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Cryopreservation of mouse embryo using vitrification method

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Abstract

Cryopreservation plays an important role in storing the embryos of unwanted strains of laboratory animals in liquid nitrogen and maintaining the lines in smallest available space. This not only helps in curtailing the unwanted expenditure of maintaining these lines but also saving time and energy. Laboratory Animal Facility of ACTREC is maintaining various strains of mice, rats and hamsters. Our facility has established rodent embryo cryo-bank which acts as a backup for the valuable strains. Initially, we have made a successful attempt by slow freezing and fast thawing method. Considering the recent advances in cryopreservation technique and success rate, we report here an attempt of the cryopreservation of mouse embryos by vitrification technique. In the present study, in all 851 embryos were collected from 132 donor female mice from four strains of different background. An average gain of 7 embryos per female was recorded by natural mating. Collected embryos were stored in 45 cryovials. As a quality control measure and to check the efficiency of newly adopted vitrification protocol, we thawed few vials at periodic interval. The percentage of recovery of embryos was ranging from 91.67% to 97.5%. The lower percentage of 91.67% was found in Smo/Smo strain, while higher percentage of 97.5% was found in outbred, Crl: CD1 (ICR) strain. These embryos were kept in CO2 incubator for 1 hr for maintaining morphology. After incubation, the percentage of embryos maintained morphology in these four strains was ranging from 57.33% to 100%. The lowest percentage 57.33% of morphology was found in spontaneous mutant S/RV/Cri-ba strain. The morphology maintained embryos were surgically transferred in 1.5 day post-coitus pseudo-pregnant female mice of CD1 strain. Out of 17 females, 12 females became pregnant and delivered total 51 live pups which were subsequently weaned and allowed to breed further. The percentage of embryo transferred Vs live births was ranging from 14.89% to 31.03%. Based upon the results, it is evident that there is an increase in overall success of the newly adopted technique of vitrification which is simple and less time consuming than slow freezing and fast thawing method.

Key words : Cryopreservation, Embryo, Ethylene glycol, Vitrification, Asynchronous transfer

Introduction

Generation of animal models by genetic modification or by targeted mutations is an ongoing procedure. The value of these animal models to study the human diseases is now well known fact. With the advancement in genetic manipulation technologies, there are thousands of new strains created by the scientists. But these valuable strains are on high risk due to either by microbiological contamination, genetic contamination or reproductive failures (Mochida *et al*., 2013). It is impractical to keep all the mouse strains as breeding colonies due to high cost involved in live animal maintenance. To protect theses strains, Animal Facilities need to establish cryobank of germplasms of available strains. As per the need, the facilities may adopt either cryopreservation of sperms, embryos, oocytes, ovaries or all. Hence, cryopreservation of germplasm is a strategy not only for saving costs and space, but also for avoiding the microbiological and genetic contamination of valuabl strains, when not in use.

Whittingham *et al*., in 1972 published the first reproducible results of mouse embryo cryopreservation by using slow freezing and slow thawing method. In this method

embryos were cooled slowly after equilibration in suitable cryoprotectant. The slow controlled rate is conventionally achieved by commercially available controlled rate freezer. This method requires, ice seeding, expensive programmable control rate freezer and is very time consuming (Jin *et al*., 2010). Also, if the cooling rate is not maintained properly then the percentage of intracellular ice formation due to insufficient dehydration is very high (Mochida *et al*., 2010). Since the first report, many technical improvements have been made by scientists to make the entire procedure simple and more effective. Scientists mainly emphasize on how to increase the success rate, survivability and reduce the cost and time required to complete the entire procedure. Rall and Fahy in 1985 developed the novel technology called "Vitrification". Vitrification as described is a simple method of directly transferring embryos into liquid nitrogen after a brief exposure to a cryoprotectant solution without intra- and extra-cellular ice formation. It is a thermodynamic procedure where viscosity of the fluid is increased by using high concentration of cryoprotective agent. Vitrification offers two important advantages over slow freezing method. First, cryoinjury associated with formation of intracellular ice is eliminated because no ice formation occurs in vitrified suspension during freezing. Second, vitrification permits a substantial reduction in the time and there is no costly equipment required for cryopreservation (Mello *et al*., 2001). However, the cryoprotectants contained are highly toxic and may affect subsequent embryo development. Therefore, the technique was not applicable to certain strains of mice, even when the solutions are cooled to 4°C to mitigate the toxic effect during embryo handling (Mochida *et al*., 2010).

