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Embryogenesis of vitrified mature bovine oocytes is improved in the presence of multi-layered cumulus cells

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Abstract. This study was aimed at evaluating the effects of multi-layered cumulus cells (MCCs) during vitrification and *in vitro* fertilization (IVF) of mature bovine oocytes and embryogenesis after IVF. The rates of cleavage and blastocyst formation were higher in vitrified and fertilized oocytes with MCCs than in denuded oocytes ($P < 0.05$), but were comparable to the rates in fresh oocytes with MCCs or without (denuded). When the MCC-enclosed oocytes were denuded before IVF, blastocyst formation rate reduced compared with that in vitrified oocytes with MCCs ($P < 0.05$). This suggested that the MCCs surrounding the mature bovine oocytes play important roles during cryopreservation: protecting them against freezing and promoting their survival and development post IVF, thereby increasing the success rates of IVF and embryonic development. Herein, we showed for the first time that calves could be produced using only 14–19 vitrified mature oocytes with MCCs from the ovaries of individual cows post slaughter.

Key words: Cattle, Cryopreservation, Metaphase II, Offspring

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Similar to semen preservation, oocyte cryopreservation is important for maintaining its genetic resources. Calves were first produced from frozen mature oocytes in 1992 [1]. In mice [2] and humans [3, 4], mature oocytes are more frequently cryopreserved than immature oocytes, because in these organisms, immature oocytes are more susceptible to chilling injuries than mature oocytes [2, 5, 6].

Whether cumulus cells are required for the successful vitrification of mature oocytes is controversial. In some studies, vitrified mature bovine oocytes with a few layers of cumulus cells were compared to oocytes without cells, and the presence of these few cumulus cell layers did not affect development [7, 8]. In contrast, mature oocytes with multi-layered cumulus cells (MCCs) underwent highly efficient embryonic development after vitrification in both mice [9, 10] and humans [11]. Moreover, in mature equine oocytes surrounded by MCCs, the meiotic spindle and chromatin were protected from chilling injuries [12]. However, in mature bovine oocytes, the presence of MCCs reduced survival after vitrification, because the cumulus cells likely interfered with the diffusion of water and cryoprotective agents, resulting in inadequate cell protection [8].

To clarify the role of cumulus cells in oocyte cryopreservation for practical calf production, we re-evaluated the effects of MCCs on the vitrification of mature bovine oocytes. We also investigated whether calves could be produced using a small number of vitrified mature oocytes collected from the ovaries of individual cows after slaughter.

We compared the rates of embryonic development among four groups of oocytes: fresh oocytes with MCCs (fresh MCC group, Fig. 1A), fresh denuded oocytes (fresh DN group, Fig. 1B), vitrified oocytes with MCCs (vitrified MCC group), and vitrified denuded oocytes (vitrified DN group). The rates of cleavage and blastocyst formation in the fresh MCC, fresh DN, and vitrified MCC groups were comparable and were higher than these rates in the vitrified DN group ($P < 0.05$, Table 1). In addition, there was no difference in the development of mature bovine oocytes between the fresh and vitrified MCC groups. The results of this study suggested that in cows, the MCCs surrounding mature oocytes play a cryoprotectant role during oocyte cryopreservation, and these cells protect oocytes, promoting their survival and development after *in vitro* fertilization (IVF). This finding is in agreement with that of a previous study in humans [11], wherein vitrified mature oocytes with cumulus cells showed rates of cleavage and blastocyst formation that were similar to those of fresh oocytes. However, the rates of cleavage and blastocyst formation of bovine oocytes were reported previously to be lower in vitrified mature oocytes with cumulus cells than in fresh mature oocytes with cumulus cells [8]. The reason for this difference could be due to variations in the vitrification protocols, e.g., different cryoprotectants, as the study in humans [11] and ours used ethylene glycol (EG) alone, whereas the study in cattle [8] used a 1:1 mixture of EG and dimethyl sulfoxide.

