—Mini Review—

Cryopreservation of Embryos in Laboratory Species

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Abstract: *Cryopreservation of embryos is an important strategy for the conservation of species and valuable strains of laboratory species. Historically, in the mouse, the technical development of cryopreservation started with slow freezing methods in the 1970's, which was then followed by vitrification method developed in the 1980's. Vitrification is advantageous in its quickness and simplicity, because it does not need programmable controlled-rate freezing machines. Furthermore, the survivability of embryos recovered after vitrification is significantly improved by avoiding chilling injury and intra- and extra-cellular ice formation. Recently, a series of new vitrification methods using a minimal volume of cryopreservation agent (CPA) and extremely high osmolality CPAs have been developed. For laboratory rats and mice, there is a long history of embryo cryopreservation and huge numbers of embryos have been kept frozen in cryobanks. In contrast, the techniques have only recently been optimized for other laboratory animals, e.g., rabbits, Syrian hamsters, Mongolian gerbils and mastomys (African rodent). Besides safety in cryopreservation, simple transportation of vitrified embryos on dry ice has been a challenging issue. By using the extremely high osmolality CPAs, we are now examining the feasibility and effectiveness of a simple method for the transportation of vitrified embryos. Development of new cryopreservation methods for embryos of laboratory species should be an integral part of the technological logistics supporting the development of biomedical sciences.*

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Introduction

The major purpose of cryopreservation for laboratory animals is the conservation of species or of particularly valuable genetic strains. For laboratory mice, many strains have been produced by genetic modifications (e.g., transgenesis, gene targeting and mutagenesis) and the number is constantly increasing. Our center has a duty, as the National BioResource Project (NBRP), to collect mouse strains, mainly from Japanese researchers, and to distribute them to laboratories all over the world. To now, our center has collected about 5,000 strains, but it is impossible both economically and physically to maintain all of them as live animals. Therefore, cryopreservation of embryos and/or spermatozoa is urgently needed. We have distributed mouse strains as live mice produced by embryo transfer after thawing of frozen embryos, or as frozen embryos sent directly to researchers on request. Thus, cryopreservation is an efficient and economical strategy for preserving valuable strains and for transporting them to other facilities.

For laboratory rats, a similar strategy for preserving strains as frozen stocks has been supported by the NBRP and they are now available for distribution to scientific communities worldwide. There is a long history of embryo cryopreservation for laboratory rats and mice and huge numbers of embryos have been kept frozen in cryobanks. By contrast, the techniques for cryopreserving embryos of other laboratory animals have only recently been optimized and applied to preserving the species or strains as frozen stocks. A series of vitrification methods using a minimum volume of cryopreservation agent (CPA) and extremely high osmolality CPAs, which enable us to transport vitrified embryos in dry ice packages, have now been developed. In this review, we describe the development of cryopreservation methods in laboratory species in the order of their development. We also present a list of

Table 1. Laboratory species yielding offspring following the transfer of cryopreserved embryos

Species	Cryopreservation method	Cryoprotective agents	Author	Year	Reference
Mouse	slow freezing	1 M DMSO	Whittingham DG, *et al.*	1972	[1]
Rabbit	slow freezing	1.6 M DMSO	Bank H, *et al.*	1974	[2]
Rat	slow freezing	1.5 M DMSO	Whittingham DG, *et al.*	1975	[3]
Mastomys	vitrification (2-step)	20%EG+F+S and 40%EG+F+S	Mochida K, *et al.*	1998	[4, 5]
Hamster	vitrification (2-step)	10%DMSO+10%EG and 20%DMSO+20%EG+F+S	Lane M, *et al.*	1999	[6, 7]
Mongolian gerbil	vitrification (2-step)	20%EG+F+S and 40%EG+F+S	Mochida K, *et al.*	1999	[8, 9]

DMSO: dimethyl sulphoxide, EG: ethylene glycol, F: Ficoll, S: sucrose.

laboratory species yielding offspring following the transfer of cryopreserved embryos in Table 1.

