Effect of Ovary Storage on Development of Bovine Oocytes after Intracytoplasmic Sperm Injection, Parthenogenetic Activation, or Somatic Cell Nuclear Transfer

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Abstract: *The purpose of this study was to examine the effect of ovary storage on the development of bovine oocytes after intracytoplasmic sperm injection (ICSI), parthenogenetic activation, or somatic cell nuclear transfer (SCNT). Oocytes were obtained from ovaries stored in PBS for 2 to 6 h (control group) or 26 to 30 h (stored group) at 15℃. The maturation rate of the oocytes was significantly lower in the stored group (67%) than in the control group (78%). The degeneration rate of the oocytes was significantly higher in the stored group (24%) than in the control group (2%). ICSI and parthenogenetic oocytes from stored ovaries had a significantly decreased development to the blastocyst stage compared with the control (ICSI 8% vs. 24%, parthenogenetic activation 15% vs. 31%). However, the development rate to blastocysts of SCNT embryos derived from cumulus cells was not different between the two groups (38% vs. 38%). Also, the storage period of ovaries did not decrease the pregnancy rate of SCNT embryos, and cloned calves were produced in both groups with the same efficiency (21% vs. 21%). In summary, ovary storage at 15℃ for 26 to 30 h reduced the maturation rate and in vitro development rate of bovine oocytes after ICSI or parthenogenetic activation, but did not decrease the blastocyst formation rate or survival rate after embryo transfer in SCNT.*

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Introduction

The quality of oocytes is an important factor affecting the developmental competence of embryos produced *in vitro*. In cattle, ovaries collected at slaughterhouses are the main source of oocytes for *in vitro* embryo production from *in vitro* fertilization (IVF) and nuclear transfer (NT). In Japan, since an outbreak of bovine spongiform encephalopathy (BSE), it has been difficult to use bovine ovaries immediately after slaughter because the use of any bovine materials, including ovaries, is prohibited by the Abattoirs Law until BSE testing results prove negative. Therefore, it is necessary to store ovaries until testing for BSE is completed.

There are some reports examining the developmental competence of bovine IVF embryos derived from oocytes after ovary storage. Ovary storage for 8 h at 38°C [1], for 16 to 24 h at 25°C [2], or for 24 h at 20°C [3] decreased the rates of cleavage and blastocyst formation. On the other hand, the storage of ovaries for 24 h at 10°C [4] or 15 to 21°C [5] did not affect the rates of cleavage and blastocyst formation after IVF. These reports suggest that the temperature of the storage solution affects the quality of oocytes derived from stored ovaries.

There have been no reports of bovine

intracytoplasmic sperm injection (ICSI) using oocytes after ovary storage. In bovine NT, ovary storage at 10°C for 24 h did not affect the *in vitro* developmental competence of cumulus cell NT embryos [4], but it did decrease the blastocyst formation rate of NT embryos derived from bone marrow mesechymal stem cells [6]. And no study has examined the effects of ovary storage with respect to the survival rate after transfer of somatic cell nuclear transfer (SCNT) embryos. In this study, we examined the effect of storage of bovine ovaries for 26 to 30 h at 15°C on the maturation rate of oocytes and *in vitro* developmental competence after ICSI, parthenogenetic activation, or SCNT. Furthermore, pregnancy and calving rates after embryo transfer of SCNT blastocysts were examined.

Materials and Methods

All animals received humane care as outlined in the Guide for the "Care and Use of Experimental Animals" (National Institute of Livestock and Grassland Science Animal Care Committee).

