Rapid method for the bovine zygotes vitrification

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Abstract. For the first time, rapid vitrification method was used for bovine zygotes. According to the results of zygote survival, as well as the rate of embryos cleavage obtained after warming of zygotes cryopreserved by rapid vitrification, no significant differences were found between the modified and standard vitrification methods. Survival rates were similar in both groups (92.0% and 93.0%, respectively). The rate of blastocysts formation was 180.40 ± 6.17 hours in the control group during vitrification of zygotes according to the standard protocol, 179.85 ± 9.12 according to the modified protocol; there were no statistically significant differences between groups. The rapid vitrification method reduce the time oocytes and zygotes are hold at suboptimal temperature and osmolarity, thereby preserving the potential suitability of oocytes for gene editing and long-term cryopreservation in cryobanks.

1 Introduction

Cryopreservation is a convenient and widely used method for storing and facilitating access to biological materials. Cryopreservation of cells, tissues and organs is important for human medicine and veterinary, in such areas as reproductive technologies and banking of gametes and embryos, transplantation, as well as in fundamental research work on laboratory animals, including genetic engineering and cloning. Currently, a variety of methods have been developed for the cryopreservation of gametes and embryos, which can generally be divided into two main groups: slow freezing and vitrification [1, 2]. The main damage to cells during cryopreservation occurs due to osmotic changes in cell volume, intracellular ice formation, changes in cytosol concentration and thermal stress due to cooling and warming [5]. During cryopreservation, extracellular water is divided into two parts. The first part forms ice, which does not dissolve intracellular substances. The second part is unfrozen water, which dissolves the substances in the cytoplasm. Therefore, the concentration of a solution of substances in cells cytoplasm increases with the ice forming. But high concentration of the solution is toxic to cells [4].

The main principle for slow freezing of gametes and embryos is a controlled gradual decrease in temperature from -5 -7°C to approximately -30-35°C at a rate of 0.5°C/min.

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This cooling rate provides sufficient time for water to leave cells so that damaging intracellular ice crystals do not form. Seeding, which prevents excessive cooling, is used in order to prevent spontaneous ice formation, induction of ice crystals formation. The second aspect is to minimize the osmotic swelling of embryonic cells during thawing. Glycerin and/or ethylene glycol are most often used as cryoprotectants for this freezing method. However, this method requires a lot of time – 3-5 hours, as well as specialized equipment – a freezer that controls the rate of temperature decrease [1, 2].

Another method of cryopreservation is vitrification. In this case, cooling occurs extremely quick, without ice forming. The idea of vitrification, achieving a glass-like state [9]. The glass formation depends mainly on the rate at which cooling and warming occurs, as well as on the viscosity and concentration of cryoprotectors. Therefore, during vitrification, cells and tissues are exposed to high concentrations of cell-penetrating cryoprotectants, such as dimethyl sulfoxide (DMSO) and ethylene glycol, which quickly dehydrate cells before the cooling process begins [9]. Currently, the vitrification method is widely used in assisted reproductive technology (ART) clinics for the treatment of human infertility due to the low cost and reduced time of the vitrification protocol (12-15 minutes). However, there are risks using this method, including damage due to the toxicity of cryoprotectants, which can lead to negative consequences, for example, a decrease in the level of embryo cleavage and blastocyst formation, or cause epigenetic and transcriptomic changes in oocytes and embryos [10]. Despite this, most vitrification protocols currently include an initial prolonged exposure phase of 10 to 15 minutes to equilibrate cells. Under the influence of hypertonic solutions of cryoprotectants, cells lose water and quickly shrink to a minimum volume. After this, cells, being in the same solution, slowly swell – equilibrate – to their original volume. Next the cells are immersed in a vitrification solution that contains an even higher concentration of penetrating cryoprotectants. In this solution, cells are applied to the carrier and cooled to -196°C. The duration of exposure to high concentrations of cryoprotectants is critical and should not exceed 60-90 seconds. Thus, the total time of holding oocytes and embryos at suboptimal temperature and osmolarity during vitrification according to the standard protocol ranges from 11 to 16 minutes. However, prolonged exposure to suboptimal conditions significantly reduces the cell suitability for traumatic manipulations such as gene editing, cloning, biopsy of embryos, intracellular injections, etc. Ultimately, this affects the safety of cells after thawing, level of blastulation and embryo survival.

