—Research Note—

Cryopreservation of Bovine Somatic Cell Nuclear-Transferred Blastocysts: Effect of Developmental Stage

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Abstract. The effect of developmental stage on the survival of bovine somatic cell nuclear-transferred blastocysts after freezing and thawing was evaluated. We also investigated how freezing affects nuclear-transferred (NT) embryos and *in vitro* fertilized (IVF) bovine embryos. Advanced-stage bovine NT blastocysts survived freezing better than early-stage NT blastocysts (86 vs 14%). The trend was similar with IVF embryos (87 vs 30%). At the stages tested, there was no significant difference in the survivability of NT and IVF embryos from advanced (86 vs 87%) or early-stage blastocysts (14 vs 30%). The average survival rate did not differ between NT and IVF bovine embryos (50 vs 51%). The higher survival rate of advanced-stage blastocysts compared to early-stage blastocysts in NT and IVF bovine embryos might be due to their higher cell number. In NT (128 ± 25 vs 53 ± 20) and IVF (128 ± 29 vs 75 ± 22) groups, advanced-stage blastocysts contained a significantly higher total cell number than early-stage blastocysts. There was no difference in total cell number between advanced-stage NT and IVF blastocysts (128 ± 25 vs 128 ± 29), however, early-stage NT and IVF blastocysts (53 ± 20 vs 75 ± 22) differed significantly.

Key words: Cryopreservation, Nuclear transfer, Bovine

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Since the birth of Dolly [1], a large number of cloned animals have been produced by somatic cell nuclear transfer using a variety of somatic cells in sheep, cattle, mice, goat and pigs [2]. Current research in this area is focused on improving the overall efficiency of this technique to the levels acceptable for the practical application of nuclear transfer in biomedicine, animal husbandry, and conservation practices [3].

Cryopreservation is the prerequisite for long term-storage, continuous availability, and transport of embryos over a long distance. It is also often used as a measure to check the quality of *in vitro* produced embryos. Unlike *in vitro* fertilization (IVF), nuclear transfer (NT) usually results in a

small number of blastocysts due to its labor-intensive procedure and lower level of development. Therefore, it would be advantageous to select good quality NT embryos and safely cryopreserve them for either embryo transfer or further studies. An efficient cryopreservation method for NT-derived embryos would thereby provide an attractive means to enhance the application of NT in biomedicine and animal industry.

There are many reports on cryopreservation of bovine IVF embryos, however, the knowledge gained might not be applicable to NT-derived embryos as they differ from IVF embryos in having a broken zona pellucida, aberrant allocation of cells between the inner cell mass (ICM) and trophectoderm (TE) [4], aberrant gene expression [5], and methylation patterns [4], and poor *in vivo*

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development [2].

There are only two published reports on cryopreservation of bovine NT embryos; one using embryonic cells [6] and the other using somatic cells [7]. Among the numerous factors that affect cryosurvival of IVF embryos, age, stage, and quality of the embryos, as well as their interactions, are very important [8–12]. To the best of our knowledge, there have been no attempts to systematically study the influence of developmental stage of cryopreserved NT bovine blastocysts on their post-thaw *in vitro* survival. Therefore, in this study we attempted to identify the developmental stage best suited for cryopreservation of NT bovine blastocysts by slow freezing using ethylene glycol as the sole cryoprotectant. We also evaluated whether there is any difference between equivalent-stage NT and IVF blastocysts in their ability to survive cryopreservation.

Materials and Methods

Collection of oocytes

Immature oocytes were isolated from ovaries collected and transported to the laboratory in normal saline at 10 to 15 C within 24 to 30 h from the time of slaughter. The cumulus-oocyte-complexes were matured in TCM 199 (Gibco Inc., Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco Inc.) and human menopausal gonadotropin (hMG; 0.1 IU/mL, Teikokuzoki, Japan) in a 5% CO₂ incubator at 39 C for 18 to 24 h. Cumulus cells were removed from oocytes by repeated pipetting after brief exposure to hyaluronidase (30 IU/mL). Only oocytes that had extruded the first polar body were used as cytoplasm for NT.

