Development of the optimal conditions for vitrification of mammals cumulus-oocyte complexes

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Abstract. The aim of the study was to develop an optimal vitrification technology for the preservation and improve the survival of bovine and porcine cumulus-oocyte complexes (COCs). The objective of this study was to compare the efficacy of the vitrification of bovine COCs and porcine COCs by analysis of morphology after warming. Freezing of oocytes was carried out by vitrification in medium with different concentrations of various cryoprotectants (DMSO, EG and sucrose). The oocytes were transferred to a cryotube, which was transferred to liquid nitrogen. The warming procedure of oocytes was carried out in a solution of sucrose after 3-14 days of storage. Morphological assessment of oocytes and fluorescence microscopy were performed after the warming procedure. Survival of porcine COCs — 67.74%, bovine COCs — 81.25%. It was found that to assess the viability of oocytes should use fluorescent dye CAM. These results indicate that the our vitrification method without direct LN2 contact is more effective for vitrification of bovine oocytes.

1. Introduction
At the present time, two methods of cryopreservation are used to conserve oocytes and embryos, both animals (cows, pigs, etc.) and humans: slow and instantaneous freezing (vitrification). Vitrification was recognized as the most effective method for freezing oocytes and embryos [1]. During the vitrification procedure, cells and tissues are exposed to a high concentration of cryoprotectants, which effectively dehydrate the cells prior to initiation of the cooling process.

However, despite the fact that vitrification technology has become a breakthrough in cryobiology, the problems associated with the freezing and revitalization of animal oocytes and embryos have not yet been solved [2, 3].

In addition, there are still no standard protocols for vitrification of mammalian oocytes due to the number of physical patterns (cooling and freezing of liquid, formation of extracellular and intracellular ice), and features of the structure of female gametes [4]. Also, due to the structural features of oocytes, there is still no informative analysis of cell survival.

Therefore, the development of optimal technology for vitrification of mammalian oocytes is relevant for both farming sector and animal husbandry. Optimization of vitrification technology will allow increasing the success in selection of rare breed's animals by 5-7 times compared with method of artificial insemination.
1.1. Preparation of oocytes from ovaries

For our research 20 bovine ovaries and 20 porcine ovaries were transported from slaughterhouse to the laboratory in NaCl with an antibiotic in a thermos bottle within 2 h after slaughter of the animals. The age of bovine ranged from 2 to 9 years. The age of pigs ranged from 5 months to 3 years. Cumulus-oocyte complexes (COCs) were aspirated from follicles (2–8 mm in diameter) using a 5 ml syringe connected to a 19-gauge needle. COCs were washed twice in Dulbecco’s phosphate buffered saline (DPBS; Life Technologies Gibco-BRL Division) supplemented with 5% of FCS, transferred in holding medium (HM), which consisted of 25 mM Hepes-buffered TCM 199 supplemented with 20% (v/v) FCS. Thereafter, they were vitrified.

1.2. Vitrification of COCs

The groups of 5–10 oocytes were placed in equilibration medium, which was composed of HM supplemented with ethylene glycol (EG) and DMSO (Wako Pure Chemical Industries), for 10–12 min and then transferred into a vitrification solution composed of HM supplemented with EG, DMSO and 0.5 M sucrose. Medium for the vitrification procedure were prepared on the basis of PBS with addition of 40 μg/ml gentamicin, 1 mM sodium pyruvate, 2 mM L-glutamine and 20% FCS (all Sigma-Aldrich, USA).

Freezing of oocytes was carried out by vitrification. Then, a group of 5–10 oocytes was placed in a small volume of vitrification solution and transferred to a cryotube, which was plunged into liquid nitrogen (LN2) in the cryo-storage XT-34 (Taylor-Wharton, USA). Each cryotubes containing COCs was kept in LN2 for at least 48 h.

Thereafter, the devices were transferred into 0.5 ml of warming solution, which was composed of HM supplemented with 1 M sucrose, at 38.5 °C. Two minutes later, oocytes were consecutively transferred to 500-µl droplets of HM supplemented with 0.5 M sucrose at 38.5 °C for 5 min. They were washed three times with HM at 38.5 °C and were analyzed.

