EFFICIENCY OF TWO CRYOPRESERVATION METHODS USING DIRECT IN-STRAW REHYDRATION AFTER REPEATED VITRIFICATION of Mouse Embryos

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ABSTRACT

Experiments were conducted to determine which of two direct in-straw rehydration methods was optimal for obtaining high survival of mouse embryos after repeated vitrification. The first vitrification method compared was a one-step design, designated "House method," where embryos were equilibrated in 3.5 M ethylene glycol for 3 min before rapid plunge vitrification in 7 M ethylene glycol/0.5 M glucose/18% w/v Ficoll 70. The second method was the commercially available BoviPRO Embryo Vitrification Kit ™(MiniTube of America, Verona, WI), which first exposed embryos to 1.4 M glycerol for 5 min, then 1.4 M glycerol/3.6 M ethylene glycol for 5 min, followed by vapor vitrification in 3.4 M glycerol/4.6 M ethylene glycol. Survival rates for morulae and early blastocysts once-vitrified by the BoviPRO were superior to that of the House method. However the re-expansion rate decreased significantly when re-expanded blastocysts were vitrified a second time with the BoviPRO method. The House method proved to be the superior method for the re-vitrification of expanded blastocysts when compared to the BoviPRO method but was only moderately successful for the vitrification of in-vivo produced mouse morulae and early blastocysts. .

1. INTRODUCTION

Vitrification is a fast developing technique for cryopreservation of both gametes and embryos and since the first report of mouse embryo vitrification by Rall and Fahy in 1985, many applicable methodologies have been developed and tested to increase viability of post-warmed embryos, but no one study is universally applicable cell and species-wide. Vitrification can be defined as a rapid solidification of liquid to a glass-like state without ice-crystal formation[7]. This technique for simplifying and improving cryopreservation of cells is appealing because expensive equipment, necessary for controlled-rate cooling, is not required and potential cell damage associated with formation of extracellular and intracellular ice crystals is nearly eliminated [29]. Alternatively to traditional slow cooling cryopreservation methods, vitrification requires a cryoprotectant solution that can remain viscous throughout the process of cooling and warming to encapsulate the cells in a matrix. In 1990, Kasai *et al*. developed a simple vitrification method for mouse morulae characterized by the direct exposure of embryos to high concentration solutions at room temperature which uses ethylene glycol/ficoll/sucrose solutions.

Since then many variations of this approach have been tested with high concentrations of one permeable cryoprotectant or a combination of lower concentrations of cryoprotectants [1-6,12-15,32-35]. However, there is a risk of increased toxic exposure to the embryo from elongated exposure times to the high concentration of cryoprotectants during the procedure. Ethylene glycol (EG) is commonly used in embryo vitrification as a cryoprotectant because of its low molecular weight, high membrane permeability, and is less toxic to mammalian oocytes and embryos than other cryoprotectants [1,12,14-15,30,35]. Vitrification solutions may also contain macromolecules to aid in facilitating the viscosity of the matrix as well as sugars such as sucrose or glucose to prevent excessive swelling and osmotic damage of cells during rehydration by

slowing water in-flow and to facilitate the removal of intracellular cryoprotectants during the dilution and to help maintain structural and functional integrity of cellular membrane during low water activity [3,17,20]

For this experiment, the methods compared utilize ethylene glycol and glycerol, two of the known least toxic cryoprotectants to date and utilize 0.25mL insemination straws commonly used for direct transfer by way of in-straw dilution [5-6]. In the animal field, in-straw dilution and direct transfer allows the embryo to be transferred to the recipient without additional handling. Two methods were selected that were previously developed with the intent to simplify and market an in-field mammalian embryo vitrification protocol. They varied slightly in their equilibration and vitrification solutions, equilibration times, embryo encapsulation volumes, and warming temperatures. Embryos in each sub group were vitrified twice by the same protocol to increase the stress and possible damage that can be induced by vitrification process therefore testing true viability. The ability for an embryo to withstand repeated vitrification is potentially appealing because post-warming procedures can be done without the need to then implant the embryo into a surrogate dam but have the ability to then re-cryogenically store it for future use, when at various stages of development.

2. MATERIALS and METHODS

Except where otherwise indicated, all chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

2.1 Statistics

Minitab was used to run a Fisher's exact test to compare the survival rates of embryos after they had been subjected to the two treatments and subsequent repeated vitrification, compared to a control group. A probability of $p<0.05$ was considered to be statistically significant.

