The Effect of Time in Vitrification Solution on Mouse Embryo Development, Birth Rate and DNA Damage

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Introduction

Vitrification is a process by which cells can be frozen in such a way that a glass-like or vitrified state is obtained. This process eliminates the formation of intracellular ice crystals which can damage organelles within the cell and cell membranes. Vitrification of mouse embryos was first reported in 1985 (Rall and Fahy). Since then, this technique has been extended to early-cell embryos (two- to eight-cells) in cattle (Vajta et al., 1997) and humans (Mukaida et al., 1998) as well as blastocysts for cattle (Park et al., 1999), mice (Lane et al., 1999), humans (Yokota et al., 2001), monkeys (Yeoman et al., 2001) and pigs (Misumi et al., 2003).

One concern regarding vitrification is the exposure of the embryos to high-osmolarity cryoprotectants because of the detrimental effects that have been observed. Rall (1987) determined that eight-cell mouse embryos remaining in vitrification solution containing dimethyl sulfoxide, acetamide and propylene glycol at 4°C for 10 to 15 minutes were able to survive, but none were able to survive after remaining in the same solution for 30 minutes. Exposure of Day-4 mouse embryos to glycerol, dimethyl sulfoxide or propylene glycol for 20 minutes, was shown to be toxic (Ali and Shelton, 2007).

To avoid embryo exposure to high concentrations of toxic cryoprotectants for extended periods, vitrification protocols generally require that embryos remain in the vitrification solution for only 1 to 2 minutes (Lieberman et al., 2002; Kuwayama et al., 2005). This can be difficult, especially since some vitrification devices have an extensive learning curve before the technique is mastered. Even with practice, one may not be able to quickly pick up embryos and vitrify within the time required; therefore, determining the length of time that embryos can safely reside in vitrification solution is important.

One way to determine the toxicity of a vitrification solution is to analyze embryos using the Comet Assay to detect DNA damage following exposure to these high osmotic solutions. This assay was first performed by Ostling and Johanson (1984) to observe murine lymphoma cells and later modified by Singh et al. (1988) to observe "single-stranded DNA breaks and alkali-labile sites." The premise for the Comet Assay is that damaged DNA strands will migrate out of a cell during electrophoresis to create the tail segment of the comet (the longer the tail, the more damage present); whereas, undamaged DNA will remain in the cell creating the head of the comet. This assay has been used to detect DNA damage in bovine oocytes (Chung et al., 2007) as well as hamster (Takahashi et al., 1999), bovine (Takahashi et al., 2000) and mouse (Fabian et al., 2003) embryos. Stowinska et al. (2008) and Kalthur et al. (2008) used the Comet Assay to analyze DNA damage in cryopreserved sperm. To our knowledge, our study is the first to use the Comet Assay to detect DNA damage in cryopreserved mouse embryos, although other researchers (Sohn et al., 2002; Ramezani et al., 2005; Kader et al., 2009) have used the terminal

deoxynucleotidyl transferase mediated dUTP nick-end labeling (TUNEL) Assay to detect DNA damage in mouse embryos.

The objectives of this study are 1) to determine the length of time in vitrification solution needed to observe a reduction in blastocyst rate; 2) to determine the effect of time of exposure in vitrification solution on blastocyst rate and birth rate; and 3) to determine the blastocyst rate and percentage of embryos demonstrating DNA damage due to extended exposure in vitrification solution.

Materials and Methods

Male and female mice (B6C3F1) were purchased from Jackson Laboratories (Bar Harbor, ME). This strain of mice was chosen because it has demonstrated good survival results when the embryos were slow-cooled and thawed. All mice were handled according to an Institutional Animal Care and Use Committee protocol for this project. Once acclimated, the female mice were injected with pregnant mare serum gonadotrophin on Day 1, and then injected with human chorionic gonadotrophin and mated on Day 3. On Day 5, the female mice were euthanized, the oviducts removed and embryos were retrieved at the two-cell stage. The same procedure to collect two-cell mouse embryos was used in each of the three experiments.

Experiment 1: Determine the length of time in vitrification solution needed to observe a reduction in blastocyst rate.