1.3. Assessment of survival rate of COCs

Morphological assessment of oocytes and fluorescence microscopy were performed for the analysis of survival rate. Two special kits for died/apoptosis cell (PI/Alexa Fluor 488 Annexin, Molecular Probes, USA) and LIVE/DEAD® Viability Assays (Calcein AM (CAM)/ethidium homodimer-1(EthD-1), Molecular Probes, USA) were used for the staining of COCs by instruction. Briefly, the COCs were simultaneously treated with Alexa Fluor 488 Annexin and 0.1 mg/ml propidium iodide (PI) in DPBS for 60 sec and stain the nuclei of cumulus cells or with CAM and EthD-1 for detection of viability cumulus cells. The COCs were then treated for 5 min with 25 μg/ml Hoechst 33342 (Calbiochem, San Diego, CA, USA) dissolved in 99.5 % ethanol, and then mounted on glass slides in glycerol droplets that were flattened by coverslips. The COCs were examined under UV light with an excitation wavelength of 330–385 nm using an epifluorescence microscope (Nikon, Japan). The nuclei of COCs cells labeled with both PI (EtHd-1) and Hoechst 33342 appeared pink or red, whereas the nuclei of cells labeled only with CAM or Annexin appeared green, and Hoechst 33342 appeared blue. A digital image of each COCs was captured, and the cell numbers of all cell types were counted using the software.

2. Results and Discussion

After aspiration of antral and preovulatory follicles from ovaries of 20 pigs 402 oocytes with different morphology and maturity (from GV to MII) were obtained and 255 oocytes with different morphology and maturity (from GV to MII) were obtained by aspiration of antral and preovulatory follicles from 20 bovine ovaries. From all the obtained COCs 31.8 % porcine COCs and 30.6 % bovine COCs were degenerative. Oocytes with normal morphology were selected for vitrification procedure.

It was also found that for one pig at the age of 5-6 months has an average of 41 oocytes, at the age of 11-12 months — 13 COCs, from 2 to 3 years — 4 COCs.
After prolonged storage the 31 porcine oocytes and 32 bovine oocytes were thawed used for the staining of COCs by PI/Alexa Fluor 488 Annexin (Figure 1) or for CAM/EtHd-1 (Figure 2).

![Figure 1](image1.png)

**Figure 1.** Morphology of bovine oocytes after vitrification with EG and DMSO and subsequent revitalization. Dyeing PI — A. Dyeing AlexaFluor 488 Annexin — B. Without dyeing — C.

![Figure 2](image2.png)

**Figure 2.** Morphology of bovine oocytes after vitrification with EG and DMSO and subsequent revitalization. Dyeing CAM — A. Without dyeing — B, D. Dyeing EtHd-1 — C.

Morphological assessment of oocytes and fluorescence microscopy were performed after the thawing procedure. Oocytes from pigs showed lower survival rates 67.74% (10/31) than those from the bovine oocytes with normal morphology — 81.25% (26/32). The remaining porcine and bovine oocytes had structural changes characteristic of degenerative oocytes. The results obtained after fluorescence microscopy correspond to the proportion of porcine and bovine oocytes with normal morphology.

As a result of staining of COCs with fluorescent dyes, it was found that to assess the survival of oocytes should use fluorescent dye CAM. Since fluorescent dyes PI and EtHd-1 did not display the process of cell death of the oocyte. In this regard, PI and EtHd-1 can’t act as indicators of the true death of oocytes.

In this study, it was demonstrated for the first time that both bovine and porcine COCs can be successfully cryopreserved by the vitrification. Although vitrification with EG and DMSO has been found to be a highly efficient method for cryopreservation of bovine oocytes then porcine. Compared with other species, oocyte cryopreservation in pigs is still considered a challenge because of the enormous sensitivity of porcine oocytes to low temperatures.
Similar results have been reported previously in porcine oocytes [5], but for bovine usually to compare the efficacy of oocyte development rates after in vitro-produced embryos at the blastocyst stage [6; 7].

3. Conclusion
Thus, the optimal vitrification technology developed as a result of the research can be successfully applied both in agriculture and animal husbandry, and for reproductive medicine (transfer of this vitrification technology from the animal model to the freezing of human oocytes). However, it is necessary for future studies to investigate live calf production from oocytes and embryos after vitrification. The developed vitrification technology can be used to preserve genetic resources (creation of cryobanks) of rare animals, genetically modified and cloned animals.

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