2.2 Embryo Production

All research activities have been approved by the University Institutional Animal Care and Use Committee (IACUC). Female mice (Swiss Webster outbreds and C57B x Swiss Webster; 10-20 wk old; California Polytechnic University, San Luis Obispo, CA) were induced to superovulate by intraperitoneal injections of 8 IU pregnant mare serum gonadotropin (PMSG) and 8 IU human chorionic gonadotropin (hCG) administered 47 h apart at which time females were mated singly with Swiss Webster outbred Males. Embryos were flushed from the excised uteri and oviducts using a modified phosphate-buffered saline (PBS) supplemented with .5% bovine serum albumin (BSA) 85-92 h post hCG administration. The embryos were washed in fresh PBS+BSA medium, pooled and held at room temp (20 +/- 1°C) and only morphologically normal, compact morulae and early blastocysts of grade 1 quality were used. The standard for embryo grading used was the morphological assessment implemented by the Society for Assisted Reproductive Technology (SART)[24]. Experiments involving suspension of embryos in cryoprotectant solutions were conducted in a room at 20 +/- 1°C.

2.3 Vitrification Solutions

For Experiment 1 embryos were held in a base medium similar to SOF + 25 mM Hepes and .25% fatty acid free bovine serum albumin (FAF-BSA) (designated as HCDM2) until vitrification (designated as HCDM2). The equilibration solution (designated as V1) consisted of 3.5 M ethylene glycol in HCDM2, and the vitrification solution (designated V2) consisted of 7 M ethylene glycol, 0.5M glucose, 18% w/v Ficoll 70 in HCDM2. For in straw rehydration a Dilution media (D) consisted of 0.5M glucose in HCDM2. Non-permeable agents used were Ficoll 70 (average molecular weight 70,000) as a macromolecule to assist vitrification, and glucose as a low molecular weight compound which causes embryo shrinkage by osmosis and slows water in-flow during rehydration [14,16,17].

In Experiment 2 all solutions were provided by the BoviPRO Embryo Vitrification Kit ™ (MiniTube of America, Verona, WI). Embryos were held in a zwitterion-buffered solution containing D-glucose, Na-pyruvate, 0.4% BSA, amino acids, growth factors, vitamins, Kanamycin, and Gentamicin (designated HM). Embryos were exposed to increased concentrations of permeable cryoprotectants in Equilibration A containing 1.4 M glycerol in PBS (designated A) followed by Equilibration B containing 1.4 M glycerol and 3.6 M ethylene glycol (designated B). The vitrification solution contained 3.4 M glycerol, 4.6 M ethylene glycol, and 0.5 M galactose (designated Vit). Embryos were rehydrated in a dilution solution containing 0.5 M galactose in PBS (designated DT).

Random, grade 1 embryos were selected from each group of mice harvested as control subjects and were not exposed to cryoprotectant but were directed cultured in-vitro in 6% CO2 in air at 38.6 +/- .5°C, in pre-gassed 30 µL drops of embryo culture medium G2 supplemented with 0.5% FAS-BSA, under mineral oil. Survival of the embryos was assessed by their ability to subsequently develop into expanded or hatching blastocysts.

2.4 Experiment 1: House Vitrification Protocol

Embryos were transferred individually from HCDM2 into 100µL V1 (3.5M ethylene glycol in HCDM2) in 1µL or less using a P10 Pipetman to equilibrate for 3 min. During the first exposure step, 0.25mL plastic insemination straws (0.25 ml, No. F06; Agtech, Kansas, USA) shown in Fig. 1, are pre-loaded using a 1mL syringe fitted with a 100 µL pipet tip on the cotton-polyvinyl end. A 1cm column of dilution medium (D), then 0.5cm column of air is aspirated, another 7cm of D is drawn up followed by 0.5cm air. After initial exposure time, embryo is transferred to a 10-µL drop of vitrification solution (V2) for 45 s and immediately loaded into the pre-loaded straw followed by 0.5cm air and 1cm (~15µL) Dilute. The straw is then heat-sealed, plunged vertically, sealed end first, into liquid nitrogen LN_2 (-196°C) to cover the embryo (Image 1). The rest of the straw is then immersed slowly. The total exposure time to the vitrification media is <1min. Straws were stored in LN_2 (-196°C) for 1-30 d.