Slow Freezing Method: The First Generation of Cryopreservation Technology

Success in the cryopreservation of mouse embryos using the slow freezing method was first reported in 1972 [1]. This documented the development to normal young after the transfer of frozen-thawed embryos to pseudopregnant recipients. In this approach, embryos are cooled very slowly (0.3–0.5ºC/min) after equilibration in 1–1.5 mol/l cryoprotectant. This method typically requires a programmable controlled-rate freezing machine and uses manual seeding at around –7ºC for the formation of pure water ice crystals in the CPA. During slow cooling, the cellular contents become concentrated by gradual dehydration in response to the concentration of the extracellular unfrozen fraction during the growth of extracellular ice. Intracellular ice formation caused by insufficient dehydration in the embryonic cytoplasm results in death, if the cooling rates become too high. After cooling to –40ºC, the intra- and extracellular CPA is sufficiently concentrated by progressive dehydration and ice formation and the straws or cryotubes containing embryos are plunged directly into LN_2.

In this slow freezing method, the absence of technical complications is one of its merits, because the thawing procedure is also slow. The straws or cryotubes are taken from LN_2 and placed at room temperature until the ice thaws, then the embryos are recovered in a thawing solution of modified phosphate-buffered saline (PB1 [10]) [11]. However, it requires a programmed freezer to control the cooling rate and takes a long time (> 2 h) to reduce temperature to –40ºC. These are the disadvantages in a slow freezing method. We can expect good results to produce a large number of offspring from frozen embryos in strains for which the embryos show high levels of resistance to manipulation and freeze-thawing. However, for embryo strains sensitive to manipulation *in vitro*, only a few offspring can be produced [12]. In particular, differences in embryo sensitivity during the procedure of freeze-thawing in each strain or species lead to significant differences in results.

Vitrification: The Second Generation of Cryopreservation Technology

In 1985, Rall and Fahy devised a vitrification method [4], in which embryos are suspended in a highly concentrated CPA solution, which can be cooled to LN_2 temperature directly, without intra- and extracellular ice formation. This method eliminated the slow cooling process and the use of a programmable controlled-rate freezing machine. However, the initial vitrification solution (VS1) was highly toxic and embryos needed to be exposed to this solution at refrigeration temperature. Since the original report, numerous vitrification solutions have been devised using various cryoprotectants. Nakagata modified the VS1 formula and created a solution with 2 mol/l DMSO, 1 mol/l acetamide and 3 mol/l propylene glycol, named DAP213 [14]. However, this solution was still very embryotoxic and the procedure was modified by equilibrating embryos were in DAP213 at 0ºC [15].

Kasai *et al*. later created a low-toxicity vitrification solution named EFS40 [16]. They tested the effects of permeating cryoprotectants on mouse morulae and chose ethylene glycol (EG) as the least toxic agent. EFS40 consists of EG, Ficoll as a macromolecule to prevent devitrification, and sucrose, which exerts a considerable osmotic effect. This EFS40 consists of

40% (v/v) EG, 18% (w/v) Ficoll and 0.3 M sucrose in PB1. It is used as a CPA for mouse embryo cryobanking at our center, after equilibration for 2 min at room temperature in EFS20 consisting of 20% (v/v) EG, 24% (w/v) Ficoll and 0.4 M sucrose in PB1. We have successfully cryopreserved more than 2,000 mouse strains with this two-step vitrification procedure [8].

Although the optimal conditions for vitrifying embryos differ among species, strains and developmental stages [17–20], the selection of the cryopreservation container is more important in practical terms. In general, embryos vitrified using plastic straws with about 10 μl of CPA show higher survival rates than those frozen in cryotubes. For straws, it is possible to decrease the concentration of CPA, because the lower volume means that the cooling speed is increased after plunging into LN_2. On the other hand, the handling of a cryotube is easier than a straw for inexperienced operators.

Minimum Volume Vitrification: The Third Generation of Cryopreservation Technology

