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THE TECHNIQUE FOR CRYOPRESERVATION OF CATTLE EGGS

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ABSTRACT

The aim of this investigation was to establish a methodology of cryopreservation of cattle eggs (oocytes) under our laboratory conditions. For cryopreservation of *in vitro* matured oocytes, the freezing in minimum volume by ultra-rapid cooling technique was used. Oocytes with at least three layers of cumulus cell were placed into the equilibration solution (ES: 3 % ethylene glycol in M199-HEPES, supplemented with 10 % foetal calf serum) for 12 min. Following equilibration, the oocytes were transferred to vitrification solution (30 % EG + 1M sucrose in M199-HEPES with 10 % foetal bovine serum) at room temperature for 25 sec. Then the oocytes were placed onto nickel grid (electron microscopy grade) and plunged into liquid nitrogen. After thawing the oocytes were fertilized *in vitro*. Development of produced embryos (cleavage on day 2, and blastocyst yield on day 7) and total cell number of blastocysts after DAPI staining were determined. We obtained a relatively high cleavage rate (55.81 %) of fertilized oocytes after thawing; of them 11.24 % developed to the blastocyst stage. The quality of blastocysts obtained from vitrified oocytes was similar to the control blastocysts, as evidenced by a comparable total cell number (84.45 vs 97.29, resp.). In conclusion, the designed freezing technique proved to be suitable for cryopreservation of cattle oocytes, nevertheless further optimization is required.

Key words: bovine; oocyte; embryo; blastocyst rate; vitrification

INTRODUCTION

Vitrification has for several years been considered as a promising option in oocytes cryopreservation, because it was successfully used previously to freeze embryos. Fuku *et al.* (1992) was the first to publish successful vitrification of bovine oocytes. However, the developmental results after fertilization were not satisfactory. A significant progress in this area was achieved later when Martino *et al.* (1996) and Vajta *et al.* (1998) applied an approach of minimizing the vitrified sample to obtain a much faster cooling rate. Since then, several papers have been published worldwide describing different techniques of vitrification of bovine oocytes. Several authors deal either with an appropriate stage (GV, MII) during maturation, where it is safest to freeze oocytes (Diez *et al.*, 2005; Sprícigo *et al.*, 2014; Bulgarelli *et al.*, 2018), or with the presence/absence of cumulus cells during vitrification (Ortiz Escribano *et al.*, 2016). Moreover, various formulations of the vitrification medium were tested (Chian *et al.*, 2004; Magnusson *et al.*, 2008). Several different devices have been used for the vitrification, which specifically minimize the volume of frozen vitrification medium, such as open-pulled straw (OPS; Vajta *et al.*, 1998), micro-drop (Papis *et al.*, 2000), nylon loop (Lane a Gardner, 2001), "hemi-straw" (HS; Vanderzwalmen *et al.*, 2003), electron-microscopic grid (Martino *et al.*, 1996) or Cryotop (Kuwayama *et al.*, 2005). However, the results of all these works are considerably varied and the described protocols are often unreproducible. There is still no consistent methodology to ensure high survival of bovine oocytes after thawing and good embryo development after fertilization.

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The aim of this work was to establish a methodology of cryopreservation of female cattle gametes for the purpose of cryostorage in a bank of animal genetic resources, as well as possibilities to improve the survival of cryopreserved eggs.

MATERIAL AND METHODS

Oocyte retrieval and *in vitro* maturation (IVM)

The ovaries were isolated from undefined cows at a local abattoir and transported to the laboratory. The oocytes were recovered from antral follicles (2-8 mm) by the aspiration of follicular fluid using sterile syringe with a needle. Cumulus-oocyte complexes (COC) were collected into a Petri dish with a holding medium (M199-HEPES with 10 % fetal bovine serum-FBS) and only COC with several layers of cumulus cells and homogeneous ooplasm were selected for *in vitro* maturation. COCs intended for vitrification were matured for 21 hours and those selected for control group were matured for 23 hours in a maturation medium containing TCM 199 (Gibco), sodium pyruvate (0.25 mmol. L^{-1}), gentamycin (50 µg.ml^{-1}), FBS 10 % and FSH/LH (1/1 I.U., Pluset) at 38.5 °C and 5 % CO_2.

Cryopreservation of oocytes

For cryopreservation of *in vitro* matured oocytes, ultra-rapid cooling technique in minimum volume was used. Selected matured oocytes were stripped off an excessive cumulus layers by vortexing during 30 s. Oocytes with approx. three remaining cumulus cell layers were placed into equilibration solution (ES: 3 % ethylene glycol (EG) in M199-HEPES, supplemented 10 % FBS) for 12 min. Following equilibration, the oocytes were transferred to vitrification solution (30 % EG + 1M sucrose in M199-HEPES with 10 % FBS) at room temperature for 25 sec. The oocytes (10-15) in a small drop were placed with a glass micropipette onto 300 mesh nickel electron microscopy grids (EM grids; Figure 1.), an excessive medium was removed by a filtration paper and then the oocytes were immediately plunged into liquid nitrogen for storage (several weeks).

