

Gel-Loading Tip Vitrification of In Vitro-Matured Bovine Oocytes and Subsequent Embryo Production by IVF and Nuclear Transfer

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Abstract: The purpose of this study was to cryopreserve bovine oocytes for subsequent blastocyst production by in vitro fertilization (IVF) and somatic cell nuclear transfer (SCNT). A vitrification procedure using gel-loading tips as containers was applied to cryopreserve in vitro-matured and denuded oocytes. In Experiment 1, oocytes were vitrified-warmed in vitrification solution (VS) containing 25, 28, 31, or 40% ethylene glycol (EG) and 1.0 M sucrose. The proportions of survived oocytes that appeared to be morphologically normal after warming, and cleaved oocytes after IVF were lower with 25% EG-based VS when compared with 28–40% EG-based VS. Blastocyst yields 8 days after IVF of oocytes vitrified-warmed in 28 and 31% EG-based VS (12 and 17%, respectively) were not significantly different from those of the fresh control group (32%). Day-7 blastocysts derived from vitrified oocytes were composed of a smaller number of inner cell mass (ICM) and trophectoderm cells than the fresh Day-7 blastocysts. In Experiment 2, oocytes vitrified-warmed in 31% EG-based VS were subjected to enucleation and SCNT. The proportions of oocytes fused and cleaved in the vitrified group were comparable to those in the fresh control group. Blastocyst yields 6 and 7 days after SCNT of vitrified oocytes were lower than those of control oocytes, but 8 days after the SCNT, the difference became statistically comparable (45 versus 58% in control group). ICM and trophectoderm cell numbers in Day-7 blastocysts derived from vitrified oocytes were smaller than those of control blastocysts

due to a slower developmental rate. In conclusion, bovine oocytes cryopreserved by vitrification in gel-loading tip were capable of developing into blastocysts after conventional IVF and SCNT, with slightly smaller cell numbers and a slower developmental rate.

Key words: Bovine oocytes, Gel-loading tip, Nuclear transfer, Vitrification

Introduction

The survival rate of cryopreserved bovine oocytes and subsequent developmental potential is low, as only a few pregnancies or calves have been produced [1–3]. During the last decade, a novel vitrification procedure characterized by an ultra-rapid cooling rate became promising for successful cryopreservation of bovine oocytes, since Martino *et al.* [4] first reported that bovine cumulus-oocyte complexes (COCs) survived vitrification and warming using electron microscope grids. An ultra-rapid cooling rate has been achieved with cryodevices such as open-pulled straws (OPS) [5, 6], cryoloop [7] and cryotop [8, 9], or without using any devices (the micro-droplet method [10] and solid-surface vitrification method [11]). Several modifications of the devices include the use of glass-pulled capillaries [12] and nylon mesh [13]. We have developed an improved OPS system using commercially available gel-loading tips for cryopreserving *in vitro*-produced bovine embryos at various developmental stages [14, 15]. In most cases [5–9, 14, 15], the vitrification solution (VS) contains a permeable mixture of ethylene glycol (EG) and dimethyl sulfoxide (DMSO) at a total concentration of 30–40%,

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and non-permeable sucrose at 0.5–1.0 M. In contrast, Papis *et al.* [10] reported a high developmental rate into blastocysts of *in vitro*-matured bovine oocytes after micro-droplet vitrification in VS containing 31% EG +1.0 M sucrose and *in vitro* fertilization (IVF). Dinnyes *et al.* [11] reported a high yield of bovine blastocysts from *in vitro*-matured oocytes after solid-surface vitrification in VS containing 35% EG +5% polyvinylpyrrolidone (PVP) +0.4 M trehalose and subsequent somatic cell nuclear transfer (SCNT), comparable to the yield from fresh control oocytes.

The present study was undertaken to extend the utility of gel-loading tip vitrification to *in vitro*-matured bovine oocytes. Following the determination of the optimal concentration of EG in VS under a conventional IVF system, the quality of blastocysts produced by IVF and SCNT of post-warm oocytes was evaluated from the developmental kinetics and the total/inner cell mass (ICM) cell number.

