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Effect of cryotop vitrification method on oocytes at different meiotic stages and embryo development *in vitro*

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ABSTRACT

The effectiveness of existing procedures based on viable embryos per oocyte frozen remains low compared to use of fresh oocytes in cattle, and the probable causes of the freezing-induced reduction in oocyte developmental competence include chromosome, cytoskeleton disruption, or even the choice of the frozen stage of the oocytes. In the present study, after vitrification, the survival rate of GV and MI oocytes were significantly lower than those in MII. The vitrifid GV oocyte showed some chromosomes dispersed and less condensed, The vitrifid MI oocytes with "Fading'' and discontinuous staining pattern were as having an abnormal microfilament distribution. The stage of GV and MI, both cleavage rate and blastocyst formation rate were reduced significantly. Therefore, the cryopreservation of GV or MI oocytes seems could not instead of the MII oocytes.

Key words: Bovine, Embryos, Oocyte, Vitrification

In livestock, large numbers of ovaries can be obtained from a slaughterhouse, and from these ovaries immature germinal vesicle (GV) stage oocytes can be obtained for producing bovine embryos by *in vitro* maturation/*in vitro* fertilization (IVM/IVF; Brackett and Zuelke 1993, Trounson *et al.* 1994). There is no doubt that embryos produced *in vitro* are more sensitive to low temperature and have less cryo-tolerance than those produced *in vivo* (Diez *et al.* 2001, Rizos *et al.* 2001, Dobrinsky *et al.* 2002), hence, success has been achieved in freezing oocytes from cattle by adapting embryo cryopreservation protocols (Fuku *et al.* 1995, Martino *et al.* 1996),but the effectiveness of existing procedures based on viable embryos per oocyte frozen remains low compared to use of fresh oocytes in cattle, perhaps due to deficiencies in the *in vitro* culture conditions, or the stage oocytes at freezing stage (Dobrinsky *et al.* 2002).

Kubota *et al.* (1998) reported effects of cryopreservation stage on the cytology of bovine oocytes at the GV, MI and MII stages, moreover, the opinions in the selection of oocytes for freezing stage are different now — some believe that cryopreservation of GV stage oocytes is usually more difficult than that of mature oocytes, zygotes or later stage embryos. GV stage oocytes cryopreservation was not advantageous, as the method is related to an increased incidence of chromosomal abnormalities despite the absence of the MII spindle, and metaphase II (MII) oocytes appear to be less susceptible to freezing damage than immature (GV) oocytes (Lim *et al.* 1992, Otoi *et al.* 1995). Others agree that the survival of mammalian oocytes and embryos after cryopreservation varies with the stage of maturation and development (Pedro *et al.* 2005, Magnusson *et al.* 2008) and the cell cycle stage during meiosis affects the outcome due to varying sensitivity to cooling procedures (Kubota *et al.* 1998). In MII oocytes during meiosis, their microtubular spindles, are highly sensitive to temperature changes (Rho *et al.* 2002), and hence chromatid disjunctions possibly occur during cooling, resulting in aneuploidy after fertilization (Eroglu *et al.*1998). Contrarily, immature oocytes at the germinal vesicle (GV) stage do not have any microtubular spindle (Wu *et al.* 1999), therefore, cryopreservation of GV oocytes seems to be an alternative approach to the storage of female gametes. Evidence suggested that the probable causes of freezing-induced reduction in oocyte developmental competence include chromosome and cytoskeleton disruption (Chen *et al.* 2003) and damage to mitochondria (Jones *et al.* 2004).The present study was undertaken to evaluate the probable causes of vitrification on microtubule and microfilament configurations of bovine oocytes at GV, MI and MII stage before and after the vitrification, and also to

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study the recovery rate, fertilization and embryo development after vitrification of these 3 stage of oocytes.

MATERIALS AND METHODS

Oocytes collection and culture: Ovaries were collected from bovine and transported to the laboratory at 30°C within 2–3h in Dulbecco's PBS (D-PBS) containing antibiotics. After one wash each with 75% alcohol for 10s and fresh normal saline (0.9% sodium chloride) the ovaries were sliced using a razor blade in sterile Petri dishes containing TCM-199 supplemented with 0.1% (W/V) polyvinyl alcohol (PVA). *In vitro* maturation of bovine oocytes was carried out as per Huang *et al.* (2001). Only cumulus oocyte complexes (COCs) with a compact, non-atretic, multilayer cumulus oophorus corona radiata, and homogenous ooplasm were selected for maturation. The selected COCs were matured in tissue culture medium (TCM)-199 supplemented with 10% fetal bovine serum (FBS), 10 µg/ml luteinizing hormone, 1 µg/ml E2 and 1 µg/ml follicle-stimulating hormone, and 1% (V/V) penicillin/streptomycin in 50 µl microdrops under mineral oil. Maturation was performed in an incubator with 5% CO_2 under humidified air at 38.5°C. The COCs were cultured for different period of time, and then the cumulus cells were removed by treatment with 300IU/ml hyaluronidase in TCM-199. Cumulus free oocytes were used in the immunofluorescence experiments. Freshly collected oocytes (GV stage), oocytes cultured for 12 h (MI stage) and 24 h (MII) were used for vitrification.

