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Mice Embryos Cryopreservation:Vitrification or Ultra-Rapid Freezing?

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ABSTRACT

***Background*:** The importance of mice as animal model for research has promoted the surge of many strains with important characteristics which need to be preserved. Embryo cryopreservation appears as the most suitable technique. However, until now there is not an effective methodology for this specie. This study aimed to evaluate three methods for mice embryos cryopreservation.

***Materials, Methods & Results*:** Morulae (n = 565) were obtained from 42 F1 females (Balb C males X C57 Black females) 6 to 8 weeks old, super-ovulated with 10 IU of eCG, and 10 IU hCG 46 h later. Recovered embryos at morulae stage were randomly allocated in the experimental groups: Fresh controls (CG), vitrification in glass micropipettes (GMV), vitrification in 0.25 mL straws (SV) and ultra-rapid freezing (UR). For vitrification, embryos were exposed for 60 s to 10% ethylene glycol (EG) + 10% propylene glycol (PROP), followed by exposure for 25 s to 20% EG + 20% PROP, while were loaded according to the experimental group (GMV or SV) and immersed in liquid nitrogen (LN_2). Re-warming was performed with decreasing sucrose (SUC) solutions (0.3 M and 0.15 M), for 5 min each. In UR group, embryos were exposed for 5 min in 3.0 M glycerol + 0.5 M SUC in D-PBS + 20% EMS, loaded in straws and maintained for 1 min 2 cm above the LN_2, and then plunged in it. Thawing was performed in a water-bath at 37ºC, for 20 s, followed by exposure for 5 min in 0.5 M SUC solution. *In vitro* culture was performed in D-PBS + 20% EMS for 72 h, at 39ºC and 5% CO_2. The first evaluation at 24 h of culture considered blastocyst stage as viability criteria. The second evaluation was performed at 72 h of culture and considered hatching rate as viability criteria. Data were analyzed by χ^2 test (P <0.05). The GMV group presented the highest blastocyst rate among all treatments (95.6%), as well as the highest hatching rate (94.9%), similar to the CG (97.8% and 93.4% respectively). The UR group presented 91.9% of blastocysts, which was higher than SV, but lower than CG. The SV group had lower blastocyst rate (78.5%) than other treatments. Conversely, the hatching rate presented (76.0%) was higher than the UR (61.3%). Additionally, 126 morulae vitrified in GMV were transferred to 7 pseudo-pregnant recipient females (18/recipient), with two of the recipients (28.5%) delivered six and nine pups, respectively.

***Discussion*:** The most commonly employed methodologies for mouse embryos cryopreservation are the ultra-rapid freezing and vitrification. In previous studies we obtained high blastocyst rates (83.6 to 92.9%) with the ultra-rapid freezing of mice morulae. The embryo stage of development influences viability of cryopreserved embryos, with the highest viability at the morula stage. In this study, the blastocyst rate obtained after the ultra-rapid freezing (91.9%) characterized the high repeatability of the technique, as well as highlighted the morula stage as suitable for cryopreservation. The GMV group showed the highest development rate among the treatments (95.6%), which did not differ from control group (97.8%). Since the GMV and SV groups used the same cryoprotectant solution, we can infer that divergences in viability between the two groups were determined by the loading container type, as well as the volume of solution used during cryopreservation. The glass micropipettes provide higher cooling rates than plastic straws, even though it is possible that the lower cooling rate provided by the straws, associated to a major volume of extender determined the lower viability of such treatments. It is possible that the SV had occasioned injuries that might have prevented a significant percentage of morulae from developing to the blastocyst stage. Conversely, once they had reached this stage, the viability was higher (96.8%). Whereas for the UR treatment, apparently the injuries did not prevent their development to the blastocyst stage, being the negative effect thus been manifested later, by reducing hatching rates (61.3%). Data allow us to conclude that vitrification in glass micropipettes is the most suitable method for cryopreservation of mice morulae, providing viability rates similar to those obtained with fresh embryos, and health live puppies, after embryo transfer.

Keywords: glass micropipettes, OPS, freezing, murine, morulae.

