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Vitrification for bovine embryos with low-quality grade

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Abstract

This study was conducted to examine the utility of vitrification for bovine embryos with low-quality grade, and simple cryoprotectants dilution method for practitioners. In Experiment 1, survival of frozen embryos was compared with that of vitrified embryos using minimum volume cooling (MVC). Then, vitrified embryos were used to confirm the optimum sucrose concentration in Experiment 2. The survival rates of embryos that had been vitrified following diluted cryoprotectants with the one-step in-straw method were compared with those of fresh control embryos in Experiment 3. Frozen-thawed or vitrified-warmed blastocysts were cultured with TCM-199 supplemented with 100 μmol/L beta-mercaptoethanol +5% fetal bovine serum at 38.5°C in an atmosphere of 5% CO_2 in air, their survival after 24 hr were compared. The development to term of fair quality in vivo embryos after vitrification was examined in Experiment 4. Results show that survival rates of frozen-thawed embryos were lower ($p < .05$) than that of vitrified-warmed ones. When vitrified embryos were warmed in 0.3 mol/L sucrose in straws, their survival rate was 100%. The total cell numbers of vitrified-warmed embryos were comparable to those of fresh control embryos. The six calves from 13 vitrified embryos were delivered in Experiment 4. These results indicate that MVC vitrification following one-step cryoprotectants dilution is utilized to preserve low-quality bovine embryos.

KEYWORDS
bovine blastocyst, cryopreservation, minimum volume cooling, one step cryoprotectants dilution, vitrification

1 | INTRODUCTION

Multiple ovulation and embryo transfer (MOET) is a powerful technology for the genetic improvement and increase of livestock species. In Japan, MOET programs have been used for genetic improvement of Holstein cattle and to increase meat production of Japanese Black cattle. The strategies to transfer low-quality embryos into recipient cattle freshly and to cryopreserve high-quality embryos are recommended for increasing productivity of MOET (Otoi et al., 2000). Although about 60,000 bovine embryos are collected in Japan, only 30% of the embryos are used for embryo transfer (ET) of fresh embryos. The remaining 70% of embryos including low-quality embryos are used for ET after cryopreservation (Perry, 2014). The cryopreservation of low-quality embryos before transfer to recipients decreased productivity of the MOET program. Unfortunately, no efficient technologies to collect only high-quality embryos have been accomplished (Bo et al., 2002; Chebel, Demétrio, & Metzger, 2008; Hasler, 2001). In addition, the cryotolerance of in vitro produced (IVP) embryos remains low with current technologies (Abdalla et al., 2010; Hamano et al., 2006). Therefore, preservation methods with higher survival rates for low-quality embryos are necessary to increase MOET productivity.

Since the first successful vitrification for embryo cryopreservation was conducted by Rall and Fahy (1985), modified vitrification protocols have been developed (Ishimori et al., 1993; Vajta et al., 1998). The minimum volume cooling (MVC) method developed by

Kuwayama and Kato (2000), characterized by lower concentrations of cryoprotectants, achieves extremely high rates of cooling (< −20,000°C/min) and warming (40,000°C/min<) rates (Kuwayama, 2002). Vitrification by MVC method was developed originally for the cryopreservation of human oocytes and embryos (Kuwayama, 2007), and subsequently applied to vitrification of embryos in pigs (Ushijima, 2005) and dogs (Abe et al., 2011), all of which have high sensitivity to low temperatures. An advanced version of the MVC procedure might be useful for low cryotolerance embryos such as IVP bovine blastocysts with low quality.

Direct transfer of bovine embryos that are frozen-thawed in the presence of ethylene glycol is most commonly used by the embryo transfer industry (Dochi, Imai, Matoba, Miyamura, & HamanoS, 2006). Therefore, to facilitate spread of vitrification embryos in the Japanese cattle industry, it is necessary to employ a simple cryoprotectants dilution method that does not manipulate embryos under a microscope. One-step or direct dilution of cryoprotectants for ET methods have already been used for vitrified bovine embryos; treated embryos support pregnancy (Caamano et al., 2015; Inaba et al., 2011; Sano et al., 2010; Vieira, Forell, Feltrin, & Rodrigues, 2007). Our final objective of this study is the modification of MVC vitrification and cryoprotectants dilution using the one-step in-straw method for bovine blastocysts with low cryotolerance. We designed four experiments for modification of vitrification with the simple cryoprotectants dilution.

