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Instructions for use

Dynamics of intracellular phospholipid membrane organization during oocyte

maturation and successful vitrification of immature oocytes retrieved by ovum

pick-up in cattle

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Abstract

The objective was to determine if immature bovine oocytes with cumulus cells at the germinal vesicle (GV) stage, could be vitrified by aluminum sheets (AS; pieces of sheet-like aluminum foil). Cleavage rates in fertilized oocytes previously vitrified by the AS procedure were higher than those vitrified by a nylon-mesh holder (NM) procedure ($89.3 \pm 2.1\%$ vs $65.0 \pm 3.7\%$). Cleaved embryos derived from the AS but not from the NM procedures, developed to blastocysts. Furthermore, in order to investigate the effects of vitrifying GV oocytes on cytoplasmic structure and on the ability to undergo cytoplasmic changes, the intracellular phospholipid membrane (IM) was stained with the lipophilic fluorescent dye DiO. After vitrification by AS, the IM remained intact relative to that of oocytes vitrified by NM. During in vitro maturation, reorganization of the IM was also undamaged in oocytes vitrified by AS before oocyte maturation, whereas the IM within oocytes vitrified by NM procedure was evidently impaired. Finally, vitrification (AS) was used for GV oocytes collected by OPU. A bull calf was born following IVP and subsequent embryo transfer. The vitrification techniques described herein should facilitate generation of viable IVP bovine blastocysts using oocytes recovered by OPU.

Key words: Bovine immature oocytes; Vitrification; Intracellular phospholipid membrane

1. Introduction

Oocyte cryopreservation has great potential as a reproductive technology for livestock production. Moreover, storing female gametes would contribute to advances in treatment of mammalian infertility, conservation of endangered species, and also increased availability of oocytes for research applications, e.g. IVF or animal cloning [1,2]. Cryopreservation of immature oocytes with cumulus cells at the germinal vesicle (GV) stage appears to be more appropriate for storage of female gametes compared to matured oocytes at the second metaphase (MII) stage. Since genetic material is enclosed within the nuclear envelope, damage to the meiotic spindle causing successive chromosomal aberration and aneuploidy could be prevented [3,4]. In that regard, Zhou *et al.* reported that GV oocytes vitrified using a cryotop holder had significantly higher cleavage and blastocyst rates than those vitrified at the MII stage [5]. However, this freezing container can only contain a limited number of oocytes. Therefore, we demonstrated that the vitrification procedure using a nylon-mesh holder (NM) has some feasibility for vitrification of bovine GV oocyte [6]. This holder would be expected to facilitate vitrification of bovine GV oocytes as a freezing container, since the NM can accommodate

large numbers of GV-stage bovine oocytes compared to other representative freezing

containers, e.g. cryotop holder. However, production of cattle using GV oocytes vitrified by

the NM procedure has apparently not been reported. Additionally, cleavage and

developmental rates after IVF of vitrified GV oocytes were drastically lower compared to

those for non-vitrified oocytes. Clearly, vitrification of bovine immature GV oocytes is not

yet a well-established procedure to facilitate gamete storage for efficient animal production.

In cattle, OPU is commonly used to match recipient availability in commercial IVP

programs. Furthermore, OPU and IVF are useful for producing large numbers of

pre-implantation embryos [7,8]. Although OPU would facilitate repeated collection of GV

immature oocytes from live cows, there are still problems to be solved [9]. For example,

this procedure is often compromised by some factors, such as seasonal variations, absence

of appropriate sperm for IVF, and location issues. However, vitrification of GV oocytes

would fundamentally overcome the intrinsic issues of using OPU. Both OPU- and

abattoir-derived oocytes have advantages and disadvantages. In that regard, the former

enables the production of oocytes that are genetically similar and allows comparative

studies at the individual level to be performed, but there is potential for animal-induced

variations in number of oocytes recovered and in blastocyst rate [10]. In contrast, the latter

permits collection of a great number of oocytes, although that is limited to ovaries derived from carcasses. Therefore, cryopreservation, which enables oocytes from livestock to be available at all times, may overcome problems that commonly arise in both of these oocytes sources. However, successful cattle production from cryopreserved OPU oocytes has apparently not been reported.