Embryo production by nuclear transfer

Nuclear transfer was performed as described previously [13] with a modification in the enucleation protocol [14]. Oocytes with a first polar body were treated with 0.5 µg/mL demecolcine (Wako, Japan) for 30 to 60 min to induce membrane protrusion. The protrusion containing maternal chromosomes and first polar body were pushed out through a cut made in the zona pellucida with a sharp needle. Cumulus cells were prepared as described previously and passaged 5 to 10 times,

and induced to G₀/G₁ by contact inhibition [15]. A single cumulus cell was introduced into the perivitelline space of the enucleated oocyte, and electrically fused and simultaneously activated by two direct current (150 V/mm) pulses for 25 µs with a 0.1 sec interval in Zimmerman's fusion medium. Fused oocytes were cultured in CR1-aa medium with 0.3% BSA (fatty acid free) containing cycloheximide (10 µg/mL) for 5 h followed by cycloheximide-free medium till day 3 (day 1=day of nuclear transfer). On the third day, embryos were moved into CR1-aa medium supplemented with 10% FBS and cultured till day 7 under 5% CO₂, 5% O₂, and 90% N₂ at 39 C. On the seventh day of culture, the early blastocysts and late morulae were moved into Dulbecco's modified Eagle's medium supplemented with 10% FBS and cultured till freezing on Day 8.

Embryo production by in vitro fertilization

In vitro fertilization was performed according to a previous report [7]. Oocytes (10–15) were matured for 22 to 24 h and then moved into 50 µL of Brackett-Oliphant medium [16] after being washed twice in the same medium. Frozen semen straws were thawed in a water bath at 37 C. Spermatozoa were washed twice by centrifugation at 600 ×g for 5 min in modified Brackett-Oliphant medium containing caffeine (5.5 M) and heparin (2.6 IU/mL), and the concentration was adjusted to 2 ×10⁶/mL. A 50-µL aliquot of semen suspension was added to each 50-µL droplet containing oocytes and co-cultured together for 6 h in a 5% CO₂ incubator at 39 C. After 6 h of co-incubation, cumulus cells were removed by repeated pipetting and cultured for 8 d (day 1=day of *in vitro* fertilization) as described above.

Classification of blastocysts

While IVF embryos can be clearly classified into different developmental stages, viz., early, mid or expanding, expanded, and hatched blastocysts [17], it is difficult to classify the NT blastocysts without some ambiguity due to escape of the blastocoel at an early-stage through the cut in the zona pellucida made during nuclear transfer. On the day of freezing, the NT blastocysts were classified into advanced and early-stages based on the development and extent of the escape of the blastocoel through the zona pellucida. Along similar lines, a small portion of the zona pellucida

of IVF embryos was also cut at the 8-cell stage, and the blastocysts developed were classified into advanced and early-stage blastocysts. For both NT and zona-pellucida-cut IVF, blastocysts containing well developed blastocoel exhibiting a distinct inner cell mass with single layer of trophoctoderm were considered as advanced-stage blastocysts. The blastocysts with less defined blastocoel (less than 50% of embryonic mass) without a clear distinction between inner cell mass and trophoctoderm were considered as early-stage blastocysts. In addition advanced- and early-stage blastocysts usually exhibited more than 60% or less than 40% escape of blastocoel through zona pellucida, respectively.

Cell numbers of blastocysts

Total cell number, inner cell mass and trophoctoderm cell numbers of advanced- and early-stage blastocysts obtained from NT and IVF on Day 8 of *in vitro* culture were counted using a chemically defined staining procedure [18]. Briefly, the embryos were incubated for 30 sec in TCM 199 containing 1% (v/v) Triton X-100 (Sigma Chemical Co., St. Louis, MO) and 100 $\mu\text{g}/\text{mL}$ propidium iodide (Sigma Chemical Co.), and then incubated in ethanol containing bisbenzimidazole (25 $\mu\text{g}/\text{mL}$; Sigma Chemical Co.) at 4 C overnight. Fixed and stained whole blastocysts were mounted in glycerol and assessed for cell number using an inverted microscope (Nikon, Tokyo, Japan) equipped with epifluorescence.

