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High Developmental Rates of Vitrified Bovine Oocytes Following Parthenogenetic Activation, In Vitro Fertilization, and Somatic Cell Nuclear Transfer[1]

András Dinnyés,[3] Yunping Dai, Shie Jiang, and Xiangzhong Yang[2]

Department of Animal Science, University of Connecticut, U-4040, Storrs, Connecticut 06269

ABSTRACT

Successful cryopreservation of mammalian oocytes would provide a steady source of materials for nuclear transfer and in vitro embryo production. Our goal was to develop an effective vitrification protocol to cryopreserve bovine oocytes for research and practice of parthenogenetic activation, in vitro fertilization, and nuclear transfer. Bovine oocytes matured in vitro were placed in 4% ethylene glycol (EG) in TCM 199 plus 20% fetal bovine serum (FBS) at 39°C for 12–15 min, and then transferred to a vitrification solution (35% EG, 5% polyvinyl-pyrrolidone, 0.4 M trehalose in TCM 199 and 20% FBS). Oocytes were vitrified in microdrops on a precooled (−150°C) metal surface (solid-surface vitrification). The vitrified microdrops were stored in liquid nitrogen and were either immediately thawed or were thawed after storage for 2–3 wk. Surviving oocytes were subjected to 1) parthenogenetic activation, 2) in vitro fertilization, or 3) nuclear transfer with cultured adult fibroblast cells. Treated oocytes were cultured in KSOM containing BSA or FBS for 9 to 10 days. Embryo development rates were recorded daily and morphologically high-quality blastocysts were cryopreserved for nuclear transfer-derived embryos at Day 7 or Day 8 of culture. Immediate survival of vitrified/thawed oocytes varied between 77% and 86%. Cleavage and blastocyst development rates of vitrified oocytes following in vitro fertilization or activation were lower than those of the controls. For nuclear transfer, however, vitrified oocytes supported embryonic development as equally well as fresh oocytes.

fertilization, IVF/ART, oocyte development

INTRODUCTION

Interest in oocyte cryopreservation has recently increased with the growing importance of in vitro embryo production, nuclear transfer, and gene banking. Cattle oocytes are sensitive to low temperatures, and despite the efforts of numerous research groups (for review [1]), cryopreservation of oocytes remains a difficult task. Only a limited number of publications reported blastocyst and subsequent calf development from cryopreserved oocytes and the results remain inefficient [2–8]. Currently, as a consequence of the limited cryosurvival of oocytes, all in vitro embryo production and nuclear transfer experiments in cattle must rely on freshly collected oocytes. This is a major limitation to numerous research teams and presents an obstacle to efficient planning and organizing of experiments.

According to Martino et al. [9], efforts to improve survival rates have been focused on comparing different cryoprotectants [10, 11] and different freezing [12] or vitrification methods [10, 13]. It is believed that one of the obstacles in vitrification technology is the insufficient cooling rate of oocytes in the current vitrification scheme [14]. In order to overcome this problem, several methods have been proposed that use very small amounts of solution. Minimum drop vitrification on a special cryostage, which was quickly cooled, allowed breakthrough results with bovine and porcine oocyte cryopreservation [15]. This method, however, is not convenient for preserving large numbers of oocytes. Methods that used a few microliters of vitrification solution loaded into glass capillaries [11], or into open-pulled plastic straws [8] and then quickly plunged into liquid nitrogen (LN_2) were successfully tested for bovine oocyte vitrification. Dropping oocyte-containing vitrification solution directly into LN_2 was proven successful [16–18] by eliminating the insulation effect of the container wall. Similarly, vitrification success was achieved by plunging oocyte-containing vitrification solutions with a small loop [19]; however, plunging a warm object into LN_2 results in boiling the liquid and, for a short time, creates an isolating layer of N_2 vapor around the object. Metal surfaces cooled with the aid of LN_2 provide a more efficient method of heat transfer and further increases the cooling rates. *Drosophila* embryos were successfully preserved by placing them in a metal grid on a cold metal surface [20].