2 | MATERIALS AND METHODS

2.1 | Basic experimental conditions

In compliance with the applicable domestic laws and regulations governing animal experiments, the designed animal experimental protocol was submitted to the Institutional Animal Experiment Committee and the Animal Ethics Committee of Nippon Veterinary and Life Science University for review and approval (Approval No. S25K-7). Because of precautions required under the special law for preventing the spread of bovine spongiform encephalopathy (BSE), bovine ovaries were collected according to the direction of the Chiba Prefectural Meat Inspection Center (Shino et al., 2004) and used for experiments after confirmation of their BSE-free status. Unless otherwise stated, all chemicals and reagents used for this study were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

2.2 | IVP of bovine embryos

Bovine ovaries were transported to the laboratory in saline at 10°C–15°C within 12 hr after slaughter. Cumulus-oocyte complexes (COCs) collected from 2–8 mm follicles of ovaries were washed with 25 mmol/L Hepes-buffered Tissue Culture Medium (TCM)-199 (12340-030; Life Technologies, Grand Island, NY, USA) supplemented with 5% fetal bovine serum (FBS; Sigma-Aldrich Co., St. Louis, MO, USA) and 10 μg/mL gentamycin (15750060; Life Technologies). In a single drop of the same culture medium (TCM199 + 5% FBS) under mineral oil in a Petri dish (Nunclon; Nalge Nunc International, Roskilde, Denmark), 50–60 COCs were in vitro matured (IVM) at 38.5°C in an atmosphere of 3% CO_2 in air for 22 hr (Shino et al., 2004). Commercially available frozen semen of Japanese Black bulls (Livestock Improvement Association of Japan Inc., Tokyo, Japan) was thawed at 38.5°C for 14 s, and the straw content was washed with BO (Brackett & Oliphant, 1975) medium twice. IVM oocytes were subsequently fertilized in vitro (IVF) with 5×10^6 sperm during a 5 hr incubation period in a 500 μl droplet of BO medium supplemented with 10 mg/ml bovine serum albumin (BSA, fraction V), 5 μg/ml heparin, and 5 mmol/L caffeine. After IVF, the oocytes were cultured at 38.5°C in a 500 μl droplet of the culture medium with cumulus cells in an atmosphere of 3% CO_2 in air for 5 days (Ushijima & Nakane, 2006). Then, embryos were transferred to a culture medium supplemented with 100 μmol/L beta-mercaptoethanol and were cultured in an atmosphere of 5% CO_2 in air for 3 days. Blastocysts obtained at 7–8 days after the beginning of IVF were used for Experiments 1–3 after evaluation of their embryo quality. High-quality embryos with degenerated blastomeres less than 10% were evaluated as good grade, whereas low-quality embryos with the degenerated blastomeres less than 30% and 50% were evaluated as fair and poor grades (Lindner & Wright, 1983).

2.3 | Conventional freezing and thawing of embryos

IVP blastocysts were frozen in basal solution (TCM-199 supplemented with 20% FBS and 10 μg/ml gentamycin) supplemented with 10% ethylene glycol and 0.1 mol/L sucrose (Sigma, S-1888). The embryos were equilibrated into the 10% ethylene glycol for 10 min and transferred to freezing medium constituted of 10% ethylene glycol and 0.1 mol/L sucrose in basal solution at room temperature. Then, an embryo was loaded into a 0.25 mL straw (Plastic Cassou AI mini straw; IMV Technologies, L'Aigle, France). The straws were placed into a −7°C methanol chamber of a programmable freezer (ET-1; Fujihira Industry Co. Ltd., Tokyo, Japan), seeded at −7°C and held at −7°C for 10 min. The embryos in straws were cooled at −0.3°C/min until reaching −30°C. Then they were plunged into liquid nitrogen (LN_2) and stored in LN_2 for a week. The straws were thawed by warming for 10 s in air at room temperature and 10 s in a 38.5°C water bath with gentle shaking (Dochi et al., 2006). Straw contents were expelled into a Petri dish. Then, embryos were washed in beta-mercaptoethanol supplemented culture medium twice and were incubated sequentially in the same medium at 38.5°C under 5% CO_2 in air for 24 hr.