Materials and Methods

Preparation of oocytes

Bovine COCs were matured *in vitro* as described previously [16]. Briefly, abattoir-derived ovaries from Holstein, Japanese Black, and crossbred cows and heifers were transported to the laboratory in Ringer's solution at 30–37°C within 2 h of slaughter. The COCs were aspirated from follicles 2–5 mm in diameter and cultured in TCM199 medium (Gibco BRL, Grand Island, NY, USA) supplemented with 5% (v/v) fetal calf serum (FCS; Cansera International Inc., Etobicoke, ON, Canada), 0.002 AU/mL FSH (Antrin; Denka Pharmaceutical Co., Ltd., Kawasaki, Japan), and 1 µg/mL estradiol-17β (Sigma Chemical Co., St. Louis, MO, USA) for 19–20 h (Experiment 1) or 18–19 h (Experiment 2) at 38.5°C in 5% CO₂ in air, with a density of 30–40 COCs per 750 µL of the IVM medium. The oocytes were freed from cumulus cells by vortex mixing (IVF) or pipetting (SCNT) in Ca²⁺ and Mg²⁺-free Dulbecco's phosphate buffered saline (D-PBS; Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) containing 0.025% hyaluronidase (Sigma).

Vitrification and warming

Oocytes were cryopreserved by vitrification using gel-loading tips as containers, according to the method of Tominaga and Hamada [14] with some modifications. Briefly, 5–7 oocytes with an extruding first polar body were exposed to 3% EG in TCM199 +20% FCS for 10–15 min (Experiment 1) or 8–12 min (Experiment 2) at

37°C. The oocytes were then equilibrated to a VS composed of 25, 28, 31, or 40% EG +1.0 M sucrose in TCM199 +20% FCS for 30 sec at 37°C, and then were aspirated with 0.6 µL VS into a gel-loading tip (NK-tip; NK System, Osaka, Japan) equipped with a 0.1/10-µL micropipette (Eppendorf, Hamburg, Germany). After being plunged into liquid nitrogen, the gel-loading tips were set in holding 0.5 mL plastic straws (IMV, L'Aigle, France) and stored for at least 1 day. Oocytes were warmed by immersing the tip part of the gel-loading tip into TCM199 +20% FCS at 37°C, and were expelled from the gel-loading tip and held for 3 min. The oocytes were then washed twice with the fresh TCM199 +20% FCS at 5 min intervals, and were cultured in the IVM medium at 38.5°C in 5% CO₂ in air for 2–3 h prior to IVF and for 1 h prior to SCNT.

IVF

A frozen-thawed sperm suspension (1 × 10⁷ sperm/mL) was prepared in Brackett and Oliphant (BO) medium [17] supplemented with 20 mg/mL BSA (Sigma) and 20 µg/mL heparin (Novo-Heparin 100; Novo Industry A/S, Osaka, Japan) after being washed with a discontinuous gradient of 90/45% Percoll (Pharmacia, Uppsala, Sweden) in BSA-free BO medium supplemented with 5 mM theophylline (Sigma) for 5 min at 500 × g. For IVF, a 50-µL sperm suspension was added to 20 oocytes in a 50-µL microdrop of the BO/BSA/heparin medium under mineral oil (Nacalai Tesque, Inc., Kyoto, Japan). Starting 6 h after IVF, the presumptive zygotes were cultured for 3 days in CR1aa medium [18], supplemented with 3 mg/mL fatty acid-free BSA (Sigma) and 0.25 mg/mL linoleic acid-albumin (LAA; Sigma), and for 2 days in CR1aa medium supplemented with 5% FCS in a 4-well multidish at 38.5°C in 5% CO₂, 5% O₂, and 90% N₂ (15 to 20 zygotes per 750 µL medium). Embryos were cultured for an additional 3 days in TCM199 + 20% FCS supplemented with 0.1 mM β-mercaptoethanol (Sigma) at 38.5°C in 5% CO₂ in air.

