A Vitrification Method by Means of a Straw to Prevent Infections in Mouse Pronuclear Embryos

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Abstract: *Mouse pronuclear embryos were cryopreserved by a simple and safe vitrification method. In the process, Vitrification Media VT101, Thawing Media VT 102 (KITAZATO. Co. Japan) and the embryos were loaded into a straw; then they were cryopreserved. Different loading methods were examined to determine the safety levels of crystallization for the embryo's survival after thawing. The best condition attained, after thawing, was a 75% embryo survived rate of which 66% developed to the two-cell stage, 71% developed to the morula stage and 27% developed to the blastosyst stage. This development of embryos after vitrification was not significantly different to that of a control group without freezing and thawing. The vitrification method was considered to protect embryos against various infections via liquid nitrogen during cryopreservation. It is expected that the method can be applied to human embryos.*

Key words: *Vitrification, Pronuclear embryos, Straw, Mouse*

Introduction

Slow freezing and vitrification methods have been used for the cryopreservation of embryos. Conventionally, the slow method of freezing human embryos has been performed at *in vitro* fertilization (IVF) institutions, but the method needs an expensive program freezer and requires a long cryopreservation time. On the other hand, the vitrification method can do the treatment quickly without the program freezer and at low cost. In 1985, Rall and Fahy showed for the first time that mouse embryos could be successfully cryopreserved by vitrification [1]. And in recent years vitrification has come to be applied at all the growth stages of mammalian embryos [2, 3]. Many researchers have studied low toxicity vitrification solutions. The vitrification solution can have cell toxicity reduced by mixing two kinds together instead of using just one [4, 5]. Mukaihashi *et al.* have succeeded in the vitrification of mouse embryos by using ethyleneglycol (EG) and dymethyl sulfoxide (DMSO) [6] and now we can easily obtain ready created solutions for vitrification without taking the trouble to prepare them, and we can have high probability of survival and development after vitrification. In the protocol, the embryos must be rapidly exposed to much lower temperatures in order to have glasslike solidification of the embryos and the outside solution, so that embryos must vitrify with the minimum volume of vitrification solution; special tools must be used with which to make contact with the liquid nitrogen (for example, open pulled straw (OPS) [7], cryoloop [8], cryotop (Kitazato. Co.)). In liquid nitrogen, however, the existence of various bacteria and viruses should be considered, and the possibility of infection of cryopreserved embryos via liquid nitrogen cannot be denied [9]. But Bielanski *et al.* have reported that the standard sealed straw prevents various viral problems even though embryos cryopreserved in OPS were infected with some viruses [10] This study examined the vitrification method simply by using Vitrification Media VT101 and Thawing Media VT 102 (Kitazato. Co.) and safely used a straw to prevent various infections. Since the straw has a large capacity, it is not avoided much vitrification solution containing embryos. Therefore, it

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was thought that the toxicity would be high for the embryos and the fall in temperature would be too slow. Consequently, it was predicted that the probability of survival after thawing was low. In order to determine the safest crystalisation condition for embryos; we examined how best to load the vitrifaication solution into the straw.

Materials and Methods

Pronuclear embryos were collected from mouse oocytes fertilized in vitro. Female mice (BDF_1) of 7~10 weeks old were superovulated with 5IU pregnant mare serum gonadotropin (PMSG; Teikoku Zoki. Co.) followed by administration of 5IU human chorionic gonadotropin (hCG; Teikoku Zoki. Co.) 48~50 hrs later. Oocytes were obtained 15–16 hrs after the hCG injection. Spermatozoa were collected from males (BDF_1) of 7~10 weeks old and suspended in 500 μl of Human Follicular Fluid medium (HFF; Fuso Pharmaceutical Industries. Co.) [11, 12].