2.4 | Vitrification of blastocyst by MVC

IVP blastocysts were vitrified according to the method described previously by Kuwayama (2002) with minor modifications. In brief, basal solution, equilibration solution, which consisted of 7.5% ethylene glycol, and 7.5% dimethyl sulfoxide in basal solution, and vitrification solution consisting of 15% ethylene glycol, 15% dimethyl

sulfoxide, and 0.5 mol/L sucrose in basal solution, were warmed at room temperature. Equilibration solution and two drops of vitrification solution were distributed at 300 μl each into spots of a dish (IFP 9670; Reproplate, Wako).

Cryotec for animal specifications (Figure 1a; Repro Life, Tokyo, Japan), a special tool consisting of a narrow, polypropylene thin film strip (0.75 mm wide, 1.88 ± 0.02 mm long, and 0.1 mm thick and top of the strip trimmed diagonally), and a hard plastic holder constitute an exclusive device for the one-step in-straw method. To protect the tool from mechanical damage during storage, a 3 cm long plastic tube cap can be attached to cover the film part (Figure 1b).

An embryo was transferred to the surface of the equilibrated solution for 12–15 min. The embryo was washed with the first spot of vitrification solution three times. Then it was equilibrated in the middle of the next vitrification solution spot. During this 60 s, an embryo was loaded onto a Cryotec strip with minimal volume (<0.1 μl) of the vitrification solution. The Cryotec was transferred quickly into LN_2 with gentle shaking. Finally, the tube cap was pulled over the Cryotec strip. The strip was stored for several weeks under LN_2.

2.5 | Stepwise warming and cryoprotectants dilution of vitrified blastocysts

A diluted solution (0.5 mol/L sucrose in basal solution) and washing solution consisting of basal solution were warmed at room temperature. Diluted solution and two drops of basal solution were distributed at 300 μl each into spots of a dish. A plastic culture dish with 3 ml warming solution (1 mol/L sucrose in basal solution) was warmed in an incubator at 38.5°C for 1 hr. The strip was removed from the tube cap in LN_2, and immersed directly into a 38.5°C warming solution dish and kept for 1 min to counterbalance the osmotic shock caused by the permeable cryoprotectants accumulated intracellularly. Subsequently, the embryo with 2 μl warming solution was transferred to the bottom of diluted solution and was equilibrated for 3 min. Then the embryo with 2 μl diluted solution was transferred to the bottom of basal solution and equilibrated for 5 min. Finally, the embryo was transferred to the basal solution surface and was washed several times with the same solution (Kuwayama, 2007). In culture medium supplemented with beta-mercaptoethanol, these vitrified–warmed embryos were incubated sequentially at 38.5°C under 5% CO_2 in air for 24 hr.

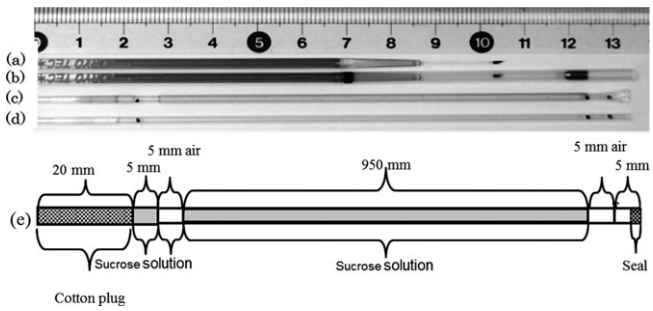

FIGURE 1 Devices for minimum volume cooling vitrification and one-step in-straw cryoprotectants dilution procedure. (a) Cryotec for animal specification constructed with a film strip and a holder. (b) Cryotec attached with a tube cap (total 135 mm long). (c) Straw loaded into 0.3 mol sucrose solution according to marks. (d) Straw with 5, 10, and 110 mm marks from the top of a straw for loading into 0.3 mol sucrose solution medium, and a 130 mm mark from end of a straw for cutting position. (e) Illustration of straw column

2.6 | Warming and cryoprotectants dilution of vitrified embryos with one-step in-straw method

