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Viability of Vitrified Biopsied Bovine Embryos after In-straw Dilution

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Abstract: The objective of the present study was to evaluate the viability of vitrified biopsied bovine embryos. Bovine embryos ranging from the compacted morula to blastocyst stage on day 7 (day 0=day of estrus) were collected from donor females, biopsied for sexing, and vitrified with ethylene glycol (EG) 25% (v/v), dimethylsulfoxide (DMSO) 25% (v/v) and 0.3% (w/v) bovine serum albumin dissolved in PBS using 0.25-ml straws. We assessed the concentrations of cryoprotectants in the straw after in-straw dilution, and the percentages (v/v) were 6.9–7.7% for EG and 2.8–3.3% for DMSO. The pregnancy rate of vitrified-warmed embryos in the in-straw dilution group (ISD group) was 57.9% when embryos were expelled from the straws after diluting the cryoprotectant in the straws, and that the survival of vitrified-warmed embryos were observed before transfer. In contrast, the pregnancy rate in the directly transfer group (DIR group) was 62.5% when embryos were transferred directly to recipients without expelling embryos from the straws. There were no differences among the pregnancy rates of the ISD group, the DIR group and the non-vitrified biopsied embryos (NV group) (56.3%). These results suggest that it was possible to warm the vitrified-biopsied bovine embryos on farms and to transfer them immediately to recipients, yielding a practical pregnancy rate.

Key words: Bovine, Biopsied embryo, Vitrification, In-straw dilution, Direct transfer

Introduction

It has been possible to control sex of offspring in cattle by transferring sexed embryos [1–3]. To put this technology to practical use on farms, it is necessary to establish a technique for cryopreservation of sexed embryos. It is

desired that a high and stable pregnancy rate is secured, in addition, to some capability of transfer to recipients easily such as direct transfer [4, 5]. Cryopreservation of sexed bovine embryos has been attempted by slow freezing [6, 7], vitrification [8–11], and ultra-rapid vitrification [12, 13]. Previous reports have described that a higher pregnancy rate is obtained by vitrification and ultra-rapid vitrification rather than by slow freezing using ethylene glycol (EG) or glycerol as a cryoprotectant [8, 12]. In these methods, vitrified and warmed embryos were moved into a solution containing a low concentration of the cryoprotectant and sucrose to dilute the cryoprotectant after warming. Then, survival of the warmed embryos is morphologically checked and the surviving embryos are transferred after being re-loaded into straws for transfer. This method requires the processing of vitrified bovine embryos in a laboratory. Leibo [14] and Suzuki *et al.* [15] reported that a high pregnancy rate was achieved in a farmyard using a simple thawing procedure that incorporated slow freezing with glycerol, and that the freezing and diluting solutions were separated by an air layer loaded into the straw. After thawing, these solutions were mixed so that the cryoprotectant was removed from embryos within the straw, and the embryos were transferred directly to recipients (one-step straw method). In addition, a few reports have described vitrified bovine embryos transferred directly to recipients using the direct transfer method or the one-step straw method [16–18].

The objective of the present study was to evaluate the viability of vitrified, biopsied bovine embryos after in-straw dilution.

Materials and Methods

The animal experiments in the present study were approved by the Institutional Animal Experiment Committee of Kanagawa Agricultural Center.

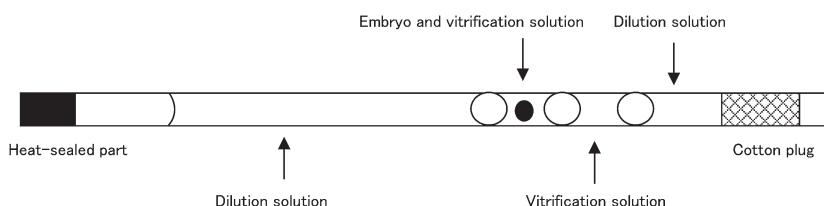


Fig. 1. The loading of the straws for in-straw dilution. Dilution solution 5% (v/v) ethylene glycol and 0.15 M sucrose solution (105 μ l), Embryo included in vitrification solution, 25% (v/v) ethylene glycol and 25% (v/v) dimethylsulfoxide solution (11 μ l), Vitrification solution (11 μ l), Dilution solution (50 μ l).