Ovary storage and oocyte maturation

Bovine ovaries were collected from a local slaughterhouse (there was no available information regarding the breed, age, health or physiological status of the cows). Ovaries were placed in PBS without washing and stored at 15°C [5] for 2 to 6 h (control group) or 26 to 30 h (stored group) after slaughter. Cumulus-oocyte complexes (COCs) were collected from ovarian follicles of 2–6 mm in diameter, and then were cultured in *in vitro* maturation (IVM) medium at 38.5°C in 5% CO_2 in air. The IVM medium was TCM-199 (Gibco-BRL, Grand Island, NY) with 10% fetal bovine serum (FBS; Tissue Culture Biologicals, Tulare, CA), 100 IU/ml penicillin G potassium (Meiji Seika, Tokyo, Japan), and 100 μg/l streptomycin (Meiji Seika). At 18-20 h after the initiation of the maturation culture, cumulus cells were removed from COCs in M2 medium containing 300 IU/ml hyaluronidase (Type I-S: from bovine testes; Sigma Chemical Co., St. Louis, MO) by pipetting with a finebore, glass pipette. To assess their nuclear configuration, oocytes were fixed in acetic acid: ethanol (1 : 3, v/v) for 24 h, and stained with 1% orcein. After staining, oocytes were examined under a phase-contrast microscope (×200). Oocytes extruding the first polar body (presumptive metaphase-II stage oocytes) were selected for ICSI, parthenogenetic activation, and SCNT.

Intracytoplasmic sperm injection (ICSI)

Straws of frozen spermatozoa from a bull (Japanese black) were thawed in hot water (35°C) for 30 s. The thawed spermatozoa were washed twice by centrifugation at 800 g for 5 min with Brackett and Oliphant's medium (BO medium) and were resuspended in BO medium supplemented with 3 mg/ml bovine serum albumin (initial fractionation by cold alcohol precipitation; Sigma). Denuded oocytes with a visible first polar body after 18 to 20 h of IVM culture were selected for ICSI, and centrifuged at 12,000g for 5 min to facilitate sperm injection. The ICSI was performed in M2 medium supplemented with 3 mg/ml BSA and 7% polyvinylpyrrolidone (PVP, MW 40,000; Sigma), according to a method previously reported [7], using a piezo micromanipulator (Prime Tech, Ibaraki, Japan). Sperm-injected oocytes were cultured in the IVM medium for 4 h. The oocytes with distinct second polar bodies were then treated for 5 min with 7% ethanol in PBS containing 1 mg/ml PVP.

Parthenogenetic activation

After IVM, oocytes with a first polar body were put in Zimmerman mammalian cell fusion medium, and a single direct current pulse of 25 V for 10 μsec was applied by sandwiching with a pair of electrodes. Then, oocytes were incubated in TCM-199 with 2.5 μg/ml cytochalasin D (Sigma), 10 μg/ml cycloheximide (Sigma), and 10% FBS for 6 h.

Nuclear Transfer (NT)

Bovine cumulus cells were obtained from a Japanese black cow by ovum pick up (OPU) and cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma) with 10% FBS, 100 IU/ml penicillin G potassium (Meiji Seika) and 100 μg/l streptomycin (Meiji Seika) at 38.5°C in 5% CO_2 in air. As donor cells for NT, the cumulus cells of passage 5 were cultured in serum-starved medium (DMEM with 0.5% FBS) for 5–7 days. The NT technique was performed as described previously [8]. The first polar body and cytoplasm presumptively containing metaphase chromosomes were pushed out through a slit in the zona pellucida with a fine glass needle. Successful enucleation was confirmed by 10 μg/ml Hoechst 33342 (Sigma) staining under a fluorescence microscope. Donor cells were transferred to the perivitelline space of enucleated oocytes. Enucleated oocytes were fused with donor cells at 24 h post maturation. The oocyte-cell complex was sandwiched with a pair of electrodes in Zimmerman mammalian cell fusion medium and a single direct

current pulse of 25 V for 10 μsec was applied for oocyte-cell fusion. Chemical activation [9] was accomplished by treatments of 10 μM calcium ionophore A23187 (Calbiochem-Novabiochem Corporation, San Diego, CA) in PBS for 5 min, and 2.5 μg/ml cytochalasin D (Sigma) and 10 μg/ml cycloheximide (Sigma) in TCM-199 with 10% FBS for 1 h, and then 10 μg/ml cycloheximide for 4 h.