Oocyte vitrification and warming: The vitrification and warming procedures were followed as per Rienzi *et al.* (2010). Oocyte survival was evaluated on the basis of the integrity of the oocyte membrane and the zona pellucida. The surviving vitrified thawed oocytes were subjected to *in vitro* fertilization.

In vitro *fertilisation (IVF):* The COCs were washed 3 times in modified Tyrode's solution (TALP) (IVF medium) as per Rho *et al.* (2002). Ten to fifteen oocytes were transferred into a 45 µl droplet of IVF medium overlaid with paraffin oil. Fresh semen collected from bulls at an artificial insemination centre was centrifuged twice at 1500 g for 5 min. The spermatozoa were resuspended in IVF medium at the concentration of 1.0×10^6 cells/mL. Finally, 5 µl of the sperm suspension was added to each droplet and cultured in a humidified atmosphere with 5% CO_2 in air at 39.8 °C for 12–18h.

Embryos cultured: The IVF embryos were cultured in 20 µl medium drops individually, and in 3 ml drops of synthetic oviductal fluid SOF with 4 mg/mL BSA (Wee *et al.* 2006), in a humidified atmosphere with 5% CO_2 at 38.5°C. The cleavage rates and the blastocyst rates were determined at 48 h and 7 days after fertilization respectively.

Indirect immunofluorescence and scanning confocal microscopy: The immunofluorescence procedure was carried out as per Li *et al.* (2011). The oocytes were immunostained with antibodies and microbule antibody diluted 1:100 in blocking buffer overnight at room temperature. Instrument settings were kept constant for each replicate, and experiments were replicated at least 3 times. The final procedure was counter staining by incubation of 10µg/mL propidium iodide (PI) for 10 min and rinse with PBS. The result of immunofluorescence was observed under a laser scanning confocal microscope, and relative fluorescent intensities were determined using an image analyzer system, by calculating the signal of α-tubulin and F-actin for microbule and microfilament.

Statistical analysis: At least 3 replicates were performed for each treatment. The proportions of embryos at each developmental stage were compared by chi-square χ^2 analysis. With SPSS software, a paired *t*-test was used to compare the values of different nuclear immunofluorescence intensities. A value of $P<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Recovery of IVM bovine oocytes.: After vitrification and warming, MII stage oocytes showed significantly higher ($P<0.05$) survival rate (50%), than that of GV stage (34.1%) and MI stage (39.3%) of the oocytes (Table 1). After 48-h culture, 24.6% of the MII stage oocytes developed to 2-cell stage and 10.5% reached blastocyst stage after 7d culture. However, in GV and MI stage oocytes, both cleavage rate and blastocyst formation rate were reduced (Table 1).

In the present study, after vitrification, the recovery and

Table 1. Recovery and development level of different frozen stages of oocytes

Stage of oocytes	No.of oocytes vitrified	No. of oocytes morphologically normal (%±SEM)	Survival rate (%±SEM)	Rate of cleavage (%±SEM)	Number of blastocysts (%±SEM)
GV	123	110 (89.4±4.1)	42 34.1±5.1	11 (10±1.4)	3(2.4±3..3)
M I	112	100 (89.3±5.6)	44 (39.3±2.8)	13 (11.6±3.6)	5(4.5±4.1)
M II	114	103 (90.4±3.3)	57 (50±3.3)*	28 (24.6±4.1)*	12(10.5±1.1)
Control	0	120 100%	106 88.3±1.9	**	81 67.5±5.1
**	27 22.5±4.2	*			

SD, Standard deviation. *Superscripts in the same column are significantly different ($P<0.05$; **Superscripts in the same column are distinct difference $P<0.01$. The cleavage rates and the blastocyst rates were determined at 48 h and 7 days after IVF respectively.