After pre-incubation for 1.5 hrs at 37°C under 5% CO_2 in air, a small amount of suspension sperm was added to the human follicular fluid medium(HFF) containing oocytes (final sperm concentration: about 100/μl). After 6 hours, pronuclear embryos were checked for the presence of two pronuclei in a second polar body and then they were used for the vitrification experiments. For the vitrification and the thawing solution Vitrification Media (ES: Equilibration Solution, VS: Vitrification Solution (Kitazato. Co.)) and Thawing Media VT102 (TS: Thawing Solution, DS: Diluent Solution, WS: Washing Solution (Kitazato. Co.)) were used. The vitrification and thawing protocol were modified from the original protocol recommend by Kitazato Supply (Japan).

The embryos were equilibrated in a drop of Equilibration Solution (ES: VT101) containing 7.5% EG and 7.5% DMSO and washed in fresh ES several times within 15 minutes. Then the embryos were exposed to Vitrification Solution (VS: VT101) containing 15% EG, 15% DMSO and 0.5 M sucrose and loaded into a 0.25 ml straw (I.M.V., L'Augle. Co.) within 1 minute. We examined how to load vitrification solutions into the straw, the quantity of vitrification solution and the number of embryos, shown in the following four experiments (experiments 1~4); And we compared probabilities of survival and rates of development after thawing to determine of the conditions for the best vitrification method.

Control group

The control groups were mouse oocytes fertilized *in vitro* without vitrification and a thawing procedure, and they were cultured in HFF medium for 5 days.

Experiment 1 (Exp 1)

Into the straw was drawn Thawing Solution (TS: VT102) containing 1.0 M sucrose, a breath of air, a drop of VS, a breath of air, 5 μl VS with 2 embryos, a breath of air and TS. Immediately after heat sealing the straw, the 2 embryos were held in the vapor of the liquid nitrogen for 3 minutes and then plunged into liquid nitrogen. During the thawing procedure, the straw was warmed by being plunged into 37°C water, after being held in the air for 10 seconds. Subsequently the straw was shaken to mix the VS and TS. Recovered embryos were exposed to a drop of Diluent Solution (DS: VT102) containing 0.5 M sucrose, washed in fresh solution within 3 minutes, and further washed with a drop of Washing Solution containing Tc199 (WS: VT102) several times. The embryos were cultured in HFF medium for 5 days.

Experiment 2 (Exp 2)

The vitrification and thawing solution were loaded in the following procedures: TS, a breath of air, a drop of VS, a breath of air, a drop of VS, 5 μl VS with 2 embryos, a breath of air and TS. Other procedures were performed as in Exp 1.

Experiment 3 (Exp 3)

Ten μl VS containing two embryos were loaded into the straw. Other procedures were performed as in Exp 1.

Experiment 4 (Exp 4)

Three embryos were vitrified and loaded into the straw as in the procedure in Exp 1.

Results

Control group

In this experiment, 94 (66%) of the 143 cultured embryos developed to the two- cell stage in the non-treated control group. Sixty-one (65%) of the 94 cleaved embryos developed to the morula stage. Thirty-two (34%) of the 94 cleaved embryos developed to the blastocyst stage (Table 1).

Experiment 1

In this vitrification procedure group, the rate of

Table 1. Post-thaw survival and development of mouse zygotes

Treatment	Zygotes treated (n)	Recovered (%)	Survived (%)	Developed to two-cell stage (%)	Developed to morula stage (%)	Developed to blastosyst stage (%)
control	143	—	—	94/143 (66)	61/94 (65)	32/94 (34)
Exp 1	24	19/24 (79)[b]	12/19 (63)	12/12 (100)[a,b]	8/12 (67)	0/12 (0)[a,b]
Exp 2	106	103/106 (97)	77/103 (75)	51/77 (66)	36/51 (71)	14/51 (27)
Exp 3	68	67/68 (99)	57/67 (85)	44/57 (77)	22/44 (50)[b]	3/44 (7)[a,b]
Exp 4	42	41/42 (98)	28/41 (68)	16/28 (57)	8/16 (50)	3/16 (19)

Control; no-treatment of vitrification and thawing procedure. Exp1; Each solution were inhaled in order of the following. TS, VS, 5 μl VS containing two embryos, TS. Exp 2; TS, VS, VS, 5 μl VS with two embryos, TS in the straw. Exp 3; TS, VS, VS, 10 μl VS with two embryos, TS. Exp 4; TS, VS, VS, 5 μl VS with three embryos, TS. Each group differences were compared by using the χ^2 test. [a]Significantly different from control ($p<0.05$). [b]Significantly different from Exp 2 ($p<0.05$).