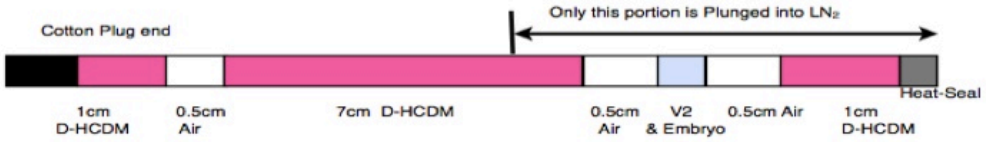

Image 1: A schematic representation of the loaded straw in Experiment 1. From Left to Right: ~10µL of dilution medium, 0.5cm air followed by 7cm dilution medium and 0.5cm air is preloaded. Then 10µL V2+ embryo, another 0.5cm air aspirated followed by 1cm dilution medium. The straw is heat sealed and the portion indicated by the double headed arrow is immediately submerged in liquid nitrogen. The rest of the straw is then slowly lowered into the liquid nitrogen.

2.5 Experiment 2: BoviPRO ™ by MiniTube Vitrification Protocol

Embryos were vitrified following the instructions provided in the BoviPRO Embryo Vitrification Kit ™. Embryos were first transferred from holding medium in 1µL or less using a P10 Pipetman into 100µL Equilibration medium A for 5 min. After initial exposure embryo is moved in 1µL or less using a P10 Pipetman into 100µL Equilibration medium B for 5 min. During the second exposure 0.25mL straws are pre-loaded with a 90µL column of DT medium followed by ~1.5cm air. The embryo is transferred to a 30µL drop of Vitrification medium and immediately loaded into the straw followed by ~1.5cm air and another 90µL of DT medium (Image 2). The straw is then heat-sealed and placed into an empty, pre-chilled 10 mm goblet held in liquid nitrogen (LN_2) vapor (-150 to -180°C) (Image 3). After 1 min of vapor exposure the goblet is lowered into liquid nitrogen (-196°C) and the straw is submerged. The total exposure time to the vitrification media is <1min. Straws were stored in LN_2 (-196°C) for 1-30 d.

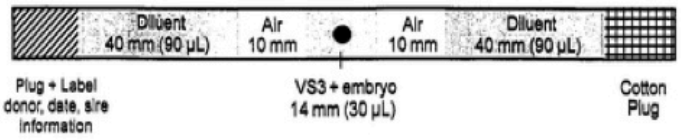

Image 2: A schematic representation of the loaded straw in Experiment 2. From Right to Left: 90µL of DT-dilution medium and 1.5cm air is preloaded followed by 30µL of V3 + embryo, another 1.5cm air aspirated followed by 90µL DT-dilution medium. The straw is heat sealed and vapor cooled for 1 min before submerging in liquid nitrogen.

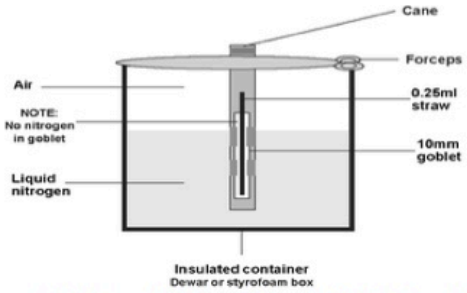

Image 3: Visual of Experiment 2 LN_2 tank for vapor exposure. Cane holding goblet supported by forceps, exposed to but not filled with liquid nitrogen.

2.6 Warming and Culturing Procedures used in Exp. 1 and 2

Vitrified embryos were warmed at one of two temperatures. For Exp.1 straws removed from LN_2 were held in air for 10 s and then placed in a water bath maintained at 37°C for 10 s. For Exp. 2 straws were held in air for 10 s before submersion in water between 20-24°C for 10 s. Immediately after initial warming, all straws were held at the sealed end and flicked 3-5 times, dislodging the air pockets to mix the contents. Embryos were allowed to rehydrate via in-straw-

dilution for up to 1 min before the sealed end of the straw was cut and its contents emptied into a Petri dish. Embryos were recovered and washed through four small drops (~50μL) of holding medium, and cultured in pre-gassed 30μL drops of G2 supplemented with 5% Bovine Serum Albumin (BSA) at 6% CO2 in air at 38.6 +/- .5°C. Re-expansion was evaluated at 12, 24, and 36 h, respectively. Survival was assessed by their ability to re-expand and subsequently develop a blastocoel as expanded blastocysts.