At the annual meeting of the International Embryo Transfer Society in 1999, the successful development to offspring after the cryopreservation of 2-cell hamster embryos was reported [6, 7]. The cryopreservation method used a cryoloop—a small loop of metallic wire 0.3–1.0 mm in diameter—as a vehicle for the CPA containing the embryos. The volume of CPA was only 1 μl and the embryos were recovered without any loss in viability. In this congress, several new technologies to preserve the embryos or oocytes in a minimum volume of CPA were presented, including methods with open-pulled straws. In these techniques, a droplet of CPA containing embryos in a minimum volume of CPA (> 1 μl) is put on the wall of a straw which is then plunged into LN_2. The aims of these methods are to accelerate the cooling speed and prevent any decrease in embryo survivability resulting from chilling injury, ice formation, toxicity of CPA and cell damage during the cooling and warming processes. Before these reports, minimum volume vitrification strategies had been developed using glass straw [21], electron microscope grids [22] and open-pulled straws [23]. Subsequently, the Cryotop method was established by Kuwayama *et al*. [24]. This method has been used for the efficient cryopreservation in many species of mammals, especially humans and domestic species. We reported that vitrified mouse oocytes of four major inbred strains and one F1 hybrid strain, which were cryopreserved using Cryotop, developed to offspring after intracytoplasmic sperm injection [25] and verified that the Cryotop method was also effective even for large numbers (20 to 30) of oocytes in species such as the mouse.

Vitrification in Laboratory Species

Many reproductive technologies such as superovulation, embryo culture, embryo transfer and embryo cryopreservation have been established successfully for the mouse. However, some strains show varied developmental ability *in vitro* [26] and for some strains, frozen–thawed embryos develop to term with low efficiency. Embryos from the A/J, AKR and C57L strains show low developmental ability *in vitro* and embryos from the GR, DDY and ddY strains show slow development to the morula stage. Also there is a great variation in the number of oocytes ovulated following the injection of gonadotropins for superovulation. In particular, obtaining sufficient number of embryos from wild-derived strains for cryopreservation is a major problem.

Similarly, rat reproductive performance is strain dependant, especially the number of oocytes ovulated after hormone treatment. Successful vitrification of rat embryos was first reported using blastocysts of the Fisher strain [27]. Embryos of the Wistar strain are easier to manipulate *in vitro* than other strains, and the developmental results of vitrified embryos have been were reported in detail through the preimplantation stages [20].

The rabbit was the first mammalian species for which offspring were produced by *in vitro* fertilization, using spermatozoa capacitated *in vivo* [28]. Rabbit embryos are relatively easy to collect because ovulation in this species is induced by the stimulation of copulation, and hormonally induced superovulation is effective. The first successful vitrification of rabbit embryos was achieved using glycerol and propylene glycol as CPAs [29]. However, the embryos must be equilibrated in multiple steps before being plunged into LN_2, because of the toxicity produced by high concentrations of permeable cryoprotectants. Therefore, Kasai *et al.* cryopreserved rabbit morulae with EFS40, similar to the method used for mouse embryos. They observed that Ficoll dehydrated the zona pellucida and the mucin coat during the vitrification procedure and most of the embryos survived with an intact zona and mucin coat after freeze-thawing; 65% of the embryos developed to live offspring following transfer into the oviduct of pseudopregnant recipients.

In the Mongolian gerbil, the cryopreservation of embryos is efficient for preserving their characteristics, because there are several strains with different coat colors and differences in sensitivity to epilepsy. To collect embryos, hormonally treated virgin females are mated with sexually mature males, because it is difficult to produce embryos by *in vitro* fertilization [30, 31]. Superovulation using gonadotropin injection is effective, especially for immature females aged 4–5 weeks. We reported the first successful vitrification of embryos in this species [27]. The best results for producing offspring were obtained from blastocysts vitrified using EFS40 as the CPA followed by transfer into the uteri of pseudopregnant females, which were prepared without hormone treatment, before mating with a vasectomized male [9].

Mastomys (*Praomys coucha*) is a murine rodent native to Africa with a body size that is intermediate between the mouse and rat. Mastomys has large phenotypic variation in many organs and in coat color. About seven species are found, including a few recombinant inbred strains in Japan [32]. The collection of embryos from mastomys is not easy because it depends on the strain, age and breeding conditions. *In vitro* fertilization [33], intracytoplasmic injection with elongated spermatid [34] and the production of offspring after vitrification of embryos with EFS40 [5] have all been successfully performed in a few laboratories. However, one problem with mastomys is the difficulty of carrying out embryo transfer, As pseudopregnant females cannot be produced because the estrous cycle and mating parameters have not been characterized. Therefore, we transferred treated embryos into the oviducts or uteri of pregnant females using coat color combinations as genetic markers.