Extensive research comparing the effect of controlled slow freezing and vitrification on embryo survival and live births is being undertaken and the results are contradicting. Some studies have reported no statistical difference between two procedures in blastocyst formation and implantation capacities of mouse (Rall and Wood, 1994) or bovine embryos (Van Wagtendonk *et al*., 1995). Dinnyes *et al*., 1995 reported that vitrification yielded significantly higher rate of implantation than those achieved after slow freezing of mouse embryos. Whereas Uechi *et al*., 1997 reported that the controlled rate slow freezing- thawing procedure of mouse embryos decreases not only development rate of blastocyst stage, but also decreases glucose incorporation of the developed blastocyst due to decreased expression of GLUT1 suggesting that cryopreservation may have ulterior consequences on the functional development of embryos by this method.

ACTREC Laboratory Animal Facility has established an embryo cryobank for various available rodent strains by using slow freezing and fast thawing method (Thorat and Ingle 2012; Thorat and Ingle 2013). In this study, we found that overall success rate varies as per genotypes and hence we decided to study the efficacy of vitrification protocol of cryopreservation of mouse embryos of different strains by assessing morphology of thawed embryos as well as percentage of live births after asynchronous transfer of thawed embryos.

Materials and Methods

Animals and Husbandry

Embryos from 6-8 week old nulliparous four mice strains from ACTREC, Navi Mumbai were used in the present study. Original stock of breeders of Crl:CD1(ICR) mice and C57BL/6NCrl mice were procured from the Charles River Laboratories, USA. S/RV/Cri-ba strain is a result of spontaneous mutation in our Swiss mice colony. Smo/Smo mice were procured from Fred Hutchinson Cancer Research Centre, USA. We bred Crl:CD1(ICR) by outbreeding method while C57BL/6NCrl mice by inbreeding method. This study was duly approved by the Institutional Animal Ethics Committee (IAEC) of ACTREC, Navi Mumbai (proposal no. 08/2011). All strains of mice were housed in an Individually Ventilated Animal Caging System (Citizen Industries, Ahmedabad, Gujarat, India) and provided with commercially available corn cob bedding material (ATNT Laboratories, MS, India). The animals were housed in a controlled environment at 23±2°C with 40-70% relative humidity and a 12-h/12-h dark/light cycle. The animals were provided with sterile water ad libitum and autoclaved balanced diet prepared in-house from natural ingredients like wheat, roasted Bengal gram, casein, milk powder, groundnut oil, vitamins, and mineral supplements, which provided approx. 21% crude proteins. Timed pregnant females were obtained by natural mating and checking for the presence of a vaginal plug (VP) the next morning.

Collection of embryos

We collected the embryos as per our previously described method (Thorat and Ingle, 2012). In short, on day 2.5 post coital, VP-positive females were sacrificed by cervical dislocation to collect the 8-cell to compacted morula-stage embryos. Both oviducts were excised along with a small piece of the adjoining uterus and placed in a drop of M2 medium (Cat No. Sigma-Aldrich, USA) in a sterile Petri dishes (Cat No. 150288; Nunc, Rochester, NY, USA). The oviducts were slowly flushed with M2 medium using 30 G needle by holding the infundibulum under a stereomicroscope (model SMZ-1500; Nikon, Tokyo, Japan) to expel the embryos. The embryos were siphoned, transferred to a fresh drop of M2 medium, and washed thrice with the same medium to get rid of the debris.

