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Factors affecting cryosurvival of nuclear-transferred bovine and swamp buffalo blastocysts: effects of hatching stage, linoleic acid–albumin in IVC medium and Ficoll supplementation to vitrification solution

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Abstract

The objective was to determine whether the hatching stage of cattle and swamp buffalo somatic cell nuclear-transferred (SCNT) blastocysts affected cryosurvival after vitrification, and whether addition of linoleic acid–albumin (LAA) to the IVC medium and Ficoll to the vitrification solution improves cryosurvival. Fused couplets were activated with ethanol and cycloheximide-cytochalasin D (day 0), and were allowed to develop in the presence of 0.3% BSA or 0.1% LAA + 0.2% BSA. Hatching blastocysts were harvested at day 7.0 (cattle) or day 6.5 (buffalo), and classified into one of three categories, according to the ratio of extruding embryonic diameter from zona to embryonic diameter inside the zona. The blastocysts were vitrified in 20% DMSO + 20% ethylene glycol + 0.5 M sucrose, with or without 10% Ficoll in TCM199 + 20% FBS, using Cryotop as a cryodevice. The post-thaw survival of the blastocysts was assessed by in vitro culture for 24 h. In cattle, when the LAA-supplemented IVC medium and the Ficoll-free vitrification solution were used, cryosurvival of the early-hatching blastocysts (77%) was not different from those of middle- and late-hatching blastocysts (74 and 80%, respectively). Inclusion of Ficoll in the vitrification solution did

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not improve the cryosurvival of SCNT blastocysts (54 to 68%). Early-hatching SCNT blastocysts produced in the absence of LAA were sensitive to the vitrification procedure (cryosurvival 56%; $P < 0.05$ versus 80% in the late-hatching blastocysts). The full-term developmental potential of SCNT blastocysts was proven only in the non-vitrified control group. In buffalo, the mean cryosurvival of hatching SCNT blastocysts produced with LAA (89%) was not different from that of those produced without LAA (87%). In conclusion, bovine SCNT blastocysts, regardless of their hatching stage, were relatively resistant to vitrification by the ultra-rapid cooling procedure when the blastocysts were produced in the presence of LAA. Furthermore, swamp buffalo SCNT blastocysts were more tolerant of vitrification than bovine SCNT blastocysts.

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1. Introduction

Recent advances in embryo cryopreservation in bovine species include high survival rates after vitrification by ultra-rapid cooling procedures. With IVF-derived bovine embryos, ultra-rapid cooling in cryodevices such as open-pulled straws (OPS) [1] and gel-loading tips [2] has made it possible to cryopreserve embryos at various developmental stages ranging from 1-cell zygotes to expanded blastocysts. Embryos produced by nuclear transplantation have mechanical slits in their zonae pellucidae, and therefore initiate hatching earlier than non-manipulated embryos. Nguyen et al. [3] were the first to achieve high in vitro survival of bovine somatic cell nuclear-transferred (SCNT) blastocysts cryopreserved by a combination of partial dehydration and vitrification. They used conventional 0.25-mL French straws as embryo containers and a vitrification solution consisting of 40% ethylene glycol (EG) + 18% Ficoll + 0.3 M sucrose (EFS40), originally reported by Kasai et al. [4]. Gong et al. [5] used the same EFS40 solution for vitrification of SCNT embryos and successfully produced a cloned calf following transfer of nine vitrified-warmed embryos. Another cloned calf has been delivered from a SCNT blastocyst vitrified in a solution consisting of 20% EG + 20% DMSO + 0.6 M sucrose, using the OPS system [6].

Removal of serum from the IVC medium for culturing presumptive zygotes improved the resistance of blastocysts to cryopreservation [7–9]. Abe et al. [9] reported that cytoplasmic lipid droplets were highly accumulated in the bovine morulae and blastocysts when the IVF zygotes were cultured in IVC medium that contained serum. Supplementation with the unsaturated fatty acid-conjugated BSA (linoleic acid–albumin, LAA), to the IVM and IVF media [10] and IVC medium [11–13] produced bovine zygotes and embryos resistant to the conventional two-step freezing procedure.

