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Downsizing cumulus cell layers to improve cryotolerance of germinal vesicle-stage bovine oocytes

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ABSTRACT

This study was undertaken to investigate whether complete removal or downsizing of the cumulus cell layers in germinal vesicle (GV)-stage bovine cumulus-oocyte complexes (COCs) can improve blastocyst development rate following Cryotop vitrification. Downsized COCs (196 μm in mean diameter) and denuded oocytes (141 μm in mean diameter) were prepared by vortex-mixing of full-sized COCs (330 μm in mean diameter) retrieved from abattoir-derived ovaries. Nuclear maturation rates, assessed by the first polar body extrusion, after vitrification and the subsequent 22-h IVM were comparable (61.9–62.9%). Approximately one-third (30.5–31.2%) of the matured oocytes derived from the downsized COCs could develop into high quality blastocysts after 6-h IVF and 8-d IVC, while 13.4 and 23.7% of the matured oocytes derived from denuded oocytes and full-size COCs reached to the blastocysts, respectively. Cytoplasmic lipid droplets of matured oocytes in vitrification group were more clustered with decreased number and increased size of the droplets, when compared to those in fresh control group. However, individual oocyte culture in well-of-the well system suggested that change of lipid droplet distribution in the matured oocytes had no adverse effect on their subsequent developmental competence up to the blastocyst stage. In conclusion, Cryotop vitrification of downsized GV-stage bovine COCs allowed blastocyst yields as high as >30%.

1. Introduction

Gamete cryopreservation can be an integral part of various assisted reproductive technologies (ART) in cattle. But, cryopreservation of bovine oocytes is still at the experimental stage even after ultra-rapid vitrification protocols have been applied to improve their developmental potential following in vitro fertilization (IVF) and in vitro culture (IVC) [1–3]. On the other hand, oocyte vitrification has become routine for banking huge number of transgenic strains in rodents [4] and therapeutic or conservative application to human infertility [5,6]. Enrichment of lipid droplets in oocytes from domestic species including bovine and porcine species is considered as a factor negatively influencing to their cryotolerance [7,8], thus making cryopreservation of bovine oocytes less practical. Supplementation of L-carnitine to in vitro maturation (IVM) medium of bovine oocytes has been reported to improve the cryotolerance of the oocytes by changing the distribution of cytoplasmic lipid droplets [9], but it is still controversial whether the positive effect of L-carnitine supplementation is reproducible [10].

Oocytes ovulated or retrieved immediately before ovulation, or in vitro-matured oocytes have been cryopreserved with or without the surrounding cumulus cells. They are assumed to be at metaphase-II (M - II) stage, and spindle apparatus in the M - II oocytes is known to be temperature-sensitive [11,12]. Blastocyst yields from vitrified-warmed bovine M - II oocytes after IVF and IVC ranged 11–36% [1–3,9,10,13–15]. We have recently achieved the high blastocyst yield (36%) when cumulus-free matured oocytes were vitrified-warmed using Cryotop as device for ultra-rapid cooling and treated with α-tocopherol prior to the IVF [15]. Since chromosomes of immature oocytes are packed in germinal vesicle (GV) without spindle apparatus, the GV stage oocytes would be an alternative stage for cryopreservation to avoid chromosomal abnormality in cryopreserved M - II stage oocytes [16]. Research for

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GV oocyte cryopreservation is important because of the increasing demands to retrieve human GV oocytes in cases of ovarian hyperstimulation syndrome (OHSS) or cancer [17].

Cryopreservation of GV-stage oocytes has not yet been established even in small rodents or humans [18]. The GV-stage oocytes are connected with the surrounding cumulus cell layers across the zona pellucida via gap junction (connexin-37), so-called cumulus-oocyte complexes (COCs). While such a functional unit, COC, is essential to harvest developmentally competent oocytes after the IVM [19], the presence of several layers of cumulus cells in the COC may result in the delayed exchange between intracellular free water and permeable cryoprotective agent (CPA) during equilibration and dilution phases [20]. In addition, hyperosmotic conditions and ultra-rapid cooling during vitrification procedure had detrimental impact on functional integrity of gap junctional communication in some species including cattle [21–23]. Blastocyst yields from bovine COCs vitrified-warmed at GV-stage were extremely low as 2–19% [24–31]. A few reports described the effect of full or partial denuding of bovine COCs on their cryotolerance, with limited success [25,29–31].

The objective of this study was to determine whether complete removal or downsizing of the cumulus cell layers in GV-stage bovine COCs can improve blastocyst development rate and the resultant blastocysts were normal. Since distribution of ooplasmic lipid droplets was different between vitrification and fresh control groups, it was also investigated how this change was associated with developmental potential of the vitrified GV oocytes.