Vitrification and Thawing Solutions

We used vitrification protocol developed by Mochida *et al*., 2011 based on EFS solution which is composed of Ethylene Glycol (EG), Ficoll PM 70 (Ficoll 70000) and Sucrose in PB1 medium. In short, to make the Ficoll-Sucrose (FS) solution, we mixed 6 gm Ficoll 70 and 3.424 gm Sucrose in 14 ml of PB1 solution. Sucrose was completely dissolved after shaking the mixture well. After ascertaining complete dissolution, 42 mg BSA was added on the surface of the solution and kept at 4°C until BSA is completely dissolved (> 4 hours or overnight). To make the equilibration solution EFS 20, we added 1 ml EG in 4 ml of FS solution. According to this dilution, EFS20 contains 20 % (v/v) ethylene glycol, 24% (w/v) Ficoll, and 0.4 mol/L sucrose in PB1 with BSA. Final

vitrification solution was prepared by addition of 2 ml of EG in 3 ml FS solution. The final concentration of EFS40 was 40 % (v/v) ethylene glycol, 18% (w/v) Ficoll, and 0.3 mol/L sucrose in PB1 with BSA. All the solutions were sterilized by filtration using a 0.45 µm filter (Millex –HV, Merck Millipore, USA) and kept at 4^0C until further use.

Thawing protocol was also adopted from work published by Mochida *et al*., 2011. Two thawing solutions, TS1 and TS2 containing 0.75 M and 0.25 M Sucrose were used, respectively. In short, TS1 was prepared by dissolving 7.7 g of sucrose in PB1 and brought the total volume to 30 ml. Once sucrose was completely dissolved we added 90 mg of BSA onto the surface the solution and left it to stand until completely dissolved. All the solutions were sterilized by filtration using a 0.45 µm filter (Millex –HV, Merck Millipore, USA). TS2 containing 0.25 M sucrose was prepared by diluting 10 ml TS1 with 20 ml volume of PB1.

Vitrification of embryos

Embryos were vitrified by a two-step method. In the first step, we transferred approximately 20-30 embryos at the bottom of drop of 50 µl of EFS20 containing 20 % (v/v) ethylene glycol, 24% (w/v) Ficoll, and 0.4 mol/L sucrose in PB1 with BSA using a glass capillary with minimum amount of the M2 medium. Embryos were exposed in EFS20 for 2 min. In the second step, we transferred all embryos from EFS20 drop into the EFS40 containing 40 % (v/v) ethylene glycol, 18% (w/v) Ficoll, and 0.3 mol/L sucrose in PB1 with BSA in the cryovials (Cat. No. CLS430488-500EA Corning Life Sciences, USA) without forming bubbles for 1 min. After the treatment of 1 min, the cryovials were directly plunged into the liquid nitrogen (LN2) container (Jumbo-45, IBP, IOCL Co. Nashik, India).

Thawing of embryos

Before thawing, a 35 mm petri plate containing 50 µl drops of embryo culture medium, Sydney IVF Blastocyst medium (K-SIBM50 William A. Cook Australia Pvt. Ltd., Brisbane, Australia) covered with paraffin oil was kept in a CO_2 incubator (Model 3111, Thermo Scientific, MA, USA) until further use.

With the help of cryogloves (Cat. No. 371060, Tarsons Products Pvt. Ltd., Kolkata, India) cryovials were removed from the liquid nitrogen container and quickly removed the cap of the vial. After 30 seconds of exposure at room temperature, added 850 µl of pre warmed TS1 solution into the cryovials. TS1 solution was pre-warmed at 37°C and mixed by gentle pipetting without formation of bubbles. Transferred the entire solution in 60 mm petri plates and held there for 3 minutes. After 2 min the plates was gently rotated to settle down the embryos at the bottom of the plate. Embryos were transferred to 50 µl drop of TS2 solution where exposure for 3 minutes was given. After 3 min, embryos were transferred in another drop of TS2 solution. Likewise three washes were given using TS2 solution.

Making pseudo-pregnant females:

Pseudo pregnant Crl: CD1 (ICR) outbred female mice were created as per the procedure described by Thorat and Ingle, 2012.

In vivo survival of vitrified embryos

To check the *in vivo* efficiency, thawed embryos were directly transferred surgically into oviduct of 1.5 day old pseudo- pregnant mice.

Statistical analysis

Statistical analysis was performed using the Chi-square exact test.