Then, we evaluated the effects of denudation of vitrified mature oocytes prior to IVF. The rate of blastocyst formation 8 days after IVF was higher for vitrified oocytes with MCCs (MCC group) than for denuded oocytes that had been vitrified with MCCs (DN group; $P < 0.05$, Table 2). The cleavage rate was also higher in the MCC group than in the DN group; however, the difference was not

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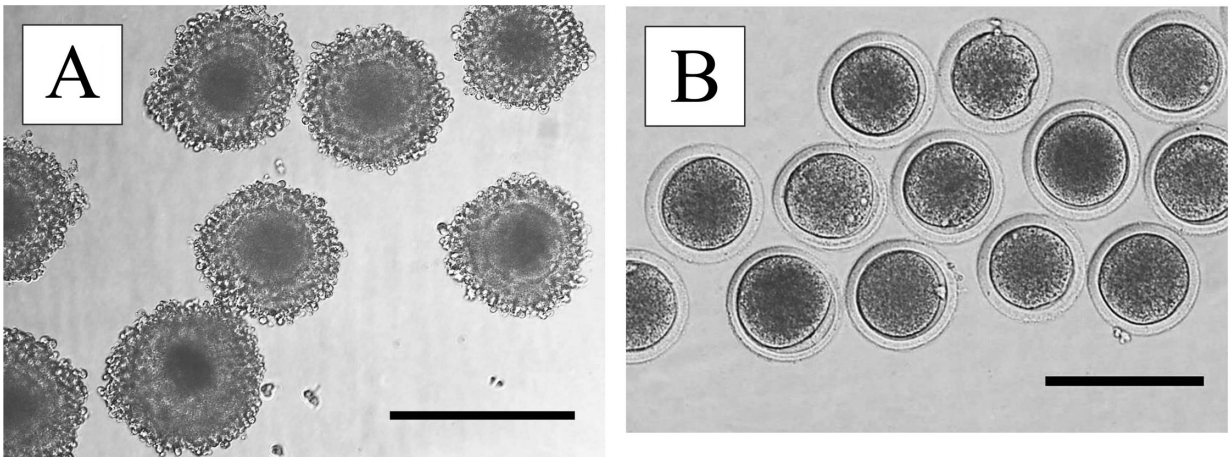

Fig. 1. Photomicrographs of mature bovine oocytes prepared for vitrification. (A) Oocytes with associated multi-layered cumulus cells (MCCs). (B) Oocytes after denudation (DN). Bars = 200 µm.

Table 1. Effects of multi-layered cumulus cells (MCCs) surrounding fresh or vitrified mature bovine oocytes on the rates of cleavage and blastocyst formation

Treatment	Number of oocytes	Cleaved (%)	Blastocyst formation rate	
			Day 7 (%)	Day 8 (%)
Fresh MCC	201	63.8 ± 4.2 [a]	27.4 ± 2.8 [a]	37.4 ± 2.3 [a]
Fresh DN	156	62.2 ± 5.7 [a]	31.9 ± 4.4 [a]	36.5 ± 5.1 [a]
Vitrified MCC	132	56.7 ± 4.9 [a]	28.1 ± 3.2 [a]	35.8 ± 4.2 [a]
Vitrified DN	141	35.8 ± 5.7 [b]	11.8 ± 1.4 [b]	15.5 ± 1.9 [b]

Mature oocytes were assigned randomly into one of the following four groups: 1. fresh oocytes with MCCs (fresh MCC), 2. fresh denuded (DN) oocytes (fresh DN), 3. vitrified oocytes with MCCs (vitrified MCC), and 4. vitrified denuded oocytes (vitrified DN). This experiment was repeated six times on different days. Values shown are means ± standard error of the mean (SEM). Different superscript letters (a, b) within the same column indicate significantly different values ($P < 0.05$).

Table 2. Effects of removing multi-layered cumulus cells (MCCs) from vitrified mature bovine oocytes before IVF on the rates of cleavage and blastocyst formation

Treatment	Number of oocytes	Cleaved (%)	Blastocyst formation rate	
			Day 7 (%)	Day 8 (%)
DN	79	39. 0 ± 5.5	13.9 ± 3.0	21.3 ± 2.9 [a]
MCC	75	53.6 ± 2.4	23.3 ± 3.3	36.2 ± 2.3 [b]

Mature oocytes were assigned randomly into one of the following two groups: 1) DN (vitrified oocytes that had the associated MCCs removed by gentle pipetting before IVF) 2) MCC (vitrified oocytes with MCCs that were fertilized by IVF). This experiment was repeated four times on different days. Values shown are the means ± SEM. Different superscript letters (a, b) within the same column indicate significantly different values ($P < 0.05$).