The objective of the present study was to develop a vitrification protocol that can produce viable blastocysts by OPU-vitrified GV oocytes and to determine if cytoplasmic structure is preserved during vitrification of GV oocytes. In order to explore a novel freezing container, we first compared the NM to an aluminum sheet (AS), as freezing containers for oocyte cryopreservation (Supplemental Fig. 1; on-line version only). After GV oocytes were vitrified by the NM or AS procedures, we further assessed meiotic capability, in vitro development, and the distribution state of intracellular phospholipid membranes by staining with the lipophilic fluorescent dye DiO [11]. In that regard, cryopreservation-induced defects in cytoplasmic structures involving intracellular membranes could contribute to poor development following oocyte vitrification.

2. Materials and methods

Methods for in vitro embryo production, ovum pick-up and embryo transfer have been described [6,7].

2.1. Oocyte collection and preparation

Ovaries of Japanese Black cattle were collected at a local abbatoir and transported to the laboratory in a vacuum (thermos) flask. Cumulus-GV oocyte complexes (GV-COCs) were aspirated from visible follicles (2–8 mm in diameter) with an 18-gauge needle attached to a 10-mL syringe. After three washes, approximately 20 GV-COCs were placed into a 100-μL droplet of a maturation medium consisting of TCM199 supplemented with 5% (v/v) fetal calf serum (FCS) in a humidified atmosphere of 5% in air at 38.5°C under a layer of mineral oil. After culturing for 1 h (to stabilize GV-COCs), they were allocated to two groups, namely, nonvitrified and vitrified oocytes.

2.2. Immature oocyte cryopreservation and thawing

The GV-COCs were vitrified using a nylon-mesh holder (NM) and an aluminum sheet (AS). In brief, an NM (pore size 60 μm; DIN100, Sefar Inc., Ruschlikon, Switzerland) was cut into triangular holds, with each base approximately 10 mm. For easy handling, a cotton-covered polyester thread (10 mm long) was attached to one corner of each holder. We also cut aluminum sheet (AS) in rectangles, approximately 5 by 20 mm.

The GV-COCs were randomly allocated into two groups, NM and AS. First, the GV-COCs were exposed for 7 min to 200-μL droplets of solution A , which was composed of 10% (v/v) ethylene glycol and 0.125 M sucrose in PB1 [12]. Thereafter, they were exposed (2 min) to 200-μL droplets of solution B, which was composed of 20% (v/v) ethylene glycol and 0.25M sucrose in PB1. Finally, they were exposed (1 min) to 200-μL droplets of solution C, which was composed of 40% (v/v) ethylene glycol and 0.5 M sucrose in PB1. After stepwise exposure to these cryoprotectants, ~ 20 GV-COCs were transferred onto each NM or an AS. Thereafter, excess cryoprotectant solution was removed by either placing the NM on a filter paper or aspirating (with a glass pipette) from the AS, and then each apparatus was directly plunged into liquid nitrogen (LN_2) by holding them in the freezing container for 40–60 s. Finally, each apparatus carrying all GV-COCs was

placed into a 2-mL polypropylene tube. After 1 h of storage in LN_2, the GV-COCs were

ultra-rapidly thawed and cryoprotectants removed in a stepwise manner. In brief, each

freezing container containing GV-COCs was transferred from LN_2 into 300-μL droplets of

PB1 warmed to room temperature in culture dishes (sequential series of 0.5, 0.25, and

0.125 M sucrose; 1 min in each solution), and finally they were transferred into PB1 for 5

min. Thereafter, the GV-COCs were cultured for 21–22 h in 100-μL droplets of fresh

maturation medium (as described in oocyte collection and preparation).