Cryopreservation

NT and IVF blastocysts on Day 8 were classified as advanced or early stage, and were frozen following the procedure described by Voelkel and Hu [19]. Embryos selected for freezing were placed into freezing medium (FM) at room temperature. The FM was Dulbecco's phosphate buffered saline (Gibco Inc. modified by adding 1 mM CaCl_2 , 1 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 5.5 mM glucose and 0.33 mM pyruvic acid) containing 0.3% BSA and 20% FBS. Embryos were then equilibrated for 5 min each in 0.5 and 1.0 M ethylene glycol (Wako, Osaka, Japan), followed by 15 min in 1.5 M ethylene glycol, during which 1 to 7 embryos depending on the availability each day, were loaded into 0.25 mL plastic straws. The straws were then transferred to an alcohol bath programmable freezer (ET-1, Fujihira, Tokyo, Japan) pre-cooled to -6.5°C , and held for 8 min.

Two minutes later, seeding was induced by touching the straws with a forceps pre-cooled in liquid nitrogen. Cooling was then continued to -35°C at $0.3^\circ\text{C}/\text{min}$, and then the straws were plunged into liquid nitrogen. The straws were held for a period of 1 d to 2 wk before thawing.

Thawing and culture of embryos

The straws were thawed by holding in air for 10 sec, and followed by warming in a water bath at 30°C for 20 sec. The straws were emptied directly into FM at 39°C and the embryos were located. The embryos were then transferred into fresh FM followed by CR1-aa medium supplemented with 0.3% BSA for 10 min each at 39°C in a 5% CO_2 incubator. The embryos were then washed twice in CR1-aa medium supplemented with 10% FBS and cultured in the same medium for 48 h under 5% CO_2 , 5% O_2 , and 90% N_2 at 39°C . Blastocysts with the original volume of the blastocoel 24 h after culture and with enlargement of the blastocoel 48 h after culture were considered to have survived [7].

Statistical analysis

Data on embryos surviving after freezing and thawing were analyzed using the Chi-square test and cell numbers were compared using the Student's t-test.

Results

Data in Table 1 present the cell numbers of Day 8 NT and IVF bovine blastocysts. In both NT and IVF groups, advanced-stage blastocysts contained significantly more ICM and TE cells than did early-stage embryos. While there was no significant difference in the cell number between NT and IVF blastocysts at the advanced-stage, there were significant differences in total and TE cell numbers of blastocysts at the early-stage.

The effect of developmental stage of blastocysts on the viability of embryos following freezing and thawing is shown in Table 2. In both NT (89 vs 18% at 24 h, 86 vs 14% at 48 h) and IVF (87 vs 33% at 24 h, 87 vs 30% at 48 h) groups, advanced-stage blastocysts survived freezing significantly better than early-stage blastocysts. There was no significant difference in survival rate in the advanced (89 vs 87% at 24 h, 86 vs 87% at 48 h) or early-stage embryos (18 vs 33% at 24 h, 14 vs 30% at

Table 1. Cell numbers of NT and IVF bovine blastocysts before freezing

Group	Blastocyst stage	No of embryos examined	Mean cell number ($\pm$ SD)		
			Total	ICM	TE
NT	Advanced	16	128 $\pm$ 25 ^a	65 $\pm$ 26 ^a	64 $\pm$ 20 ^a
	Early	16	53 $\pm$ 20 ^b	29 $\pm$ 13 ^b	25 $\pm$ 16 ^b
IVF	Advanced	13	128 $\pm$ 29 ^a	53 $\pm$ 21 ^a	74 $\pm$ 21 ^a
	Early	16	75 $\pm$ 22 ^c	30 $\pm$ 12 ^b	45 $\pm$ 18 ^c

Values with different superscripts in a column differ significantly ($P < 0.01$).