statistically significant. In a previous study in mice, more cumulus cell-surrounded oocytes were fertilized and developed to the 8-cell stage after vitrification and thawing than denuded oocytes [13]. Similarly, we observed that denudation of vitrified mature bovine oocytes before IVF led to reduced rates of cleavage and blastocyst formation. Cryopreservation-induced hardening of the zona pellucida has been observed in mammalian oocytes, which may explain the reduced rates of sperm penetration [14, 15]. The cumulus cells may minimize cortical granule release and prevent premature zona hardening, thus maintaining the fertilization ability of cryopreserved oocytes [16]. Another possibility is that the cumulus cells protect bovine oocytes against oxidative stress during fertilization, because cryopreservation has been reported to induce oxidative stress in swine oocytes [17], and oxidative stress in bovine oocytes results in death and complete inhibition of the first cleavage [18]. Overall, the results of previous studies and the present study suggest that cumulus cells likely maintain the fertilization ability of vitrified bovine oocytes, which can be damaged by chilling injury.

To evaluate the usefulness of the results of this study, oocytes were collected from the ovaries of three individual Japanese Black beef cows after slaughtering, and mature oocytes with MCCs were vitrified. After IVF and *in vitro* culture, embryos were vitrified, and transferred to Holstein heifers by nonsurgical methods. After embryo transfer, one female calf (Fig. 2A) was born using 15 vitrified mature oocytes collected from donor cow number 1, two male calves (Fig. 2B) were born using 19 vitrified mature oocytes from donor cow number 2, and one female calf (Fig. 2C) was born using 14 vitrified mature oocytes from donor cow number 3 (Table 3). These results demonstrated that calves can be successfully produced using a relatively small number of mature, vitrified oocytes with MCCs.

In conclusion, the presence of MCCs surrounding mature oocytes increased the success rates of IVF and embryonic development after vitrification of mature bovine oocytes. We successfully produced calves, for the first time, using only 14–19 vitrified mature oocytes with MCCs collected from the ovaries of individual cows post slaughter. The results of this report will help improve embryogenesis after oocyte vitrification and genetic resource preservation in cattle.

Fig. 2. Delivered Japanese Black calves following embryo transfer (ET) of fertilized vitrified mature bovine oocytes with multi-layered cumulus cells (MCCs). (A) A female calf (body weight, 30 kg) from donor cow no. 1, which was born 276 days after embryo transfer (ET). (B) A male calf (body weight, 32.5 kg) from donor cow no. 2, which was born 274 days after ET. (C) A female calf (body weight, 31.7 kg) from donor cow no. 3, which was born 283 days after ET.

Table 3. Embryo transfer to produce offspring using vitrified mature bovine oocytes with multi-layered cumulus cells (MCCs) collected from the ovaries of individual cows after slaughter

Donor cow	Number of vitrified oocytes	Cleaved (%)	Blastocyst formation rate		Number of transferred embryos	Number of offspring
			Day 7 (%)	Day 8(%)		
1	15	86.6	40.0	53.3	5	1
2	19	78.9	57.8	57.8	8	2
3	14	64.3	35.7	35.7	4	1

Oocytes were individually collected from three cows. Parentage was determined by genetic diagnostic methods.

Methods

All reagents used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise indicated.

Oocyte collection

Ovaries were obtained from Japanese Black beef cows at a local slaughterhouse, and were transported to the laboratory within 2 h stored in physiological saline containing 500 ng/ml kanamycin sulfate at 25°C. From the ovaries, cumulus-oocyte complexes (COCs) were collected from 2–6 mm-wide follicles with 18-gauge needles containing HEPES-buffered TCM199 (M199; Thermo Fisher Scientific, Waltham, MA, USA) with 10 ng/ml gentamicin sulfate.

In vitro maturation (IVM)

The IVM medium consisted of M199 supplemented with 10% newborn calf serum (NBCS; newborn calf serum, heat-inactivated, New Zealand origin; Thermo Fisher Scientific), 0.02 AU/ml follicle-stimulating hormone (Antorin-R10; Kyoritsu Seiyaku, Tokyo, Japan), 50 ng/ml EGF, 5.0 µg/ml dbcAMP, 0.6 mg/ml L-carnitine (Wako Pure Chemical Industries, Osaka, Japan), and 10 ng/ml gentamicin sulfate. COCs with two or more layers were washed thrice with IVM medium. Groups of 30–50 COCs were cultured in 4-well dishes in 500-µl droplets of IVM medium, covered with mineral oil, and incubated for 21 h at 38.5°C in 5% CO_2 and saturated humidity.

