Slow and ultrarapid cryopreservation of biopsied mouse blastocysts and its effect on DNA integrity index

Amr Kader · Tommaso Falcone · Rakesh K. Sharma ·
Devna Mangrola · Ashok Agarwal

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Abstract

Purpose To evaluate the effect of slow and ultra-rapid freezing on biopsied blastocysts' DNA integrity.

Methods Forty eight mouse blastocysts were biopsied of which 16 were cryopreserved by slowly freezing and 17 by vitrification. Fourteen intact blastocysts were slowly cryopreserved and 24 were vitrified. Eighteen fresh intact blastocysts and fifteen biopsied blastocysts served as controls. The DNA integrity index of all blastocysts was evaluated using (TUNEL) staining and confocal imaging.

Results Both slow freezing and vitrification of biopsied blastocysts induced apoptosis to a similar extent. Biopsying blastocysts before vitrification resulted in less apoptosis than vitrification of intact blastocysts.

Conclusion Slow freezing and vitrification are equal options for preservation of biopsied blastocysts as regards the DNA integrity index (DII). Biopsied blastocysts better tolerate vitrification than intact expanded blastocysts.

Keywords Blastocyst · Vitrification · Cryopreservation · Media protocol · TUNEL · Apoptotic index · DNA integrity index (DII)

Introduction

Preimplantation genetic screening (PGS) is a clinical tool to investigate aneuploidy in selected cases. The success of this method in improving assisted reproductive techniques (ART) outcome is still debatable [1–3]. In order to perform PGS, nuclear material is first obtained most commonly from eight cell stage blastomeres, or more recently from blastocyst trophectoderm cells [4, 5]. Removal of more than one blastomere is usually recommended to avoid misdiagnosis from allele drop out, PCR failure, or mosaicism [6]. However, removal of more than 25% of cleavage stage embryo blastomeres can have serious consequences on embryo development [4, 7, 8].

Removal of multiple trophectodermic cells from a blastocyst does not disturb the inner cell mass and therefore is less likely to affect the embryo development [4, 5]. Implementing PGS on blastocysts resulted in better pregnancy and implantation rates compared to the transfer of genetically screened cleavage stage embryos. This is related to the higher accuracy of genetic diagnosis at the blastocyst stage from the availability of more nuclear material from multiple cells and performing the screening on a more

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Capsule Biopsied blastocysts are negatively affected by slow freezing and vitrification to the same extent. Biopsied blastocysts can better tolerate vitrification than intact blastocysts as regards their DNA integrity.

A. Kader · T. Falcone · R. K. Sharma · D. Mangrola ·
A. Agarwal (✉)

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, USA
e-mail: Agarwaa@ccf.org

A. Kader
Department of Obstetrics and Gynecology,
West Virginia University,
Morgantown, WV, USA

A. Kader
Department of Obstetrics and Gynecology, Alexandria University,
Alexandria, Egypt

selected pool of embryos than that available at cleavage stage [9]. Delaying PGS to the blastocyst stage limits the number screened embryos to only more competent embryos minimizing the costs of screening and maximizing the accuracy of diagnosis [9–11].

The blastocyst biopsy procedure is an invasive procedure that may result in disruption of the early embryonic cells homeostasis either mechanically or affecting its future development. Though the clinical outcome of transferring genetically screened blastocysts has been investigated, the effect of the required blastocyst manipulation on the DNA integrity of remaining embryo pool of cells has not. DNA integrity may have an immediate effect on the pregnancy and implantation rates or even more remotely on the neonatal outcome or in adult life [12–15].

Following PGS, it is cost effective to preserve the non transferred screened embryos. Two cryopreservation options are currently available: slow freezing and vitrification. Slow cryopreservation of biopsied cleavage stage embryos has shown low survival rate [16] and poor developmental potential after thaw [16, 17]. Zheng et al found that vitrification of biopsied cleavage stage embryos resulted in higher survival rate compared to different slow freezing methods [18]. Parriego et al reported the first pregnancy achieved after vitrification of a biopsied cleavage stage embryo for the purposes of PGS [19]. Transferring biopsied cleavage embryos cryopreserved after in vitro culture and blastulation has shown improved results over the immediate transfer at the cleavage stage [19, 20].

