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Stepwise In-straw Dilution and Direct Transfer Using Open Pulled Straws (OPS) in the Mouse: A Potential Model for Field Manipulation of Vitrified Embryos

Qi-En YANG¹⁾, Yun-Peng HOU¹⁾, Guang-Bin ZHOU^{1,3)}, Zhong-Qiang YANG¹⁾
and Shi-En ZHU^{1,2)}

¹⁾Laboratory of Animal Embryonic Biotechnology, ²⁾State Key Laboratory of Agrobiotechnology, College of Animal Science and Technology, China Agricultural University, Beijing 100094 and ³⁾Dujiangyan campus of Sichuan Agricultural University, Dujiangyan 611830, P.R. China

Abstract. In the present study, mouse blastocysts were employed to investigate the feasibility and efficiency of stepwise in-straw dilution and direct transfer using the open pulled straw (OPS) method. In experiment I, the effects of various vitrification solutions (VS) on embryo survival were examined. After thawing, the expanded blastocyst rates (97.59 and 95.05%) and hatching rates (80.48 and 78.95%) achieved in the EDFS30 [15% ethylene glycol (EG), 15% dimethyl sulfoxide (DMSO), Ficoll, and sucrose] and EFS40 [40% EG, Ficoll, and sucrose] groups were no different from those (96.15% and 83.33%) of the control group. However, the rates in the EFS30 [30% EG, Ficoll, and sucrose] (87.80 and 55.43%) and EDFS40 [20% EG, 20% DMSO, Ficoll, and sucrose] (95.69 and 70.97%) groups were significantly lower than those (96.15 and 83.33%) of the control group ($P < 0.05$). In the experiment II, the effects of the volume of VS in the OPS on the survival of embryos after in-straw thawing were investigated. When the length of the VS in the column was less than 1 cm, the *in vitro* viability of embryos thawed by stepwise in-straw dilution was no different among the experimental and control groups. The embryos could be successfully thawed by immersing the OPS in 0.5 M sucrose for 3 min and then 0.25M sucrose for 2 min. In experiment III, the effect of immersion time of the OPS in diluent (PBS) on the viability of vitrified embryos was investigated. After in-straw thawing, OPSs were immersed immediately in 1 ml PBS for 0 to 30 min. When the immersion time of the OPSs in PBS was less than 12 min, *in vitro* development of the in-straw thawed embryos was no different from that of the controls. In experiment IV, in-straw thawed blastocysts were directly transferred to pseudopregnant mice to examine their *in vivo* developmental viability. The pregnancy (91.67%) and birth rates (42.42%) of embryos in-straw thawed and directly transferred were no different from those of the unvitrified controls (90.90 and 40%) and embryos thawed by the conventional method (84.61 and 46.94%). These results demonstrate that mouse embryos vitrified with OPS could be successfully thawed by stepwise in-straw dilution and transferred directly to a recipient and that this method might be a model for field manipulation of vitrified embryos in farm animals.

Key words: Blastocyst, In-straw dilution, Mouse, Open pulled straw (OPS), Vitrification

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embryos of humans [3]. Compared with the conventional freezing method, vitrification is relatively simple, rapid, and inexpensive. During freezing, high cooling rates are achieved by plunging containers into liquid nitrogen, and ice crystal formation is avoided by supplementation with highly concentrated cryoprotectants. Therefore, the vitrification method can be applied successfully to cryopreserve goat oocytes at the germinal vesicle stage [4] and porcine embryos [5] that have proven to be very sensitive to cryoinjury caused by the conventional protocol.

To improve the efficiency of the vitrification method, different cryoprotectants and vitrification tools have been studied. The most commonly used cryoprotectants for mammalian embryo vitrification are limited to five reagents, including DMSO, glycerol, ethylene glycol, propylene glycol and acetamide [1]. One of the most effective containers for vitrification is the OPS [6], which has been used successfully for vitrification of embryos and oocytes [2].

Meanwhile, to simplify the thawing and transferring procedure and make it more suitable for field use, some researchers have examined the feasibility of in-straw rehydration and direct transfer in the bovine [7], rabbit [8], ovine [9, 10] and equine [11]. One feature of this thawing procedure is in-straw dilution, embryo thawing, and direct transfer of the thawed embryos. This in-straw dilution and direct transfer method reduces the requirements for equipment and technical skill, which are obstacles to application of embryo transfer technology at many farms where the appropriate conditions are not available. Therefore, this modified method could be more suitable for wide application under field conditions.