In 2019, Gallardo M proposed a rapid vitrification method for human oocytes, in which the duration of the process from transfer the oocyte in a cryoprotector to liquid nitrogen was no more than two minutes [4]. Gallardo's work shows that this time reduction is feasible for human oocytes. Observations of the osmotic behavior of oocytes show that dehydration when exposed to standard solutions of cryoprotectants occurs very quickly: the minimum volume point of the shrinkage-swelling curve is reached in 60 seconds [4]. Subsequently, good results were obtained using a rapid vitrification protocol for mouse oocytes [3].

In this work, we were the first to apply a rapid vitrification protocol for the cryopreservation of bovine zygotes.

2 Materials and Methods

2.1 Oocyte collection

Bovine ovaries were collected postmortem and transported to the laboratory while maintaining a temperature of +37-38ºC for 3–4 hours after collection [8]. Visualized follicles from 2 to 8 mm were aspirated using an 18G needle. To carry out manipulations

outside the incubator oocyte-cumulus complexes (OCC) were placed in a G-MOPS buffer medium (Vitrolife, Sweden) preheated to 38.5ºC.

2.2 Oocyte maturation *in vitro* maturation (IVM)

We used a specialized medium for the maturation of bovine oocytes BO-IVM (IVF-Bioscience, UK) and Oil for tissue culture (Sage, USA). OCC were in a maturation medium for 24-26 hours at a carbon dioxide level of 6.5 vol. %, oxygen – 5.0 vol. % at a temperature of 38.5ºC.

2.3 Sperm processing

For *in vitro* fertilization, cryopreserved bovine sperm was used, frozen in 0.5 ml straws. The straws were thawed in a water bath at 37ºC for 30 seconds. After thawing sperm were processed by density gradient centrifugation (Sperm Grad, Vitrolife) for 15 minutes 400g. After centrifugation, the sediment was washed for 10 minutes at 200g in a buffer medium (Sperm Rinse, Vitrolofe) with the additioin 3 IU of heparin [6].

2.4 *In vitro* fertilization

Sperm was added to the BO-IVF fertilization medium (IVF-Bioscience, UK) under oil with oocyte-cumulus complexes at a concentration of 1.0–2.0 x 10^6 motile spermatozoa [6]. Incubation was for 16-18 hours at a temperature of 38.5ºC, carbon dioxide level of 6.5 vol. %, oxygen – 5.0 vol. %. After 16-18 hours, the oocytes were denuded from cumulus cells and cryopreserved.

2.5 Vitrification of oocytes

Oocytes were vitrified using two methods. The first group of oocytes was vitrified using the Vitrification Media kit (Kitazato, Japan) and Cryotop (Kitazato, Japan) in accordance with the manufacturer's instructions (Fig. 1). The vitrification procedure began with equilibration of oocytes for 10–12 minutes in a solution with a low concentration of penetrating cryoprotectants (ES7.5% v/v DMSO + 7.5% v/v EG). After equilibration, oocytes/zygotes were placed in vitrification medium containing (15% v/v DMSO + 15% v/v EG) for 1 minute. Oocytes should remain in the vitrification environment for no more than 1.5 minutes before they are immersed in liquid nitrogen, including the time of application to the vitrification device. According to the manufacturer's recommendation, no more than 3 oocytes were placed on one straw. Vitrified biomaterials were stored in liquid nitrogen -196°C in Dewar flasks.

The second group of oocytes was cryopreserved in similar solutions of cryoprotectors, but the time in the solution for equilibration was only 1 minute (Fig. 1).

2.6 Warming of oocytes

This procedure was conducted by using the Thawing Media kit (Kitazato, Japan) according to the manufacturer's instructions (Fig. 1). For warming, the vitrification straw was immersed in 2–5 ml of medium containing 0.5–1 mol/L sucrose at 37 °C for 1 minute. Oocytes/zygotes were then transferred to media with decreasing concentrations of sucrose for 3–5 min in each media as recommended by the manufacturer.

