NOTE *Theriogenology*

Nuclear Transfer in Cattle : Effect of Linoleic Acid-Albumin on Freezing Sensitivity of Enucleated Oocytes

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ABSTRACT. The effect of linoleic acid-albumin (LAA) supplementation to the media for IVM, enucleation, and activation on the developmental potential of bovine embryos produced by nuclear transfer (NT) into frozen-thawed cytoplasts was investigated. Blastomeres derived from morulae was placed in the perivitelline space of frozen-thawed cytoplasts, which were then fused by a DC pulse. The proportion of fused embryos was similar between groups with and without LAA (87 vs. 90%). The proportion of development to blastocysts of NT embryos derived from the media with LAA (14%) was higher than that without LAA (4%), indicating that LAA treatment of bovine oocytes during IVM, enucleation and activation can improve the ability of such cytoplasts after freezing and thawing to develop into blastocysts after NT.

KEY WORDS: cloning, cryopreservation, linoleic acid-albumin.

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The use of cryopreserved oocytes in nuclear transfer (NT) protocols would alleviate the logistical problems associated with matching the availability of donor cells. However, the developmental potential of frozen-thawed bovine oocytes following NT has been reported to be poor [2, 12, 16, 17]. We have reported that 6% of enucleated and activated bovine oocytes, followed by two-step freezing and NT, could develop into blastocysts, while none of the frozen-thawed IVM oocytes, followed by enucleation, activation, and NT, developed to that stage [11].

To improve the post-thaw viability of *in vitro*-produced bovine morulae [9] and blastocysts [10], an unsaturated fatty acid, linoleic acid in the form bound to BSA (linoleic acid-albumin: LAA, 0.1% w/v) was added to the medium for culturing IVF-derived bovine embryos. Furthermore, the positive action of LAA on the freezing sensitivity of 1-cell stage bovine zygotes was also investigated by adding it to IVM and IVF media [8]. The objective of the present study was to investigate the effect of LAA supplementation to the media for IVM, enucleation, and activation on the developmental potential of bovine NT embryos derived from frozen-thawed cytoplasts.

Bovine oocytes with compact cumulus cells were recovered by slicing of ovaries obtained from an abattoir and washing of their surfaces with Ham's F10 (Gibco BRL, NY, U.S.A.) supplemented with 2% heat-inactivated fetal bovine serum (FBS; Equitech Bio, TX, U.S.A.). They were then cultured for 22 hr in 25 mM Hepes-buffered TCM199 (Earle's salts; Gibco) supplemented with 5% FBS at 39°C in 5% CO_2 in air [7]. The IVM oocytes were then denuded from cumulus cells by Vortex mixing for 5 min, and only oocytes with a visible first polar body were transferred to a microdrop of Hepes-buffered Tyrode's (TALP-Hepes) medium [1] containing 10 μg/m*l* cytochalasin B (Sigma Chemical Co., MO, U.S.A.). Enucleation of the oocytes was accomplished by aspiration of the first polar body and a small volume of the adjoining cytoplasm with a micropipette under a microscope equipped with Nomarski interference-contrast optics at 200 × magnification. Parthenogenetic activation was induced by immersing the enucleated oocytes for 45 sec in Ca^{2+}- and Mg^{2+}-free TALP-Hepes medium containing 10 μM ionomycin (Calbiochem-Novabiochem Co., CA, U.S.A.), followed by 5 hr incubation in TALP-Hepes medium supplemented with 20% FBS and 10 μg/m*l* cycloheximide (Sigma) [11]. LAA (L8384; Sigma) at a concentration of 0.1% (w/v) was added to each medium throughout the process of cytoplast preparation, except for the ionomycin treatment.

Cytoplasts were frozen-thawed according to the procedures reported previously [6] with some minor modifications. Briefly, cytoplasts were loaded into 0.25-m*l* plastic straws (Fujihira Industries Co., Japan) containing 1.5 M ethylene glycol (Wako Pure Chemical Industries Co., Japan) and 0.1 M sucrose (Wako) in PB1 medium. After 10 min, the straws were placed into a –7°C alcohol bath of a programmable freezer (MPF-1000; EYALA, Japan) and seeded at this temperature using a thin spatula cooled in liquid nitrogen. Five minutes later, the straws were cooled to –35°C at the rate of 0.3°C/min and plunged into liquid nitrogen. After storage for at least 1 week in liquid nitrogen, the straws were warmed for 5 sec in 25°C air, followed by 15 sec in a 35°C water bath. The contents of the straws were expelled into 2.5 m*l* PB1 medium and kept for 15 min. The recovered cytoplasts were then washed 3 times with fresh PB1 medium, and the number of surviving oocytes which appeared morphologically normal was recorded.

Donor embryos for NT were produced *in vitro* as described previously [15]. Early morulae were harvested on

Day 5 (the day of IVF = Day 0). The zonae pellucidae of the morulae were removed with a metal blade. The single blastomeres were separated in 0.25% trypsin / 1 mM EDTA solution (Gibco). A blastomere was injected into the perivitelline space of a post-thaw surviving cytoplast, and the donor-cytoplast units were treated with a DC pulse of 150 V/mm for 20 μsec in Zimmermann cell fusion medium [19]. The NT embryos were washed three times with m-SOF medium and cultured in 50-μl microdrops of the m-SOF medium (20 to 30 embryos per microdrop) at 39°C in 5% CO_2, 5% O_2, and 90% N_2. The numbers of fused units were recorded 1 hr later. Twenty-four hours after NT, cleavage of the embryos to 2- to 8-cells was recorded. The development to blastocysts with a visible inner cell mass was observed once a day during the period from the fifth to eighth day after NT. On the seventh day after NT, the blastocysts were harvested, and the total cell numbers were determined after 10 min treatment in 0.9% tri-sodium citrate, fixation in a mixture of methanol: acetic acid: distilled water (3: 2: 1, v/v), and staining with 10% Giemsa solution.