2.7 Experiment 3: Re-Vitrification of Expanding Blastocysts

At 12, 24, and 36 h after warming, embryos in culture from Exp. 1 and 2 were observed for survival. Re-expanded and hatching blastocysts from each sub group House and BoviPRO were vitrified a second time and re-warmed, repeating the protocols. Re-expansion and hatching rates for both treatments were recored 24 and 48 h after the second warming, respectively.

3. RESULTS

3.1 Vitrification of Embryos

A total of 157 morulae and early blastocysts were vitrified in Exp. 1, utilizing the one-step House protocol and the proportion of survival was 36% (n=57). A total of 117 morulae and early blastocysts were vitrified in Exp. 2 using the BoviPRO Vit Kit resulting in a moderate post-warming proportion of survival of 58% (n=68). This comparison of vitrification methods indicates that significantly fewer morulae and early blastocysts survived the House vitrification and warming in-vitro ($p<0.001$) (Table 1).

3.2 Re-Vitrification of Embryos

From the survived embryos in Experiment 1 a total of 40 re-expanded and hatching blasts were re-vitrified, re-warmed and cultured following the House protocol. A significantly higher rate of survival (53%) and subsequent development into expanding and hatching blastocysts (n=21) was found when expanded and hatching blastocysts were vitrified a second time by the House method. In contrast, of the 68 re-vitrified embryos from Experiment 2, the proportion survived was found to be only 12.5% (n=8). Results indicate that the House method superior for the re-vitrification of warmed, re-expanding blastocysts ($p<0.001$) (Table 2).

3.3 Control

A total of 80 Grade 1 morulae and early blastocysts were randomly selected from each group of female mice harvested and cultured as a control. The proportion of survival was 97% and can be seen as a proportion of total embryos harvested and cultured to expanded and hatched.

Table 1. In-vitro re-expansion rates of Grade 1 embryos after vitrification

Vitrification Protocol	Number of Embryos			
	n	Vitrified	Re-expanded	P
Experiment 1 House	157	157	57	0.363
Experiment 2 BoviPRO	117	117	68	0.581 *
Non-treated Control	80	-	77	0.963
Total	354	274	125	

* Values are significantly different from other means in the column (p <0.001)

Table 2. In-vitro re-expansion rates of Grade 1 embryos after repeated vitrification

Re-Vitrification Protocol	Number of Embryos		
	Re-Vitrified	Re-expanded	P
Experiment 1 House	40	21	0.525 *
Experiment 2 BoviPRO	68	8	0.125
Total	108	29	0.2685

* Values are significantly different from other means in the column (p <0.001)

4. DISCUSSION

The objective of this study was to compare two methods of in-straw dilution embryo vitrification to determine which method produced superior post-warming viability of mouse embryos after initial and repeated vitrification. Embryos are routinely vitrified in cryoprotectant solutions containing ethylene glycol and glycerol because of their minimal toxicity compared to other cryoprotectants [1,12,14-15,30,35]. The combination of ethylene glycol, sucrose and Ficoll 70 (EFS) first established by Kasai in 1984 for vitrifying mouse embryos, has proved to be very versatile and relatively nontoxic as it is effective for embryos of many, but not all, species. Still, despite the seemingly harmless process and the ability of embryos to regenerate after being damaged, we are aware from studies of the effects of vitrification on the morphological properties of embryos that profound sub-cellular damage indeed occurs [9,13,24,32-33].

When compared to the BoviPRO method, House vitrified morula and compact blastocysts had a lower post-warming survival rate (58% and 36% respectively). This difference in viability could be associated with toxicity because of improper exposure time to high concentrations of cryoprotectants used or osmotic damages suffered in response to sudden movement of water and solutes into or out of the embryo [19,21,24]. What was more interesting was that exposure to high concentrations (3.5M and 7M relatively) of cryoprotectants for 3 min prior to vitrification resulted in a unique improvement in survivability after *repeated* vitrification (from 36% to 53% respectively). This method was also found to be exceptionally superior (53% vs. 12% respectively) to the BoviPRO subgroup for re-vitrifying warmed embryos in expanded blastocyst and hatching phases. The cause for this increase in survival rate is unclear but could possibly be due to varying characteristics of the embryos. A previous study by S.E. Zhu, et.al., involving ethylene glycol-based vitrification and in-straw dilution of expanding mouse blastocysts,

produced similar survival rates when comparing varying concentrations and exposure times [35]. In the present study, the shorter exposure time and higher concentration of protectant may have proven to be advantageous to the *re-vitrification* of warmed expanding blastocysts, possibly due to increased permeability through the damaged cell membrane, allowing cryoprotectant to sufficiently fill the blastocoel cavity at a comparable rate to that of the cells, reducing ice-crystal formation and thereby increasing survival rates.