The first experiment consisted of three trials. Trial 1 had two-cell mouse embryos remaining in vitrification solution for 1, 2, 4 or 8 minutes. Trial 2 had embryos remaining in vitrification solution for 1, 2, 4, 8, 16 or 32 minutes. Trial 3 had embryos remaining in vitrification solution for 1 or 32 minutes. For all trials, two-cell mouse embryos were exposed to a medium consisting of Dulbecco's Phosphate Buffered Saline without calcium and magnesium (DPBS; In Vitro Care, San Diego, CA), 7.5% ethylene glycol (Sigma, St. Louis, MO) and 7.5% dimethyl sulfoxide (DMSO; Sigma) for 3.5 minutes. The embryos then were transferred to a vitrification solution consisting of DPBS with 15% ethylene glycol and 15% DMSO (Graves-Herring and Boone, 2009). Both media were at 4°C immediately prior to use and remained at room temperature thereafter.

After remaining in vitrification solution for the specified amount of time, the embryos were collected and moved through a series of four thawing solutions at 37°C. The first solution consisted of DPBS with 1 M sucrose (Sigma) and 20% Synthetic Serum Substitute (SSS; Irvine Scientific, Santa Ana, CA). The second solution consisted of DPBS with 0.5 M sucrose and 10% SSS. The third solution consisted of DPBS with 0.25 M sucrose and 5% SSS and the fourth solution consisted of DPBS with 0.125 M sucrose and 2.5% SSS. The amount of time that the embryos remained in each solution was 2 minutes, 3 minutes, 5 minutes and 5 minutes, respectively.

Embryos then were placed into 50 µL drops of Human Tubal Fluid (HTF; Irvine Scientific) overlaid with washed mineral oil and cultured in an incubator at 36.7°C with 5% CO_2 and air. After 72 hours, blastocyst rates were determined.

Experiment 2: Determine the effect of vitrification solution on blastocyst rate and birth rate when embryos remain in vitrification solution for 32 minutes.

This experiment used the same media and methods as described for Experiment 1. The difference was that all embryos remained in vitrification solution for 32 minutes prior to their exposure to the thawing solutions. Also, this experiment used control embryos which were collected and placed into HTF without being exposed to any vitrification or thawing solutions. After culturing for 72 hours, the blastocyst rate was determined for the controls and 32-minute exposure embryos.

Cultured embryos were divided into three groups. The first group was control blastocysts. The second group consisted of early and expanded blastocysts from the 32-minute exposure group, and the third group consisted of four-cell to morula-stage embryos from the 32-minute exposure group. Embryos from all three groups were transferred into psuedopregnant synchronized recipient mice.

Experiment 3: Determine the blastocyst rate and percentage of embryos demonstrating DNA damage due to exposure to vitrification solution for 32 minutes followed by vitrification and warming.

Similar to Experiment 1, embryos were exposed to the vitrification solution; however, for this experiment they remained in vitrification solution for either 1 minute or 32 minutes. The embryos then were placed 10 at a time into 150 µm Stripper Tips® (MidAtlantic

Diagnostics Inc., Marlton, NJ), which were sealed at both ends using a Cryo Bio Systems SYMS Sealing System (Cryo Bio Systems, L'Aigle, France). Within 1 minute of transferring the embryos into the 15% ethylene glycol and 15% DMSO vitrification solution, the sealed Stripper Tips® were placed vertically into a goblet on an aluminum cane, which resided in 4 liters of liquid nitrogen in a Styrofoam container. A second goblet was inverted and attached to the top of the cane to secure the Stripper Tips® within the goblet. The canes were covered with cardboard sleeves and transferred to a storage tank containing liquid nitrogen.

To thaw the vitrified embryos, Stripper Tips® were removed from liquid nitrogen and exposed to air. The area within the Stripper Tip® containing the embryos was thawed quickly by rubbing this location between the thumb and index finger for 2 to 3 seconds. Both ends of the Stripper Tip® were cut off and the embryos were expelled from the device with the aid of a 0.1 mL bolus of media using a 25 gauge needle attached to a 1 mL syringe. The embryos were collected and moved through the same series of four thawing solutions at 37°C described for Experiment 1.

Once thawed, embryos from the 1-minute and 32-minute exposure groups were placed into designated 50 µL drops of Human Tubal Fluid (HTF; Irvine Scientific, Santa Ana, CA) overlaid with washed mineral oil and cultured in an incubator at 36.7°C with 5% CO_2 and air. On the same days that the embryos were thawed, fresh two-cell mouse embryos were collected for controls (cultured in HTF) and for positive controls (cultured in HTF with 1% hydrogen peroxide to induce DNA damage per instructions using Comet Assay Kit). Controls and positive controls embryos were cultured in the same environment as described above. After 72 hours, blastocyst rates for controls, positive controls, 1-minute and 32-minute exposure groups were determined. Chi-square analyses were performed on the blastocyst rate for each group.