Due to the novelty of the technique, little is known about the outcome of transferring blastocysts that are biopsied and cryopreserved at the blastocyst stage. Tominaga et al. (2007) first described the successful cryopreservation of biopsied in vitro produced bovine blastocysts [21]. A single report on transferring slowly cryopreserved biopsied human blastocysts showed implantation rate of 26% following transfer [22]. However, to the best of our knowledge there are no reports on the vitrification of blastocysts biopsied following blastulation.

We have previously demonstrated that both slow cryopreservation and vitrification can induce DNA damage to the blastocysts [23]. We have also demonstrated along with other investigators that having an opening in the zona pellucida may improve the vitrification outcome [24, 25]. Therefore, we hypothesized that the DNA integrity of biopsied cleavage stage embryos or blastocysts might be affected by the presence of a zonal opening which is an integral part of the biopsy procedure when exposed to slow freezing or vitrification.

The objective of this study was to evaluate the effect of biopsy as well as slow freezing and vitrification of biopsied blastocysts on their DNA integrity index (DII).

Materials and methods

Embryos

Commercially available cryopreserved eight cell mouse embryos (Embryotech Laboratories, Inc., Wilmington, MA) were thawed according to the manufacturer's protocol. After thawing, embryos were incubated at 37°C and 5% CO₂ for 72 h and daily examined for development. Embryos showing signs of fragmentation, delayed or accelerated development were discarded [23].

One hundred and four good quality blastulated embryos were distributed into the following different study groups:

1. Fresh intact blastocysts ($n=18$)
2. Slow cryopreserved intact blastocysts: ($n=14$)
3. Vitrified intact blastocysts: ($n=24$)
4. Fresh biopsied blastocysts ($n=15$)
5. Slowly cryopreserved biopsied blastocysts ($n=16$)
6. Vitrified biopsied blastocysts ($n=17$)

Blastocyst biopsy

Blastocyst biopsy was done with the aid of two hydraulic micromanipulators (IM 9-B Narishige, Tokyo, Japan), microsyringes (IM-6; Narishige) mounted on an inverted microscope (Leica Microsystems GmbH, Wetzlar, Germany) and laser pulse (SaturnActive, Research Instruments Ltd, UK), and a laser controlling software (Cronus 3.3.3 research Instruments Ltd, UK). A blastocyst was held still with a holding micropipette. The inner cell mass was oriented at the 7 O'clock position. Laser pulse was used to create an opening in the Zona, at 3 O'clock position. Blastocysts were allowed 2 min to 3 min after zona hatching to allow extra-embryonic cells to herniate. Herniating cells were removed by gradual aspiration and dislodgement using the biopsy needle. Laser pulse was used to complete detachment of the removed cells using same previous settings in single or multiple shots as needed using laser pulse and biopsy needle (Fig. 1).

Vitrification and warming

Vitrification was performed using the Irvine vitrification kit with the cryotip loading device (Irvine Scientific, Santa Ana, CA). As previously described [23], blastocysts were vitrified with incubation in the 1st equilibration media for 4 min then vitrification media for 60 s. Two to three blastocysts were then loaded in cryotips which were immediately heat sealed and transferred into liquid nitrogen.

After 24 h, the cryotips were warmed in a 37°C water bath for 3 s and cut from both ends to expel the embryos.

Fig. 1 Steps for blastocyst biopsy: **a** laser is adjusted to target the zona pellucida at 3 O'clock position. **b** zona pellucida is open, **c** trophoctodermic cell starts to herniate. **d** biopsied herniated trophoctodermic cells **e** biopsied blastocyst showing partial collapse and trophoctodermic cells still at the site of zonal opening and **f** collapsed biopsied blastocyst

Released blastocysts were passed into a sequence of three media with a decreasing concentration of sucrose and containing no cryoprotectants as recommended by the manufacturer (Irvine Scientific, Santa Ana, CA) and previously described. Warmed blastocysts were finally incubated in HTF medium in 5% CO₂ for 4 h before further processing [23].

