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= Original Article =

Cryotop facilitates high developmental ability of re-vitrified mouse embryos

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ABSTRACT

Vitrification is well known as an effective means of preserving not only germ cells but also reproductive organs. Although various devices are used to vitrify mammalian embryos, it has been reported in other mammalian species that the Cryotop method develops vitrified-warmed embryos better than other devices. However, information about multiple rounds of vitrification (re-vitrification) of embryos is very limited even in mice. In this study, we examined the effect of repetitively vitrified mouse embryos (2-cell stage, 4-cell stage, morula and blastocyst) at the same stage on the embryos' developmental ability. Cumulus-oocyte complexes were collected from the oviducts and fertilized with frozen-thawed epididymal spermatozoa. After *in vitro* fertilization, the embryos were cultured up to 120 h. Embryos at the 2-cell stage (24 h), 4-cell stage (47 h), morular stage (72 h), and blastocyst stage (96 h) were collected and vitrified by Cryotop. After warming, embryos were cultured for 2 h and then re-vitrified. Vitrification-warming was repeated up to three times. Our results showed that re-vitrification up to three times did not affect the developmental ability of embryos vitrified at 2-cell, 4-cell, morulare, or blastocysts. Taken together, the results show that re-vitrification of mouse embryos by Cryotop did not have a detrimental effect on embryonic development of the embryos.

Key words: Cryotop, vitrification, mouse, embryo

INTRODUCTION

Cryopreservation of germ cells is a useful and important technology for the efficient production of transgenic, mutant, and gene-targeted (knockout and knockin) animals. Especially, embryo cryopreservation is routinely used for not only efficient production of experimental animals but also clinical medicine. Many assisted reproductive technologies, including embryo cryopreservation, have been applied to address human infertility. Whittingham and his colleagues [38] first succeeded in the cryopreservation of mammalian embryos at –196°C. In their method, the mouse embryos are eventually cooled at a controlled rate to temperatures below –80°C and then plunged into liquid nitrogen at –196°C for long-term storage (reviewed by Leibo [20]). Later, Willadsen [39] modified the original method by Whittingham et al. [38] and succeeded in embryo cryopreservation using an intermediate subzero

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temperature of –36°C, instead of –80°C, before plunging the embryos directly into liquid nitrogen. The method has been improved by some modifications and has been called the 'slow-freezing method' or the 'standard method'. This slow-freezing method is widely and routinely applied for embryo cryopreservation in many species [10,19,25,29].

Rall and Fahy [27] also succeeded in preserving mammalian embryos at –196°C by an alternative called the 'vitrification method' or the 'ultra-rapid freezing method'. Although Rall and Fahy [27] reported that vitrified mouse embryos yielded a survival rate as high as that of cryopreserved ones by slow-freezing, specific studies clearly showed that the vitrification method was superior to the slow-freezing method in embryo preservation [13,32]. Contrary to the slow-freezing method, the major advantage of the vitrification method is that it eliminates physiological damage caused by intracellular or extracellular ice crystal formations, and reduces damage from chilling by shortening the exposure to a suboptimal temperature [15]. In addition, the vitrification method does not require a programmable freezer, which is generally expensive. The vitrification method is also simpler and quicker than the slow-freezing method because embryos are out of the incubator for less than 5 min in the vitrification method, whereas with slow-freezing equilibration alone takes more than 20 min [14]. Thus, vitrification can supplant slow-freezing as a method of preserving mammalian oocytes and embryos.

In general, the use of high concentrations of cryo-protective agents (CPAs) potentially increases toxicity to the vitrified embryos. Increasing the volume of vitrification solution also interferes with the survival of vitrified-warmed embryos because a large volume of solution decreases the cooling rate [14]. Therefore, a smaller volume of vitrification solution in a container is a key to obtaining a higher vitrification rate. Indeed, many devices or methods have been developed to produce a small volume of vitrification solution. An electron microscope grid [7], a gel-loading tip [9], the open pulled straws (OPS) [37], CryoLoop [18], solid surface vitrification [4,31], microdrops [26], nylon mesh [1], and metal mesh [6] were developed to minimize the volume of vitrification solution.