Once blastocyst rates were determined, the Comet Assay Kit (Trevigen®, Gaithersburg, MD) was used to evaluate the four groups of embryos at the blastocyst stage (controls, positive controls, 1-minute exposure and 32-minute exposure) for the presence of comet tails. Manufacturer's recommendations to perform the assay were optimized for our laboratory. Embryos were placed into 75 µL of melted agarose. The melted agarose containing the embryos then was placed on a Comet Assay slide. The slide was held at 4°C in the dark for 30 minutes and then placed into a 4°C lysis solution for 1 hour. After this incubation, the slide was placed into an alkaline solution at 4°C for 30 minutes. This was followed by two 5 minute rinses of the slide with Tris-Borate-EDTA (Fisher Scientific, Pittsburg, PA).

Electrophoresis was performed for 20 minutes at 20 volts and 300 amps. The slide was rinsed in alcohol for 5 minutes, allowed to dry and then placed into a desiccator. At the time of analysis, 50 µL of SYBR® Green I was added to the slide and the slide was then observed using fluorescence microscopy with a fluorescein isothiocyanate filter.

Each embryo was observed and an image was captured using Slide Book Software (Intelligent Imaging Innovation, Inc., Denver, Colorado). All embryo images were printed and examined for the presence or absence of a comet tail. Chi-square analyses were performed on the percentage of observed comet tails for all four embryo groups.

Results

Experiment 1: The first trial produced a 100% (10/10) blastocyst rate for each of the 1-, 2- and 8-minute exposure time (in vitrification solution) groups. The 4-minute exposure time group produced a 78% (7/9) blastocyst rate. The second trial produced a 100% blastocyst rate for the 1 (n=9), 2 (n=8), 4 (n=10) and 8 (n=10) minute exposure time groups. The 16-minute

exposure time group produced a 95% (19/20) blastocyst rate and the 32-minute exposure time group produced a 44% (8/18) blastocyst rate. The third trial produced 100% (22/22) blastocyst rate for the 1-minute exposure time group and a 72% (38/53) blastocyst rate for the 32-minute exposure time group (Table 1).

Experiment 2: The blastocyst rate was significantly different (P < 0.001) between the control embryos (no exposure) and the 32-minute exposure time embryos (Table 2). After the embryos were split into three groups (control, 32-minute blastocyst stage and 32-minute multi-cell and morula stage) and transferred to recipient females, there was no significant difference ($P = 0.3$) in birth rates between the control embryos (no exposure) and the second group of embryos, which included early and expanded blastocysts (Table 3). However, there was a significant difference ($P < 0.05$) in birth rates between the second group and third group of embryos, which included four-cell to morula group. There was also a significant difference ($P < 0.05$) in birth rates between the control embryos and the third group of embryos.

Experiment 3: There was no significant difference ($P = 0.316$) between the blastocyst rates for the 1-minute exposure group and 32-minute exposure group of vitrified and thawed embryos (Table 4). In contrast, the control group did demonstrate a difference in blastocyst rates ($P < 0.001$). In addition, the percentage of embryos that presented comet tails for the controls, positive controls, 1-minute exposure and 32-minute exposure group are shown in Table 4. The 1-minute exposure group was not significantly different than the control group ($P = 0.174$) but was significantly different from the 32-minute exposure group ($P < 0.001$) and the positive controls ($P < 0.001$). The 32-minute exposure group was significantly different from the control group ($P < 0.001$) and the positive control group ($P = 0.016$).

Discussion

This research explored the effect of time of two-cell mouse embryos in vitrification solution. There is a plethora of published research of vitrification that described various devices,

Table 1. Time in Vitrification Solution (Experiment 1).