Table 2. Effect of developmental stage on the viability of NT and IVF bovine blastocysts after freezing and thawing

Group	Blastocyst stage (replicates)	No. of embryos		No. of embryos surviving after (%)	
		thawed	cultured	24 h	48 h
NT	Advanced (7)	28	28	25 (89) ^a	24 (86) ^a
	Early (6)	30	28	5 (18) ^b	4 (14) ^b
	Total	58	56	30 (53) ^c	28 (50) ^c
IVF	Advanced (3)	23	23	20 (87) ^a	20 (87) ^a
	Early (3)	33	30	10 (33) ^{bc}	9 (30) ^{bc}
	Total	56	53	28 (53) ^c	27 (51) ^c

Values with different superscripts in the same column differ significantly ($P < 0.001$).

48 h) between NT and IVF embryos. The total number of blastocysts surviving cryopreservation in the NT and IVF groups was not significantly different (Table 2).

Discussion

The present study clearly demonstrated that advanced-stage NT blastocysts survived freezing better than early-stage blastocysts (Table 2). This observation was consistent with those for in IVF blastocysts [8, 9, 11, 12, 20, 21]. To our knowledge, this is the first report comparing advanced- and early-stage NT blastocysts with respect to their survival after cryopreservation. In both the NT and IVF groups advanced-stage blastocysts survived freezing significantly better than early-stage embryos. While the reason(s) for better cryosurvival of advanced-stage embryos remains to be established, we speculate that in addition to the embryo quality, it might be related to the higher total cell number in these embryos. In the present study, early-stage IVF embryos survived freezing better (though not significantly) than early-stage NT blastocysts. While no significant differences

were observed in total, ICM and TE cell numbers between NT and IVF advanced-stage blastocysts, IVF early-stage blastocysts had significantly higher total and TE cell numbers than the NT early-stage blastocysts. Slow freezing markedly reduced the number of TE cells (38 vs 27%) compared to ICM cells in bovine IVF blastocysts [22]. Although we did not examine the cell numbers of freeze-thawed blastocysts, TE cell numbers of NT early-blastocysts might be too small to survive after freezing and thawing. Rapidly developing blastocysts have a higher cell number [23] and early developing blastocysts are more likely to survive freezing and establish pregnancies [17]. It is possible that in our study the advanced-stage embryos frozen on Day 8 might have developed from rapidly cleaving/developing blastocysts. In view of the high cryosurvival rate and high total cell numbers observed in this study, and the small proportion of ploidy errors in large expanded bovine NT blastocysts [24], the large size expanded/advanced-stage NT bovine blastocysts appear to be better quality blastocysts for selection of NT blastocysts in bovine.

Bovine embryos produced *in vitro* are characterized by their higher chilling sensitivity

and lower freezability compared to their *in vivo* counterparts [25]. In the present study, however, the post freeze-thaw developmental potential of NT and IVF embryos of similar stages was not significantly different. This is consistent with our previous reports on cryopreservation of NT embryos [6, 7]. These reports, along with the present study, suggest that the method of *in vitro* production (NT vs IVF) has no influence on freezability or chilling sensitivity of bovine blastocysts. Therefore, the *in vitro* culture conditions could be mainly responsible for the higher chilling sensitivity observed with embryos produced *in vitro*, as suggested earlier [26, 27]. This is supported by an earlier observation that culture of *in vitro*-produced bovine zygotes in ewe oviduct can dramatically increase their cryotolerance, to a level similar to that of embryos produced totally *in vivo* [26, 27].

The results of this study also support the hypothesis that an intact zona pellucida is not essential to for achieving good cryosurvival, as reported earlier [28]. However, further studies are required to examine the developmental potential of frozen-thawed nuclear-transferred blastocysts into calves.

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