Recently, some studies concerning direct thawing and transfer of embryos vitrified in OPSs have been reported [12, 13]. However, details essential for successfully thawing and transferring vitrified embryos were not provided, for instance, the volume of VS in the OPS and the optimal time for successful thawing. Moreover, these previous reports were limited to a one-step dilution, which is less effective in reducing osmotic injury than stepwise dilution [14, 15].

Therefore, the present research was designed to investigate different cryoprotectants, different volumes of vitrification solution (VS) in the OPS,

and different immersion times in the dilution solution on the viability of vitrified mouse blastocysts. This study will provide the basic technical parameters for in-straw manipulation of vitrified embryos for other laboratories and for farm animals under field conditions.

Materials and Methods

The hormones (eCG and hCG) used for superovulation were purchased from Huafu Advanced Biotechnology Company (Tianjin, P. R. China). Unless otherwise stated, all other chemicals for the present study were purchased from Sigma Company (Sigma Chemical, St. Louis, MO, USA). Embryo handling was performed in the laboratory at ambient temperature (25 ± 0.5 C); all vitrification media, diluents, and embryos were maintained at 37 C on a hot plate.

Preparation of solutions

Pretreatment media: PBS was supplemented with 10% (v/v) EG or 10% EG and 10% DMSO to be used as pretreatment media.

Vitrification solutions: The vitrification solutions used in this study included EFS30 containing 30% (v/v) EG in FS solution and EFS40 containing 40% (v/v) EG in FS solution, as described by Zhu *et al.* [15] and Kasai *et al.* [16]. EDFS30 [17] containing 15% (v/v) EG and 15% (v/v) DMSO in FS solution and EDFS40 containing 20% (v/v) EG and 20% (v/v) DMSO in FS solution were also used. The FS solution consisted of PBS medium containing 300 g/l Ficoll and 171.2 g/l sucrose.

Diluents: PBS was supplemented with 0.5 M or 0.25 M sucrose (0.5 MS or 0.25 MS) to be used as diluent.

Manufacture of OPSs

Pulled straws were manufactured as described by Vajta *et al.* (1997) with the following modifications. The plugs of 0.25-ml plastic straws (IMV, L'Aigle, France) were removed, and the straws were heat-softened at the midpoint over a hot plate and pulled manually. The pulled straws were cut at the tapered end with a surgical blade. The inner diameter of the tip was 0.15–0.19 mm, and the wall thickness was no greater than 0.05 mm (measured with a microforge; MF900; Narishige, Tokyo, Japan). The thin part of each OPS was

approximately 2.5 cm long.

Embryo collection

Kunming (KM) mice (Academy of Military Medical Sciences, Beijing, P. R. China) were kept in a room at 20 ± 2 °C under a 14 h (0600–2000 h) light and 10 h (2000–0600 h) dark schedule. Female mice 7 to 11 weeks of age were induced to superovulate by i.p. injection of 10 IU of eCG followed by 10 IU of hCG 48 h later. The following morning (designated day 1 of pregnancy), the females were examined for mating as determined by the presence of a vaginal plug. Mated females were euthanized by cervical dislocation at 88 to 90 h after hCG injection to collect blastocysts. The embryos were recovered by flushing the excised uteri with PBS medium. Some of the blastocysts were cultured at 37 °C with 5% CO₂ in humidified air in an incubator for 40 h to collect hatching blastocysts in mCZB medium [18]. Embryos with normal morphology were used for the cryopreservation experiments. The blastocysts were defined as having morphologically survived if the embryos possessed an intact zona pellucida and refractive blastocoel.

Experimental design

Experiment I: Effects of different vitrification solutions on the *in vitro* development of OPS-vitrified mouse blastocysts

Vitrification of embryos: *In vivo* derived blastocysts were vitrified with various types of VS in open pulled straws. The embryos were pretreated in 10% EG+10% DMSO, or 10% EG for 30 sec, loaded into EDF30, EDF40, EFS30, or EFS40 in the narrow end of an OPS, and left for 25 sec. Then, the straws were immediately plunged into liquid nitrogen (LN₂). Five to six embryos were loaded into each OPS.

Thawing by conventional method: After storage for at least 7 days in liquid nitrogen, the embryos were thawed for *in vitro* culture. During thawing, OPSs containing embryos were kept at room temperature for 1 sec to remove the liquid nitrogen, and then the narrow tip was immersed in 500 μ l of 0.5 MS. The embryos were expelled from the straws into medium. The embryos were transferred into another 100 μ l of 0.5 MS under oil after 0.5 min and were kept there for 2.5 min. Subsequently, they were transferred into 0.25 MS and kept there for 2 min. Finally, the embryos were

washed three times with PBS. The recovered embryos were cultured in modified CZB [18] (mCZB) at 37 °C in 5% CO₂ in humidified air. After 24 or 48 h of culture, the number of embryos that developed to expanded or hatching blastocysts was recorded.