In this paper, we report the development of promising success rates with a new vitrification method that uses a precooled metal surface (solid-surface vitrification; SSV). This method combines the advantages of containerless vitrification in microdrops and the increased heat exchange of a cold metal surface. Furthermore, a clean metal surface facilitates sterile handling of droplets. The findings of this study may help researchers compensate for the obstacles they face through fluctuations in oocyte availability and seasonal variations in quality. Furthermore, preservation of endangered cattle breeds could be facilitated by the successful cryostorage of oocytes.

MATERIALS AND METHODS

Source of Oocytes

Ovaries were collected from slaughterhouses and shipped to the laboratory in maturation medium at 37 to 39°C within 19 h of the start of maturation. Matured cumulus oocyte complexes were selected on the basis of their morphology. They were then cryopreserved for 20–23 h in postmaturation medium for nuclear transfer, parthenogenetic activation, and in vitro fertilization assays.

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[2]Correspondence: Xiangzhong (Jerry) Yang, Department of Animal Science, University of Connecticut, 3636 Horsebarn Rd. Ext. U-4040, Storrs, CT 06269-4040. FAX: 860 486 4375; e-mail: xiangzhong.yang@uconn.edu
[3]Current address: Roslin Institute, Roslin, Midlothian EH25 9PS, United Kingdom.

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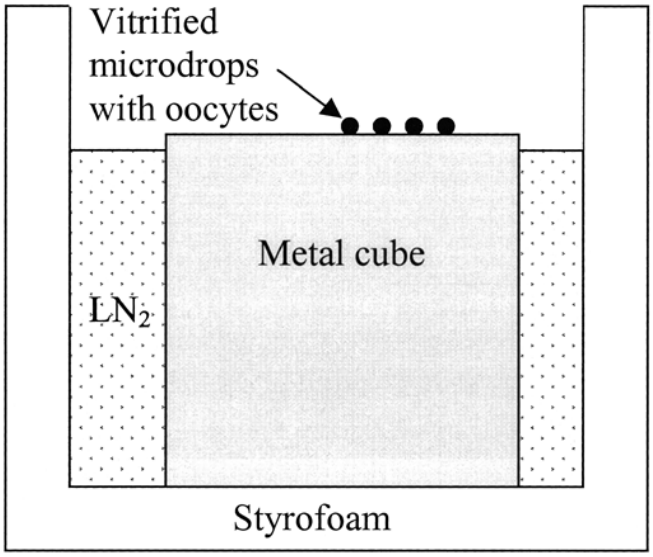

FIG. 1. The solid surface vitrification (SSV) device. A metal cube covered with aluminum foil is partially submerged into liquid nitrogen. Microdrops of vitrification solution, containing the oocytes, are dropped onto the cold upper surface of the metal cube and are instantaneously vitrified.

Vitrification and Thawing

Matured oocytes were partially (except for the 3–5 inner layers) or fully stripped of their cumulus cells by a short exposure to 0.1% hyaluronidase (Sigma Chemical Company, St. Louis, MO) followed by pipetting. Oocytes were washed three times in TCM 199 with Earles salt (Gibco BRL, Paisley, Scotland, UK) supplemented with 20% (v:v) fetal bovine serum (FBS; Hyclone, Gibco BRL) and then suspended in an equilibration medium consisting of 4% (v:v) ethylene glycol (EG; Sigma) in TCM 199 supplemented with 20% FBS at 39°C for 12–15 min. Following equilibration, groups of 5 to 10 oocytes were rinsed three times in small drops of vitrification solution consisting of 35% EG, 5% polyvinyl-pyrrolidone (PVP; Sigma), 0.4 M trehalose (Sigma) in TCM 199, and 20% FBS for 25–30 sec. They were then either placed into a trehalose solution (solution toxicity control) or dropped on the surface of a steel cube that was covered with aluminum foil and cooled to around −150 to −180°C by partial immersion into LN_2 (Fig. 1). The drops varied in size of between 1 and 2 μl, and were instantaneously vitrified. The vitrified droplets were moved with a nitrogen-cooled forceps into 1-ml cryovials either for long-term storage (2–3 wk) or they were immediately thawed by being dropped into a 39°C 0.3 M trehalose solution for 3 min. Oocyte survival was then evaluated on the basis of the integrity of the oocyte membrane and the zona pellucida. The surviving vitrified-thawed oocytes were subjected to activation, in vitro fertilization, or nuclear transfer experiments.