2.3. Assessment of meiotic competence, fertilization, and pronuclear formation in

cryopreserved oocytes

After culture for IVM, oocytes were denuded of cumulus cells in

phosphate-buffered saline (PBS) supplemented with 0.1% (w/v) hyaluronidase (H3506;

Sigma) by repeated pipetting (internal diameter was 300 to 400 μm). Then, the

cumulus-free oocytes were fixed in acetic acid/ethanol (1/3, v/v) for 3 d, stained with 1%

orcein (w/v) in 45% acetic acid, and nuclear configuration in each oocyte was assessed with

phase-contrast microscopy. To assess fertilization and pronuclear formation orcein staining

was done 6 h after IVF. Penetration rates were calculated from the proportion of oocytes

forming single or multiple penetrated sperm nuclei or male pronuclei. Furthermore,

pronuclear formation rate from the proportion of oocytes retaining both a single female

pronucleus and a single male pronucleus were also determined.

2.4. In vitro fertilization and subsequent culture of embryos

Straws containing frozen bull semen of Japanese Black cattle were thawed (for 20

s in a 35 °C water bath). After warming, sperm were washed twice by centrifugation at

600×g for 8 min in Brackett and Oliphant (BO) medium [13], containing 2.5 mM

theophylline. Subsequently, sperm were extended in fertilization medium (concentration,

5×10^6 cells/mL). Groups of 10 to 20 COCs were co-incubated with sperm in 100-µL

droplets of fertilization medium consisting of BO medium with 3 mg/mL bovine serum

albumin and 2.5 mM theophylline for 18 h in a humidified atmosphere of 5% CO_2 in air at

38.5°C under a layer of mineral oil.

In vitro culture (IVC) of embryos was done in a modified synthetic oviduct fluid

medium (SOF) supplemented with 22 amino acids (1 mM glutamine, 5 mM glycine, 2 mM

taurine, essential amino acids for basal medium Eagle, and nonessential amino acids for minimum essential medium), 10 μg/mL insulin, and 1 mg/mL polyvinyl alcohol in a humidified atmosphere of 5% CO_2 in air at 38.5°C under a layer of mineral oil. At 18 h after insemination, presumptive zygotes were denuded of cumulus cells by repeated pipetting of COCs (internal diameter of the pipette, 150 to 180 μm). Then, zygotes were washed three times with SOF and cultured for in vitro development to the blastocyst stage. After observing embryo development, some blastocysts were stained with 25 μg/mL Hoechst 33342 (Sigma) in PB1, and subsequently, the number of total cells was assessed.

2.5. Ovum Pick-Up and embryo transfer

Ovum pick-up (OPU) was done as described [7] with a real-time B-mode ultrasound scanner (ECHOPAL II,Hitachi Medical, Tokyo,Japan) equipped with a 6.5-MHz micro convex transducer and a disposable 54-cm 17-gauge needle (COVA Needle, Misawa Medical Industry, Tokyo, Japan) . Briefly, each cow was sedated with 10 mg xylazine hydrochloride im (Skillpen®, Intervet, Tokyo, Japan), and then given 75 mg prifinium bromide iv (Padorin®, Taiyo Pharmaceutical Industry, Tokyo, Japan), a synthetic

gastrointestinal antispasmodic agent, to prevent abdominal straining. After emptying the

rectum and cleaning the perineum, the transducer was advanced into the anterior vagina.

Ovaries were manipulated by transrectal palpation and positioned so that the follicles were

aligned with the path of the needle. A disposable needle was inserted (via the needle guide)

through the wall of the vagina and into the ovarian follicles (diameter > 3 mm). Follicular

contents were aspirated into a tube containing Ringer's lactate solution (Nippon Zenyaku

Kogyo, Fukushima, Japan) supplemented with heparin (10 IU/mL, Ajinomoto, Tokyo,

Japan) and 0.5% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA). An electric

suction pump (FV4, Fujihira Industry, Tokyo, Japan) at a constant negative pressure of

95-105 mmHg, and a flow rate of 20-25 mL/min was used. The collected material was

searched with a dissecting microscope and GV oocytes were subjected to vitrification by

the AS method, followed by IVC culture, as described above. Subsequently, three

blastocysts were nonsurgically transferred (one blastocyst per recipient) to three

synchronized recipient cows (prepared as described [7]).