SCNT

The SCNT was performed according to the method of Akagi *et al.* [19] with minor modifications. Briefly, oocytes were enucleated by piercing the zona pellucida with a glass needle and squeezing out the polar body and a small volume of cytoplasm lying beneath the polar body. Successful enucleation of each oocyte was confirmed by Hoechst 33342 (Sigma) fluorescent staining of the corresponding karyoplast that was squeezed out. At passage 12–17, 80 percent confluent

cumulus oophorus cells were individually inserted into the perivitelline space of the cytoplasts. The donor cell-cytoplast couplets were fused in Zimmermann cell fusion medium using a direct current pulse (25 V/mm, 25 μ sec) generated by a BTX200 Electro Cell Manipulator (BTX, San Diego, CA) with needle-type electrodes. Fifteen minutes after the electrofusion treatment, the fused oocytes were activated by a combined treatment of direct current pulse (20 V/mm, 20 μ sec) in a 1-mm gap electrofusion chamber and 10 μ g/mL cycloheximide (Sigma) and 0.3% BSA in CR1aa medium in 5% CO₂, 5% O₂, and 90% N₂ at 38.5°C for 5 h. The reconstructed embryos were cultured for up to 8 days, as described above for culturing the IVF-derived embryos.

Immunosurgery and differential staining

The number of ICM and trophoblast cells of Day-7 blastocysts was determined by a differential cell staining technique, as described previously [14], allowing determination of the total number of cells for blastocysts and the percentage of ICM cells based on the total number of blastocyst cells. Briefly, blastocysts were treated with 0.5% pronase (Pronase E, Sigma) in D-PBS for 5 to 10 min and zona-free embryos were incubated for 10 min in 10 mM trinitrobenzene sulphonic acid (Nacalai) in Ca²⁺ and Mg²⁺-free D-PBS containing 4 mg/mL PVP (Sigma) on an ice plate. The embryos were then incubated for 30 min in TCM199 containing 0.1 mg/mL anti-dinitrophenol BSA (ICN Biomedicals, Inc., Aurora, OH, USA) and for a subsequent 20 min in TCM199 containing 20% guinea pig complement (Rockland Immunochemicals, Inc., Gilbertsville, PA, USA), 10 μ g/mL propidium iodide (Sigma), and 10 μ g/mL Hoechst 33342 at 38.5°C. Finally, embryos were washed twice in D-PBS, mounted on a glass slide with a mounting reagent (Mounting medium: Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD, USA), and examined at \times 100 magnification under a fluorescence microscope at 330–380 nm.

Experimental design

In Experiment 1, the optimum concentration of EG for VS supplemented with 1.0 M sucrose was examined. Oocytes were vitrified-warmed in a VS of 25, 28, 31, or 40% EG and 1.0 M sucrose. The post-warm oocytes were subjected to conventional IVF and *in vitro* culture for 8 days. Blastocysts harvested on Day-7 (Day-0 was defined as the day of IVF) were subjected to differential cell staining. In Experiment 2, the utility of post-warm oocytes for embryo production by SCNT was examined.

Oocytes were vitrified-warmed in a VS of 31% EG and 1.0 M sucrose. The post-warm oocytes were enucleated and subjected to SCNT. Day-7 blastocysts (Day-0 was defined as the day of cell fusion) were also subjected to the differential cell staining.

Statistical analysis

The experiments were repeated at least four times. The percentage data for each replicate were arc-sine-transformed before being subjected to one-way ANOVA. Data were compared using the Tukey's test. A value of $P < 0.05$ was chosen as an indication of statistical significance.

Results

Experiment 1

When oocytes were vitrified-warmed in VS containing 25, 28, 31, or 40% EG and 1.0 M sucrose, the proportions of surviving oocytes that appeared morphologically normal after warming and the cleaved oocytes after IVF were lower with 25% EG-based VS than with 28–40% EG-based VS (Fig. 1, Table 1). Blastocyst yields 8 days after IVF of oocytes vitrified-warmed in 25 and 40% EG-based VS (8 and 10%, respectively) were significantly different from the yield of the fresh control group (32%), while the yield for 28 and 31% EG-based VS (12 and 17%, respectively) were intermediate and not significantly different from others. Day-7 blastocysts derived from vitrified oocytes (Fig. 2) were composed of a smaller number of ICM and trophoblast cells than the fresh Day-7 blastocysts, but the percentages of ICM cells based on the total number of blastocyst cells were comparable among the groups (Table 1).