Parthenogenetic Activation of Oocytes

The methods described by Susko-Parrish et al. [21] were modified and employed for parthenogenetic activation. After a total of 22 h post in vitro maturation culture (IVM) (30 min additional culture for oocytes in metaphase II after thawing), oocytes were fully denuded of cumulus cells by pipetting. Oocytes were activated by a 5-min exposure to Ca-ionophore A23187 (Sigma) at room temperature followed by culture in 2.5 mM 6-dimethylaminopurin (DMAP; Sigma) for 3.5 h [22]. After washing, the treated oocytes were cultured in KSOM [22] plus 0.1% BSA (Fraction V, Sigma) for an additional 48 h. The culture environment consisted of 5% CO_2 in humidified air or 5% O_2, 5% CO_2, and 90% N_2 at 39°C. Morphological survival and cleavage rates were recorded. All cleaved embryos were further cultured in KSOM medium supplemented with 1% BSA for 7 days.

In Vitro Fertilization and Culture

Vitrified-thawed oocytes and nonvitrified fresh control oocytes were fertilized with frozen-thawed bull semen from a single ejaculate. Frozen-thawed sperm were washed twice by centrifugation in modified Brackett-Oliphant (m-BO; [23]) medium supplemented with 10 mM caffeine and 4 mg/ml BSA and then resuspended in the fertilization medium (m-BO medium supplemented with 10 μg/ml heparin and 4 mg/ml BSA). Sperm concentration was adjusted to 1×10^7 sperm/ml, and then about 25 oocytes were added to 100 μl fertilization-medium droplets. After 6 h of sperm-oocyte incubation, the oocytes were washed and cultured in KSOM medium supplemented with 0.1% BSA for 48 h at 39°C in a humidified atmosphere of 5% O_2, 5% CO_2, and 90% N_2. After 48 h in culture, the in vitro fertilized eggs were freed of cumulus cells by pipetting and cleavage rates were recorded. The cleaved embryos were further cultured in KSOM supplemented with 1% BSA in an atmosphere of 5% O_2, 5% CO_2, and 90% N_2 for another 7 days. The medium was changed every 2 days throughout the culture period.

Nuclear Transfer

After IVM for 20 h, vitrified-thawed oocytes in metaphase II as well as fresh control oocytes were denuded from cumulus cells and selected by the presence of the first polar body. Enucleation was achieved by piercing the zona pellucida with a glass needle and pushing out the polar body and the surrounding cytoplasm [7]. Successful enucleation was confirmed by Hoechst 33342 (Sigma) fluorescent staining of the pushed-out karyoplasts. Confluent fibroblast cells at passage 29–31, serum starved for 4 to 6 days, were then individually transferred into the perivitelline space of the enucleated oocyte (cytoplast). Cytoplast-fibroblast complexes derived from the vitrified-thawed oocytes vs. fresh control oocytes were then fused and activated with two direct current pulses (50 V/mm, 10 μsec, 1 sec apart) in Zimmerman cell fusion medium [24] in a 1-mm gap electrofusion chamber by a BTX200 Electro Cell Manipulator (BTX, San Diego, CA). They were then cultured in KSOM medium supplemented with 10 μg/ml cycloheximide (Sigma), 2.5 μg/ml cytochalasin-D (Sigma), and 0.1% BSA for 1 h, and for an additional 4 h in KSOM medium with cycloheximide (10 μg/ml) and BSA without cytochalasin-D. After activation, embryos were further cultured in KSOM and 0.1% BSA medium for 48 h in an atmosphere of 5% O_2, 5% CO_2, and 90% N_2. The cleaved embryos were then selected and cultured for an additional 7 days in KSOM supplemented with 5% FBS on cumulus cell monolayers in an atmosphere of 5% CO_2 in air. The medium was changed every 2 days throughout the culture period. Noncleaved eggs were stained to check the rate of nonfused cytoplast-fibroblast complexes. Morphologically high-quality nuclear transfer blastocysts were vitrified with the VS3a method [25, 26] and stored for future embryo transfer purposes. As a control for the oocyte cryopreservation process, some oo-

TABLE 1. Parthenogenetic development of vitrified-thawed matured bovine oocytes cultured under 5% CO_2 in air.