For warming, nickel grids were directly transferred into thawing solution (0.5M sucrose in M199-Hepes, at 37 ˚C) for 1 min. The warmed oocytes were transferred across the three diluent solutions (0.25M, 0.125M and 0.0625M sucrose in M199-HEPES) for 3 min in each, and then washed twice in M199-HEPES with 10 % FCS for 5 min. Oocyte survival was evaluated on the basis of the integrity of the ooplasm and the *zona pellucida* after 2 h culture post-thawing.

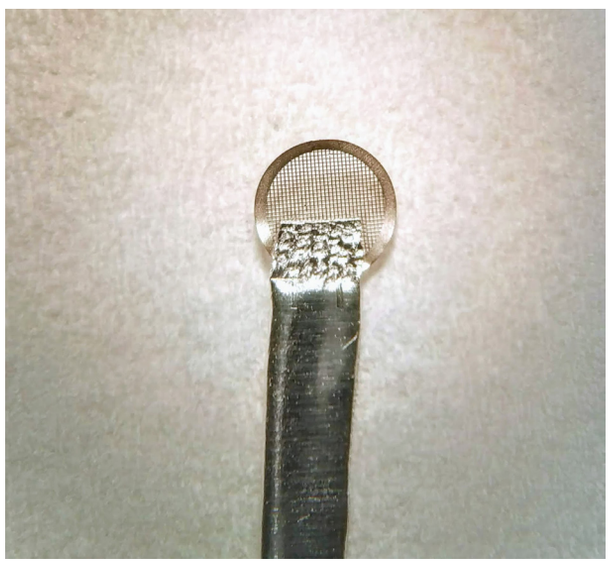

Figure 1. Electron microscopy 300 mesh grid (EM grid) fixed in a holder

***In vitro* fertilization (IVF) of vitrified-warmed oocytes and embryo culture**

Warmed morphologically good-looking oocytes were washed in IVF-TALP medium (TALP solution, 10 µg.ml^{-1} heparin, 50 µg.ml^{-1} gentamycin) and put into 100-µl droplets of IVF medium under a mineral oil, where the sperm (at 2×10^6 per ml) and PHE solution (20 µM penicillamine, 10 µM hypotaurine, 1 µM epinephrine) was previously added, and incubated for 18 h at 39 °C in 5 % CO_2. Following insemination, presumptive zygotes were vortexed in centrifuge tubes containing 0.5 ml holding medium for 30 s to remove residual cumulus cells. Denuded zygotes were transferred to the dish with the Buffalo Rat Liver (BRL) – cell confluent monolayer in B2 medium with 10 % FCS.

On the Day 2 since insemination – the cleavage rate, and on Day 7, 8 and 9 – the blastocyst rate were determined.

Fluorescent staining

Randomly selected blastocysts on day 7 were fixed and stained to count the total cell number. The blastocysts were washed twice in medium and in the PBS with 0.6 % of polyvinylpirrolidone and immediately fixed in 4 % formalin for 10 min. Then the embryos were covered with a drop of Vectashield anti-fade medium containing DAPI fluorochrome (chromatin staining; Vector Laboratories, Burlingame, CA, USA), mounted between a coverslip and microslide and shortly stored at 4 °C until fluorescence analysis. Stained embryos were checked under a Leica fluorescence microscope using specific filter with wavelength for blue fluorescence and x 20 magnification objective.

RESULTS

In our study totally 378 oocytes were frozen using minimum volume vitrification technique. Fewer oocytes (25) were lost or damaged during freezing or thawing and 352 oocytes were successfully thawed. From these, 85 oocytes were excluded after 2 hours of culture, because they morphologically appeared as not surviving freezing/thawing procedures. Remaining 267 oocytes were fertilized *in vitro*. Results of cleavage and blastocyst rate of vitrified and *in vitro* fertilized oocytes are presented on Table 1.

Randomly selected blastocysts on Day 7 were fixed and stained with DAPI fluorescent nuclear dye. Total numbers of nuclei/cells were counted using a Leica fluorescence microscope. The mean total cell counts in D7 blastocysts from control and vitrified oocytes were not significantly different (Table 2).