Experiment II: Effects of different volumes of vitrification solution in OPSs on the *in vitro* development of vitrified/stepwise in-straw thawed embryos

Embryo vitrification: The narrowed section of each OPS was labeled with lengths of 0.3, 0.5, 0.7, 1.0, 1.2, 1.5, 1.7 and 2.0 cm starting from the narrow tip using different marker colors to designate the volume of VS loaded into the OPS. The VS (EDFS30) with the highest *in vitro* survival of treated embryos in experiment I was used for embryo vitrification in experiment II. Embryos were loaded with various volumes of vitrification solution into the narrow section of a pulled straw and vitrified as described above.

Thawing using a new stepwise in-straw dilution method: After storage, the cryoprotectants were diluted by a new method in which an OPS was used as a container for dilution (Fig. 1, left).

Two modified tubes (the top half of 15 ml tubes was cut off) containing 0.5 MS and 0.25 MS were placed upright in 37 °C water bath (Fig. 1, right). The modified tubes will hereafter be referred to solely as “tubes”. The OPS was immersed and left to stand in a tube containing 0.5 MS for 3 min and then 0.25 MS for 2 min. The embryos were expelled into PBS and washed twice for *in vitro* culture. Embryos thawed by the conventional method (as described in the Materials and methods section of this paper) were used as a control.

Experiment III: Effect of immersion time in PBS on the *in vitro* development of in-straw thawed blastocysts

Based on the data obtained from experiment II, embryos were vitrified with the optimal volume of VS and thawed as described above. After dilution, OPSs containing thawed blastocysts were transferred and left to stand in tubes containing PBS for 0, 2, 3, 4, 6, 8, 10, 12, 15, 20 or 30 min, respectively. Then, the embryos were recovered and cultured for examination of their viability. Embryos thawed by the conventional method (as described in the Materials and methods section of this paper) were used as a control.

Experiment IV: *In vivo* development of in-straw

Fig. 1. The simple equipment required for in-straw dilution of embryos vitrified by the OPS method. Left side: (1) Tube containing sucrose solutions or PBS, (2) OPS, (3) 0.5 MS, (4) 0.25 MS, (5) Vitrification medium containing embryos, and (6) PBS. Right side: a 37°C bath chamber.

Table 1. *In vitro* development of mouse blastocysts vitrified with different solutions and thawed by the conventional method

Pretreatment		Vitrification		Vitrified	Recovered (%)	Developed to	
Solution	Time	Solution	Time			EB* (%)	HB* (%)
10% E+10% D	30 sec	EDFS30	25 sec	82	82 (100)	81 (97.59)	66 (80.48) ^{AB}
10% E+10% D	30 sec	EDFS40	25 sec	93	93 (100)	89 (95.69)	66 (70.97) ^B
10% EG	30 sec	EFS30	25 sec	82	82 (100)	72 (87.80)	45 (55.43) ^C
10% EG	30 sec	EFS40	25 sec	76	76 (100)	73 (95.05)	60 (78.95) ^{AB}
Control	–	–	–	78	78 (100)	75 (96.15)	65 (83.33) ^A

This experiment was repeated 6 times. Values with different superscripts within the same column are significantly different ($P < 0.05$).

*EB=expanded blastocyst; HB=hatched blastocyst.

thawed blastocysts after direct transfer

This experiment examined the *in vivo* development of blastocysts vitrified and thawed by this in-straw dilution method. After in-straw dilution in tubes, OPSs were left to stand in PBS for 6 min. Then, the OPSs containing embryos were used as catheters for transferring embryos into each uterine horn of recipients (12 embryos per animal) that had mated with a vasectomized male 4 days previously. Fresh embryos and vitrified embryos that were thawed by the conventional method (as described in the Materials and methods section of this paper) were transferred to recipients as controls.

Statistical analysis

All experiments were repeated at least three times. Data were subjected to arcsine transformation prior to ANOVA (PROC GLM; SAS Institute, Cary, NC, USA). Statistically significant differences between treatment groups ($P < 0.05$)

were determined using the Tukey Test. A P value of less than 0.05 was considered to be statistically significant.

Results

Experiment I

As shown in Table 1, no significant differences ($P > 0.05$) were detected for the expanded blastocyst rates between the treated (87.80–97.59%) and control (95.16%) groups. There were no significant differences in hatching blastocyst rates between the control (83.3%) and EDFS30 (80.48%) or EFS40 (78.95%) groups; however, the hatching blastocyst rates of the EDFS40 and EFS30 groups were significantly lower than that of the control group ($P < 0.05$).