In contrast to the highly reproducible SCNT procedure in cattle, efficiency in producing SCNT buffalo embryos is not satisfactory [14,15], despite an increased demand for cloned buffaloes. We have previously reported that efficiency in producing SCNT blastocysts in swamp buffalo (19–22%) was approximately half of that in cattle (39–41%) [15]. On the other hand, both bovine- and buffalo-enucleated oocytes receiving buffalo fibroblasts equally developed into blastocysts (33 and 39%, respectively) [16]. There have been only a few reports on the cryopreservation of buffalo embryos [17–21], including the birth of calves after transfer of vitrified-warmed water buffalo IVF-derived embryos [21].

The present study was undertaken to determine whether the hatching stage of bovine SCNT blastocysts affects cryosurvival after vitrification by the minimum volume cooling (MVC) procedure, and whether addition of LAA to the IVC medium and of Ficoll to an EG + DMSO-based vitrification solution improves cryosurvival (experiment 1). An additional vitrification study was conducted for swamp buffalo SCNT hatching blastocysts produced by IVC, with or without LAA (experiment 2).

2. Materials and methods

2.1. Chemicals and media

All chemicals were purchased from the Sigma Chemical Company (St. Louis, MO, USA) unless stated otherwise. For culturing donor cells, alpha minimal essential medium (α -MEM) supplemented with 10% fetal bovine serum (FBS; Gibco BRL, Grand Island, NY, USA), defined hereafter as α -MEM/FBS, was used. For cryopreserving the cultured donor cells, 10% DMSO was added to the tissue culture medium 199 (TCM199) supplemented with 25 mM HEPES and 20% FBS, defined hereafter as HEPES-buffered M199/FBS. The IVM medium for oocyte maturation was TCM199 supplemented with 10% FBS, 50 IU/mL hCG (Chorulon; Intervet, Boxmeer, Netherlands), 0.02 AU/mL FSH (Antrin; Denka Pharmaceuticals, Tokyo, Japan) and 1 μ g/mL estradiol 17 β . The Emcare holding medium (ICP Bio, Auckland, New Zealand) was used as the basal medium throughout the process of enucleation to ethanol activation, except during electrofusion, when the Zimmermann medium [22] was used. The IVC medium for culturing SCNT embryos was modified synthetic oviduct fluid with amino acids (mSOFaa) [23] supplemented with 0.3% fatty acid-free BSA (Sigma, A6003) or 0.1% LAA (Sigma, L8384) + 0.2% fatty acid-free BSA.

2.2. Production of SCNT blastocysts

2.2.1. Preparation of donor cells

The ear skin of adult Holstein cow and swamp buffalo ($n = 1$ each) was biopsied and transported to the laboratory. Skin tissues were removed from cartilage and cut into small pieces before being placed in 60-mm culture dishes (Nunc, Roskilde, Denmark) and covered with a sterile glass slide. An amount of 5 mL of α -MEM/FBS was added into the dishes and the tissue was cultured for 8–10 days in a humidified atmosphere of 5% CO₂ in air at 37 °C. The fibroblasts outgrowing from ear skin tissues were harvested using 0.25% Trypsin-EDTA and seeded in 5 mL of α -MEM/FBS in a 25-cm² culture flask (Nunc). At sub-confluence, fibroblasts were harvested by standard trypsinization and subjected to passages. The fibroblasts were frozen at the third cell culture passage and stored in liquid nitrogen (LN₂). The post-thaw fibroblasts were cultured in α -MEM/FBS and used for nuclear transfer between passages 3 and 8 of culture. A few minutes before donor cell injection, a single cell suspension of the fibroblasts was prepared in an Emcare holding medium.

2.2.2. Preparation of recipient cytoplasm

Abattoir-derived bovine (Holstein) and buffalo ovaries were transported to the laboratory within 4 h of slaughter. Cumulus-oocyte complexes (COCs) were obtained by aspiration from follicles 3–6 mm in diameter using an 18-gauge needle attached to a 10 mL syringe, and washed four times with PBS supplemented with 0.1% polyvinyl pyrrolidone (PVP). Each of 20 COCs was cultured in 100 μ L droplets of IVM medium covered with mineral oil in a humidified atmosphere of 5% CO₂ in air at 38.5 °C for 21 h.