Preparation of oocytes with multi-layered cumulus cells (MCCs) and denuded oocytes

After IVM, COCs were randomly allocated and either partially or completely denuded of cumulus cells. Certain COCs were placed in M199 containing 0.01% hyaluronidase at 25°C. These COCs were transferred to M199 containing 10% NBCS at 25°C within 5 sec, and washed five times. Some cumulus cells were removed, leaving three or more layers over the surface of the zona pellucida by gentle pipetting with a fine pipette. These mature oocytes were used in subsequent experiments as oocytes with MCCs.

Other COCs were placed in M199 containing 0.1% hyaluronidase at 37°C. These COCs were transferred to M199 containing 10% NBCS at 37°C within 30 sec, and washed thrice. The cumulus cells were completely removed by gentle pipetting with a fine pipette. These mature oocytes were used in subsequent experiments as denuded oocytes.

Oocyte vitrification

ES solution consisted of M199 supplemented with 10% NBCS, 3% ethylene glycol (EG; Wako Pure Chemical Industries), and 10 ng/ml gentamicin sulfate. VS solution consisted of M199 supplemented with 10% NBCS, 30% EG, 1.0 mol/l sucrose (Wako Pure Chemical Industries), and 10 ng/ml gentamicin sulfate.

Mature oocytes were vitrified using previously described methods [19] with some modifications. In brief, the mature oocytes were incubated in 100-µl droplets of ES solution for 12 min at 25°C. After equilibration, the mature oocytes were transferred to 100-µl droplets of VS solution for 30 sec at 25°C. Then, up to 10 mature

oocytes were loaded into a Cryotop device (Cryotop-AG; Kitazato, Shizuoka, Japan) with a minimum quantity of solution and immediately immersed in liquid nitrogen.

Oocyte warming and repair culture

TS solution consisted of M199 supplemented with 10% NBCS, 0.5 mol/l sucrose, and 10 ng/ml gentamicin sulfate. DS1 solution consisted of M199 supplemented with 10% NBCS, 0.25 mol/l sucrose, and 10 ng/ml gentamicin sulfate. DS2 solution consisted of M199 supplemented with 10% NBCS, 0.125 mol/l sucrose, and 10 ng/ml gentamicin sulfate. DS3 solution consisted of M199 supplemented with 10% NBCS, 0.0625 mol/l sucrose, and 10 ng/ml gentamicin sulfate.

The vitrified mature oocytes were warmed by immersing the Cryotop device in 2 ml of TS solution for 1 min at 37°C. After warming, the mature oocytes were first transferred into 500 µl of DS1 solution and incubated for 3 min at 37°C, and then transferred into 500 µl of DS2 solution and incubated for 3 min at 37°C, and finally transferred into 500 µl of DS2 solution and incubated for 3 min at 37°C. Then, the oocytes were washed in 100-µl droplets of M199 containing 10% NBCS and incubated for 5 min at 37°C.

After warming, the oocytes were incubated with 500 µl of M199 containing 10% NBCS, covered with mineral oil, and incubated for 2 h at 38.5°C in 5% CO_2 and saturated humidity.

In vitro fertilization (IVF)

Frozen semen from Japanese Black bulls stored in straws (Livestock Improvement Association of Japan, Tokyo, Japan) was thawed in water at 37°C for 30 sec, and washed twice with IVF100 (Research Institute for the Functional Peptides, Yamagata, Japan) by centrifugation (2,000 rpm, 5 min). Spermatozoa were removed from the pellet, and added to IVF100 to obtain a suspension with a final sperm concentration of 5.0×10^6/ml. This suspension served as the IVF medium.

After IVM, the COCs were removed from the maturation medium, and washed thrice with IVF100. Up to 30 COCs were incubated in 35-mm dishes containing 100-µl droplets of IVF medium covered with mineral oil for 6 h at 38.5°C in 5% CO_2 and saturated humidity.

In vitro culture (IVC)

The IVC medium used was Charles Rosenkrans 1 medium with amino acids [20] containing 5% NBCS and 10 ng/ml gentamicin sulfate. After IVF, cumulus cells and spermatozoa were removed from the surface of the zona pellucida by gentle pipetting with a fine pipette. The putative zygotes were washed thrice with IVC medium. Up to 40 zygotes were cultured in 4-well dishes in 500-µl droplets of IVC medium covered with mineral oil for 8 days at 38.5°C in 5% CO_2-5% O_2-90% N_2 and saturated humidity.