Embryo collection

Embryos were collected from superovulated Japanese Black and Holstein cows by a non-surgical flushing technique 7 days after the onset of estrus. Follicle stimulating hormone (FSH, Antrin, Kawasaki Mitaka Pharmaceuticals, Tokyo, Japan) was given twice daily at decreasing doses (7, 7, 6, 6, 5 and 5 AU) over 3 days to Holstein cows, and (5, 5, 3, 3, 2 and 2 AU) over 3 days to Japanese Black cows. Doses of 0.75 mg cloprostenol (PGF₂ α analogue, Estramate, Sumitomo Pharmaceuticals, Osaka, Japan) were administered to induce luteolysis 2 days after the first FSH treatment. The cows were observed for behavioral estrus and inseminated twice at 2.5 and 3 days after injection of PGF₂ α analogue.

The embryos were examined microscopically to determine their qualities and developmental stages as previously described [19]. Embryos ranging from the compacted morula to blastocyst stages graded code 1 (morphologically excellent or good embryos) [19] were used in the present study.

Biopsy and culture of embryos

Embryos were placed into microdrops of Dulbecco's PBS supplemented with 0.2 M sucrose under an inverted microscope equipped with a micromanipulator (Narishige Co, Ltd., Tokyo, Japan). A small piece of each embryo was removed from the edge of the compact morula or from the trophoblast of blastocysts by cutting with a microrazor blade (Bio-cut blade, Feather Safety Razor Co., Ltd., Osaka, Japan) attached to the micromanipulator. The biopsied embryos were cultivated for 3–5 h in TCM199 (Gibco, Invitrogen, Carlsbad, CA, USA) with 20% fetal bovine serum (FBS, Gibco, Invitrogen, Carlsbad, CA, USA) and 0.1 mM β -mercaptoethanol (Sigma-Aldrich Chemical, St. Louis, MO, USA) (ME199) at 38.5 °C under 5% CO₂ in humidified air.

Vitrification of biopsied embryos

The vitrification procedure was based on the method originally designed for intact bovine embryos by Ishimori

et al. [20], with slight modification. The biopsied embryos were exposed to 200 μ l of equilibration solution (VS50, 50% vitrification solution) consisting of 12.5% (v/v) EG (Nacalai Tesque Inc., Kyoto, Japan), and 12.5% (v/v) dimethylsulfoxide (DMSO, Nacalai Tesque) dissolved in PBS supplemented with 0.3% (w/v) bovine serum albumine (BSA, FractionV, Sigma) at room temperature for 60 sec. The embryos were then exposed to vitrification solution, 25% (v/v) EG, and 25% (v/v) DMSO dissolved in PBS supplemented with 0.3% (w/v) BSA (VSED), and kept for 30 sec. The biopsied embryos were then loaded into 0.25-ml plastic straws (IMV, L'Aigle, France) containing the dilution solution, 5% (v/v) EG, and 0.15 M sucrose (Nacalai Tesque) in PBS supplemented with 20% (v/v) FBS (EGS), then with two 11 μ l droplets of VSED. The straws were immediately heat-sealed, placed horizontally in liquid nitrogen vapor at –180 °C for 2 min and then immersed in liquid nitrogen for storage. The dilution procedure was based on our previous report [18]. In brief, straws containing embryos were warmed in air for 5 sec and then warmed in 20 °C water. The straws were then removed from the water and shaken to mix the four columns of liquid (11 μ l, 11 μ l VSED and 105 μ l, 50 μ l EGS) in the straws. They were then held vertically with the cotton plug facing up and placed in the same water bath for 3 min (Fig. 1).