According to the method described by Sano et al. (2010), with minor modifications, cryoprotectants were diluted with the one-step in-straw method. A stainless-steel vacuum bottle (JNL-350; Thermos, Niigata, Japan) was filled with 38.5°C warm water, and the inner lid of the bottle was closed (Figure 2). A straw filled with 0.3 mol sucrose solution was cut at 130 mm from the cotton plug (Figure 1e). To insert easily a Cryotec strip into the straw column filled with warming solution, a micropipette tip (T-1005; Axygen Scientific Inc., Union City, CA, USA) was pushed into the straw of the cutting position as a guide. Then, the 0.25 ml straw (Figure 1c) with the micropipette tip was put into the bottle through the air vent of the inner lid. The top of the strip, which was loaded on embryos, was inserted into the straw through the micropipette tip and was immersed for 1 min. The Cryotec strip was gently shaken and removed from the straw. For in vitro culture, the straw was placed at room temperature for 9 min, then embryos in straws were expelled into plastic dishes and were washed twice in beta-mercaptoethanol supplemented culture medium. These vitrified–warmed embryos were incubated in the same medium at 38.5°C under 5% CO_2 in air for 24 hr. For ET, embryos in the straws attached into ET guns (Cassou-gun; IMV Technologies) were transferred to recipients 6–8 days post natural estrus via the cervical canal to the uterine horn on the corpus luteum side (Yoshida et al., 2012). Then the pregnancy rate was examined at 60 days after ET and then newborn offspring were observed.

2.7 | Cell count of embryos

The surviving embryos were assessed in terms of re-expansion blastocysts incubated for 24 h after preservation. These embryos were fixed using air-drying (Ushijima & Nakane, 2006) and were stained with 5% Giemsa stain to allow cell counting. The total cell numbers of the embryos were counted under a microscope at 400× magnification.

3 | EXPERIMENTAL DESIGN

3.1 | Experiment 1

The objective was to compare in vitro survival of vitrified IVP embryos using the MVC method with that of cryopreserved embryos

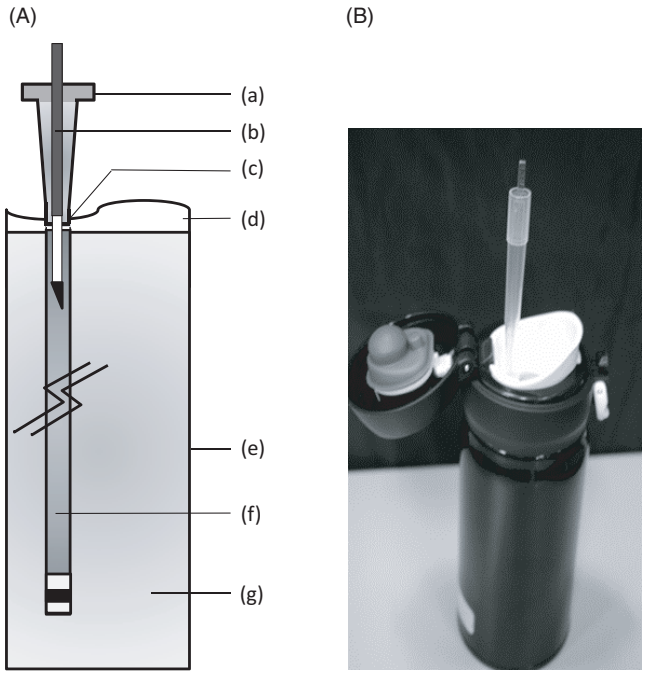

FIGURE 2 Warming and cryoprotectants dilution with the one-step in-straw method for bovine vitrified embryos. (A) A straw with a chip is put into a stainless bottle through its narrow air hole. (B) Illustration of Cryotec position in a straw. Top of the Cryotec strip dipped completely into thawing solution: (a) micropipette tip, (b) Cryotec, (c) air vent, (d) inner lid, (e) stainless steel vacuum bottle, (f) 0.25 ml straw, and (g) 38.5°C water

using conventional slow freezing. The good, fair and poor grade IVP embryos were cultured for 24 hr after preservation treatments. The survival rates and total cell numbers of the resultant embryos were compared.

3.2 | Experiment 2

In Experiment 2, sucrose concentrations adapted for one-step cryoprotectants dilution were evaluated. The IVP blastocysts evaluated as fair grade were immersed directly into a 38.5°C dish containing 0.5, 0.3 or 0.2 mol/L sucrose in basal solution for 1 min. The vitrified–warmed blastocysts were transferred to the same medium at room temperature for 9 min, washed in beta-mercaptoethanol supplemented culture medium twice and were incubated sequentially in the same medium at 38.5°C under 5% CO_2 in air for 24 h and then the embryo survival was observed.

3.3 | Experiment 3

The good, fair and poor grade IVP embryos were vitrified with diluted cryoprotectants with the one-step in-straw method in 0.3 mol/L sucrose. In vitro survival and development of embryos vitrified following dilution cryoprotectants with the one-step in-straw method were compared with that of IVP fresh control embryos of the same embryo quality.