A Cryotop is an alternative device that consists of a thin strip of plastic film [15]. In the protocol using Cryotop, embryos are loaded with the use of a glass capillary under the control of a stereomicroscope [14]. Since almost all of the medium is removed before cooling, the embryos (or oocytes) are covered with only a very thin solution layer, and then capped Cryotop is plunged directly into liquid nitrogen [14]. In rabbits, it has been shown that the vitrification with Cryotop yielded a higher rate of post-warming survival of pronuclear-stage (PN) embryos than either the gel-loading tip or CryoLoop [10]. In the pig, it was reported that the Cryotop method was superior to the OPS technique for vitrification of matured porcine oocytes [22]. Furthermore, we also found that the Cryotop method is effective for the vitrification of PN embryos in rats [28]. Thus far, the Cryotop method has been used for oocytes and/or embryos of vitrification in other species, including the rabbit [10], the human [7, 16], the cattle [3, 23], the minke whale [11], the pig [5], the buffalo [24], the cat [36], the horse [2], and the sheep [12,33]. These results strongly suggest that Cryotop is a superior device, and one of the most powerful, for vitrification of mammalian oocytes and embryos. However, only two papers have been found in which the Cryotop method is applied to the vitrification of mouse embryos [21,40].

Although many papers have focused on vitrification using various devices or protocols, the efficacy of repeated vitrification is not well understood. Recently, it was reported that mouse embryos at the 1-cell stage were vitrified using the CryoLoop and then were re-vitrified at the successive stages (2-cell, 8-cell, and blastocyst stages) [30]. From the results, those authors concluded that re-vitrification did not affect development in either the 8-cell stage or the blastocyst stage [30]. Thus, re-vitrification of embryos at successive stages seems to maintain a high developmental ability of the vitrified/warmed embryos. However, no one has reported the effect of re-vitrification at the same stages on embryonic development *in vitro*. Such information will be useful for many researchers in not only basic biology but also clinical medicine, because spare embryos may be re-vitrifiable at the same stage after embryo transfer. The objective of the present study was to clarify the effects of re-vitrification by Cryotop up to three times at the same stages (2-cell stage, 4-cell stage, morular stage, or blastocyst stage) on the development into blastocysts and/or hatched blastocysts.

MATERIALS AND METHODS

All chemicals and reagents were purchased from the Sigma-Aldrich Corporation (St. Louis, MO, USA) unless otherwise stated. All procedures for the handling and treatment of the animals were conducted according to the guidelines established by the Animal Research Committee of Azabu University.

Animals

MII oocytes were collected from the oviducts of ICR female mice (4–8 weeks) that were superovulated by i.p. injection of 5 IU equine chorionic gonadotropin (eCG; Nippon Zenyaku Kogyo Co., Tokyo, Japan) followed by 5 IU human chorionic gonadotropin (hCG; Asuka Pharmaceutical Co., Tokyo, Japan) 48 h later. Fourteen hours after the hCG injection, the females were killed and their oviductal ampullae were removed. The oviductal ampullae were placed in oil and cumulus-oocyte complexes (COCs) were collected from the oviductal ampullae. COCs were transferred in paraffin-oil-covered 100 µl drops of TYH medium [35] equilibrated at 37°C under 5% CO2 in air.