Slow freezing

Slow cryopreservation was done using glycerol based blastocyst freeze media (Irvine scientific, Santa Ana, CA) as previously described [23]. Two to three blastocysts were loaded into conventional 0.25 mL cryopreservation straws (IMV-Technologis-L'aigle, France). After loading, the straws were transferred to Cryoplanner device (Kryo 360 1.7, TS Scientific, PA) and cooled as per a standard protocol [23]. After at least 24 h, blastocysts were thawed using blastocyst thaw media (Irvine scientific, Santa Ana, CA) as previously described. Cleavage embryos were

thawed using embryo thaw media (Irvine scientific, Santa Ana, CA) as previously described [23].

Thawed blastocysts were incubated in HTF medium in 5% CO₂ for 4 h before further processing.

DNA damage by TUNEL staining

Blastocysts were fixed in 4% paraformaldehyde in PBS (pH 7.4) at room temperature for 1 h, followed by washing in PBS with 0.3% PVP. They were permeabilized with chilling 0.5% Triton X-100 for 3 min. Following 3 additional washes in the PBS/PVP solution, blastocysts were incubated in TUNEL (terminal deoxynucleotide transferase (TdT)-mediated dUTP nick-end labeling) reaction solution (Roche Diagnostics Corporation, Indianapolis, IN) at 37°C for 1 h in the dark. Blastocysts were then washed in PBS and mounted in DAPI (4', 6-diamindino-2-phenylindole) containing Vectashield anti-bleaching compound (Vector Labs, Burlingame, CA). Mounted blastocysts were examined for DNA integrity using confocal microscopy [23].

Confocal imaging

Leica TCS-SP2 laser scanning spectral confocal microscope (Leica Microsystems, Heidelberg, GmbH, Germany) with HC PL Apo 20X, 0.70 NA, oil immersion objective at zoom X2 was used. DAPI, and TUNEL staining were visualized using excitation wavelengths of 364 nm 488 nm respectively. Images were collected along the Z-axis at 2 μ m intervals. Figure 2 shows compiled 2D images of the 3D confocal scanning of different intact and biopsied blastocysts with merged blue and green fluorescence channels [23].

DNA integrity index

Volocity 64 software (Improvision, Lexington, MA) was used to reconstruct the embryo in 3D. The DNA integrity index was calculated for each individual blastocyst as the percentage of TUNEL^{−ve} blastomeres / total number of blastomeres [23].

Statistical analysis

Mean $\pm$ SD of DII in the control and treatment groups were estimated and compared 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) [23].

Results

Effect of slow cryopreservation and vitrification on DNA integrity of biopsied blastocysts

Following slow cryopreservation or vitrification the biopsied blastocysts after warming, the DNA integrity indices of all experimental and control groups were analyzed and compared. (Table 1)

Slow cryopreservation of biopsied blastocysts resulted in DNA integrity index that was not statistically different than fresh blastocysts ($P=0.39$). Blastocyst biopsy procedure had no significant effect on the DNA integrity index ($P=0.4$). While no significant decrease in DNA integrity index was seen following slow cryopreservation ($P<0.18$), vitrification resulted in significant decrease in DNA integrity indices of intact blastocysts as compared to fresh intact blastocysts ($P<0.001$). There was no significant difference between slowly cryopreserved intact or biopsied blastocysts. However, biopsied vitrified blastocysts had significantly higher DNA integrity index compared to intact vitrified blastocysts. Biopsied blastocysts had statistically significant lower DNA integrity index after slow cryopreservation as compared to fresh biopsied blastocysts. Vitrifi-

cation of biopsied blastocysts on the contrary showed DNA integrity index that was comparable with fresh biopsied blastocysts. Finally, there was no significant difference comparing the DNA integrity index of vitrified or slowly cryopreserved biopsied blastocysts.

Discussion

The Sydney IVF group has reported the use of blastocyst biopsy for PGS and achieved pregnancy transferring fresh and slowly cryopreserved genetically screened blastocysts. The group could not conclude a benefit for the use of blastocyst biopsy for PGS for aneuploidy using five colour FISH whereas comparative microarray studies and DNA fingerprinting showed the predictive value of the technique [26, 27].

The effect of cryopreservation on biopsied blastocysts has not been specifically evaluated. Our study primarily focused on the effect of cryopreservation, either slow or ultrarapid on the DNA integrity of the biopsied blastocysts. We found that the blastocyst biopsy procedure did not induce DNA damage. The results also showed that cryopreservation of biopsied blastocysts through either slow cooling or vitrification did not induce additional significant damage to the DNA than it would with slow cryopreservation or vitrification of intact blastocysts. Interestingly, biopsied vitrified blastocysts showed less DNA damage than intact vitrified blastocysts. Slow cryopreservation did not induce any significant DNA damage either in intact or biopsied blastocysts.