2.6. Observation of the oocyte intracellular membrane by fluorescent staining

To evaluate the ntracellular membrane of oocytes before/after vitrification, a saturated solution of DiO (3,3'-dioctadecyloxa-carbocyanine perchlorate; Molecular Probes) was prepared by mixing several DiO crystals in 100 μL ethanol [11,14,15]. The resulting DiO solution was stored at room temperature, and used over a period of several days. First, oocytes were fixed for 30 min in PBS containing 4% (w/v) paraformaldehyde and 0.1% (w/v) PVA at room temperature. After fixation, oocytes were washed in PBS containing 0.1% (w/v) PVA, and then incubated with the saturated solution of DiO for 1 min at room temperature. After three washes with PBS-PVA, stained oocytes were mounted on glass slides and observed using a Nikon C1-Plus confocal scanning laser microscope with an argon laser for illumination.

2.7. Statistical analysis

Data for cleavage and blastocyst rates were analyzed (StatView software, Abacus Concepts, Inc., Berkeley, CA) by one-way ANOVA with Fisher protected least significant difference (PLSD). Percentage data were arcsine transformed before analysis. Differences were considered significant for $P < 0.05$.

3. Results

3.1. Availability of an aluminum sheet (AS) for GV-oocyte vitrification

To investigate effects of freezing container on viability, oocytes were vitrified in two containers (NM and AS), and compared to non-vitrified controls. In these three groups 59.5, 67.4 and 67.7%, respectively, of GV oocytes reached the MII stage after culture (no significant difference between NM and AS; Table 1). After insemination, vitrification using NM significantly reduced rates of penetration and pronuclear formation 65.0 and 55.2%, respectively), compared to the nonvitrified control (Table 2). Furthermore, the NM procedure also significantly reduced the cleavage rate (11.1%), and none developed to the blastocyst stage (Table 3). Compared to the NM procedure, oocytes vitrified by the AS procedure had significantly higher rates of penetration, pronuclear formation, and cleavage (89.3, 68.0, and 31.3% respectively), and 4.3% formed blastocysts (Tables 2 and 3). The number of cells in the blastocysts derived from oocytes vitrified by AS procedure (n = 5, 121 ± 3.7) was similar to that in controls (n = 9, 118 ± 5.5).

3.2. Distribution state of intracellular phospholipid membrane in the oocytes vitrified by the AS procedure

Vitrification often causes severe cryoinjury and malfunctions in oocytes, e.g. disruption of the meiotic spindle and the cytoskeleton and damage to the mitochondria [16]. Based on intracellular phospholipid membrane (IM) staining by the lipophilic fluorescent dye DiO, the types of IM organization within unfrozen bovine GV and MII oocytes were categorized (Fig. 1), as dynamics of IM structure in bovine oocyte during meiosis have not been well characterized [17]. The IM organization within GV oocytes included: homogenous distribution within ooplasm; no distinct IM network apart from the cortex; and no distinct IM network in the ooplasm and cortex (Fig. 1). In this study, 77.4% of oocytes had a continuous IM network (Fig. 1a), but the IM organizations of the remaining oocytes were partially or widely discontinuous (13.2 and 9.4%, respectively; Fig. 1g). Following NM vitrification and warming, 32.4 and 8.1% of oocytes contained partially or widely discontinuous IM, whereas these changes were noted in only 10.0 and 7.5% of oocytes vitrified by the AS procedure (similar to the unfrozen controls, Fig. 1g). Thereafter, the IM organization in MII oocytes derived from GV oocytes unfrozen (control) and vitrified by

NM or AS procedures before IVM were determined (Fig. 1d–f). After IVM culture, oocytes

with a first polar body were identified. The IM underwent spatial reorganization during

meiotic maturation, such that cytoplasmic localization of IM in MII oocytes concentrated to

cortical areas (Fig. 1d), whereas 63.2% of unfrozen MII oocytes retained an IM network

localized to cortical areas beneath the oolemma (Fig. 1d and h). The distribution pattern of

Fig. 1f closely resembled the GV oocyte (as shown in Fig. 1a), which might be responsible

for impaired reorganization of the IM during oocyte maturation. In oocytes vitrified by the

NM procedure, most of the MII oocytes had partially or entirely incomplete concentration

of IM structure to cortical areas (Fig. 1h). In contrast, in oocytes vitrified by the AS

procedure, the proportion of MII oocytes with disrupted IM reorganization were drastically

decreased; more than half (52.4%) of MII oocytes had an IM network localized to cortical

areas (Fig. 1h).