Observation of warmed embryos

Some embryos were expelled into ME199 and kept for 3 min at room temperature, the in-straw dilution group (ISD group). They were then washed and cultured for 3–5 h in ME199 at 38.5 °C under 5% CO₂ in humidified air. Subsequently, embryo survival was determined by morphological observation. The surviving embryos were re-loaded into 0.25-ml plastic straws containing TCM199 (Gibco, Invitrogen) supplemented with 20% (v/v) FBS and transferred to the recipients. In the directly transfer group (DIR group), embryos were transferred directly to recipients without expelled from the straws. In the non-vitrification group (NV group), biopsied embryos after *in*

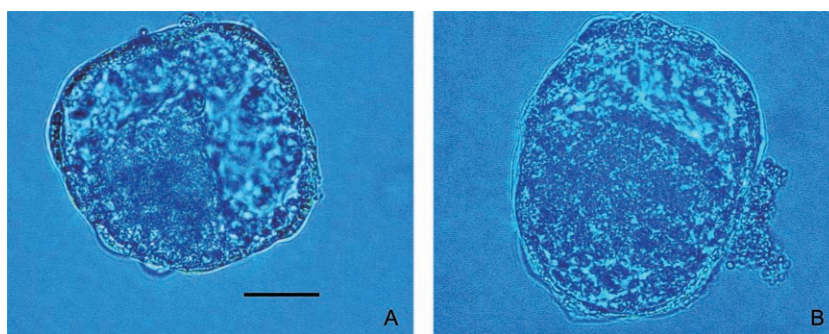


Fig. 2. Morphologies of biopsied bovine embryos before vitrification (A) and after warming (B). Scale bar=50 μ m.

Table 1. *In vitro* and *in vivo* viabilities of vitrified biopsied-embryos of the cattle

Groups*	Vitrification	Evaluation	No. of warmed embryos	No. of surviving embryos(%)	No. of recipients	No. of pregnancies (%) **	No. of offspring (%) ***
ISD	+	+	19	19 (100.0)	19	11 (57.9)	9 (47.4)
DIR	+	-	16	-	16	10 (62.6)	9 (56.3)
NV	-	+	-	-	32	18 (56.4)	16 (50.0)

*ISD: in-straw dilution, DIR: directly transfer after in-straw dilution, NV: non-vitrification. **The number of pregnancies as a percentage of the number of warmed embryos, Diagnosis by palpation per rectum on Day 35 after embryo transfer. ***The number of offspring as a percentage of the number of warmed embryos.

in vitro culture for 3–5 h were loaded into straws containing TCM199 (Gibco, Invitrogen) supplemented with 20% (v/v) FBS without vitrification. They were then transferred to the recipients.

Embryo transfer

Recipients used for this study were Holstein heifers and cows kept at our Institute. Embryos were non-surgically transferred 7–8 days after the onset of estrus into the uterine horn ipsilateral to the ovary bearing corpus luteum. Pregnancy was diagnosed by palpation via the rectum 35 days after of embryo transfer.

Measurement of CPA concentration

The solution in the straws (50 μ l) was collected from three parts of the straws immediately, 1.5 min and 3 min after mixing VSED and EGS in the straws. Then the EG and DMSO concentrations in each part were measured using a gas chromatograph with a flame ionization detector. The column used for analyses was a glass capillary column of 0.53 mm diameter \times 30 m (J&W, DB-WAXetr; Agilent Technologies Inc., CA, USA). The column temperature was initially 120 $^{\circ}$ C, elevated by 10 $^{\circ}$ C/min to a final temperature of 250 $^{\circ}$ C.

Statistical analyses

The significance of differences between means (rate of pregnancy) was compared by Pearson's χ^2 -test or Fisher's exact test using the SPSS program (SPSS 11.5 J, SPSS Inc., Tokyo Japan). Differences with probability values (*P*) of 0.05 or less were considered significant difference.

Results

Table 1 presents data for embryo survival after warming and the pregnancy rate after transfer of biopsied and vitrified bovine embryos. The morphological survival of biopsied bovine embryos after vitrification and warming was 100.0% (19/19). The pregnancy rate of biopsied bovine embryos after transfer was 57.9% (11/19) in the ISD group, 62.5% (10/16) in the DIR group, and 56.3% (18/32) in the NV group (Fig. 2).

Table 2 shows, the concentrations of cryoprotectants in the straws in the ISD group. The EG concentration in different parts of the straw ranged from 5.7–9.1% (v/v) immediately after mixing VSED and EGS, and from 6.9–7.7% (v/v) after 3 min. The DMSO concentration ranged from 0.9–5.2% (v/v) immediately after mixing, and from 2.8–3.3% (v/v) after 3 min.