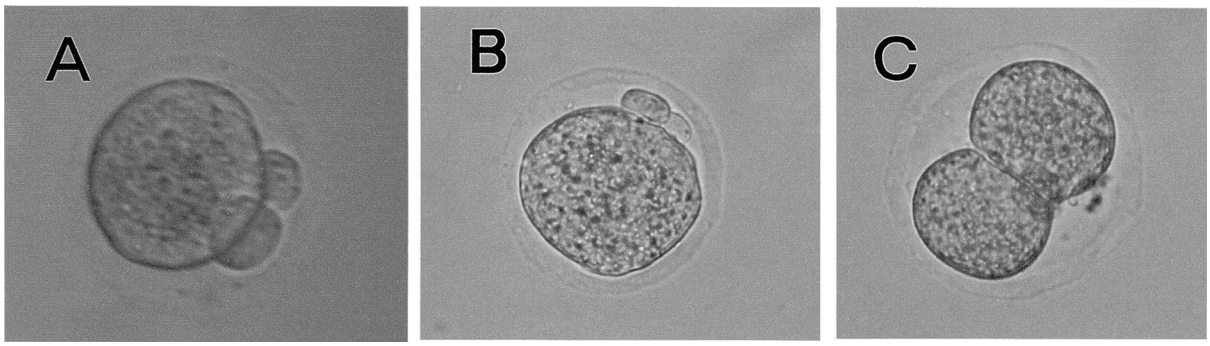

Fig. 1. (A) A mouse pronuclear embryo before vitrification. (B) A mouse pronuclear embryo after thawing. (C) An embryo of day 1 after thawing.

embryos recovered from the straw after thawing was 79%. The surviving embryos developed to the two-cell stage the next day at a significantly higher rate than in the control group, but the cleaved embryos developed after 5 days to the blastocyst stage at a significantly lower rate than in the control group.

Experiment 2

When two small amounts of VS were loaded separately on top of VS containing embryos, the rate of embryos recovered from the straw, after thawing, was 97%. The rates of development to the two-cell, morula and blastosyst stages were not significantly different from that of the control group.

Experiment 3

When 10 μl vitrification solution containing embryos was loaded into the straw, the rate of development to blastosysts was significantly lower than that of the control group.

Experiment 4

When three embryos were vitrified, the rates of development to the two-cell, morula and blastocyst stages were not significantly different from that of the control group.

Discussion

The first pregnancy after vitrification of human embryos was reported by Gordts *et al.* and Barg *et al.* in 1990 [13, 14], and in 2000 Yokota *et al.* reported a successful pregnancy after vitrification at the blastosyst stage [15]. But there are few reports of vitrification in pronuclear stage embryos except that of Jelinkova *et al.* [16]. Zygote stage embryos are more sensitive to osmotic shock than cleavage-stage embryos because they have lower permeability to cryoprotective additives [17]. Therefore, many researchers have examined low toxicity vitrification solutions. The vitrification solution can be made lower in cell toxicity by mixing two kinds together rather than using one. It is thought that the toxicities of EG and DMSO are reduced when they are

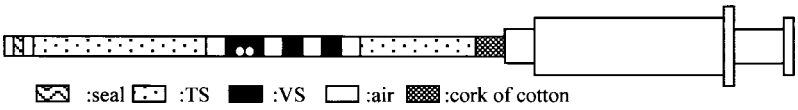

Fig. 2. The vitrification method by using commercial solution. Each solution were inhaled in order of the following. TS, VS, VS, 5 μl VS containing two embryos and TS. The procedure detailed described in the text.