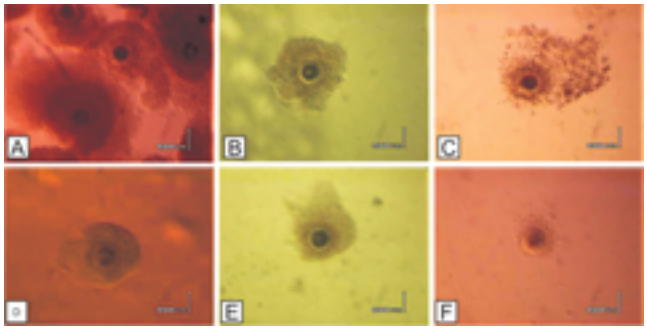

Fig. 1. Morphological figures of the COCs at different stage before (A-C) and after (D-F) vitrification (A) and D : GV-COCs; B and (E): MI COCs;(C) and (F):MII COCs. Bar=600μm.

effect of freezing on development after insemination of IVM bovine MII oocytes (Table 1) were better than another 2. Similarly, bovine oocytes reportedly tolerated chilling and cryopreservation better at the MII than the GV and MI stage (Rienzi *et al.* 2010.). Men *et al*. (2002) also observed a significantly higher proportion of cleaved bovine embryos from vitrified MII oocytes than those derived from vitrified other stage of oocytes developed into blastocysts, just as Aono *et al.* (2005) showed high rates of survival, maturation and 2-cell embryos formation.Moreover, the duration of exposure to vitrification medium seems to be very high (5 min) compared with the earlier studies; this was about less than 1 min (Ubaldi *et al.* 2010, Saragusty and Arav 2011). This may be the reason for the low survival, cleavage and blastocyst rate in current study.

The number of cumulus cells around the oocytes decreased a lot (Fig.1C vs 1F), after cryopreservation of germinal vesicle (GV) oocytes. Khosravi *et al.* (2010) also described such irreversible damage to the oocyte membrane or impaired intercellular communication between the oocyte and the cumulus cells after cryopreservation of germinal vesicle (GV) oocytes. Although a minority of cumulus free oocytes can complete meiotic maturation *in vitro,* the retention of cumulus cells appears to be important for oocytes to maintain their ability to mature to M-II (Bing *et al.* 2002) and the proportion of vitrified oocytes development was significantly lower (Park 2005).

Normalcytology observed in IVM bovine oocytes: Most oocytes had normal spindle configuration (Fig.2B and C) and chromosome (Fig.2A) alignment indicating a barrel-shaped structure and the chromosomes arranged on the equator of the metaphase plate in the control and nearly no difference in the stage of MII after vitrification (Fig.2F).

The oocytes in the stage of GV showed abnormal morphology, with some chromosomes displaced from the metaphase plate. The chromosomes are dispersed and less condensed (Fig.2D). The spindle is obvious with rounded poles and the chromosomes arranged on the plate of the equator but condensed (Fig.2E), and the microtubules are different to deserved.

In our study, the oocytes in the stage of GV showed chromosomes displaced from the metaphase plate, and the spindles did not emerge (Fig.2D), the abnormalities of chromosomes in GV oocytes *in vitro* after freezing are common (Parks and Ruffing 1992), and probably relate to freezing-induced impairment of factors critical to the assembly of the meiotic spindle. We examined in vitrified MII-stage oocytes 1–2 h after oocyte warming. The spindle is able to reform after oocyte warming, and we can find this reforming after the analysis of quantitative tubulin signals (Fig.2G). Gomes *et al.* (2008) showed that, although microtubules are disrupted immediately after cryopreservation of MII-stage oocytes, they are able to reform after culturing at 37°C, such that morphologically normal meiotic spindles become apparent.