DISCUSSION

Previously we focused our investigations on cryopreservation of eggs being in ovarian tissues from cows for the purposes of national gene bank of animal genetic resources (Makarevich *et al.*, 2017). However, using this cryopreservation strategy we did not obtain promising results, as the oocytes were damaged and did not develop further (Makarevich *et al.*, 2018). In present work we are dealing with the developing a methodology for cryopreservation of mature bovine oocyte based on published reports with the best results after fertilization. We used the technique of ultra-rapid vitrification. The critical factor for ultra-rapid freezing is the minimization of the volume of medium frozen together with the oocyte. The minimum volume of the frozen sample ensures a sufficiently rapid drop

Table 1. Development of fresh or vitrified-warmed oocytes after IVF

Groups	Oocytes totally	Oocytes vitrified	Oocytes warmed	Oocytes in IVF	Embryo cleavage n (%)	Blastocyst rate n (%)
Vitrified	378	378	352	267	149 (55.81)[a]	30 (11.24)[a]
Control	404	-	-	404	293 (72.52)[b]	93 (23.02)[b]

[a] versus [b] – difference is significant at $p < 0.05$ (Chi-square test).

Table 2. Total cell number of blastocysts *in vitro* produced from vitrified or fresh bovine oocytes

Groups	No. blastocysts	Cell number (x ± SEM)
Vitrified/warmed	11	84.45 ± 10.16
Control (fresh)	14	97.29 ± 6.43

Differences between groups is not significant (t-test).

in the temperature (cooling rate) for the creation of amorphous ice without crystal formation. While in the plastic straw, traditionally used for embryo cryopreservation, the cooling rate was set at approximately 4000 °C.min^{-1}, with the minimum volume method this cooling rate increased to 22800 °C.min^{-1} (Kuwayama *et al.*, 2005), which is sufficient for solidification without crystallization.

As a carrier enabling the volume of frozen medium to be minimized we chose an electron microscopy mesh grid (EM grid), previously reported by Martino *et al.* (1996), which we improved by adding a holder (Figure 1). The added holder allows more convenient and faster handling of the grid with oocytes. The undoubted advantage of using EM grids is the possibility of simply sucking off the excessive vitrification medium while leaving the oocytes safely retained on the grid. The whole process is done very quickly. It has been reported that the time during which the oocytes are exposed to the vitrification solution before freezing is crucial for good oocyte survival after thawing. Papis and colleagues (2000) observed that development was substantially weaker in the group of oocytes that were in contact with the vitrification medium for 45 sec before freezing versus the group that was exposed to the vitrification medium for only 30 to 32 seconds. Similarly, Vajta *et al.* (1998), although choosing another method of equilibration, obtained promising results when oocytes were exposed to the vitrification medium for only 25 seconds. Therefore, we also chose 25 seconds as the time of exposure to the vitrification medium before freezing.

A great deal of the work done later could not repeat these good results (about 25 % of blastocysts; Vajta *et al.*, 1998; Papis *et al.*, 2000). However, numerous follow-up researchers obtained only blastocyst rates below 5 % (Chian *et al.*, 2004; Zhou *et al.*, 2010; Prentice *et al.*, 2011; Sprícigo *et al.*, 2014; Wiesak *et al.*, 2017; Bulgarelli *et al.*, 2017 a.o.). Remarkably, in all mentioned reports with low blastocyst rate (up to 5 %) the oocytes were exposed to ethylene glycol at a higher concentration (mostly 7.5 %) for several (5 to 10) minutes prior to freezing.

Generally, vitrification process impairs the blastocyst formation from bovine vitrified oocytes regardless of the protocol, the cryodevices and cryoprotectants used. The decreased developmental capacity of oocytes is mainly due to the toxic action of cryoprotectants. This effect was confirmed by the decreased developmental competence of oocytes exposed to cryoprotectants without freezing (Martino *et al.*, 1996). Papis *et al.* (2000) reported a "slight" equilibration at a low ethylene glycol concentration (3 %) that is sufficient to saturate oocytes during prolonged culture/equilibration (12 min) but minimizes toxic effects and does not significantly reduce oocyte developmental competence after fertilization compared to higher concentrations. A similar strategy was successfully used by Ishii and colleagues (2018).

Another important part of our methodology is the use of three-step decreasing sucrose concentration after thawing. Sucrose solution is used to restrict water permeation into the oocytes and to prevent excessive swelling of the oocytes as the cryoprotectant leaves the cells. Nowshari *et al.* (1998) tested three dilutions of cryoprotectant in one-, two- or three-steps of descending sucrose concentrations after thawing. Their experiments demonstrate that the three-step dilution procedure resulted in the highest number of mouse oocytes cryopreserved by ultra-rapid cooling developing to blastocysts. Good results were obtained also after thawing bovine oocytes using two-step (Kuwayama *et al.*, 2005) or three-step (Ishii *et al.*, 2018) dilution procedure.

In conclusion, using previously published knowledge in the field of vitrification of bovine oocytes, we have compiled and tested ultra-rapid vitrification in very small volume of vitrification solution under conditions of our laboratory. We obtained relatively high embryo cleavage rate (55.81 %) of *in vitro* fertilized oocyte after thawing, and 11.24 % of cleaved embryos developed to the blastocyst stage. The quality of these *in vitro* produced blastocysts was similar to the control, as evidenced by a comparable total cell count. Nevertheless, further research is needed to improve efficiency of in vitro embryo production.

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