3.3. Application of vitrification by the AS procedure to immature oocytes

retrieved by ovum pick-up

Sixty-four oocytes were recovered from three cows, and 54 GV oocytes with

cumulus cells were vitrified by the AS procedure (Table 4). Ten oocytes were removed due

to deterioration of cellular membranes. More than 40% of IVF embryos derived from

vitrified-OPU oocytes had cleaved on Day 2, and three developed to the blastocyst stage

(Table 4). At 280 d after transfer of these blastocysts, one Japanese Black bull calf (40 kg

was born), providing proof of concept that vitrified-OPU bovine oocytes can support

full-term fetal development (Supplemental Fig. 2, on-line version only).

4. Discussion

To our knowledge, characterization of dynamics of IM organization during meiosis

of bovine oocytes and production of viable blastocysts derived from vitrified-OPU

immature oocytes have not been previously reported. Structural change in the IM during

meiosis is critical for successful fertilization and early embryonic development, because the

IM is associated with varied biological functions such as protein folding and degradation,

lipid metabolism, compartmentalization of the nucleus, regulation of the Ca^{2+} ion gradient,

and membrane synthesis [18-22]. In mice, GV oocytes had a continuous endoplasmic

reticulum (ER) network throughout the oocyte. During oocyte maturation, the ER

undergoes a dramatic change; clusters of ER form in the cortex of mature MII oocytes [18].

Furthermore, reorganization of the ER during meiosis was impaired in MII oocytes vitrified

before oocyte maturation, although early embryonic development after maturation and

fertilization was not studied [18]. The ER is detected by DiO staining [11]. In the present

study, IM organization for most bovine oocytes was virtually identical to that of murine

oocytes. However, although the latter are resistant to vitrification and warming, bovine GV

oocytes vitrified using the NM procedure frequently had aberrant IM organization, with

much of that attributed to the ER [11]. The impaired form of IM organization in the NM

vitrification procedure was also more common at the MII stage (Fig. 1h).

Bovine oocytes, regardless of the meiotic state, are much more difficult to

cryopreserve than cleavage-stage embryos [6,23]. Contemporary cryopreservation

techniques for bovine GV oocytes often result in low survival and subsequent development

due to the impaired cytoplasmic structures essential for fertilization and embryonic

development. In particular, intracellular ice crystal formation during freezing is the most

serious problem with oocyte cryopreservation. Solid surface vitrification (SSV) is a

vitrification method performed on a metal surface prefrozen by partial immersion in liquid

nitrogen [24,25], suggesting that metal is appropriate for use as a freezing container.

Aluminum has low cytotoxicity, causing no toxic, immune or carcinogenic effects in rabbit

muscle and no damage to cultured macrophages and fibroblasts [26-28]. Furthermore,

aluminum is useful for metallic biomaterials with superior biocompatibility. In this study,

bovine GV oocytes vitrified by the AS procedure had higher developmental rates compared

to those vitrified by the NM procedure. However, vitrification of bovine GV-COCs using

the NM procedure has yielded blastocysts following *in vitro* embryo production [6]. This

discrepancy between the earlier and present observations may be due to, at least in part, to

differences in cell culture environment. It is well known that oxidative stress caused by a high oxygen concentration (95% air; approximately 20% O_2 concentration) under culture conditions impairs developmental ability of bovine preimplantation embryos [29]. Although cysteamine (a reducing agent) was present in the culture medium used in the present study, insufficient control of oxidative stress might have had detrimental effects on the bovine embryos. Nevertheless, oocytes vitrified by AS procedure supported development to the blastocyst stage.

In conclusion, vitrification using aluminum sheets reduced disruption of intracellular organization during meiosis. Blastocysts were produced from OPU-GV oocytes, and a full-term viable calf was born.