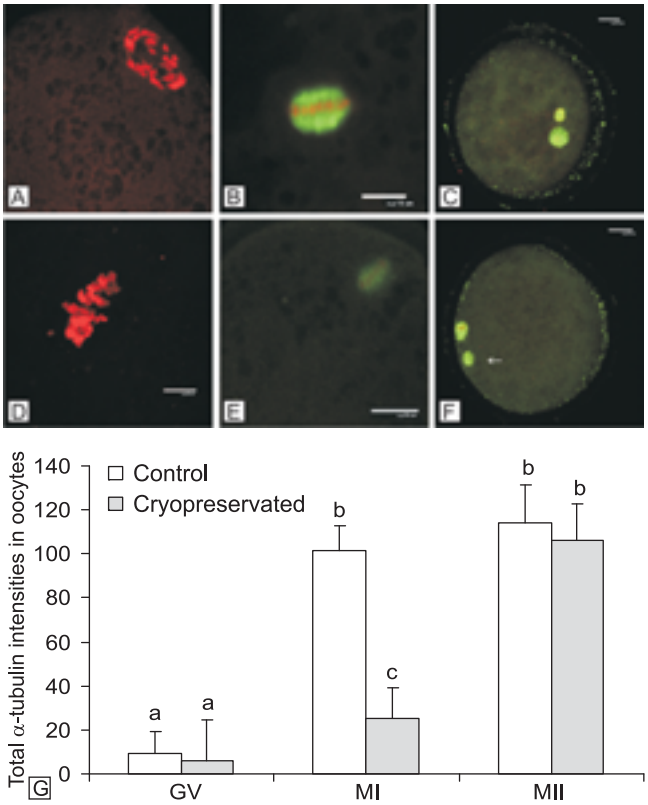

Fig. 2. Microbule distribution in bovine oocytes and effects of cryopreservation, viewed with epifluorescence microscopy. (A-C) Normal cytology observed in IVM bovine oocytes maintained at 39.8°C; (D-F) The IVM bovine oocytes after cryopreservation viewed with epifluorescence microscopy. (A) and D): GV COCs; (B) and (E):MI COCs; (C)and (F): MII COCs. G: The quantitative analysis tubulin signals of oocytes before and after cryopreservation. Oocytes stained for microtubules with anti-tubulin/FITC anti-IgG, Green; Chromosomes stained with PI,Red;.Bar=20μm. Green images represent microtubules, red images represent chromosomes, immature oocytes at the GV stage do not have any microtubular spindle, and yellow images represent the merging of green and red images. Arrowheads indicate the first polar body (PB1).