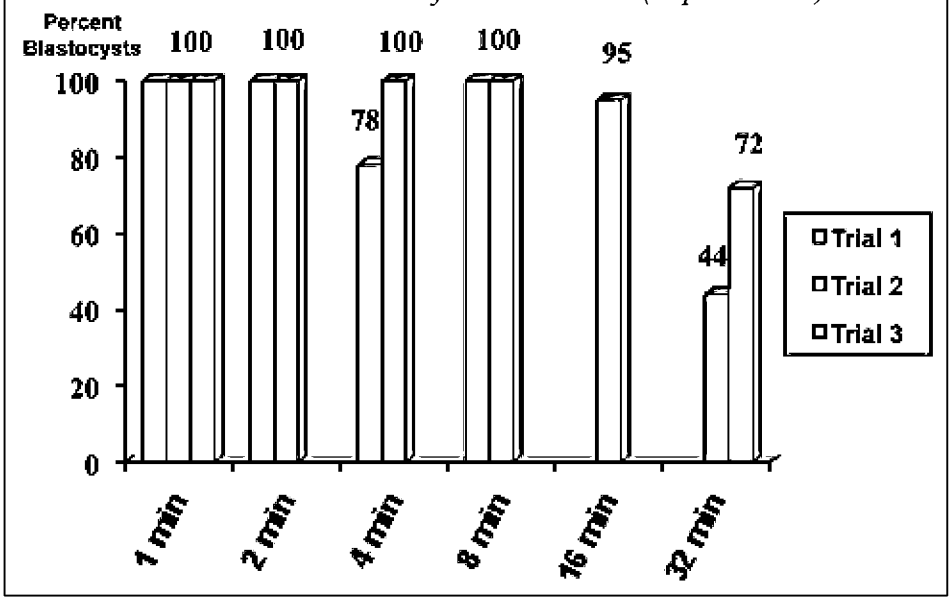
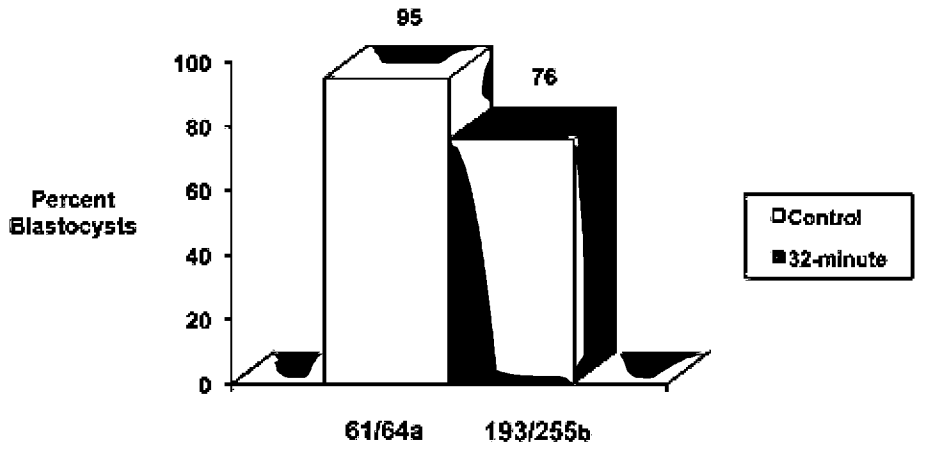
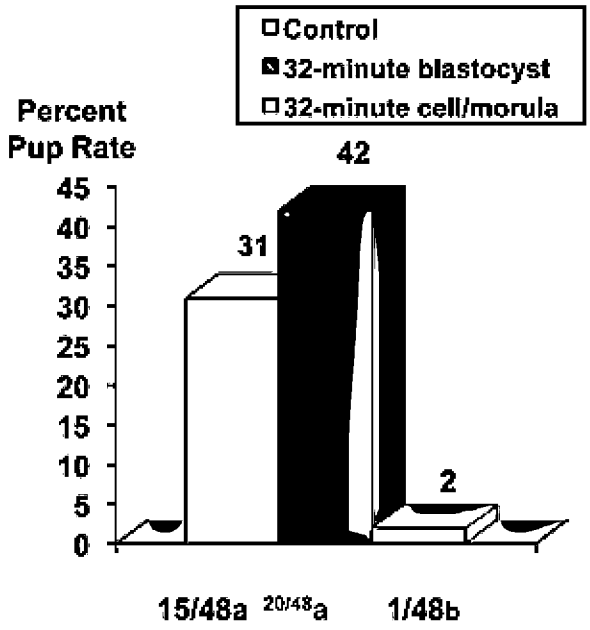


Table 2. Time in Vitrification Solution Blastocyst Rate (Experiment 2).