Results

Collection of embryos

During the period of 24 months i.e. during the year 2014 and 2015, we collected total of 851 numbers of 8-cells to morula stage compacted embryos from 132 donor females of four different strains of mice. All the embryos were found morphologically normal with intact zona pellucida and normal cytoplasmic granulation. These embryos were vitrified in 45 cryovials with an average 18.91 (=19) number of embryos per vial. The dehydrated embryos showed a shrunken morphology. Out of four strains, outbred Crl: CD1(ICR) mice yielded an average of 7.56±0.961, inbred C57BL/6NCrl mice 6.26± 0.828, spontaneous mutant S/RV/ Cri-ba mice 6.20±0.816 and knock out Smo/ Smo mice 5.63± 0.518 number of embryos. Total number of females used, embryos collected and vitrified is summarized in Table 1.

Few embryos from each strain were thawed for quality control purpose. Out of 404 thawed embryos, we could retrieve 388 numbers of embryos with the average retrieval percentage of 96.04%. Out of 388 embryos, total 293 embryos were morphologically intact and normal which amounts to 75.52%. The rate of morphologically normal embryos after thawing was recorded as 74.36% for CD1 mice, 57% for S/RV/Cri-ba, 78% for C57BL/6NCrl and 100% for Smo/ Smo strain. Strain wise summary of the embryos thawed, and retrieved is presented in Table 1.

In vivo survival

All the morphologically normal embryos were surgically transferred in the oviduct of 1.5 dpc pseudo pregnant female mice. Total 293 normal embryos were transferred in 17 pseudo-pregnant female mice with an average of 17 embryos per mouse. The average percentage of pregnancy was reported to 70.59%. The percentage of live pups born ranged from 14.89% to 31.03%. Data related to number of pseudo pregnant female mice, pregnancy percentage and live fetuses born are summarized in Table 1.

Discussion

In the present study we cryopreserved embryos by vitrification method. In our previous study we cryopreserved mice embryos by slow freezing and fast thawing method (Thorat and Ingle, 2012). Slow freezing is an expensive and time consuming as compared to vitrification. The primary advantage of vitrification method for the cryopreservation of mouse embryos are that expensive freezing apparatus is not required and overall time required for vitrification is very low (Mello *et al*., 2001). We noticed that quantity of liquid nitrogen required for vitrification is very minimal when compared to that of slow freezing method, which makes it more cost effective. Most attempts have been made to improve the technique since vitrification has been invented by Rall and Fahy, 1985. Based upon simplification of the protocol and success rate, in this study we have adopted vitrification protocol developed by Mochida *et al*., 2011. The main advantage of this method is use of EFS solution which consists of ethylene glycol, Ficoll 70 and sucrose. Ethylene glycol is less toxic and high permeability than other cryoprotectant (Miyake *et al*., 1993). Ethylene glycol has been widely used for the vitrification of embryos and oocyte from various mammalian species including rat (Han *et al*., 2003), rabbit (Kasai *et al*., 1992a), cow (Tachikawa *et al*., 1993), horse (Hochi *et al*., 1994), sheep (Martinez and Matkovic, 1998), monogolian gerbil (Mochida *et al*., 2005) and mastomys (Mochida *et al*., 2001). Ficoll 70 has beneficial effect because of high solubility and low viscosity also it facilitates non crystallization by preventing devitrification during warming (Kasai *et al*., 1990). Sucrose facilitates removal of intracellular cryoprotectant during dilution (Leibo and Mazur, 1978; Kasai *et al*., 1980) as well as reduces the toxicity by causing the embryos to shrink rapidly and reducing the amount of ethylene glycol in the cells (Kasai *et al*., 1990). The most protocol developed so far are by using plastic straws. However, plastic straws are more fragile and need certain skills during handling for freezing and thawing. So the choice of container used for vitrification is Cryotubes (Mochida *et al*., 2013).