Experiment 2

Oocytes that were the morphologically normal (95%, 99/104) after warming were subjected to enucleation, resulting in a successful enucleation rate of 88% (87/99). This proportion was similar to that for fresh control oocytes (93%, 90/97). The proportions of oocytes fused and cleaved in the vitrified group were comparable to those in the fresh control groups (Table 2). The blastocyst yields 6 and 7 days after SCNT of vitrified oocytes were lower than those of control oocytes, but 8 days after the SCNT the difference became statistically comparable (45 vs. 58% in control group). The numbers of ICM and trophoblast cells in Day-7 blastocysts derived from vitrified oocytes were smaller than those of control blastocysts, but there was no

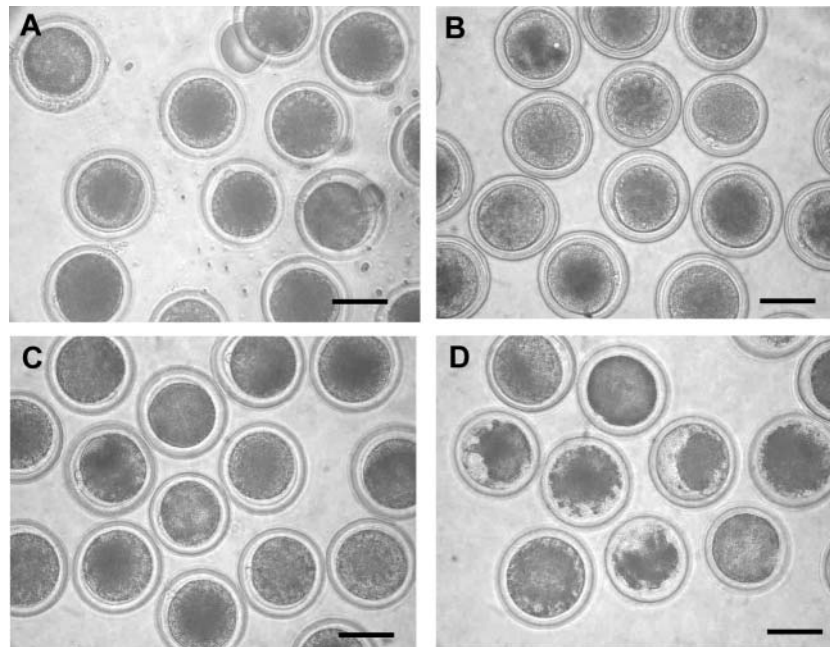


Fig. 1. Representative photographs for post-warm morphology of bovine oocytes (A: 40% EG, B: 31% EG, C: 28% EG, D: 25% EG; Scale bar = 100 μ m).

Table 1. *In vitro* development of vitrified-warmed bovine oocytes after IVF and quality of blastocysts

Concentration of EG	No./(%) of oocytes					Number of cells in Day-7 blastocysts; $\bar{x} \pm$ SD			
	Vitrified	Survived*	Cleaved	Developed to blastocysts until		N	Total	ICM cells	(% ICM)
				Day-7	Day-8				
40% EG	175	157 (90) ^a	78 (50) ^a	12 (7) ^b	16 (10) ^b	12	51.4 \pm 27.0 ^b	10.8 \pm 8.9 ^b	(18.2 \pm 8.2) ^c
31% EG	160	144 (90) ^a	70 (49) ^a	17 (12) ^b	25 (17) ^{ab}	16	57.9 \pm 19.3 ^b	14.5 \pm 5.6 ^b	(24.8 \pm 5.6) ^{abc}
28% EG	144	124 (86) ^a	60 (48) ^a	13 (10) ^b	15 (12) ^{ab}	9	60.8 \pm 16.1 ^{ab}	14.3 \pm 6.5 ^b	(23.7 \pm 8.9) ^b
25% EG	85	13 (15) ^b	5 (38) ^b	0 (0)	1 (8) ^b	—	—	—	(—)
Fresh control	145	—	74 (51) ^a	40 (28) ^a	46 (32) ^a	40	83.4 \pm 30.9 ^a	23.9 \pm 9.3 ^a	(28.7 \pm 6.0) ^a

^{a-c} Different superscripts within columns denote significant differences of $P < 0.05$ (one-way ANOVA/Tukey's test). *Oocytes that appeared to be morphologically normal. Experiments were repeated at least four times.

difference in the percentages of ICM cells based on the total number of blastocyst cells between the groups (Table 2).