Experiment II

There was no difference in the *in vitro* viability of

Table 2. Effect of length of vitrification solution in the OPS on *in vitro* development of vitrified mouse blastocysts after the new stepwise in-straw dilution method

Length (cm)	Vitrified	Recovered (%)	Developed to	
			EB* (%)	HB* (%)
0.3	66	66 (100)	66 (100) ^A	52 (78.78) ^A
0.5	73	73 (100)	73 (100) ^A	55 (75.34) ^A
0.7	75	75 (100)	75 (100) ^A	57 (76.00) ^A
1.0	79	79 (100)	79 (100) ^A	61 (77.21) ^A
1.2	74	74 (100)	59 (79.72) ^{AB}	43 (58.11) ^B
1.5	79	79 (100)	42 (53.16) ^{BC}	18 (22.74) ^C
1.7	72	72 (100)	50 (69.44) ^C	20 (27.77) ^C
2.0	65	65 (100)	26 (40.00) ^C	11 (16.92) ^C
Control	67	67 (100)	67 (100) ^A	53 (79.10) ^A

This experiment was repeated 6 times. Values with different superscripts within the same column are significantly different ($P < 0.05$).

*EB=expanded blastocyst; HB=hatched blastocyst.

Table 3. *In vitro* development of in-straw thawed blastocysts after storage in PBS for different amounts of time

Time (min)	Vitrified	Recovered (%)	Developed to	
			EB* (%)	HB* (%)
0	60	60 (100)	60 (100) ^A	47 (78.33) ^A
2	60	60 (100)	60 (100) ^A	45 (75.00) ^A
3	70	70 (100)	70 (100) ^A	50 (71.42) ^A
4	65	65 (100)	65 (100) ^A	47 (72.31) ^A
6	71	71 (100)	71 (100) ^A	52 (73.24) ^A
8	72	72 (100)	71 (98.61) ^A	51 (70.83) ^A
10	63	63 (100)	63 (100) ^A	44 (69.84) ^A
12	62	62 (100)	62 (100) ^A	46 (74.19) ^A
15	64	64 (100)	64 (100) ^A	33 (51.56) ^B
20	63	63 (100)	61 (98.82) ^A	28 (45.90) ^{BC}
30	62	62 (100)	48 (77.42) ^B	19 (30.64) ^C
Control	67	67 (100)	67 (100)	53 (79.10) ^A

This experiment was repeated 6 times. Values with different superscripts within the same column are significantly different ($P < 0.05$).

* EB=expanded blastocyst; HB=hatched blastocyst.

embryos thawed by stepwise in-straw dilution between the experimental and control groups when the length designating the volume of VS was less than 1 cm. When the length designating the volume of VS was more than 1.2 cm, there was no difference in the survival of embryos thawed by stepwise in-straw dilution among the experimental and control groups; however, the expanded (<69.44%) and hatching blastocyst rates (27.77%) of these embryos were significantly lower than those of the controls (100 and 79.10%; $P < 0.05$) (Table 2).

Experiment III

There were no significant differences among the

groups in terms of blastocyst rates or hatching blastocyst rates when the immersion time in PBS was shorter than 12 min. When the time exceeded 15 min, the expanded blastocyst rate was similar to that of the controls, but the hatching blastocyst rate was significantly reduced (51.56%) compared with that of the controls (79.10%; $P > 0.05$) (Table 3).

Experiment IV

The pregnancy (91.67%) and birth rates (42.42%) of embryos in-straw thawed and directly transferred were similar to those of fresh embryos (90.90 and 40%) and were not significantly different from the ones thawed by the conventional method

Table 4. *In vivo* development of in-straw treated mouse blasocysts after direct transfer

Treatment	Recipients pregnant/transferred (%)	Birth (%)	
		Pregnant recipients	Total recipients
Direct	11/12 (91.67)	56/132 (42.42) ^A	56/144 (38.89) ^B
Conventional	11/13 (84.61)	62/132 (46.94) ^A	62/156 (39.79) ^B
Nonfreezing	10/11 (90.90)	48/120 (40.00) ^A	48/132 (36.36) ^B

Values with different superscripts within the same column are significantly different ($P < 0.05$).

(84.61 and 46.94%) (Table 4).