Figure legends

Fig. 1. The intracellular membrane (IM) structure in bovine oocytes at the GV and MII

stages. The IM was labeled using the lipophilic fluorescent dye DiO. In each panel, the

upper panels were unvitrified oocytes at the GV stage (a-c), whereas lower panels were the

MII stage (d-f). Note that GV oocytes had a continuous IM across the broad ooplasm (a),

whereas the IM in MII oocytes was concentrated to cortical areas (d). Some GV oocytes

had discontinuous IM organizations, either partially (b) or widely (c). e, f: Representative

photographs of IM organizations at the MII stage, with defective concentration of IM in

cortical areas (e) or not at all (f). Note that the IM distribution pattern of (f) had a close

resemblance to that of GV oocyte as shown in (a). g, h: proportion of bovine oocytes

unvitrified (GV: n = 53 ; MII: n = 19) and vitrified by the NM (GV: n = 40 ; MII: n = 10)

and AS (GV: n = 37 ; MII: n = 21) procedures, showing each types of the IM organization,

which were categorized as shown (a-c) in GV oocytes and (d-f) in MII oocytes, respectively.

Three independent experiments were replicated. Bar = 20 μm (a).

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Supplemental Fig. 1. Freezing containers for vitrifying oocytes at the GV stage.

a: aluminum sheet (AS). b: nylon-mesh holder (NM). Bar = 10 mm (a) and 5 mm (b).

Supplemental Fig. 2. This male Japanese black calf was born 280 d after transfer of

blastocysts produced from bovine OPU-GV oocytes vitrified/warmed using AS. The calf

weighed 40 kg at birth and has matured without incident.

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Table 1.　Subsequent in vitro maturation of bovine oocytes vitrified by the nylon-mesh holder or aluminum sheet procedure.

Treatment	No. of oocytes examined	No. of oocytes progressed to			
		GV (% ± SEM)	MI (% ± SEM)	AI/TI (% ± SEM)	MII (% ± SEM)
Control cryoprotectant (−)	127	8 (6.3±3.4)	29 (22.8±2.0)	4 (3.1±1.1)	86 (67.7±3.4)
Control cryoprotectant (+)	98	1 (1.0±0.9)	27 (27.6±3.3)	5 (5.1±2.7)	65 (66.3±5.1)
NM	116	9 (7.8±2.2)	30 (25.9±2.8)	8 (6.9±1.2)	69 (59.5±5.2)
AS	92	3 (3.3±1.9)	19 (20.7±3.0)	8 (8.7±3.1)	62 (67.4±4.1)

[a] See details in Materials and Methods. [b] NM: nylon-mesh holder. [c] AS: aluminum sheet.

Three independent experiments were replicated using more than 30 oocytes per repeat.

Table 2. Penetration and pronuclear formation following fertilization in cryopreserved oocytes.

Treatment	No. oocytes examined	No. oocytes inseminated	No. embryos consisted of two pronulei
Control	117	90 (76.9 ± 4.7)	78 (66.7 ± 2.3)
NM	203	132 (65.0 ± 3.7)*	112 (55.2 ± 4.5)*
AS	103	92 (89.3 ± 2.1)	70 (68.0 ± 6.0)

[a] NM: nylon-mesh holder. [b] AS: aluminum sheet. * significant differences for Control ($P<0.05$)

Three independent experiments were replicated using more than 30 oocytes per repeat.

Table 3. In vitro development after IVF of bovine oocytes vitrified by either the nylon-mesh holder or aluminum sheet procedure.

Treatment	No. oocytes examined	No. embryos cleaved (% ± SEM)	No. embryos developed to the blastocyst stage(% ± SEM)
Control	209	106 (50.7±5.1)	42 (20.1±4.4)
NM	180	20 (11.1±2.9)*	0 (0.0±0.0)*
AS	115	36 (31.3±12.3)	5 (4.3±2.9)*

[a] NM: nylon-mesh holder. [b] AS: aluminum sheet. * significant differences for Control (*P*<0.05) Three independent experiments were replicated using more than 30 oocytes per repeat.

Table 4. In vitro development after IVF of oocytes that were aspirated from Japanese black cows and then vitrified/warmed by using the aluminum sheets.