Though PGS vitrified blastocysts have yet to be further investigated clinically, vitrification of open zona pellucida (ZP) or collapsed blastocysts has been studied. The two procedures showed to improve the vitrification outcome [24, 28–30]. We have previously confirmed the protective effect of both zonal hatching or blastocoele aspiration before vitrification on the DNA integrity index of vitrified-warmed blastocysts [25]. Improvement of blastocyst vitrification outcome can be attributed to better dehydration of the blastocoele, avoiding the potential formation of damaging ice crystals in the blastocelic cavity and providing direct contact of the blastomeres to the full concentration of the cryoprotectants during vitrification rather than this concentration being diluted by the blastocelic fluid [24, 25]. We can speculate that in biopsied blastocysts, the blastocelic fluid is rapidly replaced by vitrification media rather than equilibrating with a surrounding vitrification media through the zona pellucida and a continuous layer of blastomeres. In accordance with this phenomenon, our results showed biopsied blastocysts to have better DII than intact blastocysts following vitrification. The blastocyst biopsy procedure could inadvertently serve as a form of assisted hatching and blastocoele reduction explaining the improved results of vitrifying biopsied blastocysts.

Fig. 2 Confocal compiled images of TUNEL and DAPI stained blastocysts showing: **a** intact blastocyst **b** biopsied blastocyst with biopsy site revealed **c** biopsied blastocyst with herniating cells **d** biopsied blastocyst partially collapsed

Table 1 DNA integrity Index in intact and biopsied blastocysts following slow cryopreservation and vitrification

Parameter	Intact Blastocysts (<i>n</i> =56)		
	Fresh (<i>n</i> =18)	Slow Cryopreservation (<i>n</i> =14)	Vitrification (<i>n</i> =24)
DNA Integrity Index %	94.61±4.75	91.76±5.84 (<i>P</i> =0.18) ^a	78.76±16.38 (<i>P</i> <0.001) ^b
Parameter	Biopsied Blastocysts (<i>n</i> =48)		
	Fresh (<i>n</i> =15)	Slow Cryopreservation (<i>n</i> =16)	Vitrification (<i>n</i> =17)
DNA Integrity Index %	96.28±3.57 (<i>P</i> =0.4) ^c	94.03±2.36 (<i>P</i> =0.44) ^d (<i>P</i> =0.019) ^e	94.37±6.31 (<i>P</i> <0.001) ^f (<i>P</i> =0.06) ^g

P<0.05 was considered statistically

NS Non significant for the following:

^a Comparing fresh blastocysts to slowly cryopreserved intact blastocysts (NS)

^b Comparing slowly cryopreserved blastocysts to vitrified intact blastocysts

^c Comparing fresh intact to fresh biopsied blastocysts (NS)

^d Comparing slowly cryopreserved intact to slowly cryopreserved biopsied blastocysts (NS)

^e Comparing fresh biopsied to slowly cryopreserved biopsied (NS)

^f Comparing comparing vitrified intact to vitrified biopsied blastocysts

^g Comparing slowly cryopreserved to vitrified biopsied blastocysts (NS)

Conclusions: The present study provides preliminary evidence for the feasibility of cryopreserving PGS blastocysts by vitrification in a closed system. Slow cryopreservation does not induce significant DNA damage to the blastocysts. The study also showed the DII of biopsied blastocysts is not significantly affected by slow freezing or vitrification. It was remarkable that biopsied vitrified blastocysts showed significantly better DNA integrity index compared to vitrified intact expanded blastocysts. The DII of blastocysts after slow freezing was not affected by the blastocysts being intact or biopsied. The high dehydration and high viscosity requirements to achieve proper vitrification are not required for slow freezing, therefore, the impact of zonal opening or blastocoele reduction are more prominent with vitrification than with slow freezing of biopsied blastocysts. Studies would be required to further validate our findings and study suggested blastocysts cryo-behavior on the human blastocysts. Clinical trials studying the transfer of genetically screened vitrified blastocysts would also be of clinical benefit.

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