Oocytes treated	n	% Oocytes survived	% Cleaved Day 2*	% Blastocyst*			% Expanded or hatched blastocysts*
				Day 7	Day 8	Day 9	
Vitrified with cumulus cells	42	86 B	64 BC	0 B	8 B	11 B	8 B
Vitrified without cumulus cells	68	79 B	52 B	6 B	6 B	6 B	3 B
Nonvitrified	47	100 C	81 C	13 B	17 B	17 B	15 B

* Based on surviving oocytes. Groups with different letters within the same column are significantly different ($P < 0.05$).

cytes from the vitrified-thawed groups used for nuclear transfer experiments were activated as described earlier (Ca-ionophore and DMAP), and cultured the same way as the nuclear transfer embryos.

Specific Experiments

Experiment 1: Parthenogenetic development of vitrified oocytes. Matured oocytes were vitrified-thawed and parthenogenetically activated as just described. The toxicity of the vitrification solution without cooling was tested, and the effects of the presence vs. removal of the cumulus cells before cryopreservation were compared. Furthermore, culture of activated oocytes was tested in two separate trials, one in an atmosphere with a high (20%) content of O_2, the other with a low content (5%).

Experiment 2: In vitro development of fertilized vitrified-thawed oocytes. Vitrified-thawed oocytes were fertilized in vitro as described earlier. On the basis of the results from experiment 1, cumulus cells were only partially removed before vitrification. The control oocytes were treated with hyaluronidase, and their surrounding cumulus layers were similarly partially removed before fertilization. Vitrified oocytes were either immediately thawed or stored in liquid nitrogen containers for several days or weeks. Fertilized zygotes were further cultured in an atmosphere with a low O_2 content.

Experiment 3: Development of cloned embryos with vitrified-thawed oocytes. Vitrified-thawed oocytes were enucleated and used as recipient cytoplasts for somatic cell nuclear transfer. The enucleation, fusion, cleavage, and blastocyst development rates of vitrified vs. fresh cytoplasts were compared. As a control group for the vitrification and culture system, some of the vitrified-thawed oocytes were parthenogenetically activated and further cultured. The zygotes were cultured in a low O_2 atmosphere for the first 2 days, then cocultured for an additional 7 days with cumulus cells in a high O_2 content atmosphere.

Statistics

All experiments were repeated at least three times. Development of oocytes was evaluated and treatments were compared using chi-square analyses.

RESULTS

Parthenogenetic Development of Vitrified Oocytes (Experiment 1)

Results of the parthenogenetic activation experiments are presented in Tables 1 and 2. Table 1 presents the results of parthenogenetic development of matured oocytes vitrified with cumulus cells vs. those that were vitrified without cumulus cells and cultured in an atmosphere of 5% CO_2 in air. Table 2 summarizes the developmental data following similar treatments but with embryos cultured in an atmosphere of reduced (5%) O_2 content. Immediate survival of the oocytes (i.e., no lyses after thawing through the start of the culture period) varied between 77% and 86%, but were not different between oocytes with or without cumulus cells before vitrification ($P > 0.05$). When compared retrospectively, cultures in low levels of O_2 resulted in superior ($P < 0.05$) blastocyst development compared with the cultures in high (20%) O_2 levels. In the high O_2 experiment, cleavage rates of activated nonfrozen controls were significantly higher than those of the vitrified-thawed groups ($P < 0.05$), but blastocyst development was not different ($P > 0.05$). In the reduced O_2 experiment, cleavage and blastocyst development of vitrified oocytes was lower than those of controls ($P < 0.05$); however, the solution treatment alone caused no reduction in those parameters ($P > 0.05$). The development of oocytes vitrified with or without cumulus cells was not significantly different ($P > 0.05$) regardless of the culture environment. The blastocyst formation from the vitrified and solution-treatment groups appeared approximately 1 day later than it did in the fresh controls. The rate of expanded and hatched blastocysts that developed from vitrified oocytes was significantly lower than it was for controls ($P < 0.05$).

In Vitro Development of Fertilized Vitrified/Thawed Oocytes (Experiment 2)

The development rate of in vitro fertilized fresh vs. vitrified-thawed oocytes is presented in Table 3. Immediate survival of the oocytes (85% vs. 93% for controls) was slightly reduced by vitrification. Cleavage rates of the surviving oocytes did not significantly differ (58% vs. 69%, $P > 0.05$); however, blastocyst development of vitrified oo-

TABLE 2. Parthenogenetic development of vitrified-thawed matured bovine oocytes cultured under an atmosphere of 5% O_2, 5% CO_2, and 90% N_2.