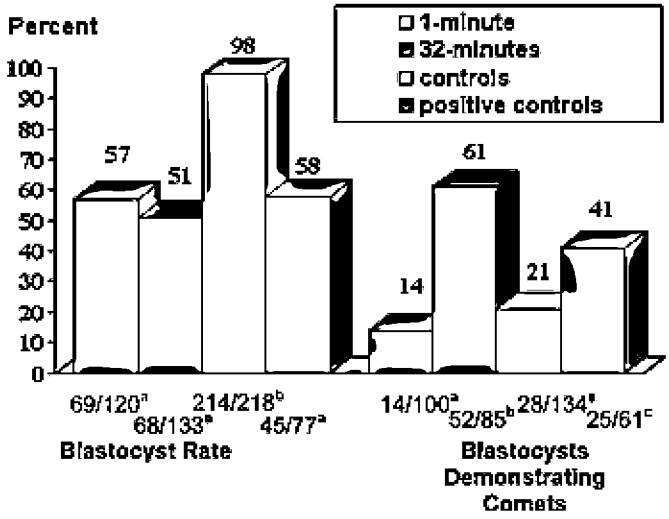
[a,b] Different superscripts indicate statistical difference ($P < 0.05$).

Table 3. Time in Vitrification Solution Birth Rate (Experiment 2).



[a,b] Different superscripts indicate statistical difference ($P < 0.05$).

Table 4. Time in Vitrification Solution Blastocyst Rate and Blastocysts Demonstrating Comet Tails (Experiment 3).



[a,b,c] Different superscripts in groupings indicate statistical difference ($P < 0.05$).

protocols and recipes. There are also various species that have been used as models and the mouse model also includes many strains. We have chosen the vitrification method and solutions that are familiar to our facility (Graves-Herring and Boone 2009) and have chosen the mouse strain that we use to perform toxicity testing. Although we will be comparing our research to others, their solutions, methods, media temperatures, cell-stage and strains of mice may not be the same; therefore, the conditions are not equivalent.

Experiment 1 explored the amount of time (1, 2, 4, 8, 16 or 32 minutes) an embryo could be exposed to vitrification solution before a reduction in blastocyst rate was observed. This experiment was necessary to determine the exposure time for Experiment 2 and 3. For this preliminary study, the blastocyst rate was considered reduced if it was less than or equal to 80%. (A blastocyst rate of 80% is considered normal development for two-cell mouse embryos cultured in our laboratory.) In this experiment embryos were not vitrified, but were instead exposed to vitrification and thawing solutions and then cultured to the blastocyst stage. In Trial 1 a reduced blastocyst rate was observed for the 4-minute exposure group (78%) but not for the 8-minute exposure group (100%). In Trial 2, a slight decrease in blastocyst rate was observed for the 16-minute exposure group (95%), but only the 32-minute exposure group demonstrated the defined reduction in blastocyst rate (44%). In Trial 3, a decrease was observed at the 32-minute exposure group (72%). The reduction in blastocyst rate observed at 32 minutes determined the extended time in vitrification solution for Experiment 2 and 3.

Rall (1987) determined that eight-cell mouse embryos remaining in vitrification solution containing dimethyl sulfoxide, acetamide and propylene glycol at 4°C for 10 to 15 minutes were able to survive, but none were able to survive after remaining in the same solution for 30 minutes. Ali and Shelton (2007) demonstrated exposure of Day-4 mouse embryos to glycerol, dimethyl sulfoxide or propylene glycol for 20 minutes to be toxic (blastocyst rate of 27.3% [12/44]).

In our study the 16-minute exposure group was able to produce a 95% blastocyst rate. Although we did not observe blastocyst rates for times between 17 to 20 minutes, we demonstrated that 44% (Trial 2) and 72%

Figure 1. Mouse blastocyst with some DNA damage (as indicated by the small comet tail).

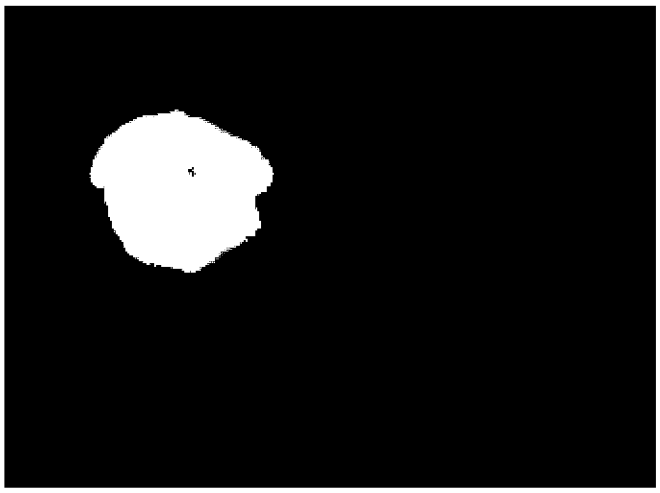


Figure 2. Mouse blastocyst with a large amount of DNA damage (as indicated by the large comet tail).