Donor cows	No. of oocytes collected	No. oocytes cultured (%)	No. embryos cleaved (%)	No. embryos developed to the blastocyst stage (%)
1	12	9 (75.0)	4 (44.4)	1 (11.1)
2	36	30 (83.3)	14 (46.7)	2 (6.7)
3	16	15 (93.8)	6 (40.0)	0 (0.0)

Table 1 Subsequent *in vitro* maturation of bovine oocytes vitrified by the nylon-mesh holder or aluminum sheet procedure.

Vitrification procedure[a]	No. oocytes examined	No. oocytes progressed to			
		GV (% ± SEM)	MI (% ± SEM)	AI/TI (% ± SEM)	MII (% ± SEM)
Control	127	8 (6.3±3.4)	29 (22.8±2.0)	4 (3.1±1.1)	86 (67.7±3.4)
NM[b]	116	9 (7.8±2.2)	30 (25.9±2.8)	8 (6.9±1.2)	69 (59.5±5.2)
AS[c]	92	3 (3.3±1.9)	19 (20.7±3.0)	8 (8.7±3.1)	62 (67.4±4.1)

[a] See details in Materials and Methods.   [b] NM: nylon-mesh holder.   [c] AS: aluminum sheet.
Three independent experiments were replicated using more than 30 oocytes per repeat.

Table 2 Penetration and pronuclear formation following fertilization in cryopreserved oocytes

Treatment	No. oocytes examined	No. oocytes penetrated	No. embryos consisted of two pronulei
Control	117	90 (76.9±4.7)	78 (66.7±2.3)
NM[a]	203	132 (65.0±3.7)*	112 (55.2±4.5)*
AS[b]	103	92 (89.3±2.1)	70 (68.0±6.0)

[a] NM: nylon-mesh holder.　　[b] AS: aluminum sheet.　　* significant differences for Control ($P<0.05$). Three independent experiments were replicated using more than 30 oocytes per repeat.

Table 3 *In vitro* development after IVF of bovine oocytes vitrified by either the nylon-mesh holder or aluminum sheet procedure.

Treatment	No. oocytes examined	No. embryos cleaved (% ± SEM)	No. embryos developed to the blastocyst stage (% ± SEM)
Control	209	106 (50.7 ± 5.1)	42 (20.1 ± 4.4)
NM[a]	180	20 (11.1 ± 2.9)*	0 (0.0 ± 0.0)*
AS[b]	115	36 (31.3 ± 12.3)	5 (4.3 ± 2.9)*

[a] NM: nylon-mesh holder. [b] AS: aluminum sheet. * significant differences for Control ($P<0.05$).

Three independent experiments were replicated using more than 30 oocytes per repeat.

Table 4 *In vitro* development after IVF of oocytes that were aspirated from Japanese black cows and then vitrified/warmed by using the aluminum sheets.

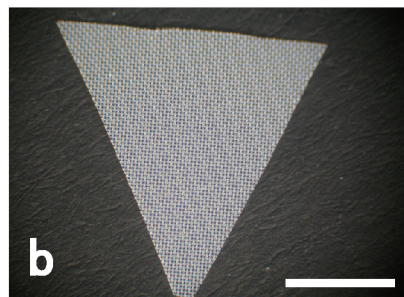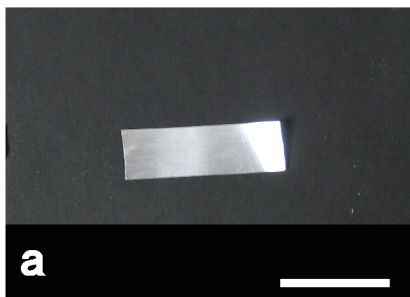| Donor cows	No. oocytes collected	No. oocytes cultured (%)	No. embryos cleaved (%)	No. embryos developed to the blastocyst stage (%)
1	12	9 (75.0)	4 (44.4)	1 (11.1)
2	36	30 (83.3)	14 (46.7)	2 (6.7)
3	16	15 (93.8)	6 (40.0)	0 (0.0)

Figure 1

Supplemental Figure 1

Supplemental Figure 2