Vitrification, warming, and transfer of embryos

ES solution consisted of M199 supplemented with 10% NBCS, 7.5% EG, 7.5% dimethyl sulfoxide (DMSO; Wako Pure Chemical Industries), and 10 ng/ml gentamicin sulfate. VS solution consisted of M199 supplemented with 10% NBCS, 15% EG, 15% DMSO, 1.0 mol/l sucrose, and 10 ng/ml gentamicin sulfate. TS solution consisted of M199 supplemented with 10% NBCS, 0.5 mol/l sucrose, and 10 ng/ml gentamicin sulfate. DS solution consisted of M199 supplemented with 10% NBCS, 0.25 mol/l sucrose, and 10 ng/ml gentamicin sulfate.

Seven to eight days after IVF, the blastocysts, which were classified into grades 1–2 according to the manual of the International Embryo Transfer Society [21], were vitrified and warmed using previously described methods [22], with some modifications. Briefly, embryos were incubated in 100-µl droplets of ES solution for 12 min at 25°C. After equilibration, one or two embryos were transferred into 100-µl droplets of VS solution and incubated for 90 sec at 25°C. Then, the embryos were loaded into the Cryotop device with a minimal quantity of medium, and immediately immersed into liquid nitrogen.

The vitrified embryos were warmed by immersing the Cryotop device in 1 ml of TS solution for 1 min at 37°C. After warming, the embryos were first transferred into 100-µl droplets of DS solution, incubated for 3 min at 37°C, and then incubated in 100-µl droplets of M199 containing 10% NBCS for 5 min at 37°C, and finally washed in 100-µl droplets of M199 containing 10% NBCS for 1 min at 37°C.

After culture for 4 h, the embryos were transferred into synchronized Holstein heifers 7–8 days after estrus using previously described nonsurgical methods [23] (one or two embryos per animal). Pregnancy was confirmed by ultrasonography twice at 30–40 days and 50–60 days after embryo transfer. After parturition, a test was performed to verify the parentage of the calves.

Statistical analysis

The rates of cleavage and blastocyst formation were expressed as the mean percentage ± standard error of the mean (SEM). Values were analyzed using one-way analysis of variance (ANOVA) and the Tukey-Kramer method. Differences were considered statistically significant at p values less than 0.05.

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References

1. **Fuku E, Kojima T, Shioya Y, Marcus GJ, Downey BR.** In vitro fertilization and development of frozen-thawed bovine oocytes. *Cryobiology* 1992; **29**: 485–492. [Medline] [CrossRef]
2. **Schroeder AC, Champlin AK, Mobraaten LE, Eppig JJ.** Developmental capacity of mouse oocytes cryopreserved before and after maturation in vitro. *J Reprod Fertil* 1990; **89**: 43–50. [Medline] [CrossRef]
3. **Chian RC, Huang JY, Tan SL, Lucena E, Saa A, Rojas A, Ruvalcaba Castellón LA, García Amador MI, Montoya Sarmiento JE.** Obstetric and perinatal outcome in 200 infants conceived from vitrified oocytes. *Reprod Biomed Online* 2008; **16**: 608–610. [Medline] [CrossRef]
4. **Cobo A, Kuwayama M, Pérez S, Ruiz A, Pellicer A, Remohí J.** Comparison of concomitant outcome achieved with fresh and cryopreserved donor oocytes vitrified by the Cryotop method. *Fertil Steril* 2008; **89**: 1657–1664. [Medline] [CrossRef]
5. **Candy CJ, Wood MJ, Whittingham DG, Merriman JA, Choudhury N.** Cryopreservation of immature mouse oocytes. *Hum Reprod* 1994; **9**: 1738–1742. [Medline] [CrossRef]
6. **Goud A, Goud P, Qian C, Van der Elst J, Van Maele G, Dhont M.** Cryopreservation of human germinal vesicle stage and in vitro matured M II oocytes: influence of cryopreservation media on the survival, fertilization, and early cleavage divisions. *Fertil Steril* 2000; **74**: 487–494. [Medline] [CrossRef]
7. **Zhou XL, Al Naib A, Sun DW, Lonergan P.** Bovine oocyte vitrification using the Cryotop method: effect of cumulus cells and vitrification protocol on survival and subsequent development. *Cryobiology* 2010; **61**: 66–72. [Medline] [CrossRef]
8. **Ortiz-Escribano N, Smits K, Piepers S, Van den Abbeel E, Woelders H, Van Soom A.** Role of cumulus cells during vitrification and fertilization of mature bovine oocytes:

