that negatively correlates with embryo development potentials (32–35).

Park et al. have evaluated the apoptotic index of bovine blastocysts after vitrification in conventional and minimal volume concentration straws (36). They reported an increase in both apoptotic index and apoptosis-related gene expressions. In a similar study, Fabian et al. studied the effect of vitrification in inducing apoptosis in porcine blastocysts (37). Those authors demonstrated an increase in the DNA fragmentation index immediately after thawing, which increased after 24 h of incubation. The apoptotic changes were not morphologically evident until after 24 h of incubation. Our findings are in agreement with those two studies but contrary to a report by Huang et al., who failed to show any difference between fresh and vitrified embryos regarding hatching rate and apoptotic index using a bovine embryo model and cryoleaves, a vitrification device (38). Those investigators did not examine the degree of blastocysts expansion before considering the DNA damage, and no evaluation was made for blastocysts that were cryopreserved by slow freezing. As reported by Rajaei et al., the mere exposure of blastocysts to any of the cryoprotectants (DMSO, ethylene glycol, propandiol) without cryopreservation increases apoptotic index and impairs blastocele re-expansion in culture (39).

The current reports of improvement after transfer of vitrified blastocysts compared with slow cryopreservation may be due to reduced damage by formation of ice crystals. Contrary to our expectations based on this hypothesis and earlier clinical reports, the present study showed that vitrification resulted in increased apoptosis compared with slow cryopreservation with glycerol. This may be explained by the introduction of other biologic changes related, but not limited, to metabolic changes, such as glucose, pyruvate, and oxygen uptake, which might be more predominant in slow freezing and minimal or absent in vitrification. These parameters were affected in bovine blastocysts by slow freezing and remained unchanged during vitrification (27). Similar findings were reported with human cleavage-stage embryos (40). It may be possible that the brief exposure to high concentrations of cryoprotectants in vitrification may induce a higher percentage of apoptosis than prolonged exposure to low concentrations as in slow freezing. On the other hand, the prolonged exposure to the cryoprotectants in slow freezing may have more residual effects through metabolic or proteomic modification than the short exposure during vitrification.

We obtained the highest levels of apoptosis when using propandiol in slow cryopreservation, which suggests its unsuitability as a cryoprotectant for blastocysts. This may be related to the development of intermediate toxic metabolites, as is the case for the closely related ethylene glycol, whose catabolism leads to formation of toxic aldehydes (41). In addition, alteration in the embryonic gene expressions cannot be ruled out with any cryopreservation or vitrification method (28, 29). The exact mechanism of this finding is unclear.
In conclusion, the present study confirms the increase in the apoptotic index as a result of slow cryopreservation or vitrification of the blastocysts. This increase in apoptotic index was highest in slow freezing with propanediol, lower with vitrification, and least in slow freezing with glycerol. Increase in apoptosis was more remarkable in EB. Further studies are needed to investigate other pathways than apoptosis that may be modulated by vitrification or slow freezing hindering embryo development. Proteomic and metabolomic analysis may be needed to further increase our fundamental understanding of the cryobiologic events that may occur in blastocysts undergoing cryopreservation or vitrification and its implications in clinical outcome.

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REFERENCES