In this study, cryopreserved sperm was used for *in vitro* fertilization. The sperm was collected from cauda epididymides of BDF1 male mice (10–15 weeks). After dissections, the epididymides were removed and placed in a 35-mm sterile plastic dish containing 400 µl R18S3 medium [34]. The epididymal sperm was counted by a hematocytometer, and sperm motility and viability were evaluated according to a previous report [28]. Namely, sperm motility was assessed visually and determined by direct observation at 37°C under light microscopy at 100 x. For cryopreservation, sperm was loaded into 0.25 ml plastic straws (Fujihira Industry, Tokyo, Japan). The straws were exposed to liquid nitrogen (LN2) vapor for 10 min (about –150°C) and then plunged into LN2 and stored for at least 1 week. For thawing, the straws were plunged in water at 37°C for 10 sec and the contents were then expelled into a 35-mm sterile plastic dish. Post-thaw sperm motility was evaluated as described above. The frozen-thawed sperm was resuspended in TYH medium for 1 h. The frozen-thawed sperm was then added to the TYH drops containing COCs (final sperm concentration was 2×10^6 sperm/ml) and co-cultured for 6 h. After culture, COCs were transferred into a 100 µl drop of KSOMaa [8] supplemented with 0.1% hyaluronidase, and cumulus cells were removed by being drawn up repeatedly into a fine pipette. The denuded oocytes were washed there times in KSOMaa and then evaluated using an inverted phase-contrast microscope (Olympus, Yokohama, Japan). Oocytes having two pronuclei were determined to be fertilized. Only fertilized oocytes were transferred into 50 µl of the same medium and cultured at 37oC under 5% CO2 in air. The embryos were collected at the PN stage (0 h from the end of co-culture), 2-cell stage (24 h), 4-cell stage (47 h), morular stage (72 h) or blastocyst stage (96 h) and used for the following experiments.

Vitrification of mouse embryos by Cryotop

The Cryotop method was carried out according to a report by Kuwayama and Kato [14] with some modifications. In brief, five embryos were placed in a small volume (5 ml) of equilibrium solution composed of 7.5% ethylene glycol (EG) + 7.5% dimethylsulfoxide (DMSO) + 20% FCS in PB1 for 3 min at 20–22oC. The embryos were then transferred into a small volume (5 ml) of vitrification solution composed of 15% EG + 15% DMSO + 0.5 M sucrose + 20% FCS in PB1 for 1 min. The embryos were placed on a sheet of Cryotop (Kitazato BioPharma, Shizuoka, Japan) in a small volume of vitrification solution. The Cryotop was plunged into liquid nitrogen when the embryos were exposed to the vitrification solution for 1 min and stored for at least 1 week. The embryos were warmed by immersing the Cryotop into warming solution composed of 0.5 M sucrose + 20% FCS in PB1 at 37°C for 3 min, and then placed with 20% FCS in PB1 at 37°C for 5 min. After washing three times with KSOMaa, these embryos were cultured in paraffin-oil-covered 100 µl drop of KSOMaa. After culture, development to the blastocyst stage and their hatching were evaluated. Re-vitrification was carried out as follows: in our preliminary study, vitrified-warmed embryos cultured for 2 h before re-vitrification showed a higher survival rate than embryos cultured less than 2 h. Therefore, the vitrified-warmed embryos were cultured for 2 h and then re-vitrified [second-time vitrification (2V)]. Some of the vitrified-warmed embryos were cultured for 2 h and then vitrified a third time (3V). After warming, the ability of embryonic development was evaluated as described above.

Statistical Analyses

Each experiment had at least three replicates. All

Table 1. The effect of repetitive vitrification on the development of 2-cell mouse embryos

Vitrification times	No. examined	Development to blastocysts (%)[a]
1V	114	99 (86.8)
2V	114	92 (80.7)
3V	117	99 (84.6)

[a]Percentage of survived embryos

Table 2. The effect of repetitive vitrification on the development of 4-cell mouse embryos

Vitrification times	No. examined	Development to blastocysts (%)[a]	Hatched blastocysts (%)[a]
1V	106	99 (93.4)	96 (97.0)
2V	114	104 (91.2)	98 (94.2)
3V	101	90 (89.1)	82 (91.1)

[a]Percentage of survived embryos

Table 3. The effect of repetitive vitrification on the development of mouse morulae

Vitrification times	No. examined	Development to blastocysts (%)[a]
1V	123	116 (94.3)
2V	124	114 (91.9)
3V	170	60 (85.7)

[a]Percentage of survived embryos

Table 4. The effect of repetitive vitrification on hatching of mouse blastocysts

Vitrification times	No. examined	Hatched blastocysts (%)[a]
1V	80	74 (92.5)
2V	80	71 (88.8)
3V	70	61 (98.1)

[a]Percentage of survived embryos

percentage data were subjected to arcsine transformation before statistical analysis. Data for *in vitro* development of vitrified/warmed embryos were analyzed by one-way analysis of variance (ANOVA) and Tukey's test. The numbers of offspring were compared using Student's t test. $P < 0.05$ was considered significant.