Discussion

We have previously demonstrated that gel-loading tip vitrification using VS of permeable 20% EG +20% DMSO and non-permeable 0.6 M sucrose, originally reported for OPS vitrification [5, 6], was effective in cryopreserving IVF-derived bovine embryos at various stages of development [14–16]. In the present study, the applicability of gel-loading tip vitrification was

successfully extended to *in vitro*-matured and denuded bovine oocytes, especially by using a VS of 31% EG and 1.0 M sucrose following a gentle pre-equilibration treatment with 3% EG for 8–15 min. The blastocyst yield 8 days after IVF of vitrified-warmed oocytes (17%) was not significantly different from that of fresh control oocytes (32%), as shown in Table 1 (Experiment 1). Loss of oocyte viability after vitrification in suboptimal concentrations of EG is probably due to chemical toxicity of the VS when the EG concentration is too high, mechanical damage caused by unstable glass-like solidification during cooling, and/or devitrification during warming when the EG concentration is too low. In our

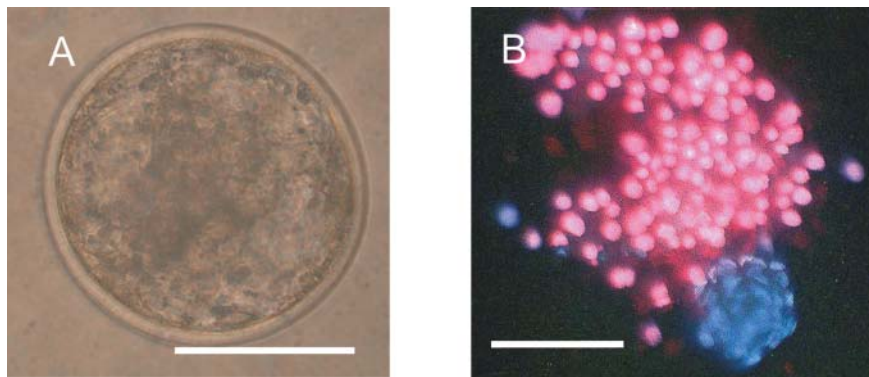


Fig. 2. A Day-7 bovine blastocyst derived from IVF of vitrified oocytes (A) and its differential cell staining (B; Blue = ICM cells, Red = trophoctoderm cells). Scale bar = 100 μ m.

Table 2. Successful use of vitrified-warmed bovine oocytes for somatic cell nuclear transplantation

Groups	No./(%) of couplets						Number of cells in Day-7 blastocysts; $\bar{x} \pm$ SD			
	Prepared	Fused	Cleaved	Developed to blastocysts until			N	Total	ICM cells	(% ICM)
				Day-6	Day-7	Day-8				
Vitrified	85	67 (79)	47 (70)	10 (15) ^b	22 (33) ^b	30 (45)	21	71.6 \pm 30.4 ^b	17.4 \pm 7.5 ^b	(23.8 \pm 0.5)
Fresh control	89	64 (72)	46 (72)	18 (28) ^a	33 (52) ^a	37 (58)	33	93.0 \pm 26.3 ^a	22.1 \pm 7.5 ^a	(23.7 \pm 0.5)

^{a, b} Different superscripts within columns denote significant differences of $P < 0.05$ (one-way ANOVA). Experiments were repeated at least four times.

preliminary experiment using a VS containing 20% EG +20% DMSO +0.6 M sucrose, only 10% of 112 bovine oocytes vitrified in gel-loading tips developed into blastocysts within 8 days of IVF (data not shown). This modification of the design of VS composition was derived from a report on micro-droplet vitrification by Papis *et al.* [10] in which 30% of vitrified bovine oocytes developed into blastocysts 8 days after IVF without a significant difference, 42% for fresh control oocytes. Other “ultra-rapid” vitrification method for cryopreserving *in vitro*-matured bovine oocytes resulted in blastocyst yields after IVF of 15% for electron microscope grid vitrification (vs. 42% for fresh control) [4], 25% for OPS vitrification (vs. 48% for fresh controls; in this case, COCs were denuded 6 h after onset of maturation culture) [5], and 19% for solid surface vitrification (vs. 33% for fresh controls) [11], all of which were better than <5% for the conventional two-step freezing method [1, 2] and 3–10% for the 0.25-mL straw vitrification method [20, 21]. Direct comparison of OPS vs. solid surface vitrification for cryoreservation of Chinese-native bovine oocytes [22] resulted in yields of Day-8 blastocysts of 13 vs. 6%, respectively, under

experiment conditions in which 23% of fresh control oocytes developed into blastocysts.