Oocytes treated	n	% Oocytes survived	% Cleaved on Day 2*	% Blastocyst*			% Expanded or hatched blastocysts*
				Day 7	Day 8	Day 9	
Vitrified with cumulus cells	127	77 B	56 B	11 BC	14 BC	17 BC	14 BC
Vitrified without cumulus cells	118	81 B	56 B	3 B	11 B	16 B	11 B
Solution alone	85	94 C	69 BC	16 CD	23 CD	30 CD	25 C
Nonvitrified	98	98 C	79 C	27 D	32 D	32 D	26 C

* Based on surviving oocytes. Groups with different letters within the same column are significantly different ($P < 0.05$).

TABLE 3. In vitro development of vitrified-thawed matured bovine oocytes following in vitro fertilization.

Oocyte treatment	n	% Oocytes survived	% Cleaved on Day 2*	% Blastocyst*			% Expanded or hatched blastocysts*
				Day 7	Day 8	Day 9	
Vitrified-thawed	175	85 BC	58 B	15 C	19 C	20 C	12 BC
Vitrified-stored-thawed	258	81 B	62 B	6 B	11 B	11 B	6 B
Nonvitrified	98	93 CD	69 B	24 CD	33 D	35 D	19 C

* Based on surviving oocytes. Groups with different letters within the same column are significantly different ($P < 0.05$).

cytes was significantly lower than it was in controls (20% vs. 35%, $P < 0.05$). Furthermore, prolonged storage of oocytes resulted in a further, significant reduction in blastocyst development (11% vs. 20%, $P < 0.05$). Development to expanded and hatched stages was not significantly reduced by vitrification (12% vs. 19%, $P > 0.05$). Cryostorage of oocytes, however, negatively affected the hatching rate (6% vs. 19%, $P < 0.05$).

Development of Cloned Embryos from Vitrified-Thawed Oocytes (Experiment 3)

The data on nuclear transfer experiments using vitrified vs. fresh oocytes as a source of cytoplasts are presented in Table 4. Most of the oocytes (252 of 269, 94%) survived the vitrification process. Enucleation rates of fresh vs. vitrified oocytes did not differ (142 of 163, 87% vs. 127 of 139, 91%, respectively; $P > 0.05$). Fusion rates with cytoplasts obtained from fresh or vitrified-thawed oocytes (74% vs. 62%, $P > 0.05$) did not differ. Subsequent development of embryos to cleavage (90% vs. 85%), blastocyst (29% vs. 27%), or hatched blastocyst stage (22% vs. 20%) did not statistically differ ($P > 0.05$). Cleavage rates of parthenogenetically activated vitrified-thawed oocytes (56%) was significantly lower than those of nuclear transfer embryos ($P < 0.01$); however, development rates to the blastocyst stage (32%) were not different. The rate that oocytes reached expanded or hatched blastocyst stages following parthenogenetic activation was lower (10%) than it was after nuclear transfer.

DISCUSSION

The major finding of this study is that vitrified-thawed oocytes can be used successfully as recipients for somatic cell nuclear transfer with high blastocyst development. This finding has important implications for nuclear transfer research and practice.

Previously, Lim et al. [12] obtained blastocyst-stage embryos following in vitro fertilization (IVF) of frozen-thawed matured bovine oocytes. Since then, several teams have investigated the possibility of using cryopreservation to preserve bovine oocytes. Oocyte survival and subsequent blastocyst development, however, have remained low, ranging from 0% to 10% [2, 4, 5, 10, 11, 13, 27, 28]. Few limited studies [2–4, 6–8] have resulted in pregnancies or births following transfer of embryos originating from frozen-thawed bovine oocytes. Encouragingly, recent developments in vitrification methods have substantially increased the success rates in blastocysts derived from IVF of vitrified matured bovine oocytes; reportedly up to 25% [8] and 30% [17]. These improved success rates were believed to be attributed to the increased cooling rate during oocyte vitrification [14].