2. Materials and methods

2.1. Experimental design

In the first experiment, effect of cumulus cell volume during Cryotop vitrification of GV-stage bovine COCs on nuclear maturation and developmental potential after IVM/IVF/IVC was investigated. In the second experiment, well of the well (WOW) culture system was applied to connect cytoplasmic lipid droplet distribution of mature oocytes with their developmental potential to blastocyst stage. Quality of blastocysts produced in the WOW culture system was also compared between fresh control and vitrification groups.

2.2. Preparation of GV-stage COCs with different cumulus cell volumes

Unless otherwise stated, all chemicals used in this study were purchased from Sigma-Aldrich Chemicals. Local abattoir-derived bovine ovaries were transported to laboratory in 20–24 °C saline within 6 h after slaughter. The contents of follicles (2–8 mm in diameter) were aspirated with an 18-G needle connected to a 10-ml syringe. Oocytes surrounded by at least three layers of compact cumulus cells were defined as GV-stage COCs and collected in HEPES-buffered tissue culture medium (TCM)-199 (Earle's salts; Gibco™) supplemented with 3 mg/ml BSA, 0.2 mM sodium pyruvate, and 50 μg/ml gentamicin sulfate (defined hereafter as TCM-199/BSA). The partial or complete removal of the cumulus cell layers was performed by vortex-mixing in the TCM-199/BSA (5 s or 3 min) combined with pipetting. Thus, the GV-stage COCs were randomly allocated to three groups; full-size COC (FS-COC), downsized COC (DS-COC) and denuded oocyte (DO), as shown in Fig. 1.

2.3. Vitrification and warming

GV-stage oocytes with or without cumulus cell layers (FS-COCs, DS-COCs and DOs) were subjected to a vitrification procedure according to the method described previously [15], with minor modifications. Briefly, oocytes were rinsed with HEPES-buffered TCM-199 with 20% fetal bovine serum (FBS; HyClone™, GE Healthcare Life Science, Logan, UT, USA) base medium, and then equilibrated with 7.5% ethylene glycol (EG; Wako Pure Chemical Industries, Osaka, Japan) and 7.5% dimethyl sulfoxide (DMSO; Wako) in the base medium for 3 min at an ambient temperature (23–28 °C) and then transferred into a vitrification solution consisting of 15% EG, 15% DMSO, and 0.5 M sucrose in the base medium. Within 60 s, up to 12 oocytes were loaded onto the polypropylene strip of a Cryotop device (Kitazato Corp., Shizuoka, Japan) with a minimal amount of the vitrification solution and quickly plunged into liquid nitrogen (LN_2). After storage in LN_2 for >1 week, oocytes were warmed by immersing the polypropylene strip of Cryotop into 3 ml of base medium containing 1 M sucrose prewarmed to 38.5 °C for 1 min. The oocytes were transferred to the base medium in a stepwise manner (0.5, 0.25, and 0 M sucrose for 3, 5, and 5 min, respectively).

2.4. IVM

Fresh and vitrified-warmed COCs or DOs oocytes were matured in HEPES-buffered TCM-199 supplemented with 10% FBS, 0.2 mM sodium pyruvate, 0.02 AU/ml follicle-stimulating hormone (FSH; Kawasaki-Mitaka Pharmaceutical, Kanagawa, Japan), 1 μg/ml 17β-estradiol, and 50 μg/ml gentamicin sulfate for 22 h at 38.5 °C under 5% CO_2 in air (10–12 COCs per 100-μl microdrop under mineral oil). Then, cumulus cells were completely removed by vortex-mixing for 3 min in TCM-199/BSA containing 1000 IU/ml hyaluronidase. Oocytes were comprehensively checked for extraction of the first polar body, and oocytes with an extruded first polar body were defined as matured. Morphological survival rates of the oocytes were evaluated at this time point.

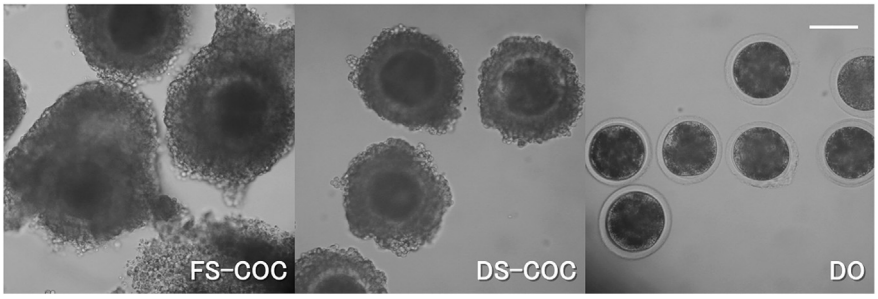

Fig. 1. GV-stage oocytes surrounded with or without cumulus cell layers. (A) Full-size COCs (FS-COCs), with 330 μm in mean diameter. (B) Downsized COCs (DS-COCs), with 196 μm in mean diameter. (C) Denuded oocytes (DOs), with 141 μm in mean diameter. Scale bar = 100-μm.