In vitro culture

After ICSI, embryos were transferred into CR1aa medium [10] and then cultured in the CR1aa medium at 38.5°C in 5% CO_2, 5% O_2 and 90% N_2 for the first 2 days. Over the following 5 days, they were cultured in CR1aa medium with 5% FBS. Parthenogenetic and NT embryos were cultured in a serum-free medium (IVD 101, Research Institute of Functional Peptides, Yamagata, Japan) at 38.5°C in 5% CO_2, 5% O_2 and 90% N_2 for 7 days.

Embryo Transfer (ET)

On day 7 after NT, embryo transfer was performed non-surgically. Holstein and Japanese black crossbred cows were used as recipient animals. Each synchronized recipient cow received a single NT blastocyst into the uterine horn ipsilateral to the corpus luteum. Diagnosis of pregnancy was made by ultrasonography at 30, 60 and 90 days of gestation.

Statistical analysis

Data were analyzed by Chi square test, or by Fisher's exact probability test when the expected value for any parameter was less than 5.

Results

The oocyte maturation (metaphase II, MII) rate after IVM in the stored group (67%, 229/344) was significantly lower than in the control group (79%, 266/336). The rate of degenerated oocytes was significantly higher in the stored group (24%; 81/344) than in the control group (2%; 8/336) after IVM. Of the remaining oocytes that did not mature, 70% were degenerated oocytes in the stored group.

In vitro development of bovine oocytes after ICSI is shown in Table 1. There were significant differences in the cleavage and development rates to the blastocyst between the stored group and the control (34% vs. 54% cleavage rate and 8% vs. 24% blastocyst formation rate. $p<0.05$).

After parthenogenetic activation, there was no difference in the cleavage rate between the two groups (73% in the stored group vs. 79% in the control, Table 2). However, the development rate to the blastocyst was significantly lower in the stored group (15%) than in the control (31%) ($p<0.05$).

The successful enucleation rate was significantly lower in the stored group (74%; 231/314) than in the control (83%; 282/350) ($p<0.05$). As shown in Table 3, there was no significant difference between the stored and control groups in the fusion rate (88% vs. 85%), cleavage rate (81% vs. 78%), or the development rate to the blastocyst stage (38% vs. 38%) of NT embryos.

Twenty-nine (control group) and 19 (stored group) NT embryos were transferred to recipient cows. On day 30 after ET, 12 (control group; 41%) and 8 (stored group; 42%) recipient cows became pregnant (Fig. 1). However, 9 of 20 (45%) recipients aborted before day

Table 1. Effect of storage of bovine ovaries on the *in vitro* development of oocytes after ICSI

Groups	No. of oocytes	No. cleaved (%)	No. of blastocysts (%)
Stored	64	22 (34.4)[b]	5 (7.8)[b]
Control	62	33 (53.2)[a]	15 (24.2)[a]

[a, b]values with different superscript in the same column differ significantly ($p<0.05$).

Table 2. Effect of storage of bovine ovaries on the *in vitro* development of oocytes after parthenogenetic activation

Groups	No. of oocytes	No. cleaved (%)	No. of blastocysts (%)
Stored	131	104 (79.4)	20 (15.3)[b]
Control	132	96 (72.7)	41 (31.1)[a]

[a, b]values with different superscript in the same column differ significantly ($p<0.05$).

Table 3. Effect of storage of bovine ovaries on the *in vitro* development of oocytes after SCNT

Groups	No. of oocytes	No. fused (%)	No. cultured	No. cleaved (%)	No. of blastocysts (%)
Stored	231	204 (88.3)	194	158 (81.4)	73 (37.6)
Control	282	239 (84.8)	229	178 (77.7)	88 (38.4)