In our study, following cryopreservation, the rate of morphologically intact oocytes was as high as 86%. The relatively high rates of cryosurvival and embryo development following vitrification of bovine oocytes can be attributed to several factors. The SSV method achieved a high cooling rate by using the combination of microdrops and improved heat exchange by direct contact with a metal surface. The warming of the oocytes was equally fast by directly dropping the vitrified samples into a warm solution. The small sample size reduced the occurrence of cracking, as has been reported by other methods (i.e., [14]). Cracking of the vitrified sample, because of temperature differences between the outer layers and the core, can result in zona pellucida ruptures during cooling or thawing. Furthermore, shortly after thawing, lysis of the plasma membranes can occur.

A toxicity test of the vitrification solution (experiment 1) showed no reduction in cleavage and blastocyst development compared with that of controls. Our earlier results showed that EG has a relatively low toxicity for bovine oocytes [11], and the gradual equilibration in a low concentration of cryoprotectant solution before vitrification was reported as beneficial by others [17]. The use of a mixture of a relatively low molecular weight and high penetration rate cryoprotectant (i.e., EG), a viscosity-increasing compound (PVP), and a membrane-protective sugar (trehalose) assured that even a short exposure to the solution would result in vitrification of the oocytes. A similar mixture was successfully used for bovine embryo vitrification [29]. The very short exposure to a high concentration of EG before cooling and after thawing reduced its toxic effects; moreover, toxic effects during thawing were minimized by the direct dilution method.

Our high success rates reported here may also be attributable to some procedural modifications on the basis of recent developments reported by others [8, 9, 11, 13, 17, 29]. Specifically, we attempted to handle oocytes in solu-

TABLE 4. In vitro development of bovine nuclear transfer embryos from fresh or vitrified-thawed recipient oocytes.

Recipient oocyte/treatment	n	% Fused	% Cleaved on Day 2*	% Blastocyst*			% Expanded or hatched blastocysts*
				Day 7	Day 8	Day 9	
Vitrified/NT	106	62 B	85 B	17 B	21 B	27 B	20 BC
Fresh/NT	106	74 B	90 B	22 B	28 B	29 B	22 B
Vitrified/activated	109	—	56 C	25 B	30 B	32 B	10 C

* Based on fused embryos or activated oocytes. Groups with different letters within the same column are significantly different ($P < 0.05$). NT, Nuclear transfer.

tions at or close to physiological temperatures, followed by quick cooling and warming to and from the vitrification temperature in order to ''outrun'' the chilling damages. Bovine oocytes are known to be very sensitive to chilling [30, 31], and immature oocytes (i.e., in the GV stage) were reported to be more sensitive than mature oocytes [2, 27]. Parks and Ruffing [32] and Aman and Parks [30] reported damage of the metaphase II spindles following cooling to temperatures between 25° and 4°C for only 1 min. Vitrification techniques allow the time to be reduced while the oocytes are exposed to critical chilling temperatures, in comparison to the freezing methods, and thus, may be superior to the conventional freezing method for oocytes and embryos produced in vitro [1, 9, 14].

Our results also showed that the presence of a few cumulus cell layers surrounding the oocytes before vitrification had no harmful effect on the cleavage and developmental rates following parthenogenetic activation. The presence of the cumulus cells can be an important factor in achieving high fertilization rates following IVF, although nuclear transfer experiments require the removal of the cumulus cells before enucleation. The vitrified-thawed oocytes seemed to be more fragile, and care must be taken to remove the cumulus cells before nuclear transfer manipulation.

In the study, we found that development following parthenogenetic activation of vitrified oocytes was reduced compared with controls. This finding suggests that, despite our efforts to improve the vitrification method, the cooling/thawing procedure negatively affected oocyte developmental competence. The exact mechanism of the damage is not known, but spindle depolimerization caused by cryoprotectants [33, 34], combined with cooling, might have been responsible. We found that the culture conditions also significantly affected blastocyst development rates, both in the treatment groups and the controls. Embryonic development in a defined KSOM plus BSA culture system without coculture can be improved by reducing the O_2 level in the incubator [35]. Our results using vitrified oocytes confirm this observation. The parthenogenetic development of vitrified oocytes was further increased (up to 32% of blastocysts) by using cumulus cell coculture with KSOM plus FBS media, which illustrates the importance of the culture system for vitrified-oocyte derived embryos.