mixed, ES and VS are made from such a combination. In addition, some tools such as OPS improved the conventional straws which were used in the vitrification of mammals [18]. The OPS can give a very high cooling and warming rate and can decrease osmotic damage [19]. Furthermore, the cryoloop can have its temperature lowered more rapidly than OPS [20]. Mukaida *et al.* reported a successful birth after the transfer of vitrified human blastocyst embryos by means of a cryoloop [21]. These newer techniques require direct contact between the embryos and liquid nitrogen. But in assisted reproductive technology, sometimes we have to treat infected patients such as those suffering from hepatitis B/C virus and HIV. Naturally, embryos collected from infected patients have the worrying possibility of being infected. It is widely acknowledged that many viral and bacterial pathogenic agents may adhere to the intact zona pellucida, and most infectious viral and bacterial agents easily survive in cryoprotectants and liquid nitrogen [22]. In spite of this, many patients' embryos were kept in the same freezing tank. Cryopreservation must only be carried out by directly contacting the liquid nitrogen, when it is confirmed as safe to do so. In order to avoid such problems, we examined, with Vitrification Media VT101 (ES and VS) and VT102 (TS, DS and WS), how to load the vitrification solution into the straw to obtain the safest possible glassy state (Exp1~4). We wanted to load TS with VS into the straw since embryos might attach to TS immediately after thawing for and avoid poisoning the embryos. If VS is loaded into the straw with TS, VS containing embryos will freeze a little because TS is inside the straw. VS and embryos must be vitrified and not be frozen at the time of cryopreservation [20]. We loaded a small amount of VS on top of VS containing embryos to flush TS from the straw as in the method of loading used by Ishimori *et al.* and Segino *et al.* [23, 24] (Exp 1). But in Exp 1, although the rate of cleavage was better, the late development to the blastosyst stage was poorer than in the control group. In Exp 1, some of the VS containing embryos became muddy white, and it was predicted that TS loaded into the straw was not able to be flushed out completely by loading a small amount of VS. In Exp 2, we tried to load a small amount of VS twice on top of VS containing embryos; this time VS containing embryos did not freeze completely and Exp 2 had equivalent results to the control group. In Exp1 and 2, VS was 5 μl, the minimum quantity with which we could operate since VS containing embryos could require a very high cooling rate for complete vitrification [25]. Although Exp 2 was considered to be the best condition method, in Exp 3 we changed the Exp 2 method into 10 μl VS. If a large amount of VS is loaded, it is easier to operate than when a small volume of VS is loaded, and many embryos may be able to be vitrified at once; but the rates of development to the morula and blastosyst stages after thawing were poor as compared with 5 μl VS in Exp 2. In Exp 3 there was too much VS for a rapid temperature fall and the formation of glasslike solidification of VS containing embryos [21]. In Exp 4, we examined the number of embryos which could be vitrified at once. Three embryos were cryopreserved by the same method as in Exp 2; and the rate of development to the stage of two-cell embryos, morulae and blastosysts were not significantly different from the control group, but the results were a little inferior to those in Exp 2. A lot of pretreatment solution carried into the following solution when many embryos were treated. This occurrence was used to predict that osmotic pressure adjustment becomes imperfect [17]. From the above experiments, when a few VS were loaded twice on top of 5 μl VS containing two embryos in a straw such as in Exp 2 (Fig. 2), it was discovered that embryos could be vitrified by using the straw, and the rates of survival and development after thawing were better, than that of the group without cryoprocedure. Since the already created solution for vitrification is more easily available, this method has made it possible to reduce the time required to produce the solution; and it prevents infection with various viruses from the outside by using the straw. It is a simple and safe method of vitrification. It is expected that clinical application can be applied to human embryos.

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