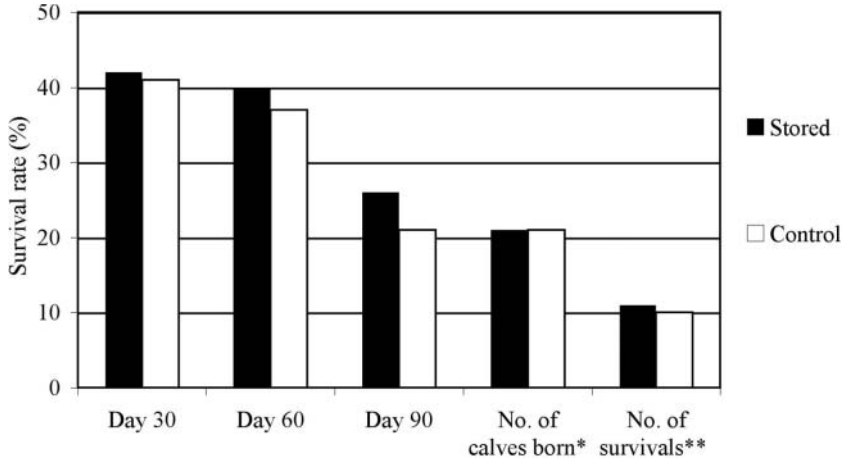

Fig. 1. Effect of storage of bovine ovaries on survival rate after transfer of somatic cell nuclear transfer blastocysts. *Including stillborn calves, **Number of survivals 60 days after birth.

90, and one in the stored group aborted around day 180. Four live calves in the stored group and 3 live and 3 stillborn calves in the control group were obtained from cumulus cells. The pregnancy rate, calving rate (21% in the stored group vs. 21% in the control group) and survival rate 60 days after birth (11% vs. 10%) did not differ significantly between the two groups.

Discussion

The present study demonstrated that the storage of ovaries for up to 30 h did not affect the *in vitro* and fetal development of SCNT embryos and it was possible to produce clone calves using MII oocytes derived from ovaries stored for 26 to 30 h. These results indicate that MII oocytes derived from ovaries stored in PBS at 15°C for up to 30 h can be used as recipients for SCNT. However, the storage of bovine ovaries for 26 to 30 h decreased the *in vitro* developmental competence of ICSI and parthenogenetic embryos.

In this study, although ovary storage for 26 to 30 h decreased the maturation rate, we used only MII oocytes for *in vitro* embryo production. In mouse oocytes, low temperature alters the zona pellucida [11, 12], resulting in a reduced fertilization rate. Cooling of bovine immature oocytes to 13°C affects the integrity of the oocyte membrane, and deceased fertilization rate and a tendency of high polyspermy rate were observed [13]. ICSI is the technique of the direct injection of a single spermatozoon into an oocyte. By performing ICSI in this study, we avoided the influence of zona hardening or polyspermy on developmental competence. However, different results were observed among ICSI, parthenogenetic activation and SCNT. The exact reason for the differences is not clear, but a possible explanation is that ovary storage for 26 to 30 h might induce some damage to the oocyte nucleus. ICSI embryos contain sperm and oocyte nuclei, and parthenogenetic embryos consist of only oocyte nuclei. On the other hand, in SCNT, the oocyte nucleus is removed after IVM. In cattle, when GV stage oocytes are cooled at 4°C for 10 min, abnormal spindle formation at the MII stage is observed [14]. In porcine oocytes, the rate of nucleus with DNA fragmentation significantly increases when ovaries are stored at 15, 25 and 35°C for 6 h [15]. In the present study, DNA fragmentation or abnormal spindle formation after maturation might have been induced in some oocytes in

the stored group. However, in NT, these influences on the subsequent development might have been avoided by replacement of the chromosomes of oocytes by the somatic cell nucleus.

In this study, four live cloned calves were obtained by SCNT using oocytes collected from ovaries stored for 26 to 30 h. It has already been demonstrated that oocytes from a one-day stored ovary support full development to term in bovine IVF [3] or SCNT [6]. Abe *et al.* [3] reported that two live calves were obtained after transfer of 12 frozen-thawed IVF blastocysts produced using oocytes from ovaries stored at 25°C for 24 h. Our study demonstrated that oocytes from ovaries stored for 26 to 30 h support development to the blastocyst stage after ICSI. However, we did not examine the *in vivo* development of ICSI embryos after ET. It will be necessary to examine whether oocytes from one-day stored ovaries support full development to term after ICSI. Recently, Kato *et al.* [6] obtained one live calf after transfer of 11 NT blastocysts from ovaries stored for one day at 10°C. However, in cattle, the effect of one-day storage of ovaries on the fetal development of NT embryos is unclear. Our study shows that the *in vivo* developmental ability after transfer of NT embryos from ovaries stored at 15°C for one day is not inferior to that from short-term storage of 2 to 6 h.