Development of in vitro fertilized oocytes was found to be significantly reduced by vitrification in our study. It has been reported that the fertilization process can be compromised by the effect of the cryoprotectants [36], the cooling [31], and the partial removal of the cumulus cells [37]. To avoid the confounding effect of the cumulus cells on fertilization, control oocytes were partially denuded of cumulus cells. We obtained high blastocyst development, similar to that of the parthenogenetically activated controls, demonstrating that the reduction of the cumulus cell layers was not compromising fertilization. Cleavage rates of vitrified and control oocytes were not different, showing that fertilization itself was not reduced by the treatment. However, significant reduction in blastocyst development showed that vitrification has long-term effects on the overall development of the embryos. Blastocyst development rates of vitrified-thawed and fertilized or parthenogenetically activated oocytes were similar, confirming our assumption that the reduced developmental competence is not attributable to impaired fertilization. This finding is in agreement with the observations of Martino et al. [31] and Kubota et al. [7] in cattle. In contrast, freezing did result in a failure of the zona block to polyspermy in mice [38]. Long-term storage of oocytes further reduced their blastocyst development rates, illustrating that the storage system needs further refinements before finding practical application. Temperature fluctuations during the storage in the gas phase of the nitrogen container and during handling before and after storage might have been responsible for the reduced competence of these oocytes. Experiments with liquid-phase storage are underway.

Nuclear transfer experiments normally utilize recipient cytoplasts from oocytes matured in vivo or in vitro. Developing a successful cryopreservation procedure for bovine oocytes would immensely benefit nuclear transfer research and practice because it would eliminate seasonal fluctuations and the dependence on the timing of slaughter and oocyte shipment. Previously, we have demonstrated that frozen-thawed oocytes can be used for nuclear transfer with embryo-derived blastomeres, and calves were produced by this procedure [7].

In this study, we examined the cryosurvival of matured oocytes and evaluated their use as recipient cytoplasts for nuclear transfer. A vast majority of the oocytes (94%) survived the vitrification process; this rate was not different from controls and was much higher than that achieved in earlier freezing experiments by Ito et al. [39] (62%) and Kubota et al. [7] (74%).

Previous experiments with frozen-thawed matured oocytes showed a significant reduction of enucleation rates when compared with fresh oocytes [7]. We detected no reduction in this study in the enucleation rates for the vitrified-thawed oocytes. This improvement is likely attributable to the vitrification procedure employed in our study. Exposure to a low temperature is known to result in depolimerization of the meiotic spindle [31, 32]. Vitrification has been hypothesized as being beneficial because the exposure time to the critical temperature zone is reduced and because instantaneously cooling the oocytes into a vitrified state allows no time for spindle depolimerization [8, 9].

Most importantly, we found that fusion, cleavage, and blastocyst development rates achieved with vitrified-oocyte-derived cytoplasts were generally high, and not different from those of the fresh controls. To our knowledge, this is the first report on the use of cryopreserved oocytes as recipients for somatic cell nuclear donors with a promising blastocyst development rate. Earlier attempts for open-pulled straw vitrification of cytoplasts and use of them in blastomere-nuclear transfer experiments resulted in a significant reduction in blastocyst development rates compared with fresh recipient cytoplasts (9.1% vs. 34.5%, respectively [33]).

Our nuclear transfer experiments also provided direct proof of which cellular components are affected by cryopreservation. The reduced rates of blastocyst development following IVF and parthenogenetic activation but following nuclear transfer indicate that the nuclear material but not the cytoplasm was adversely affected by vitrification. However, in thawed oocytes, the slower development to the blastocyst stage in all three treatments indicates that there is an alteration to the cytoplasmic components as well.

In conclusion, our data demonstrated that cryopreservation of matured bovine oocytes with the novel SSV method resulted in high survival and cleavage rates. Furthermore, we demonstrated that they were capable of developing into morphologically good-quality blastocysts following parthenogenetic activation, in vitro fertilization, and nuclear transfer. Similar rates of development were ob-

served among the somatic cell nuclear-transferred embryos using vitrified-thawed metaphase II oocytes vs. control fresh oocytes as nuclear recipients. Further experiments are needed to demonstrate the ability of the resulting nuclear-transfer embryos produced in vitro to develop into progeny.

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