At 21 h of IVM culture, the cumulus cells were mechanically removed by repeated pipetting using a fine-tip pipette in 0.2% hyaluronidase and washed five times in the Emcare holding medium. Oocytes with an extruding first polar body were placed in 5 μ g/mL cytochalasin B for 15 min, and enucleated by a micromanipulator under an inverted microscope (200 \times magnification). Briefly, the zona pellucida close to the first polar body was dissected with a glass needle and a small volume (\sim 10%) of cytoplasm lying beneath the first polar body was squeezed out of the opening of zona pellucida. The enucleated oocytes were washed five times in the Emcare holding medium and kept in the same medium until donor cell injection. The successful enucleation of each oocyte was confirmed by Hoechst 33342 fluorescent staining of the corresponding karyoplast that was squeezed out.

2.2.3. Reconstruction and IVC

An individual donor cell, 14 to 16 μ m in diameter [14], was inserted into the perivitelline space of the cytoplasm, using a slit in the zona pellucida dissected at enucleation. The donor cell-cytoplasm couplets were fused in the Zimmermann medium by two direct currents (2.4 kV/cm for 15 μ s in cattle, and 2.6 kV/cm for 17 μ s in buffalo; the distance between electrodes was about 110 μ m) generated by a hand-made fusion machine (SUT F-1, Suranaree University of Technology). The number of couplets successfully fused was recorded 1 h after electro-stimulation. The fused couplets (reconstructed embryos) were activated by a combined treatment of 7% ethanol in the Emcare holding medium for 5 min and 10 μ g/mL cycloheximide + 1.25 μ g/mL cytochalasin D in mSOFaa medium + 10% FBS in a humidified atmosphere of 5% CO₂ in air at 38.5 °C for 5 h.

The reconstructed embryos were cultured in mSOFaa medium supplemented with 0.3% BSA or 0.1% LAA + 0.2% BSA in a humidified atmosphere of 5% CO₂, 5% O₂, 90% N₂ at 38.5 °C for 2 days (20 embryos per 100 μ L droplet). Thereafter, SCNT embryos at the 8-cell stage were selected and co-cultured with bovine oviductal epithelial cells (BOEC) in a humidified atmosphere of 5% CO₂ in air at 38.5 °C for 5 days (10 embryos per 100 μ L droplet), as described previously [14]. Half of the volume of culture medium was replaced every day.

2.2.4. Classification of hatching blastocysts

The blastocysts at the hatching stage were harvested at day 7 (in the case of cattle) or day 6.5 (in the case of buffalo) and photographs were taken. As shown in Fig. 1, three hatching stages were identified according to the ratio of extruding embryonic diameter from zona (D2) to embryonic diameter inside the zona (D1); early-hatching stage: D2/D1 = 0.01–0.70, middle-hatching stage: D2/D1 = 0.71–1.00 and late-hatching stage: D2/D1 = 1.01–1.70. Hatching blastocysts that developed beyond the D2/D1 ratio of 1.71 (mostly or