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INTRODUCTION

The similarity with the human genome, easy maintenance and multiplication capacity, had accredited the mice as one of the most important animal models for research. Consequently, many lineages have been spread, some of them transgenic or mutant, which need to be preserved. Cryopreservation arises as an adequate methodology for protecting such lineages from environmental disasters, genetic alterations or infectious diseases [6,15,19]. It is also applicable for short-term storage of embryos, when these cannot be used fresh due to a lack of pseudo-pregnant recipients [15]. Despite positive results have first been shown since the decade of 70 [20,21], and fetuses/live young have been obtained with cryopreserved mouse embryos at all pre-implantation stages [15], the results are very variable and nowadays there is not yet an effective and routine methodology for this specie. The ultra-rapid freezing, in which embryos are dehydrated using high cryoprotectant solutions, is a reasonable alternative to the conventional protocols. This has been already shown by different authors [2,7,17], who obtained high survival rates freezing embryos using the technique. Similarly, vitrification [14] provides good results. It consists of an extreme high increase of solution viscosity, allowing cellular components to direct pass from the liquid to a vitrified and amorphous state, without the generation of ice crystals. Whereas the opened methodologies, like the open pulled straws (OPS) [18] and the glass micropipettes [8] increase cooling rate, potentially increasing viability after cryopreservation. Thereby, the aim of this study was to verify the viability of mice morulae after cryopreservation by ultra-rapid freezing or vitrification, being the latter either in straws or in glass micropipettes.

MATERIALS AND METHODS

This study was approved by the Santa Catarina State University Ethics Committee of Animal Research (protocol 1.16/2010).

Obtaining the structures

Embryo donors were F1 female mice obtained from the crossbreed between Balb C males and C57 Black females, with age ranging from 6 to 8 weeks. The animals were maintained in a photoperiod of 14 h of light /day, at 20ºC ± 3ºC, with water and food *ad-libitum*. Super-ovulation was performed through intra-peritoneal injections of 10 IU eCG[1], followed by 10 IU of hCG[2] after 46 h. Mice were then placed in breeding cages, where 1 male proved for fertility was maintained overnight with 2 females. Female mice were checked for vaginal plug on the following morning (Day 1) to confirm mating. The positive (bred) females were sacrificed 3 days later by cervical displacement [13]. After dissection and removal of the uterine horns, these were deposited in 0.1 mL of D-PBS added of 20% of estrous mare serum (EMS), in Petri dishes. The uterine horns were flushed from the ovarian tube junction using approximately 0.5 mL of D-PBS + 20% EMS, with the aid of a syringe and a 30 gauge needle and handled by non traumatic tweezers. Flushing was performed under stereomicroscope. Just after recovery, embryos were classified by morphologic criteria, according to IETS (International Embryo Transfer Society) standards [16]. Only compact morulae classified as excellent were used for the study, being them randomly allocated among the four experimental groups.

Cryopreservation

All treatments were performed in D-PBS based cryoprotectant solutions. The D-PBS was prepared in the laboratory using ultra-pure water and chemicals from Sigma-Aldrich. The first group was vitrified using glass micropipettes (GMV). The second was vitrified in 0.25 mL straws (SV), and the third was frozen in 0.25 mL straws by the ultra-rapid method (UR). During all replications, a control (not vitrified) group was left in DPBS + 20% EMS in a water-bath at 37°C. On GMV treatment (n=137) morulae were exposed for 60 s to a solution of 10% ethylene glycol (EG)[3] + 10% propylene glycol (PRO)[4], followed by exposure to 20% EG + 20% PRO, for 25 s, while embryos were loaded in the glass micropipette and immediately immersed in liquid nitrogen (LN_2). On group SV (n=121) embryos were exposed to the same solutions, during the same intervals as for group GMV, however the loading was done in 0.25 mL straws, being 20 embryos placed in a 1 cm long column of vitrification solution, surrounded by 2 air bubbles, being the latter surrounded by two columns of 0.3 M sucrose (SUC) solution. The straws were plunged in LN_2 immediately after loading. On group UR (n=124), morulae were exposed during 5 min to a solution of 3.0 M glycerol[5] + 0.5 M SUC, loaded in 0.25 mL

straws using the same scheme as for SV, being the solutions surrounding the air bubbles composed of 0.5 M SUC. After loading, straws were exposed to LN_2 vapor, 2 cm above the LN_2 for 1 min, and then the straws were plunged into LN_2. Embryos from all treatments were maintained in cryogenic containers until thawing or re-warming.