Effects on survival, fertilization, and blastocyst development. *Theriogenology* 2016; **86**: 635–641. [Medline] [CrossRef]

9. **Kohaya N, Fujiwara K, Ito J, Kashiwazaki N.** High developmental rates of mouse oocytes cryopreserved by an optimized vitrification protocol: the effects of cryoprotectants, calcium and cumulus cells. *J Reprod Dev* 2011; **57**: 675–680. [Medline] [CrossRef]
10. **Kohaya N, Fujiwara K, Ito J, Kashiwazaki N.** Generation of live offspring from vitrified mouse oocytes of C57BL/6J strain. *PLoS ONE* 2013; **8**: e58063. [Medline] [CrossRef]
11. **Tong XH, Wu LM, Jin RT, Luo LH, Luan HB, Liu YS.** Fertilization rates are improved after IVF if the corona radiata is left intact in vitrified-warmed human oocytes. *Hum Reprod* 2012; **27**: 3208–3214. [Medline] [CrossRef]
12. **Tharasanit T, Colleoni S, Galli C, Colenbrander B, Stout TA.** Protective effects of the cumulus-corona radiata complex during vitrification of horse oocytes. *Reproduction* 2009; **137**: 391–401. [Medline] [CrossRef]
13. **Park SE, Chung HM, Cha KY, Hwang WS, Lee ES, Lim JM.** Cryopreservation of ICR mouse oocytes: improved post-thawed preimplantation development after vitrification using Taxol, a cytoskeleton stabilizer. *Fertil Steril* 2001; **75**: 1177–1184. [Medline] [CrossRef]
14. **Carroll J, Depypere H, Matthews CD.** Freeze-thaw-induced changes of the zona pellucida explains decreased rates of fertilization in frozen-thawed mouse oocytes. *J Reprod Fertil* 1990; **90**: 547–553. [Medline] [CrossRef]
15. **Matson PL, Graefling J, Junk SM, Yovich JL, Edirisinghe WR.** Cryopreservation of oocytes and embryos: use of a mouse model to investigate effects upon zona hardness and formulate treatment strategies in an in-vitro fertilization programme. *Hum Reprod* 1997; **12**: 1550–1553. [Medline] [CrossRef]
16. **Vincent C, Pickering SJ, Johnson MH.** The hardening effect of dimethylsulphoxide on the mouse zona pellucida requires the presence of an oocyte and is associated with a reduction in the number of cortical granules present. *J Reprod Fertil* 1990; **89**: 253–259. [Medline] [CrossRef]
17. **Somfai T, Ozawa M, Noguchi J, Kaneko H, Kuriani Karja NW, Farhudin M, Dinnyés A, Nagai T, Kikuchi K.** Developmental competence of in vitro-fertilized porcine oocytes after in vitro maturation and solid surface vitrification: effect of cryopreservation on oocyte antioxidative system and cell cycle stage. *Cryobiology* 2007; **55**: 115–126. [Medline] [CrossRef]
18. **Fatehi AN, Roelen BA, Colenbrander B, Schoevers EJ, Gadella BM, Beverst MM, van den Hurk R.** Presence of cumulus cells during in vitro fertilization protects the bovine oocyte against oxidative stress and improves first cleavage but does not affect further development. *Zygote* 2005; **13**: 177–185. [Medline] [CrossRef]
19. **Papis K, Shimizu M, Izaike Y.** Factors affecting the survivability of bovine oocytes vitrified in droplets. *Theriogenology* 2000; **54**: 651–658. [Medline] [CrossRef]
20. **Rosenkrans CF Jr, Zeng GQ, MCNamara GT, Schoff PK, First NL.** Development of bovine embryos in vitro as affected by energy substrates. *Biol Reprod* 1993; **49**: 459–462. [Medline] [CrossRef]
21. **Robertson I, Nelson RE.** Certification and identification of embryos. *In*: Stringfellow DA, Givens MD (eds.), Manual of The International Embryo Transfer Society. 4th ed, Champaign: International Embryo Transfer Society; 2010: 86−105.
22. **Kuwayama M, Vajta G, Kato O, Leibo SP.** Highly efficient vitrification method for cryopreservation of human oocytes. *Reprod Biomed Online* 2005; **11**: 300–308. [Medline] [CrossRef]
23. **Kimura K, Matsuyama S.** Successful nonsurgical transfer of bovine elongating conceptuses and its application to sexing. *J Reprod Dev* 2014; **60**: 210–215. [Medline] [CrossRef]