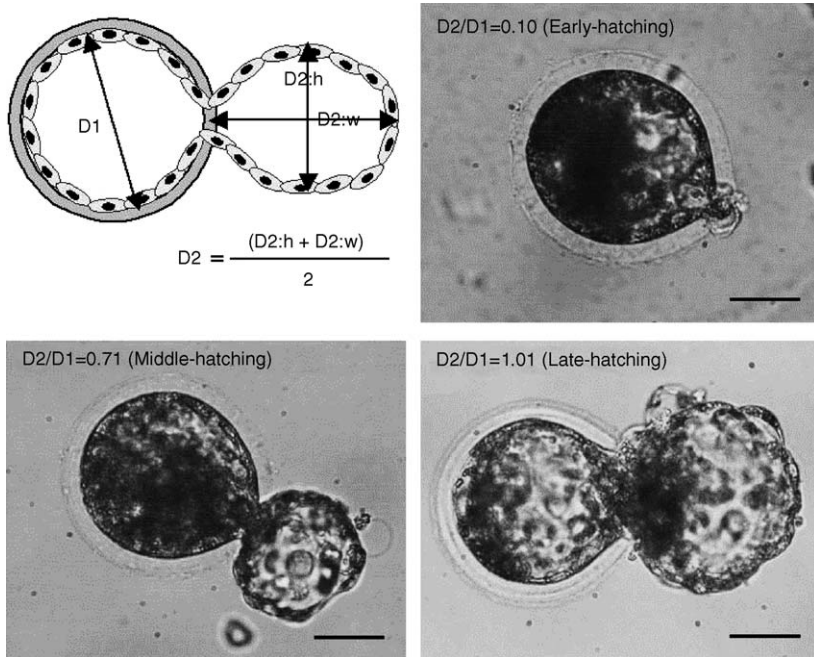


Fig. 1. Classification of hatching stage of bovine and swamp buffalo SCNT blastocysts. Hatching blastocysts were classified into one of three groups according to the ratio of extruding embryonic diameter from zona (D_2) to embryonic diameter inside the zona (D_1); Early-hatching stage: $D_2/D_1 = 0.01$ – 0.70 , Middle-hatching stage: $D_2/D_1 = 0.71$ – 1.00 , late-hatching stage: $D_2/D_1 = 1.01$ – 1.70 . Some representative photographs of hatching bovine SCNT blastocysts are shown. Scale bar = $50 \mu\text{m}$.

completely hatched) or those with extremely low morphological quality were not used in the present study.

2.3. Vitriification and cryosurvival assay

2.3.1. Vitriification by MVC procedure

Embryos were exposed to 10% (v/v) EG + 10% (v/v) DMSO in HEPES-buffered M199/FBS for 2 min at 22°C , and then transferred into a vitriification solution consisting of 20% (v/v) EG + 20% (v/v) DMSO + 0.5 M sucrose with and without 10% (w/v) Ficoll in HEPES-buffered M199/FBS. In the buffalo series, Ficoll was not added to the vitriification solution. One to three embryos were placed on a sheet of each Cryotop (Kitazato Supply Co., Tokyo, Japan) in a small volume of the vitriification solution ($<1 \mu\text{L}$). The Cryotop device was plunged into LN_2 after the embryos were exposed to the vitriification solution for 30 s at 22°C .

2.3.2. Warming and culture for survival assay

After storage in LN_2 , the embryos were thawed by immersing the Cryotop into 0.5 M sucrose in HEPES-buffered M199/FBS for 5 min at 22°C . Finally, the embryos were

placed into HEPES-buffered M199/FBS following a stepwise dilution with 0.4, 0.3, 0.2, and 0.1 M sucrose solutions at 5 min intervals. The post-warm embryos were washed three times with mSOFaa containing BSA or LAA + BSA (in which the tested embryos were produced), and co-cultured with BOEC in a humidified atmosphere of 5% CO₂ in air at 38.5 °C for 24 h (1–5 embryos per 100 µL droplet). The embryos developing to a more advanced hatching stage, with a clearly visible inner cell mass, were considered to be surviving.

2.3.3. Embryo transfer to recipients

Some bovine SCNT blastocysts were transferred to recipients after vitrification, warming, and in vitro culture for 24 h. One or two embryos were transferred into the uterine horn (ipsilateral to ovulation) of a recipient Holstein cow or heifer that had been synchronized naturally or by treatment with 500 µg of a PGF_{2α} analogue (Estrumate; Sherling-Plough, New South Wales, Australia). Fresh control day-7 SCNT blastocysts were transferred to synchronous recipients. Pregnancy was diagnosed on day 40 by ultrasonography and on days 60 and 120 by transrectal palpation.

2.4. Statistical analysis

Experiments were replicated at least three times in each treatment group. The post-warm survival rates of SCNT blastocysts were compared by chi-square test and pregnancy rates of recipients by Fisher's exact probability test, using the StatView program (Abacus Concepts, Berkeley, CA, USA). A value of $P < 0.05$ was chosen as an indication of statistical significance.

3. Results

3.1. Cryosurvival of bovine SCNT blastocysts (experiment 1)

The overall successful enucleation rate of bovine IVM oocytes was 86.4% (1614/1868) and the fusion rate of donor fibroblasts with the recipient cytoplasts was 85.4% (1196/1401). Efficiency in producing SCNT blastocysts on day 7 in the LAA-containing IVC medium (40.7%, 246/604) was higher ($P = 0.005$) than that in the LAA-free medium (32.3%, 144/446).