RESULTS

In Tables 1-4, the numbers of repeated vitrifications are shown as 1V, 2V, and 3V. Regardless of the rounds of repeated vitrification, the percentages of vitrified-warmed embryos at the 2-cell stage were 86.8% (1V), 80.7% (2V), and 84.6% (3V), respectively (Table 1). There were no significant differences among the treatments. The effect of repetitive vitrification on embryonic development of 4-cell mouse embryos is shown in Table 2. In the 1V, 2V, and 3V groups, most of the vitrified embryos developed to the blastocyst stage (93.4%, 91.2%, and 89.1%, respectively). Most of the blastocysts were hatched [97.0% (1V), 94.2% (2V), and 91.1% (3V)]. There were no significant differences among the treatments in the percentages of embryos that developed to blastocysts and hatched blastocysts. Table 3 shows the effect of repetitive vitrification on embryonic development of mouse morulae. Most of the morulae developed to blastocysts [94.3% (1V), 91.9% (2V), and 85.7% (3V)]. There were no significant differences among treatment groups. The effect of repetitive vitrification on blastocyst development is shown in Table 4. The percentages of hatched blastocysts were 92.5% (1V), 88.8% (2V), and 98.1% (3V). There were no significant differences among the treatments.

DISCUSSION

In our present study, there were no significant differences among the treatment groups for repetitive vitrification at any embryonic stages. Although no studies have determined the effect of multiple rounds of vitrification using Cryotop, one paper showed Cryoloop (another vitrification device) facilitated re-vitrification of embryos at successive stages of development (the pronuclear stage, the 2-cell stage, the 8-cell stage, and

the blastocyst stages) [30]. However, they reported better hatching rates in the non-vitrified group than in the vitrified group [30]. In the present study, we did not compare hatching rates between the non-vitrified group and vitrified or re-vitrified groups, but most of the vitrified and re-vitrified embryos were hatched (88.8%–98.1% in Table 4). Therefore, Cryotop can be applicable for vitrification including repetitive vitrification of mouse embryos. Indeed, Hochi et al. [9] compared three vitrification methods (a gel-loading tip, Cryoloop, and Cryotop) for the vitrification of rabbit pronuclear embryos. Cryotop yielded higher post-warming survival of rabbit embryos than either the gel-loading tip or Cryoloop. They concluded that one of the reasons for the difference might be the total concentration of cryoprotective agents (CPAs) in vitrification solution. Indeed, the CPA concentration of the Cryotop method [30% (v/v)] was lower than those of the gel-loading tip and Cryoloop methods [40% (v/v)] in the study. Since a low concentration of CPAs is involved in reducing cytotoxic effects, Cryotop seems to be a more suitable device for re-vitrification of mouse embryos.

Another possible reason for the high developmental ability of re-vitrified mouse embryos by Cryotop is the total volume of vitrification solution during the vitrification. The Cryotop method is probably the latest minimum-volume vitrification approach [17]. Cryotop consists of a narrow, thin film strip (0.4 mm wide, 20 mm long, 0.1 mm thick) attached to a hard plastic holder [17]. In the Cryotop method, CPAs are loaded with a narrow glass capillary onto the top of the film strip in a volume of less than 0.1 µl. After loading, almost all of the solution is removed, leaving only a thin layer covering the embryos [17]. The minimal volume approach of the Cryotop method increases the cooling and especially the warming rates, which may contribute to maintain the high developmental ability of re-vitrified embryos after warming.

Taken together, the results show that re-vitrification by Cryotop did not have a detrimental effect on the development of mouse embryos. Our present results will be useful in the development of assisted reproductive technologies in humans.

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