3.4 | Experiment 4

Embryos were collected from superovulated Japanese Black cattle 6–8 days after the first artificial insemination. The fair quality embryos with low cryotolerance were vitrified and subsequently diluted cryoprotectants added with the one-step in-straw method for 1 min at 38.5°C. The vitrified–warmed embryos in straws attached into ET guns were transplanted into recipients 6–8 days post natural estrus. The development to term of vitrified embryos was examined.

3.5 | Statistical analysis

Statistical analyses were performed using Stat View Version 5.0 (SAS Institute Inc., Cary, NC, USA). The survival rates of preserved embryos in Experiments 1–3 were assessed by Chi-square test with Holm's adjustment. For assessment of sequential data in Experiment 1 and Experiment 3 of total cell numbers, two-way analysis of variance with repeated measures was used to determine the main effects of group. When a significant interaction was detected, Scheffe's F test was used for multiple comparisons test to detect significant differences among groups. Differences with a probability value of less than 0.05 were considered significant.

4 | RESULTS

4.1 | Experiment 1

As shown in Table 1, the post-thaw survival rate of good quality embryos was 60%. The survival rates of conventional freezing embryos depended on the embryonic quality being lower ($p < .05$). In contrast, the post-warming survival rate of vitrified embryos was 100%. It did not depend on the embryo quality. The total cell numbers of vitrified embryos were significantly higher than those of frozen and thawed embryos ($p < .05$).

4.2 | Experiment 2

When IVP blastocysts evaluated as fair grade were diluted with cryoprotectants directly in different sucrose concentrations, the survival

TABLE 1 Survival rate of frozen or vitrified in vitro produced bovine blastocysts with different embryo qualities

Embryo quality	Preservation method	No. of embryos	Surviving embryos (%)	Total cell numbers of embryos ($M \pm SD$)
Good	Vitrification	20	20 (100.0)[a]	134.2 ± 17.1[a]
Good	Slow freezing	20	16 (60.0)[b]	80.6 ± 11.9[d]
Fair	Vitrification	20	20 (100.0)[a]	96.8 ± 23.2[c]
Fair	Slow freezing	20	7 (35.0)[c]	40.6 ± 3.5[e]
Poor	Vitrification	20	20 (100.0)[a]	67.9 ± 20.9[d]
Poor	Slow freezing	20	4 (13.3)[c]	26.5 ± 3.5[f]

[a,b,c,d,e,f]Values with different superscripts within columns differ significantly ([a, b]$p < .05$; [a,c,d,e,f]$p < .01$).

of embryos in 0.3 mol/L sucrose solution was 100%. In contrast, those of embryos in 0.5 and 0.2 mol/L sucrose were lower (Table 2).

4.3 | Experiment 3

All the embryos vitrified and subsequently diluted cryoprotectants by the one-step in-straw method were recovered from the straws and survived, irrespective of the embryonic quality. No significant differences were found in cell numbers of various embryo qualities after culture between vitrified blastocysts and fresh control embryos (Table 3).

4.4 | Experiment 4

Embryos collected from Japanese Black cattle and evaluated as fair quality were vitrified following diluted cryoprotectants by the one-step in-straw method with 0.3 mol/L sucrose. ET was performed on 13 cattle non-surgically; six (46.2%) of them became pregnant and delivered six Japanese Black calves, four males and two females.

5 | DISCUSSION

The present study confirmed that the viability of conventional embryo freezing decreases in an embryo quality-dependent manner. In contrast, all of the embryos vitrified by the MVC method survived irrespective of their embryo quality. This study also demonstrated that high survival rates after vitrification were accomplished across all embryo qualities, when 0.3 mol/L sucrose solution was used as the warming solution for the simplified one-step cryoprotectants dilution.

TABLE 2 Survival of bovine vitrified blastocysts after warning and cryoprotectants dilution with the one-step method in different sucrose concentrations

Sucrose concentration (mol/L)	No. embryos examined	Surviving embryos (%)
0.5	60	53 (88.3)[a]
0.3	60	60 (100.0)[b]
0.2	58	51 (87.9)[a]

[a,b]Values with different superscripts within columns differ significantly ($p < .05$).