Re-warming / Thawing

On group GMV, re-warming was performed upon 3 s exposure to air, followed by the immersion of the GMV tip in a 0.3 M SUC solution at 35°C, while the bottom was closed with the fingertip, so that a positive pressure gradient was created, pushing the embryos out of the GMV towards the dish containing 0.3 M SUC solution. After 5 min in this first solution, embryos were transferred to a second re-warming solution, with 0.15 M SUC for additional 5 min, when embryos were transferred to D-PBS + 20% EMS. On group SV, after 3 s of exposure to air, straws were immersed in water-bath at 35°C for 20 s, when the straw was spin for mixing the solutions. Soon after, the straw content was poured into a Petri dish. The time lapsed until embryos were transferred to another solution, of 0.15 M SUC was 5 min. Just after, the embryos were finally rinsed in D-PBS + 20% EMS. For thawing embryos from UR group, straws were exposed to air during 3 s and immediately immersed in a water-bath at 35°C, being the straw spin 20 s later. For cryoprotectants removal embryos were transferred to a Petri dish to be 5 min exposed to 0.5 M SUC solution, then rinsed in D-PBS + 20% EMS. Embryos used as controls (CG) were maintained in a water-bath at 37°C during same period used for treatments.

In vitro culture (IVC)

Embryos were cultured for 72 h in D-PBS + 20% EMS under mineral oil, at 38.5°C, with 5% CO_2 and saturated humidity. Two evaluations were performed, being the first one 24 h after the onset of IVC, considering development to the blastocyst stage as the viability criteria. The second evaluation, 72 h after the onset of IVC and considered the hatching rate as the viability criteria.

Additionally, 126 morulae vitrified in GMV were transferred to 7 pseudo-pregnant recipient mice (18/recipient). Recipients were bred with vasectomized males 72 h prior to embryo transfer. The interval between re-warming and transfer was of approximately 2 h. Recipients were submitted to anesthesia using xylazine hydrochloride (10 mg/Kg) + ketamine hydrochloride (50 mg/Kg). The lumbar region was submitted to trichotomy, antisepsis and incision of skin. The incision was pulled aside to the region of peripheral kidney fat, which was torn to expose the ovary, and consequently the uterine horn. Embryos were loaded in a glass micropipette, which was used to punch the uterine horn, being introduced approximately 4 mm for depositing the nine embryos corresponding to each horn. The uterine horn was re-introduced, and the incision was pulled to the other side, to repeat the same process.

Statistical Analysis

Embryo development and hatching rates were analyzed through the Chi-square test, with 5% of significance level.

RESULTS

The three methods provided high embryo survival as well as *in vitro* development competence. The GMV treatment showed the highest developmental rate among the treatments (95.6%), which was not different from control group (97.8%). The UR group yielded 91.9% of blastocysts, which was similar to MV (P >0.05), but lower than CG. On the other hand, after 24 h of culture, SV had a blastocyst yield significantly lower (78.5%) than all other treatments (Table 1).

Hatching rate was evaluated 72 h after the onset of IVC. The GMV group again showed the best result (94.9%), which was similar to control (93.4%), but significantly higher than the other treatments. On the other hand, the SV and UR treatments showed a distinct behavior, with inversion of viability observed at 24 h of culture. The SV hatching rate (76.0%) was significantly higher (P <0.05) than the UR group (61.3%).

Regarding the additional evaluation after embryo transfer, two out of seven recipients (28.5%) delivered six and nine pups, respectively, all them alive and proven to be fertile at puberty. The overall efficiency of mice obtained from the transfer of vitrified embryos was of 12% (15/126).

DISCUSSION

Since the pioneering reports of success with mouse embryos cryopreservation [20,21], many

Table 1. *In vitro* development of mice embryos cryopreserved by different methodologies.

Treatments	N	Blastocysts 24 h culture		Hatched Blastocysts 72 h culture		
		N	%	N	%/Total	%/BI*
Control	183	179	97.8[a]	171	93.4[a]	95.5[a]
Glass micropipettes (GMV)	137	131	95.6[a].b	130	94.9[a]	99.2[a]
Straws Vitrification (SV)	121	95	78.5c	92	76.0b	96.8[a]
Ultra-rapid (UR)	124	114	91.9b	76	61.3c	66.7b
Total	565	519	91.8	469	83.0	90.4

[a,b,c]Distinct letters in the same column indicated differences by χ^2 test (P <0.05).
*****Calculated on a blastocyst basis.