Although embryos vitrified once using the two-step equilibration method in Exp.2 had good post-warming viability, the re-vitrification of those survived re-expanded blastocysts resulted in significantly lower survival rates (58% and 12% respectively). Previous studies of various vitrification methods using ethylene glycol-based solutions have produced similar results where the survival rates of mouse blastocysts have been lower than that of eight-cell embryos to morulae, demonstrating that structural differences do change the rate of permeation of cryoprotectants [2,18,22,23,29,34]. As the embryo develops from a morula to a compact blastocyst, a fluid-filled blastocoel begins to enlarge and the individual cells of the embryo become smaller [8]. Sufficient permeation of the cryoprotectant is essential for embryo survival during cryopreservation, but the time necessary for this to happen must not exceed the toxic effects of the permeable cryoprotectants, which must first penetrate the cells before filling the cavity. Therefore the intracellular concentration could reach a toxic level while the blastocoel cavity remains insufficiently permeated, leading to ice-crystal formation within the once fluid-filled space and subsequent decrease in survivability [2,15,35]. Less toxic conditions combined with longer exposure times has proven to be more favorable for the vitrification of expanded mouse blastocysts, rather than more permeating cryoprotectants/higher concentrations with shorter exposure times [14, 35]. However its possible that the extended exposure time during *second* equilibration (5min) had extremely toxic effects on the previously vitrified-warmed expanding blastocysts, combined with the embryo's more vulnerable state, having been potentially severely damaged and in a state of regeneration from the initial vitrification and warming [13, 31-33]. The BoviPRO (5min+5min) equilibration times may be better suited for larger (>300μm) livestock blastocysts.

Differences in survival rates between the two methods could be because of subtleties of the vitrification process such as the size of the encapsulating matrix hosting the embryo within the straw (10 - 30 μL respectively) and the method in which the straw is exposed to the liquid nitrogen, which may have a slight effect on the cooling rates and subsequent survival rates [34]. This procedural difference is thought to be minimal because by plunging rapidly to submerge the section of the straw hosting the embryo, large bubbles are formed in the bath of liquid nitrogen and until temperatures equalize the straw is essentially in a vapor at a similar temperature range to that of the submerged pre-cooled goblet used in Exp. 2. Technician error appeared more prevalent in the House method, though all embryos were vitrified by the same technician, possible variances in micro-pipetting could occur and even a 1μL decrease in volume could have an effect on how well the vitrification solution matrix hosts the embryos. Losses did occur due to technician error, primarily with the smaller 10μL volume, where embryos presumably adhered to the sides of the straw or ends during the straw loading or expulsion and recovery.

In the present study, all embryo handling was done at ambient room temperatures. This has been sufficient for many vitrification protocols however additional studies have demonstrated an ability to overcome the toxic effects of cryoprotectants by decreasing the temperature during equilibration [26] [27] [10] [4][11]. The decreased temperature slows water activity which could have a positive effect on the survival rates for both the vitrification and re-vitrification of mouse

embryos depending on the stage of development and vitrification protocol. The optimal equilibration time is dependent on the exposure temperature, due to rate of permeation of the cryoprotectant and its toxicity, which are also temperature dependent [12]. Another elemental difference in this comparison study was the warming temperature of the water baths which boasted nearly a 10 degree difference for the two experiments. Although it is known that increased temperatures will increase the rate of dilution and rehydration, in this experiment it is difficult to determine exactly how influential this is in comparing the two methods. It is likely that the combination of the non-permeating carbohydrates [22] and macromolecules and higher temperature were all contributing factors to the survival rates, reconfirming that an optimum combination of solutions, equilibration times and temperatures to formulate a method that produces consistent, high viability for *repeated* vitrification of embryos in developing stages has yet to be found. When comparing the two vitrification methods as a whole, it is evident that even though the House method can be a viable vitrification method, easily prepared in most laboratories, it was most successful when re-vitrifying warmed-expanded blastocysts whereas the BoviPRO Vit Kit proves to be better suited for initially vitrifying in-vivo produced earlier stage embryos and not with repeated vitrification of expanded blastocysts.

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