Discussion

Blastocysts have widely been adopted for embryo production, cryopreservation and transfer in farm animals, especially in the bovine and ovine, because blastocysts have passed the critical step of genomic activation and have high developmental potential [19]. An efficient method of blastocyst cryopreservation for laboratory animals could be potential model for farm animals. Vitrification solutions containing different cryoprotectants have been used to cryopreserve animal blastocysts. Some researchers have reported that a mixture containing EG and DMSO can be used to successfully vitrify mouse blastocysts [20]. Supplementation with macromolecular reagent can reduce the toxicity of the solution by decreasing the concentration of the permeating agent required for vitrification. When Ficoll and sucrose (non-permeating cryoprotectants) were incorporated into an EG-containing VS, Zhu *et al.* [15] obtained high viability of vitrified-warmed mouse blastocysts. With the invention of the OPS method, some researchers have successfully cryopreserved blastocysts from various mammalian species using a VS consisting of EG, DMSO, and sucrose (EDS) [21, 22]. The present study investigated vitrification solutions containing the permeating cryoprotectants ED or EG + DMSO, small saccharide, and the macromolecular agent Ficoll 70. Mouse blastocysts were successfully vitrified with the EDFS30 or EFS40 solutions and thawed by the conventional procedure; *in vitro* development of the embryos showed no significant difference from those of the controls. Therefore, EDFS30 was selected for further experiments because it produced the highest *in vitro* survival of vitrified-warmed embryos within this group.

In-straw dilution and direct transfer has been used for bovine embryos frozen using the conventional procedure [23], which uses the concentration of cryoprotectants at a relatively low concentration. However, if embryos are cryopreserved by vitrification, the volume of VS in the straw must be as small as possible to avoid toxic effects on the further development of the embryos. In the experiments of Vajta *et al.* [6] and Eldridge-Panuska *et al.* [11], relatively large amounts of holding medium and small amounts of VS solution were loaded in a 0.25 ml straw to decrease the VS concentration after dilution. Although previous reports have focused on in-straw dilution and direct transfer of embryos vitrified by the OPS method [14, 15], some factors, such as the volume of VS in the OPS and the immersion time in PBS, which may affect the viability of thawed embryos, have not been studied. Our results showed that the volume of VS in the OPS has no influence on the viability of post-thawed embryos when the length designating to the volume of VS in the OPS is shorter than 1 cm. However, when the length designating to the volume of VS in the OPS is longer than 1.2 cm, the viability of post-thawed embryos is significantly decreased. In this case, the VS cannot be diluted sufficiently because not enough MS enters the OPS by capillary action, and this results in lower development of in-straw thawed embryos.

In-straw dilution of embryos vitrified by the OPS method does not resemble conventional thawing in terms of the process of removing cryoprotectants. When the OPS-vitrified embryos were thawed by stepwise in-straw dilution, the VS in the OPS was diluted gradually with two different concentrations of sucrose solution and by PBS through capillary action. Sucrose has been found to be potentially detrimental to the viability of embryos at high concentrations and high temperatures [24, 25], although sucrose is virtually non-toxic when used

at refrigerated temperatures [26]. Meanwhile, permeating agents have a strong toxic effect on embryos, and this effect would increase the longer embryos are exposed to these chemicals [27]. In the present experiment, embryos survived and maintained high developmental potential for up to 12 min in OPSs after thawing. This high developmental potential could mainly result from stepwise in-straw dilution with different concentrations of sucrose solution. During the dilution procedure, permeating agents may be removed gradually from the vitrified embryos, and osmotic swelling may be avoided to some extent.

The vitrified embryos survived in OPSs for up to 17 min after removal from liquid nitrogen (Table 3). This amount of time (17 min) is sufficient for non-surgical embryo transfer in the bovine, as this normally takes 3 to 8 min to accomplish based on our experience. So, in-straw thawing is fit for embryo transfer in the field. Our results indicate that the pregnancy and birth rates of the in-straw dilution and direct transfer groups were no different from those of the fresh control and conventional thawing groups. The birth rate (40%) was comparable to previous reports [19, 28]. In our experiment, all embryos were recovered after warming and dilution by immersing the OPSs in PBS for different amounts of time. One possible explanation for this phenomenon was that once an

OPS was plunged in diluent, the column of vitrified medium began to thaw immediately and the embryos were moved upward by capillary action. So far, the OPS method has been successfully applied to vitrify embryos from the ovine [22, 29], hircine [30], and porcine [31]. In terms of in-straw dilution and direct transfer of vitrified embryos, the OPS method has more advantages, such as a simpler procedure and higher post-thawing viability compared with the straw method [9]. Our results demonstrate that mouse embryos vitrified by the OPS method could be successfully thawed by the stepwise in-straw method under microscope free conditions and transferred directly to recipients. In summary, we established a simple and effective method of thawing mouse embryos vitrified by the OPS method, and this method may be a model for manipulation of vitrified embryos from small ruminants and swine under field conditions.

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