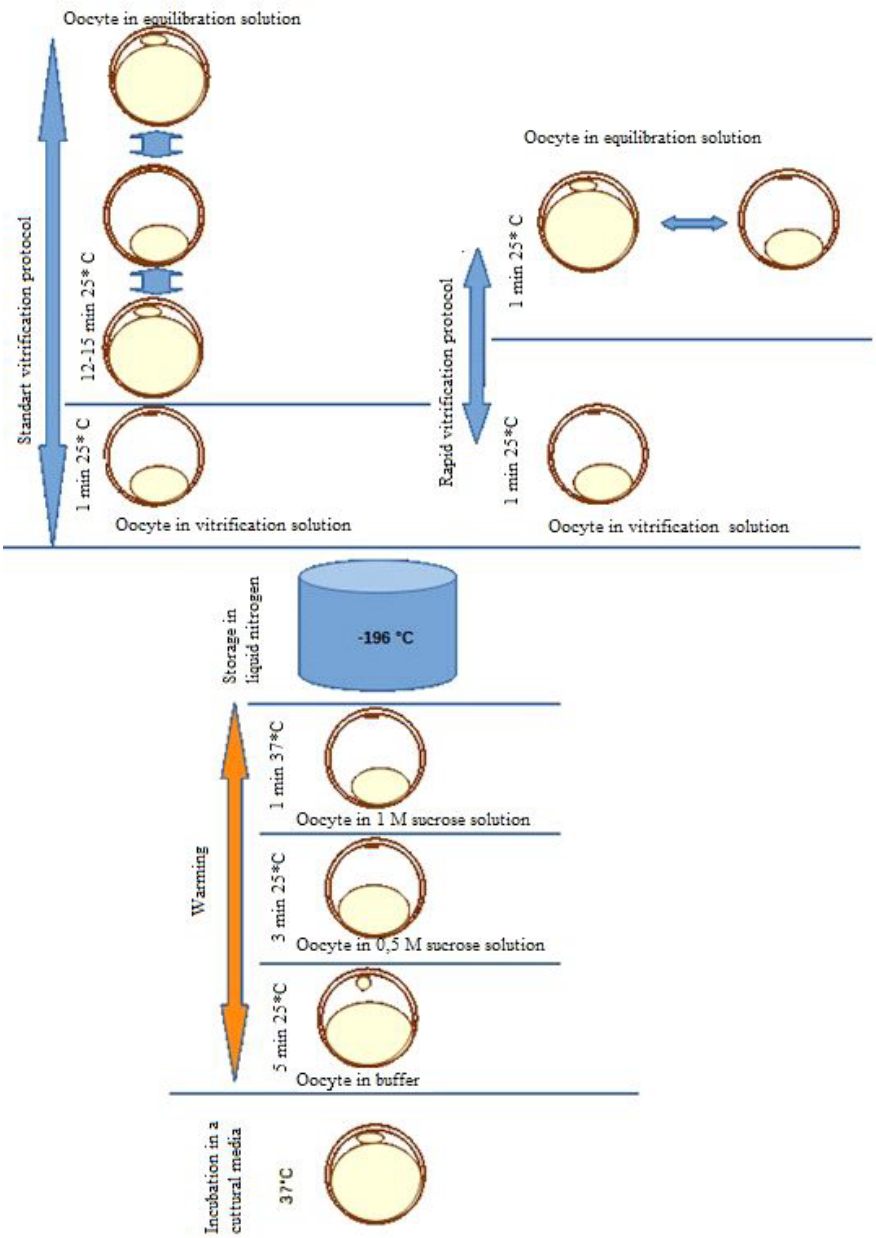

Fig. 1. Vitrification methods (standard and rapid) and bovine oocyte thawing protocol

2.7 Embryo cultivation with time-lapse monitoring

After thawing, fertilized oocytes were cultured for 170-180 hours in BO-IVC medium (IVF-Bioscience, UK) covered with Oil for tissue culture (Sage, USA) at a carbon dioxide level of 6.5 vol. %, oxygen – 5.0 vol. % at a temperature of 38.5ºC without changing the medium until the blastocyst stage.

The image was taken using the Primo Vision system (Vitrolife, Sweden) for 8 days (192 hours) after fertilization of bovine oocytes [7]. The resulting images formed a video sequence for subsequent analysis.

2.8 Statistical processing

Statistical analysis is performed using Microsoft Excel. All results are expressed as mean ± SEM or percentage. Statistical comparisons were made between two groups were performed using t-test. The significance of differences was determined at $p < 0.05$.

3 Results and Discussion

The survival rate of oocytes using rapid vitrification method was analyzed in comparison with standard vitrification method (Table 1). Comparison of survival rates between groups did not reveal a significant difference.

Table 1. Data on oocyte survival using rapid and standard vitrification methods.