In the present study (Experiment 2), bovine mature oocytes vitrified in a VS of 31% EG +1.0 M sucrose were enucleated without a reduction in the success rate (88 vs. 93% in fresh control). This is in agreement with previous reports in which successful enucleation rates for fresh vs. vitrified bovine oocytes did not differ (87 vs. 91% [11]; 66 vs. 71% [23], respectively), but is not in agreement with the results of Kubota *et al.* [3], who reported a lower success rate for enucleation in frozen vs. fresh bovine oocytes (61 vs. 83%, respectively). In a SCNT experiment using buffalo mature oocytes after cryotop vitrification [24], we have reported similar enucleation rates for fresh vs. vitrified oocytes (96 vs. 88%, respectively). A short-term culture prior to IVF (2–3 h) and SCNT (1 h) was employed for post-warm bovine oocytes in the present study, since the abnormal spindle structure reported in frozen-thawed bovine oocytes [25] may be repaired by a 2-h incubation prior to IVF [26, 27]. Although a recent study by Chian *et al.* [28] indicated that bovine mature oocytes cryopreserved by vitrification in a VS containing EG and PROH using a

plastic sticker (the device improved cryotop for large quantities of oocytes) did not require such an incubation time prior to the IVF, it is suggested that sufficient restoration of the integrity of the microtubule organization after incubation in EG-based vitrification is required to enhance the outcome of fertilization and SCNT for vitrified oocytes [29]. Here, we report a yield of bovine SCNT blastocysts from vitrified-warmed oocytes as high as 45%, statistically comparable with 58% in the fresh control group on Day-8 (Table 2). Dinnyes *et al.* [11] reported that there was no significant difference in production efficiency of bovine SCNT blastocysts between vitrified and fresh control oocytes on Day-8 (21 and 28%, respectively) and Day-9 (27 and 29%, respectively). Atabay *et al.* [23] also reported that 24% of bovine oocytes after solid-surface vitrification developed into SCNT blastocysts on Day-7, which was comparable with 33% of fresh control oocytes.

Regarding the qualitative aspects of IVF- or SCNT-derived bovine blastocysts, the developmental rate and cell numbers of the blastocysts have been used as practical alternatives to confirmation of pregnancy or birth of calves, while the birth of calves is undoubtedly the most rigorous criterion for determining the true viability of embryos. In the present study, both Day-7 IVF and SCNT blastocysts derived from vitrified oocytes were composed of a smaller number of ICM and trophectoderm cells than the corresponding fresh Day-7 blastocysts, while the percentages of ICM cells based on the total number of blastocyst cells were comparable with the percentages of fresh control blastocysts. These results indicate a slower developmental rate for SCNT blastocysts derived from vitrified oocytes. Atabay *et al.* [23] reported that the total number of cells in bovine SCNT blastocysts derived from vitrified oocytes was smaller than that in fresh oocytes (116.8 vs. 175.3, respectively). Dinnyes *et al.* also demonstrated that development to the blastocyst stage after SCNT derived from vitrified oocytes was slower than for fresh oocytes. Based on these results, when embryo quality is compared between IVF- and SCNT-derived blastocysts, the adverse effect of vitrification seems to be more detectable in IVF-derived blastocysts, suggesting that the nucleus (maternal chromosomes) and surrounding cytoplasm (microtubules of meiotic spindles) are sensitive to cryopreservation [30]. Both decreasing temperature [31] and the DMSO itself [32] induce depolymerization of spindle microtubules and abnormal aster formation, both at the spindle poles and in the cytoplasm. Therefore, gel-loading tip vitrification using a VS with a

lower concentration of EG is basically effective in allowing rapid passage through the “dangerous” temperature zone for the spindle depolymerization and in minimizing the chemical and osmotic toxicity of the VS.

In conclusion, bovine oocytes cryopreserved by vitrification using a gel-loading tip were capable of developing into blastocysts after conventional IVF and SCNT. The quality of the resultant blastocysts, judged by developmental kinetics and total/ICM cell numbers, was not completely equal to that of control blastocysts, but overall yields of IVF and SCNT blastocysts derived from vitrified oocytes were comparable to those from fresh oocytes.

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