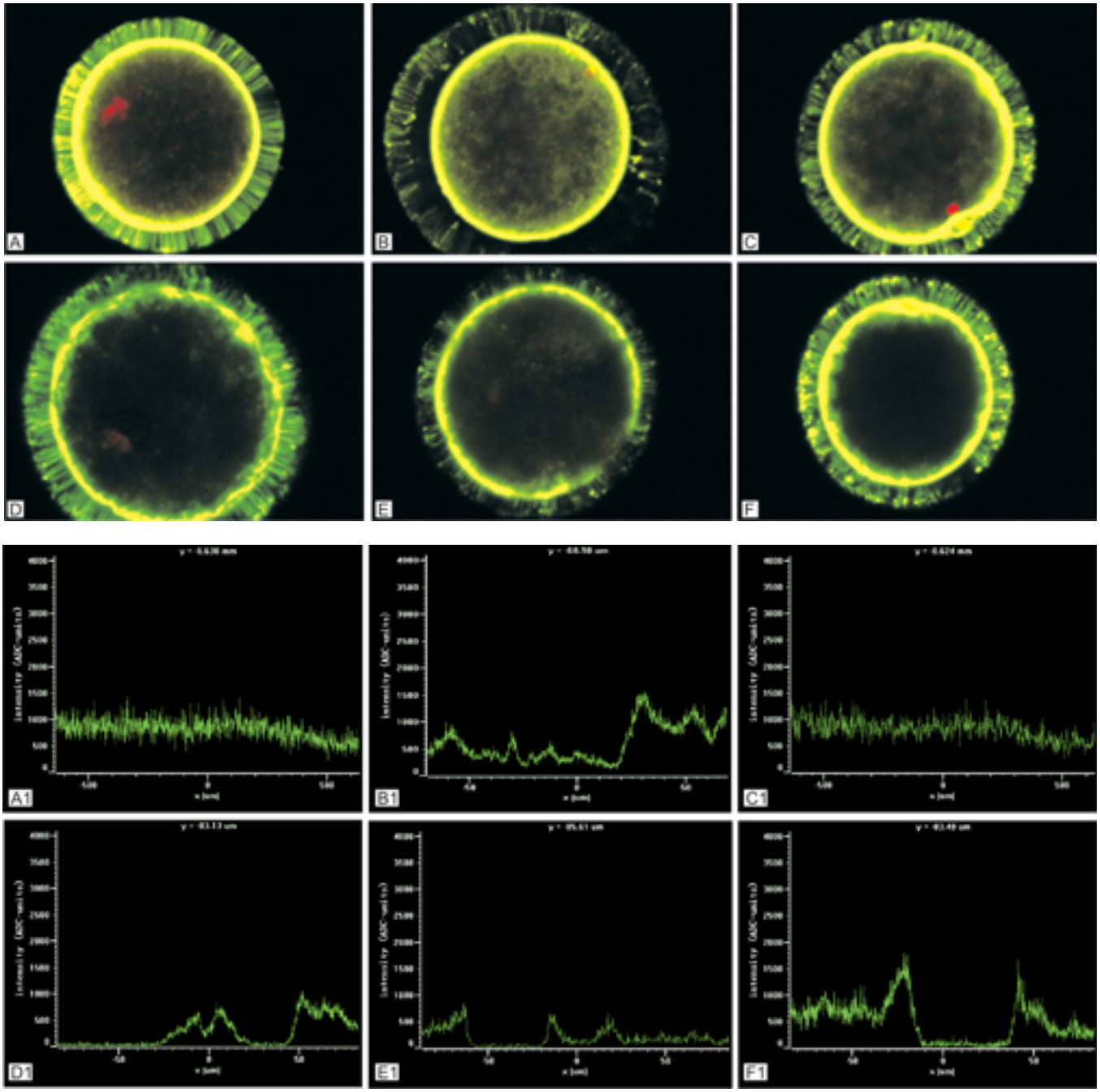

Fig. 3. Microfilament distribution in bovine oocytes and effects of cryopreservation, viewed with epifluorescence microscopy.(A-C) Normal, even distribution of microfilaments with a brighter area of fluorescence immediately beneath the plasma membrane (the actin band). (Fig.3D-E) "Fading" actin pattern; distribution of microfilaments is discontinuous and staining intensity is uneven throughout vitrified oocytes. (Fig.3A1-F1) The oscillogram of microfilament fluorescent expression. (A) and D : GV COCs; (B) and (E): MI COCs; (C) and (F): MII COCs. Oocytes stained for microfilaments with anti- actin filaments/FITC anti-IgG, Green; PI,Red; Bar=20μm.

Because GV-stage oocytes have not yet formed meiotic spindles and their chromatin is decondensed within the nuclear envelope (Fig. 2), they may be less susceptible to freezing damage that would otherwise disrupt the spindle in an MII stage oocyte (Park *et al.* 2005). Therefore, the cryopreservation of GV-stage oocytes is an attractive alternative for freezing female gametes. But in present study, the oocytes frozen at the GV stage have lower survival rates and developmental competence than oocytes frozen at the MII stage (Table 1).

Since the major concerns about spindle damage in oocyte freezing are all linked to the use of mature oocytes (MII), several attempts were made to freeze immature oocytes (germinal vesicle (GV)), in which the meiotic spindle is not yet formed. And evidence suggested that damage to the DNA and/or microtubules could explain the limited success of oocyte cryopreservation (Mark *et al.* 2006).

Microfilament distribution in bovine oocytes and effects of cryopreservation: Filamentous actin was examined separately for distribution throughout the oocyte and for the fluorescence intensity and continuity of actin immediately beneath the oolemma, referred to as the actin band. Actin in most untreated oocytes appeared homogeneously distributed with a distinctively more intense cortical actin band (Fig. 3 A-C).The vitrified oocytes with "Fading"and discontinuous staining pattern were as having an abnormal microfilament distribution.

The present study showed a dramatic change in distribution of actin in the GV and MI stage oocytes after vitrification and warming. Many oocytes with disrupted microfilament organization also had a discontinuous and a "fading'' actin pattern. Because of the association of microfilaments with other structures, it is possible that their disruption is the result of damage to another cellular component such as the plasma membrane or mitochondria. Moreover, the oscillogram of microfilament fluorescent expression reflects the "fading'' actin pattern (Fig. 3. D1-F1), the stable line was dropped sharply, which means the cryopreserved oocytes membrane integrity was destructed. Thouas *et al*. (2004) observed that no vitrified oocyte survived the process as evidenced by use of vital stains that depend on membrane integrity. Disruption of the plasma membrane and mitochondria were also observed in frozen-thawed bovine oocytes (Ohboshi *et al.* 1998).

The cleavage rate of GV and MI oocytes is 10 % and 11.6% (Table 1), respectively, while the cleavage rate of MII oocytes is 24.6%, and this phenomenon maybe caused by the disrupted microfilament, just as the "Fading'' actin pattern (Fig.3), and this irreversible change made the oocyte quite sensitive to surrounding conditions, such as the osmotic stress, solute effects, or intracellular ice formation intrinsic to the freezing process, arresting the oocytes penetrated by sperm development further (Ubaldi *et al*. 2010).

In a more general sense, cryopreservation of supernumerary oocytes instead of embryos may also be regarded as a step towards decreasing differences between genders regarding choice in reproduction (Homburg *et al.* 2009).

In conclusion, the vitrified GV and MI stage of oocytes showed chromosome and cytoskeleton disruption, an abnormal microfilament distribution with a discontinuous staining pattern, and the survival rates were significantly ($P<0.05$) lower than those of MII oocytes, the cryopreservation of GV or MI oocytes seems could not instead of the MII oocytes.

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