Ovary storage at 15°C for 26 to 30 h decreased the maturation rate (67%) compared with that of the control group (79%). This negative effect on the maturation rate has been observed after ovary storage at 20°C for 18 h ([16], 68% vs. 83%) or 24 h ([3], 56% vs. 87%). In this study, the rate of degenerated oocytes was significantly higher in the stored group (24%) than in the control group (2%) after maturation culture. A previous study reported a similar result (29% vs. 5%) using oocytes from stored ovaries at 20°C for 24 h [3]. In excised ovaries, the lack of blood circulation may produce conditions detrimental to oocytes, such as low oxygen and the accumulation of toxic metabolites [17]. Also, it has been observed that the timing of undergoing GVBD and reaching MII in stored ovaries is earlier than that in non-preserved ovaries [16]. This difference in the timing of reaching MII may affect the enucleation rate. We observed a lower enucleation rate at 18–20 h after IVM culture in the stored group than in the control. Immediately after polar body extrusion, microtubules still link to the polar body closely with the second metaphase plate in the bovine oocyte [18]. In this study, the number of oocytes immediately after the first polar body extrusion might have been larger in the control group than in the stored group.

In this study, bovine ovaries were stored at 15°C as described in a previous report, which demonstrated that the storage of bovine ovaries at 15°C for 24 h did not reduce *in vitro* development after IVF [5]. Also, we used PBS as the storage solution, because, in our preliminary experiment, the rate of first polar body extrusion after IVM tended to be higher in PBS (111/181; 61%) than in physiological saline (141/251; 56%). One-day storage of ovaries at 15°C in PBS decreased the maturation rate and *in vitro* development rates of parthenogenetically activated or ICSI embryos. On the other hand, a recent study demonstrated that bovine ovaries can be stored at 10°C in physiological saline for at least 24 h without decreasing oocyte maturation competence or *in vitro* development after IVF, parthenogenetic activation, or SCNT [4]. Also, Schernthaner *et al.* [5] reported that one-day storage at the same temperature (15°C) as used in this study did not decrease *in vitro* development of IVF embryos. In the study of Schernthaner *et al.*, ovaries were washed once immediately after collection in 70% alcohol and three times in physiological saline, and then stored; but in our study, the ovaries were immersed in PBS directly after collection without washing and stored for 26 to 30 h. These results suggest that ovary storage at 15°C in PBS is not an optimal condition for the storage of bovine ovaries, and that the state of ovary storage may be improved by suitable treatment of ovaries immediately after collection. Recently, Iwata *et al.* [19] demonstrated that the addition of magnesium and raffinose to the storage solution improved the developmental competence of IVF oocytes obtained from ovaries stored for 9 h. Further examinations of storage conditions such as temperature and components of the solution are required in order to improve oocyte quality after long-term ovary storage.

References

1) Abe, S. and Shioya, Y. (1996): Effects of temperature and duration of preservation of bovine ovaries in physiological saline on the development of bovine embryos derived from follicular oocytes matured and fertilized in vitro. Anim. Sci. Technol. (Jpn.)., 67, 633–638.
2) Yang, N.S., Lu, K.H. and Gordon, I. (1990): In vitro fertilization (IVF) and culture (IVC) of bovine oocytes from stored ovaries. Theriogenology, 33, 352 (abstr.).
3) Abe, S., Okazaki, T. and Hasegawa, K. (1996): Effect of preservation of physiological saline at 20°C for 24h on development of bovine embryos derived from follicular oocytes matured and fertilized in vitro. Jpn. J. Embryo