(Trial 3) of embryos exposed to vitrification solution for 32 minutes were able to develop. This is a higher developmental rate than either Rall (1987) and Ali and Shelton (2007) observed, indicating that the vitrification solutions in this study may not be as toxic.

Experiment 2 demonstrated that the blastocyst rate for the 32-minute exposure group was 76% (193/255) which was significantly different ($P < 0.001$) than the control group (no exposure; 95% [61/64]) indicating that the extended time in vitrification solution inhibited blastocyst growth. Although we determined in Experiment 1 the blastocyst rate for 32-minute exposure embryos was 44% (Trial 2) and 72% (Trial 3), we had a higher blastocyst rate in Experiment 2 (76%). The difference in blastocyst rates could be the result of the low number of embryos (n=18 [Trial 2]; n=53 [Trial 3]) used in Experiment 1.

In Experiment 2, the number of embryos used in the 32-minute exposure group was four times the amount of embryos used in the control group (no exposure; 255 vs. 64). In order to transfer 16 embryos to each designated recipient mouse (8 to each oviduct), we needed to ensure there would be adequate numbers of embryos available for transfers of the second group (32-minute blastocyst group), and third group (32-minute multi-cell to morula stage group). There was no significant differences ($P = 0.3$) between the birth rates for the controls (31% [15/48]) and the second group (42% [20/48]), indicating that those embryos that remained in vitrification solution for 32 minutes and produced blastocysts could produce pups at the same rate as embryos that were not exposed to vitrification solution. In contrast, those embryos that remained in vitrification solution for 32 minutes but were unable to produce blastocysts (32-minute multi-cell to morula stage group) were unable to produce pups (2% [1/48]) similar to the controls (no exposure; 31% [15/48]; $P < 0.05$).

Experiment 3 demonstrated that although there was no difference in blastocyst rates between the 1-minute exposure group and 32-minute exposure group in blastocyst rate, the 32-minute exposure group embryos had significantly more DNA damage as determined by the Comet Assay. A pitfall of the Comet Assay is its inability to specify the exact type of DNA damage. If there is damage to individual blastomeres, degeneration of blastomeres, or degeneration of polar bodies, the Comet Assay cannot delineate these differences. Even though the Comet Assay does not pin-point the origin of the DNA damage, it can provide useful information as to the extent of the damage present by the length of the comet tail.

A major concern with vitrification has been that the solutions are toxic. Those that are performing the procedure must be capable of quickly moving embryos through vitrification solution and loading/placing them into/on a device for storage, sealing the device (if applicable) then quickly plunging the device into liquid nitrogen. In some cases, it is recommended

that this process be completed in 90 to 110 seconds. The vitrification solution used in this study has shown that mouse embryos can remain in the solution for 32 minutes and the embryos have the capacity to produce blastocysts (although at a decreased rate). This gives some leeway to those individuals that are learning the procedure and may not be able to perform the vitrification process within a limited amount of time.

This study is just one of many that are needed to evaluate the exposure time to vitrification solutions. Some future studies should include a more extensive study into exposure time. We only ran three trials to determine the 32 minute exposure time. More trials with times within the 16 to32 minutes should be performed.

For Experiment 2 we observed embryos that were not exposed to vitrification solution to those that were exposed for 32 minutes. Another future study should include exposing embryos to vitrification solution for 1 minute and comparing the blastocyst and live birth results to those exposed for 32 minutes and those not exposed. Not only should the experiment include the process of exposing the embryos to solution, but should also include the vitrifying process and results of not vitrifying to vitrifying should be compared. This would help explain why, in Experiment 2, we observed a 76% blastocyst rate and in Experiment 3 we observed a 51% blastocyst rate for the 32-minute exposure groups. With this future study, we might be able to conclude if this was due to the vitrifying process. If the Comet Assay also as used, we potentially could determine if the vitrifying process causes more DNA damage than just exposure to solutions.

In summary, exposure of mouse embryos to vitrification solution for 32 minutes does cause a decrease in blastocyst rate compared to embryos that are not exposed to vitrification solution. However, if the embryos grow to the early or expanded blastocyst stage and are transferred into recipients, these embryos can produce pups at the same rate as those that are not exposed to vitrification solution despite the higher rate of DNA damage demonstrated by the 32-minute exposed embryos. ■

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