In the Syrian (Golden) hamster, the success of *in vitro* fertilization was reported in 1963 [35], but offspring from embryos fertilized *in vitro* were not obtained until 1992 [36]. Hamster oocytes are widely used for sperm activity tests because they accept the penetration of spermatozoa from many other species. However, the hamster embryo is very sensitive to environmental factors (e.g., short wavelength light, temperature and chemicals) and it is difficult to establish *in vitro* culture systems, for them as most hamster embryos show arrested development after exposure to cryoprotectants [37]. Eight-cell embryos appear to be less sensitive than 1-cell (pronuclear stage) and 2-cell embryos. So far, only one group has reported successful slow freezing of 8-cell stage embryos [38]. Bavister *et al.* demonstrated successful vitrification with a cryoloop, as mentioned above [7].

In the guinea pig, about 15 strains with various coat colors had been bred at the National Institute of Health in Japan until 1992. Today, only three strains are bred as important genetic variations. Since the first report of successful *in vitro* fertilization using the guinea pig, by Yanagimachi in 1972 [39], many studies on capacitation and the acrosome reaction of spermatozoa have been reported, because of the large size of the acrosome and the specific characters of the acrosome reaction. However, reproductive technology with the guinea pig has not advanced remarkably. In particular, it is difficult to induce superovulation in this species. We reported that the number of ovulated oocytes increased from 3.6 to 9.8 after inhibin vaccination [40]. We have made only a few attempts to cryopreserve guinea pig embryos, and our limited experience suggests there are two problems to overcome: delipidation of the embryos and the development of embryo transfer techniques for producing offspring.

High Osmolality Equilibrium Vitrification: The Fourth Generation of Cryopreservation Technology

In our center, mouse strains are distributed to researchers as live animals or as cryopreserved embryos or spermatozoa, according to request. We have transported more than 100 strains of vitrified embryos in dry shippers worldwide every year. The dry shipper is an exclusive container in which there is an adsorbent for LN_2. The temperature inside is stable at near –190°C. However, it is heavy, large and expensive. Moreover, it needs constant maintenance, so transportation incurs the full fare for a round air trip. Therefore, we examined whether it would be possible to preserve and transport vitrified embryos at dry ice temperature. Jin *et al.* established a novel vitrification method using a very high osmolality solution in plastic straws [41]. Survivability was demonstrated by the development to term of a high proportion (75%) of transferred embryos. The osmolality of the standard vitrification solution, EFS40, is 18.0 moles/kg water. However, Jin *et al.* used EFS35c at an osmolarlity of 23.3 moles/kg water. In this CPA, the percentage of EG is decreased from 40% to 35% (v/v), but the sucrose concentration is greatly increased from 0.3 mol/l to 0.975 mol/l. The survival rate of fully dehydrated and vitrified embryos in this solution is similar to that seen in embryos frozen by slow freezing to –80°C using a programmed freezer (equilibrium slow freezing), except

Table 2. Survival of C57BL/6 mouse 2-cell embryos vitrified with EFS45c solution after storing in dry ice for 2 days with or without transportation

Transportation	No. of embryos			No. of recipients pregnant /used (%)	No. of embryos		No. of offspring (%)
	vitrified	recovered (%)	morphologically normal (%)		transferred	implanted (%)	
–	60	59 (98)	59 (100)	5 / 5 (100)	59	54 (92)	46 (78)
+	125	124 (99)	123 (99)	9 / 10 (90)	123	103 (93)*	74 (67)*

*: percentages in pregnant recipients.

that there is less extracellular ice formation. This new method with a high osmolality CPA has the advantages of both vitrification and equilibrium slow freezing. Consequently, the cooling process is simple and quick and does not need a programmed freezer; and the vitrified embryos are not susceptible to the warming rate or to transportation on dry ice. This method clearly offers superior embryo cryopreservation. Recently, we determined the optimal CPA (EFS45c: 33.6 moles/kg water) for the equilibrium vitrification of embryos using a cryotube, and succeeded in transporting embryos on dry ice [42] (Table 2).

Conclusion

The cryopreservation methods of mouse embryos over the 38 years since the initial success have passed through the development of equilibrium vitrification, slow freezing, vitrification, and minimum volume vitrification. High osmolality equilibrium vitrification is expected to become the new standard, because the transportation of cryopreserved mouse embryos between facilities all over the world is gathering pace. The development and diffusion of technologies for cryopreservation of rats and other laboratory animals should be considered as a project on a global scale.

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