Parameter	Standard vitrification method	Rapid vitrification method
Number of zygotes	100	100
Zygote survival rate, %	92 (92.0)	93 (93.0)
Number of repeats	5	5

Table 2 shows the cleavage parameters of zygotes frozen using standard vitrification method and rapid vitrification method. There were no significant differences in the rate of cleavage and time of formation of blastocysts between the groups.

Table 2. Data on cleavage rate parameters for different vitrification methods in comparison with the control group without vitrification.

Parameter	Standard vitrification method*	Rapid vitrification method *
No blastocyst formation		
Duration of the first cleavage, h	1.31 ± 0.82	1.30 ± 0.75
from 2 to 3 cells, h	11.07 ± 0.58	10.83 ± 0.37
from 3 to 4 cells, h	1.80 ± 1.92	1.07 ± 0.28
from 4 to 5 cells, h	9.68 ± 0.65	10.39 ± 0.48
Successful blastocyst formation		
Duration of the first cleavage, min	0.17 ± 0.03	0.20 ± 0.03
from 2 to 3 cells, h	9.60± 0.35	10.10± 0.25
from 3 to 4 cells, h	0.25 ± 0.08	0.20 ± 0.03
from 4 to 5 cells, h	9,68 ± 0.57	10.32 ± 0.85
Blastocyst formation, h	181.40 ± 6.17	179.85 ± 9.12
Expanded blastocyst, h	189.13 ± 7.90	187.57 ± 14.23
Blastocyst formation, %	20.7	19.4
Total embryos in the group	92	93

*Data are expressed as mean ± SEM.

Rapid vitrification method is based on the study of osmotic compression of oocytes. It has been shown that dehydration of oocytes when exposed to standard solutions of cryoprotectants occurs very quick within 60 seconds [4]. Rapid vitrification method assumes a minimum volume of the oocyte at the time of its introduction into the vitrification solution with high concentrations of cryoprotectants (Fig. 1). Unlike the standard vitrification method, when the stage of cells compression is followed by the stage of equilibration and osmotic equilibrium (Fig. 1). When using a rapid vitrification method

equilibration requires only 60 seconds in the cryoprotectant solution. After this, cell dehydration is completed, and the exposure time to cryoprotectors can be reduced. However, reduction in exposure time at the equilibration stage raises certain concerns, as it may subsequently affect not only the survival of oocytes/zygotes, but also the further development of embryos after thawing. Therefore, the use of rapid vitrification method in clinical practice in medical laboratories is still limited [4].

The results of our study show that in the group with rapid vitrification there were no significant differences in the survival rate of bovine zygotes (Table 1) and in the rate of cleavage of embryos obtained from these zygotes compared with zygotes vitrified according to the standard protocol (Table 2). It should be noted that when using rapid vitrification method on human and mouse oocytes, this protocol also showed good oocyte survival [3, 4]. A rapid vitrification method used on mouse oocytes leads to reduced damage to mitochondrial distribution due to smaller changes in cell volume, in contrast to the standard method [3]. It can be assumed that the preservation of mitochondrial function promotes the supply of ATP and supports embryonic development. A one-time reduction in the volume of oocytes is necessary to remove water from the cytoplasm of the cell during vitrification, but with the standard protocol equilibration is necessary after compression, during which cells restore their volume and are subsequently compressed again (Fig. 1).

Although we did not observe a decrease in the level of developing blastocysts or a change in the rate of development of bovine embryos, it should be noted that in the design of this experiment we did not use the vitrification method for cryopreservation of unfertilized bovine oocytes. At the same time, there are a number of reports of damage to the endoplasmic reticulum during vitrification of unfertilized oocytes at the metaphase II stage, which induces the release of Ca^{2+} into the cytoplasm of the oocyte, and therefore can lead to abnormal fertilization after vitrification [3].

4 Conclusions

Thus, we applied rapid vitrification method for bovine zygotes and obtained survival rates, cleavage rates and the level of development of blastocysts comparable to the standard vitrification method and to the control group. This method allows to reduce the exposure time of cells in solutions with cryoprotectants at suboptimal temperature and osmolarity, and also decrease the time of the procedure to two minutes compared with standard vitrification (11-16 min). This method of rapid vitrification is promising for use in cell processing during gene editing, since reducing the time oocytes in suboptimal conditions allows to minimize stress at this stage and thereby positively improve the technique.

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