The experiments were repeated three or four times. Percentage data in each replicate underwent arcsin transformation before being subjected to one-way analysis of variance (ANOVA). The significant differences were determined by the Bonferroni/Dunn test using STATVIEW (Abacus Concepts, CA, U.S.A.). Mean day until initial blastocyst appearance and mean cell number of the blastocysts were analyzed by ANOVA and the Tukey-Kramer test. A value of $P<0.05$ was chosen as an indication of statistical significance.

The presence or absence of LAA in IVM medium did not affect the proportion of bovine oocytes surviving enucleation (96%, 289/302 vs. 94%, 305/323; P=0.94). The mean morphological survival of cytoplasts derived from the media with LAA after freezing and thawing (80%, 190/238) was higher than that without LAA (65%, 159/243), but the difference was not significant (P=0.24). When single blastomeres of *in vitro*-produced morulae were injected into the perivitelline space of post-thaw surviving cytoplasts, mechanical damage tended to occur less frequently in the group with LAA than in the group without LAA (96%, 159/165 vs. 80%, 116/145; P=0.13).

The proportion of fused oocytes was similar between frozen-thawed cytoplasts derived from the media with and without LAA (Table 1). The cleavage rate of fused oocytes was also similar between the groups with and without LAA. However, the proportion of development into blastocysts of NT embryos derived from the media with LAA (14%) was significantly higher than that without LAA (4%). In the non-frozen control group, 82% of NT embryos fused, and 87% of them cleaved and 39% developed into blastocysts. The quality of NT blastocysts was not different among the three groups including the non-frozen control group, as assessed by the total cell numbers and the initial day of the blastocyst appearance. The morphology of the NT blastocysts produced using frozen-thawed cytoplasts appeared to be normal (Fig. 1).

Membrane fluidity of living cells is determined by lipid composition-related factors such as the carbon-chain length and the unsaturation level of membrane phospholipids or the ratio of membrane phospholipids to cholesterol, and a higher membrane fluidity facilitates water loss from cells during slow cooling [4]. The positive action of LAA on cryopreservation of enucleated bovine oocytes obtained in the present study may be explained by increased membrane fluidity due to direct incorporation of linoleic acid into the lipid bilayer. In addition, the LAA may play a role in the depletion of membrane cholesterol, because polyunsaturated fatty acids have been reported to decrease the rate of synthesis and esterification of cholesterol in adenocarcinoma-derived cultured cells [14]. The albumin from bovine serum is a factor influencing cholesterol efflux from sperm cells as a water-soluble lipid carrier [3]. High density lipoprotein (HDL) has been shown to extract cholesterol from the plasma membrane of sperm cells [5, 18], and HDL derived from oviduct and follicular fluid appeared to extract membrane cholesterol more efficiently than albumin [5, 13]. Therefore, it is possible that the affinity of linoleic acid in LAA for the lipid bilayer of the plasma membrane facilitates access of the bound albumin to membrane cholesterol.

Regarding the qualitative aspects of NT-derived bovine blastocysts, developmental kinetics and cell numbers of the blastocysts, as examined in the present study, have been used as practical alternatives to the confirmation of pregnancy or the birth of calves. However, the birth of calves is undoubtedly the most rigorous criterion for determining the true viability of the NT embryos. In addition, further research is

Table 1. Effect of linoleic acid-albumin on the developmental potential of bovine embryos produced by nuclear transfer into frozen-thawed cytoplasts

LAA	No. of Donor-cytoplast units	% of Fused	% of Cleaved[a)]	% of Developed to blastocysts[a)]	Quality of blastocysts[b)] Cell numbers	Quality of blastocysts[b)] Days of initial appearance[c)]
+	159	87	81[d)e)]	14[d)]	96.6 ± 26.2[g)]	6.4 ± 0.8
−	116	90	66[d)]	4[e)]	112.3 ± 78.0	6.5 ± 0.6
Fresh controls	115	82	87[e)]	39[f)]	112.2 ± 39.1	6.4 ± 0.5

a) Calculated from fused units.
b) Mean ± SD.
c) Days after NT.
d)-f) Significantly different within columns.
g) Data from 2 blastocysts appearing at eighth day after NT are not included.

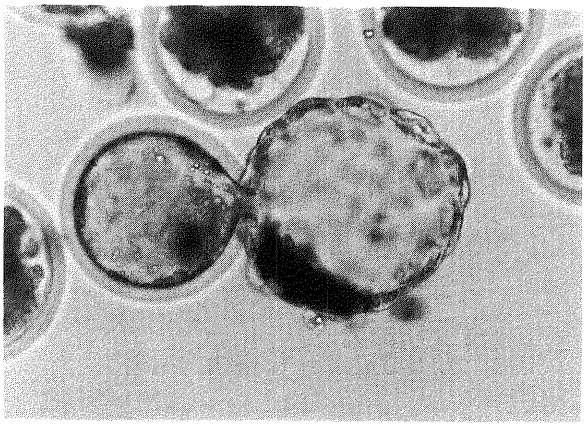

Fig. 1. A hatching bovine blastocyst developed from a frozen-thawed and nuclear-transferred cytoplast.

required for the development of a more reliable method for oocyte cryopreservation, such as ultra-rapid freezing or vitrification.

In conclusion, linoleic acid-albumin treatment of bovine oocytes during *in vitro*-maturation, enucleation and activation can improve the ability of such cytoplasts after freezing and thawing to develop into blastocysts after nuclear transfer.

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