TABLE 3 Survival of in vitro produced blastocyst after vitrification by minimum volume cooling and diluted cryoprotectant agents by the one-step in-straw method with 0.3 mol/L sucrose

Embryo quality code	Preservation method	No. of embryos examined[†]	Surviving embryos (%)	Total cell numbers of embryos ($M \pm SD$)
Good	Vitrified	30	100.0	130.8 ± 18.3[a]
Good	Fresh control	30	100.0	134.1 ± 22.4[a]
Fair	Vitrified	30	100.0	93.3 ± 11.3[b]
Fair	Fresh control	30	100.0	103.3 ± 14.2[b]
Poor	Vitrified	30	100.0	65.7 ± 14.6[c]
Poor	Fresh control	30	100.0	60.3 ± 14.5[c]

[†]Embryos loaded into Cryotec were diluted cryoprotectants by the one-step in-straw method at 38.5°C for 1 min and placed in the straw at room temperature for 9 min.
[a,b,c]Values with different superscripts within columns differ significantly ($p < .05$).

Pregnancies after the transfer of embryos that had been vitrified and warmed in a straw have been already reported (Caamano et al., 2015; Inaba et al., 2011; Sano et al., 2010; Vieira et al., 2007). It has also been reported that bovine embryo vitrification and subsequent cryoprotectants dilution with the one-step in-straw method was comparable to that of the slow freezing method (Sano et al., 2010). In addition, the vitrification methods were also useful for sex-determined embryos with low cryotolerance (Akiyama et al., 2010; Inaba et al., 2011). The vitrification and cryoprotectants dilution used in the present study might be feasible for embryos with extremely low cryotolerance.

Generally, sucrose solutions are used for the dilution of post-thawed/warmed embryos as an osmotic buffer to restrict water permeation and to prevent excessive swelling of the embryos during removal of cryoprotectants from the cells (Schneider & Mazur, 1984). In the original MVC method, cryoprotectants are diluted in 1 mol/L sucrose solution at a temperature higher than 37°C (Kuwayama, 2002). As longtime exposure in dilution solution containing sucrose with high concentration adversely affected embryo viability, lower sucrose concentration was recommended for warming solution for the one-step in-straw method (Inaba et al., 2011). Vitrified–warmed embryos should be submerged in dilution solution for 10 min, because ET procedure from one-step cryoprotectants dilution to transfer to a recipient takes at least 5 min (Tanaka, 1995). Inaba et al. (2011) also described survival of bovine embryos unaffected by the presence or absent of sucrose in the one-step in-straw cryoprotectants dilution solution. When 0.3 mol/L sucrose was used as a dilution medium, all vitrified–warmed embryos survived (Table 2) and their total cell numbers were comparable to those of non-cryopreserved embryos in this study (Table 3). In particular, as the survivability of low-quality embryos was improved by MVC vitrification and subsequent simple cryoprotectants dilution method, the series protocol may be expected to be applicable for low-quality embryos.

Sano et al. (2010) reported better precision of the warming process using micropipette tips. Caamano et al. (2015) recommend their own protocol to prevent embryo loss during the warming process. However, such troubles were not observed during experiments in the present study. The diagonally trimmed top of the Cryotec facilitated the insertion of the strip through the micropipette tip. In addition, the inner diameter of the micropipette tip was wider than the width of the Cryotec strip. The strip top of Cryotec reached the end on the surface of the warming solution inside the straw. Selection of these materials might have contributed to the stability of embryo manipulation for vitrification.

The MVC device, polypropylene thin film strip, has been frequently used for vitrification materials because of its high preservation potential and convenience. For example, vitrification volume in 2 μl of vitrification solution is recommended for the one-step

method to facilitate the release of embryos from the strip (Sano et al., 2010; Vieira et al., 2007). Kuwayama (2007) reported that the modified procedure of vitrification volume over 0.1 μl might cause adverse effects for oocyte survival after vitrification by decreasing both cooling and warming rates. On the other hand, minimizing the volume of vitrification solution containing oocytes or embryos not only increases both cooling and warming rates, but also decreases the risk of ice crystal formation (Kuwayama, 2002). The minimizing volume offers the obvious benefit of decreased concentration of cryoprotectants; it moderates toxic and osmotic hazards to the cellar plasma membrane in vitrification conditions (Arav, 2014). Loading an embryo into the vitrification solution with minimum volume might provide high embryo survivability consistent with the present study.

In conclusion, MVC vitrification following cryoprotectants dilution with the one-step in-straw method for the preservation method for bovine embryos is viable with not only low-quality grade but also high-quality grade embryos, instead of the conventional freezing method in the embryo transfer industry.

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