Table 2. Concentrations of cryoprotectants after in-straw dilution of vitrified biopsied embryos of the cattle

Part of straw*	Time	EG (%)			DMSO (%)		
		0 min	1.5 min	3 min	0 min	1.5 min	3 min
cotton plug		9.1	6.6	7.0	5.2	2.3	2.8
middle		7.8	7.7	7.7	3.7	3.2	3.3
heat seal		5.7	8.2	6.9	0.9	4.4	3.2

*The solution in the straw was collected from three parts of the straw after mixing.
EG: ethylen glycol, DMSO:dimethylsulfoxide.

Discussion

The objective of the present study was to evaluate the viability of vitrified, biopsied bovine embryos after warming. The results of the present study show no significant difference ($P > 0.05$) among the pregnancy rates of vitrified, biopsied bovine embryos after transfer both by in-straw dilution, by direct transfer and the transfer of biopsied fresh embryos.

Thiber *et al.* [6] and Hasler *et al.* [7] reported that the pregnancy rate was significantly lower in slow-frozen biopsied embryos than in intact embryos. Other reports have also described no significant reduction between the pregnancy rates of vitrified or ultra-rapidly vitrified biopsied embryos and those of fresh embryos [8, 12]. The present study found no significant difference between the pregnancy rates of biopsied embryos with and without vitrification, supporting in the vitrification earlier reports is suitable for cryopreservation of biopsied bovine embryos [8, 12].

For embryo vitrification, it is necessary to dilute the vitrification solution immediately after warming to minimize the time that embryos are exposed to high concentration of cryoprotectant [21]. In the present study, the cryoprotectants were diluted by mixing the dilution solution and the vitrification solution in the straw and held it for 3 min. Survival and pregnancy rates following warming of vitrified bovine embryos in the present study were similar to those of one-step dilution [9, 10, 20, 22, 23] and stepwise dilution [24, 25] reported in the previous studies. This result confirms that biopsied and vitrified embryos can be warmed and transferred immediately to recipients using a simple process at a remote farmyard site.

Pedro *et al.* [26] reported that exposure of mouse embryos for 30 min to 1.0 M or 1.5 M sucrose solutions reduced embryo viability at the two-cell stage to the expanded blastocyst stage, indicating the possibility of physical damage to the plasma membranes of embryos through long-term exposure to a hypertonic solution. In the present study, we assume that physical damage to the embryos during the procedures of dilution and trans-

fer was reduced through use of the dilution solution containing sucrose with concentrations lower than those described in previous reports in which the cryoprotectant was diluted in a single step after vitrification [9, 10, 18, 20, 21]. In addition, the warmed embryos were not kept in the dilution solution containing sucrose for a long time, which is one reason why negative effects on the pregnancy rate were avoided. In general, permeating agents are strongly toxic to embryos, and their toxicity is enhanced by long-time exposure [21]. The cryoprotectant concentration in the straw during the dilution process was measured in the ISD group and the DIR group in the present study. EG and DMSO in different parts of the straw were equilibrated in a short time and were diluted to a concentration lower than EG (1.5 M, 1.8 M) used in slow-freezing direct transfer [4, 5]. Therefore, it is considered to be some reasons for the lack of reduction in the viability of embryos, a high conception rate comparable to the ISD group was also achieved in the DIR group. However, Yang *et al.* [27] reported from the in-straw dilution of mouse embryos vitrified using open pulled straws (OPS) that inefficient dilution of the cryoprotectant might occur depending on the length of designating to the volume that the vitrification solution loaded into the OPS, affecting the development of embryos after warming. For that reason, it is necessary to maintain an accurate composition of the vitrification solution and the dilution solution. Similar attention might be necessary in the method used in the present study. Moreover, for popularization of the warming procedure and direct transfer on farms, it will be necessary to examine the effects of the warming temperature and the time after warming on the viability and fertility of embryos.

In conclusion, the results of the present study suggest that vitrification of biopsied bovine embryos can give a pregnancy rate that is comparable to that of fresh biopsied embryos, and that the embryos can be warmed and transferred directly to recipients on farms by diluting the cryoprotectant in the straws. The results of this study will contribute to the development of cattle breeding programs.

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