The in vitro survival rates of day-7 SCNT bovine blastocysts after the MVC-cryotop vitrification are summarized in Table 1 and the photograph of a blastocyst surviving 24 h after warming is shown in Fig. 2A. All vitrified bovine blastocysts were recovered. When the LAA-supplemented IVC medium and the Ficoll-free vitrification solution were used, cryosurvival of the early-hatching blastocysts (77%) was not different from those of middle- and late-hatching blastocysts (74 and 80%, respectively). Inclusion of Ficoll in the vitrification solution did not improve the cryosurvival of SCNT blastocysts (54 to 68%). The early-hatching SCNT blastocysts produced in the absence of LAA were sensitive to the vitrification procedure.

The in vivo developmental potential of vitrified-warmed and 1-day-cultured SCNT blastocysts as well as fresh day 7 SCNT blastocysts is shown in Table 2. Seven of 14

Table 1

Cryosurvival of bovine SCNT blastocysts harvested on day 7: effects of hatching stage, LAA in IVC medium, and Ficoll in vitrification solution (VS)

LAA during IVC	Ficoll in VS	No. survived/no. examined (%)			
		Hatching stage			
		Early	Middle	Late	Overall
+	–	23/30 (77)	20/27 (74)	24/30 (80)	67/87 (77) ^a
+	+	23/34 (68)	15/28 (54)	19/32 (59)	57/94 (61) ^b
–	–	15/27 (56) ^x	20/30(67) ^{x,y}	28/35 (80) ^y	63/92 (68) ^{a,b}

^a Within a column, ratios with a different superscript were different ($P < 0.05$).

^b Within a column, ratios with a different superscript were different ($P < 0.05$).

^x Within a row, ratios with a different superscript were different ($P < 0.05$).

^y Within a row, ratios with a different superscript were different ($P < 0.05$).

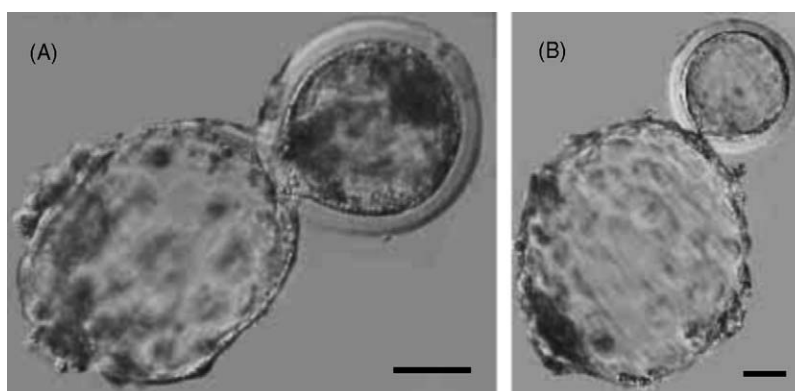


Fig. 2. Photographs of the SCNT blastocysts surviving 24 h after vitrification and warming: (A) bovine and (B) buffalo. During post-warm culture, buffalo embryos appeared to develop slightly faster than bovine embryos. Scale bar = 50 μ m.

recipients (50%) receiving a total of 25 post-warm surviving SCNT blastocysts were pregnant at day 40 while 11 of 27 recipients (41%) receiving a total of 37 fresh SCNT blastocysts were pregnant. Although four recipients that had received post-warm embryos

Table 2

In vivo survival of bovine SCNT blastocysts after vitrification and warming

Groups	No. embryos transferred/recipient females	No. (%) pregnant recipients ^a			
		Day 40	Day 60	Day 120	Full-term
Vitrified	25/14	7 (50)	4 (29)	0 (0) ^b	0 (0) ^b
Fresh control	37/27	11 (41)	7 (26)	5 (19) ^c	3 ^d (11) ^c

^a All pregnant recipients had a single conceptus.

^b Within a column, ratios with a different superscript were different ($P < 0.05$).

^c Within a column, ratios with a different superscript were different ($P < 0.05$).

^d Two calves died soon after birth, while one calf survived.

Table 3

Cryosurvival of swamp buffalo SCNT blastocysts harvested on day 6.5: effects of hatching stage and LAA in IVC medium

LAA during IVC	No. survived/no. examined (%)			
	Hatching stage			
	Early	Middle	Late	Overall
+	4/5 (80)	5/5 (100)	8/9 (89)	17/19 (89)
–	10/12 (83)	6/8 (75)	10/10 (100)	26/30 (87)

Ficoll was not added to the vitrification solution.