In the present study we have used 8-cells to compact morula-stage embryos for vitrification as this stage is less sensitive to osmotic stress and also morulae are highly permeable to ethylene glycol (Kasai *et al*., 1992b). Miyake *et al*., 1993 also reported that 8-cell to morula-stage embryos can be vitrified without significant loss in viability. Few other reports have also suggested that this stage is most preferable stage for maintaining high survival rate (Scheffen *et al*., 1986; Kono and Tsunoda, 1987; Nakagata 1989; Matsumoto *et al*., 1987; Valdez *et al*., 1990). The average gain of embryos per female by natural mating was found to be satisfactory for all strains. The average gain in C57BL/6 mice was 6.26 which is similar to our previously published results (Thorat and Ingle, 2012). We have noticed higher embryo yield in case of outbred CD1 mice when compared to other strains. The genetic backgrounds of mice are a major factor for reproductivity and embryo recovery (Dinnyes *et al*., 1995; Taketo *et al*., 1991).

For the quality control purpose and standardization of vitrification procedure at our laboratory conditions, we thawed representative embryos from all four strains. The percentage of recovery of embryos was ranging from 91.67% to 97.5%. The higher recovery percentage of 97.50% was noticed in outbred CD1 mice, followed by 97.18% in case of C57BL/6 mice, 93.75% in S/RV/Cri-ba mice and 91.67% in Smo/smo knockout mice. Our percentage of recovery corresponds to the recovery earlier reported by Kasai *et al*., 1990. Technical skill of individuals plays an important role in recovery of embryos during the thawing procedure.

In the present study, the post warming viability was assessed on the basis of original morphology retained by the embryos. Post thawing percentage of normal morphologic embryos was reported between 57.33 to 100%. The variation in percentage of normal morphology of embryos might be due to variation in number of embryos thawed for each strain.

Morphologically normal embryos were surgically transferred into pseudo-pregnant mice. Asynchronous transfer of embryos to the oviduct is reported to yield better implantation than synchronous transfer (Renard and Babinet, 1984). Therefore, in this study we adopted asynchronous transfer of embryos in 1.5 day old pseudo pregnant mice. The percentage of young ones born from asynchronous transfer in each strain varies from 14.89 to 31.03%. We reported increased percentage of live births from asynchronous transfer of embryos in oviduct when compared with our earlier report of synchronous transfer of embryos in uterus (Thorat and Ingle, 2012). However, technical skill, number of embryos transferred per female and strain differences of donor mice cannot be overlooked for overall success of the procedure.

In conclusion, vitrification of mouse embryos from various background of outbred, inbred, spontaneous mutant and knockout strain of mice is possible. However, the success rate varies within the type of strain used. Pregnancies and deliveries thereby leading to birth of normal live fetuses from transfer of thawed embryos into the pseudo-pregnant females provided confirmation that vitrification procedure is valid and effective to practice at our laboratory conditions. The resultant progeny from the present study is good enough to expand the breeding colony and cater the future need of animals. Further, asynchronous transfer has even increased the percentage of live births when compared with our earlier report of synchronous transfer. To evaluate its efficiency, further there is a need to undertake studies by using more number of embryos of these strains as well as other available strains.

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Table 1. Information on number of females utilized, total number of embryos collected and vitrified, number of cryovials, number of embryos thawed, total number of embryos retrived after thawing, number of embryos maintained normal morphology after thawing, and subsequent live births from four different strains.

Sl. No.	Strain	Number of females used = n	Total embryos collected and vitrified = n	Mean ± SD embryos collected	Number of Embryo thawed = n	Normal embryo retrieved on thawing	Embryos maintained normal morphology and transferred	Number of psuedo-pregnant females used	Number of females found pregnant	Number of live births*
1	CD1(ICR)/Cri	25	189	7.6 ± 0.8	40	39 (97.5)	29 (74.36)	2	2	9 (31.03)
2	S/RV/Cri-ba	49	304	6.2 ± 0.9	80	75 (93.75)	43 (57.33)	2	1	8 (18.60)
3	C57BL/6NCrl	50	313	6.3 ± 1.7	248	241 (97.18)	188 (78.0)	11	7	28 (14.89)
4	Smo/Smo	8	45	5.6 ± 0.1	36	33 (91.67)	33 (100)	2	1	6 (18.18)

* Calculated on no. of embryos transferred Vs. no. of live births.

Values in parenthesis indicates the percentage