methods have been described, evaluating different cryoprotectant solutions and cooling rates. The conventional freezing method, which applies a temperature decrease of 0.5°C/min from crystallization (-7°C) to -30°C, is a well established method for bovine embryos, providing fully satisfactory results. With mouse or rat embryos, there are few reports published [4,12] possibly due to the high cost of equipments [15]. The methodologies most commonly employed for mouse embryos cryopreservation are the ultra-rapid freezing and vitrification. The ultra-rapid method [17] provides dehydration similar to the obtained with the conventional curve, even without cooling, allowing embryos to be plunged directly into LN_2, and thus dispensing freezing equipments. In previous studies [7], good developmental rates (83.6 to 92.9% of blastocysts) were obtained with the ultra-rapid freezing of mice morulae, using 3.0M glycerol added of 0.25 or 0.5 M lactose. Still regarding this method, the influence of embryo developmental stage has been demonstrated [3,9], with highest viability (80% blastocyst) obtained at the morulae stage. In this study, the blastocyst rate obtained with the method was 91.9%, what confirms the high repeatability of the technique, as well as, highlights the morula stage as suitable for the cryopreservation process. Nevertheless, the method was employed as a parameter of viability to be overcome. Vitrification is another method routinely used for mouse embryos cryopreservation [14], which also gives consistent results [10], and dispenses the use of expensive equipments. In this study, out of 565 cryopreserved morulae, 519 (91.8%) reached the blastocyst stage within 24 h of culture, showing that the three methods allow high survival rates and maintain the embryo *in vitro* development competence.

Although all groups showed satisfactory development to the blastocyst stage, there were significant differences among them. The GMV group showed the highest development rate among all the treatments (95.6%), similar to CG (97.8%). Since the GMV and SV treatments used the same cryoprotectant solution, we can infer that the differences in viability observed between both these groups were determined by the type of loading container used, as well as the volume of solution used during cryopreservation. There are three ways to increase the cooling and warming rates: by reducing the volume of solution; by direct contact of the solution with LN_2 and by preventing thermal vaporization of nitrogen [11]. Many studies showed that the glass micropipettes provide higher cooling rates than plastic straws [1,5,8]. Thus, it is possible that a lower cooling rate provided by the straws, associated to a major volume of extender determined the lower viability of such groups. Moreover, this might have been due to the toxic effect caused by excessive exposure time associated to the possibility of de-vitrification phenomenon during re-warming, what results in ice crystals formation and consequent damage to embryos.

Regarding the evaluation after 72 h of culture, the GMV group showed the best result again, with

94.9% of hatching, which was not different from control (93.4%) but was higher than the other treatments. Regarding the SV and UR groups, there was a reversal effect of their viability, observed after 24 h of culture, where a greater viability in the SV group (76.0% of hatching) was showed, in comparison to UR (61.3%). It is possible that the SV treatment caused injuries that might have prevented a significant percentage of morulae from developing to the blastocyst stage. Conversely, once they had reached this stage, the viability was higher (96.8%). Apparently, for the UR treatment the injuries did not prevent development to the blastocyst stage. Thus, this negative effect might have been manifested later, by significantly reducing hatching rate (61.3%).

When hatching rate was evaluated considering the embryos that had developed to the blastocyst stage after the first 24 h of culture, no differences were observed among the control (95.5%), GMV (99.2%) and SV (96.8%) groups, being all them significantly higher than UR (66.7%).

CONCLUSIONS

According to our data, the most appropriated method for cryopreservation of mice morulae is the one that uses glass micropipettes for loading. This method allowed viability similar to that obtained with fresh embryos. Also, it provided the birth of healthy mice, after transfer of vitrified/re-warmed embryos to recipients. Moreover, vitrification in straws, as well as the ultra-rapid freezing method allows high embryo development rates, although still lower than that obtained with vitrification in glass micropipettes.

SOURCES AND MANUFACTURERS

[1]eCG - Folligon, Intervet Shering-Plought Animal Health, Cotia, SP, Brazil.

[2]hCG - Chorulon, Intervet Shering-Plought Animal Health, Cotia, SP, Brazil.

[3] Ethylene glycol, Sigma-Aldrich Brasil Ltda., São Paulo, SP, Brazil.

[4] Propylene glycol, Sigma-Aldrich Brasil Ltda., São Paulo, SP, Brazil.

[5] Glycerol, Merck S.A., São Paulo, SP, Brazil.

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