Transfer (Jpn.)., 18, 106–111.
4) Matsushita, S., Tani, T., Kato, Y. and Tsunoda, Y. (2004): Effect of low-temperature bovine ovary storage on the maturation rate and developmental potential of follicular oocytes after in vitro fertilization, parthenogenetic activation, or somatic cell nuclear transfer. Anim. Reprod. Sci., 84, 293–301.
5) Schernthaner, W., Schmoll, F., Brem, G. and Schellander, K. (1997): Storing bovine ovaries for 24 h between 15 and 21°C does not influence in vitro production of blastocysts. Theriogenology, 47, 297 (abstr.).
6) Kato, Y., Imabayashi, H., Mori, T., Tani, T., Taniguchi, M., Higashi, M., Matsumoto, M., Umezawa, A. and Tsunoda, Y. (2004): Nuclear transfer of adult bone marrow mesenchymal stem cells: developmental totipotency of tissue-specific stem cells from an adult mammal. Biol. Reprod., 70, 415–418.
7) Horiuchi, T., Emura, C., Yamauchi, Y., Oikawa, T., Numabe, T. and Yanagimachi, R. (2002): Birth of normal calves after intracytoplasmic sperm injection of bovine oocytes: a methodological approach. Theriogenology, 57, 1013–1024.
8) Takahashi, S., Kubota, C., Shimizu, M., Tabara, N., Izaike, Y. and Imai, H. (2000): Production of cloned calves by somatic cell-nuclear transplantation. Cloned animal and placentation (Roberts, R.M., Yanagimachi, R., Kariya, T. and Hashizume, K., eds.), pp. 30–35, Yokendo Publishers, Tokyo.
9) Akagi, S., Adachi, N., Matsukawa, K., Kubo, M. and Takahashi, S. (2003): Developmental potential of bovine nuclear transfer embryos and postnatal survival rate of cloned calves produced by two different timings of fusion and activation. Mol. Reprod. Dev., 66, 264–272.
10) Rosenkrans, C.F.Jr. and First, N.L. (1991): Culture of bovine zygotes to the blastocyct stage: effects of amino acids and vitamins. Theriogenology, 35, 266 (abstr.).
11) Johnson, M.H., Pickering, S.J. and George, M.A. (1988): The influence of cooling on the properties of the zona pellucida of mouse oocytes. Hum. Reprod., 3, 383–387.
12) Miralles, A., Martinez, E., Pujol, N., Garcia, A. and Garcis, J. (1989): Ultrastructure changes in mouse oocytes cooled in absence of freezing. Cryobiology, 26, 540.
13) Arav, A., Zeron, Y., Leslie, S.B., Behboodi, E., Anderson, G.B. and Crowe, J.H. (1996): Phase transition temperature and chilling sensitivity of bovine oocytes. Cryobiology, 38, 35–42.
14) Wu, B. Tong, J. and Leibo, S.P. (1999): Effects of cooling germinal vesicle-stage bovine oocytes on meiotic spindle formation following in vitro maturation. Mol. Reprod. Dev., 54, 388–395.
15) Wongsrikeao, P., Otoi, T., Karja, N.W.K., Agung, B., Nii, M. and Nagai, T. (2005): Effects of ovary storage time and temperature on DNA fragmentation and development of porcine oocytes. J. Reprod. Dev., 51, 87–97.
16) Narita, M., Goda, S., Inaba, Y., Imai, K., Matoba, S., Tagawa, M., Nagai, T., Saito, N., Yonai, M. and Dochi, O. (2005): Effects of the storage of bovine ovaries on the nuclear maturation and development of in vitro produced embryos. Reprod. Ferti. Dev., 17, 295 (abstr.).
17) Nakao, H. and Nakatsuji, N. (1992): Effects of storage conditions of bovine ovaries and oocytes on the success rate of in vitro fertilization and culture. J. Reprod. Dev., 38, 11–13.
18) Galli, C., Duchi, R., Moor, R.M. and Lazzari, G. (1999): Mammalian leukocytes contain all the genetic information necessary for the development of a new individual. Cloning, 3, 161–170.
19) Iwata, H., Hayashi, T., Sato, H., Kimura, K., Kuwayama, T. and Monji, Y. (2005): Modification of ovary stock solution with magnesium and raffinose improves the developmental competence of oocytes after long preservation. Zygote, 13, 303–308.