(29%) and seven that had received fresh embryos (26%) maintained their pregnancies until day 60, only three cloned calves (11%) were delivered from three recipients in the fresh embryo group. Two calves died soon after birth and one calf survived.

3.2. Cryosurvival of buffalo SCNT blastocysts (experiment 2)

The overall successful enucleation rate of buffalo IVM oocytes was 85.8% (1011/1179) and the fusion rate of donor fibroblasts with the recipient cytoplasts was 86.3% (676/783). Between the LAA-containing medium and the LAA-free medium, efficiency in producing SCNT blastocysts on day 6.5 (20.7%, 39/188 and 18.9%, 63/332, respectively) was similar.

All vitrified buffalo blastocysts were recovered. The mean cryosurvival of hatching SCNT blastocysts produced with LAA (89%) was not different from that of those produced without LAA (87%), as shown in Table 3. Although the number of blastocysts classified into each of three hatching stages was small, cryosurvival of buffalo SCNT blastocysts tended ($P = 0.06$) to be higher than that of bovine blastocysts without LAA. The photograph of a buffalo blastocyst surviving 24 h after warming is shown in Fig. 2B.

4. Discussion

In the present study, relatively high survival rates of bovine and buffalo SCNT blastocysts after vitrification and warming were obtained using the cryotop as a cryodevice. A variety of containers or devices, including an electron microscope grid [24], OPS [1], nylon loop [25] and cryotop [26], all of which can minimize the volume of vitrification solution for the ultra-rapid cooling rate, have been used for vitrification of mammalian embryos and oocytes. Comparative studies between OPS and cryotop in bovine and ovine morulae to blastocysts [27], pronuclear-stage rabbit zygotes [28] and germinal vesicle-stage whale oocytes [29] suggest that cryotop is a better alternative cryodevice than OPS. Recently, MVC-cryotop vitrification has been successfully applied to pre-hatching embryos in pigs [30,31], oocytes and blastocysts in humans [32,33], and IVM oocytes or enucleated cytoplasts in buffalo [34]. A possible advantage of the MVC-cryotop vitrification procedure originally reported by Kuwayama and Kato [26] may be due to the use of a lower concentration of permeating cryoprotective agents (CPA) in the vitrification solution (30%), but the composition of the vitrification solution employed in the present study was the same as that reported for the OPS system (CPA concentration 40%) [1].

Blastocysts produced by nuclear transplantation have mechanical slits in their zonae pellucidae, and therefore initiate hatching earlier than their non-manipulated counterparts. Bovine blastocysts microinjected with exogenous DNA at the pronuclear stage also initiated hatching earlier without thinning their zonae pellucidae, and survived cryopreservation by conventional freezing and vitrification as far as the blastocysts were harvested on day 7 [35]. However, day 6 blastocysts were sensitive to vitrification. Cryosurvival of IVF-derived bovine blastocysts biopsied on days 7 to 8 was relatively high (78% [36], 86% [37]). Among in vivo and IVF-derived bovine embryos, the expanded blastocyst seems to be the stage most tolerant to cryopreservation (see review by Massip [38]). Kelly et al. [27] reported that post-warm hatching rates of IVF-derived bovine and ovine embryos were improved by the progression of embryo development when days 5–7 embryos were vitrified using cryotop or OPS. Amarnath et al. [39] recently reported that bovine SCNT day 8 blastocysts at the advanced hatching stage survived conventional freezing better than the early stage blastocysts (86% versus 14%). In the present study, the cryosurvival of early hatching bovine SCNT blastocysts produced with LAA-free medium was significantly lower than that of advanced embryos (56% versus 80%, Table 1), probably due to their lower cell numbers.

There have been reports describing a positive effect of LAA in IVC medium for IVF-derived bovine zygotes on improvement in their survival rate after conventional freezing [11–13]. In the present study, the higher sensitivity of early-hatching bovine blastocysts to vitrification may be reduced by culturing SCNT embryos in the presence of LAA (Table 1). A suboptimal IVC condition for IVF-derived bovine embryos induces accumulation of intracellular lipid droplets [8,9]; these droplets are responsible for the high sensitivity of pig [40] and cattle [41] embryos to cryopreservation. The quality of blastocysts is often judged based on developmental kinetics and total cell numbers. Although these parameters were not examined in the present study, the morphological appearance (grade or cellular darkness; data not shown) seemed to be similar in blastocysts produced with LAA-containing medium and LAA-free medium. A possible contribution of LAA to improving cryotolerance may be modification of membrane lipid composition, facilitating water loss from the cell.

Composition of the vitrification solution (permeating CPAs and non-permeating macromolecules or saccharides) is among the factors influencing cryosurvival of embryos. A vitrification solution named EFS40 has been widely used for embryo cryopreservation in mice, rabbits, horses, cattle and marsupials, and in pigs and humans after replacing sucrose with trehalose (see review by Kasai [42]). Possible cryoprotective roles of macromolecules (e.g., polyethylene glycol, polyvinyl pyrrolidone, Percoll, Ficoll, and BSA) added to the vitrification solution are promoting solidification of the solution and reducing the chemical toxicity of the permeating agents. The Ficoll-70 was less toxic than polyethylene glycol when it was mixed with 40% EG [43]. In the present study, addition of 10% Ficoll-70 to a vitrification solution containing 20% EG, 20% DMSO and 0.5 M sucrose had no impact on cryosurvival of SCNT bovine blastocysts. When either two different permeating CPAs are used as a mixture, or when DMSO is included in the vitrification solution, further addition of Ficoll may have a negligible or even adverse effect on improving cryosurvival. To date, positive effects of Ficoll have been derived only in solutions containing 30 to 40% EG or 40 to 50% glycerol [42].

The full-term developmental potential of bovine SCNT blastocysts after vitrification and warming was not proven in the present study. Pregnancies of recipient cows or heifers

receiving vitrified-warmed SCNT blastocysts were maintained for no longer than 60 days. The reason for the pregnancy loss was unknown and requires further investigation. The statistical significance between fresh and vitrified embryos should not be over-emphasized due to the small number of embryos transferred. The first live calves derived from vitrified SCNT blastocysts have been reported in 2003–2004 from two independent laboratories [5,6]. Gong et al. [5] reported that transfer of nine vitrified SCNT blastocysts into nine recipients resulted in three pregnancies at day 60 and birth of a cloned calf, while transfer of eight fresh SCNT blastocysts into eight recipients resulted in two pregnancies at day 60 and two newborn calves. Tecirlioglu et al. [6] reported that transfer of 53 vitrified SCNT blastocysts into 14 recipients resulted in six pregnancies at day 40 and the birth of a cloned calf. In the latter study, none of the seven fresh SCNT blastocysts transferred into two recipients resulted in the birth of cloned calves (a larger scale experiment is on-going; personal communication, Dr. Tecirlioglu). The high frequency of embryonic loss during the first and the second trimesters of the gestation period, as well as perinatal deaths, is still an obstacle for somatic cell nuclear transfer in cattle.

Buffalo SCNT embryos appeared darker and developed a half-day earlier than bovine SCNT embryos [14]. However, buffalo SCNT blastocysts are more likely to survive vitrification than bovine SCNT blastocysts (Tables 1 and 3). The high content of intracellular lipid droplets in porcine embryos at early stages is still considered as the major cause of their high sensitivity to low temperature [40]. Nevertheless, Hayashi et al. [44] reported that expanded and hatched porcine blastocysts were capable of developing full-term after conventional freezing. Duran et al. [21] recently reported that approximately 90% of IVF-derived water buffalo embryos survived vitrification in EFS40 solution when the embryos were vitrified at the morula to expanded blastocyst stages, followed by birth of six calves after transfer of 71 post-warm embryos. In the present study (Table 3), the late-stage hatching blastocysts had a high cryotolerance (95%, 18/19), but their true survival in vivo remains to be clarified.

In conclusion, bovine SCNT blastocysts regardless of their hatching stage were relatively resistant to the MVC-cryotop vitrification procedure when the blastocysts were produced in the presence of LAA, and swamp buffalo SCNT blastocysts were more tolerant of vitrification than bovine SCNT blastocysts.

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