JMBFS
Journal of Microbiology, Biotechnology and Food Sciences
International peer-reviewed scientific online journal
Published by Faculty of Biotechnology and Food Sciences

THE RESISTANCE OF OOCYTES AND *IN VITRO* PRODUCED CATTLE EMBRYOS TO CRYOPRESERVATION

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https://doi.org/10.55251/jmbfs.10749

ARTICLE INFO

Received 3. 10. 2023
Revised 22. 11. 2023
Accepted 29. 11. 2023
Published 1. 12. 2023

Review

ABSTRACT

In vitro fertilization and production of bovine embryos *in vitro* is method, which improves utilization of animal reproduction potential. In connection with cryopreservation methods and ability of long-term cryostorage of embryos offers suitable tool for positive influence of endangered species or breeds. *In vitro* embryo production with subsequent embryo transfer contributes to the improvement of reproduction rates and bypassing some reproduction failure in high productive animal breeding. The method is generally used for *in vitro* production of transferable bovine embryos. Worldwide about 411 000 of IVP bovine embryos are annually transferred to recipients (IETS, 2013). Most of them were transferred immediately after producing, which is difficult for organization and synchronization of animals. In contrast to this possibility the storage of embryos for later using brings undisputable advantages. In order to protect animal genetic resources and to preserve the gene pool of rare and endangered animals, it is necessary to establish gene banks containing stocks of gametes and embryos of these animals. Optimization of the methods of *in vitro* production of bovine embryos and increasing their resistance to cryopreservation process is an actual challenge to ensure long-term storage in the gene bank of animal resources. The main goal of this mini-review is to provide general insight into the task of oocyte and embryo resistance to cryopreservation.

Keywords: oocyte, embryo, in vitro culture, vitrification, cryotolerance

INTRODUCTION

For the protection of genetic diversity of endangered species and breeds, it is essential to create genetic resource banks of male and female gametes and embryos **(Wildt, 2000)**. Cryopreservation of bovine oocytes and embryos in combination with *in vitro* fertilization is a key technology that allows *in vitro* embryo production (IVP) and its transfer to recipients at a convenient time for the breeder, thus, promoting utilization of animal reproduction potential. In addition, *in vitro* production of embryos from frozen oocytes can significantly contribute to the expansion and conservation of the gene pool of endangered species or breeds of animals, whose numbers are significantly reduced.
Methods for freezing oocytes must ensure their good viability after thawing. To date, several strategies have been devised for the treatment of infertility using frozen oocytes or ovarian tissues. These strategies describe the freezing of either immature oocytes isolated from the ovaries or mature (after ovulation) oocytes. Cryopreservation of embryos is more effective, in contrast to the freezing of tissues or oocytes. Freezing and long-term storage of embryos has reached at present better progress and more successful than freezing of oocytes. Oocytes are very sensitive to freezing, because of low surface-to-volume ratio, hence water and cryoprotectants cannot pass through the plasma membrane so fast **(Pereira and Marques, 2008)**. Additionally, freezing of matured oocytes can cause meiotic spindle disruption and chromosomal aberration **(Rho *et al.*, 2002)**. Freezing of mature (metaphase II) oocytes yields variable results with respect to problems that arise during fertilization and embryonic development. Upon thawing, the hardening of the *zona pellucida*, which is associated with the exocytosis of prematured cortical granules, blocks sperm penetration. The freezing procedure may also damage the oocyte cytoskeleton **(Vincent and Johnson, 1992)**. The freezing of oocytes at the germinal vesicle (GV) stage could be an alternative in order to avoid the risk of aneuploidy **(Tucker *et al.*, 1998)**, but in our previous studies it did not yield satisfactory results **(Makarevich *et al.* 2018)**. Limited success in these studies may be due to the freezing of immature oocytes found in primordial follicles that are localized in the ovarian cortex. This suggests that overcoming the high freezing sensitivity of oocytes requires further research **(Hwang and Hochi, 2014)**. Embryos, in contrast, may be frozen much better, because properties of plasma membrane, size and character of blastomeres change after the fertilization and fast dehydration is enabled to reduce ice crystal formation **(Chen *et al.*, 2005)**. However, embryos produced *in vitro* do not survive cryopreservation as well, as embryos developed *in vivo*, what reduces the pregnancy rate of cows after embryo transfer **(Seidel, 2006)**. The problem of cryopreservation of IVP embryos is a reduced cryotolerance in contrast to their *in vivo* obtained counterparts. The main aim of this mini-review is to provide an insight into the current state of the oocyte and embryo cryopreservation and its cryoresistance.

REASONS FOR REDUCED CRYORESISTANCE OF IVP EMBRYOS

Embryos produced under *in vitro* conditions significantly differ from *in vivo*-derived embryos in morphological and ultrastructural quality **(Lonergan, 2006)**. IVP embryos have markedly darker and non-transparent cytoplasm, what is caused by ultrastructural changes, mainly by accumulation of lipid droplets **(Abe *et al.*, 2002)**. The high content of lipid droplets in the cytoplasm is considered to be one of the main causes of poor freezing of oocytes and pre-implantation embryos of cattle and pigs **(Amstislavsky, 2019)**. The important role belongs not only to the fat droplet content but also to the proportion of unsaturated and saturated fatty acids in triacylglycerides, which influences at what temperature the lipid phase transition (LPT) occurs, and it leads to disconnections between fat droplets, smooth endoplasmic reticulum and mitochondria, thus, affecting the normal energy metabolism processes in the cell **(Horvath and Seidel, 2006)**.
The second problem is the lipid composition of the cell membranes. Phospholipids are major structural components of cell membranes and are involved in a wide range of signaling pathways **(Sunshine and Iruela-Arispe, 2017)**. Some studies have shown that phospholipid profiles and the proportion of phosphatidylcholines and sphingomyelins are different in embryos with different cryotolerance **(Leão *et al.*, 2017; Sudano *et al.*, 2012)**, suggesting that phospholipids may be good predictors of embryo survival after cryopreservation.
The freezing of cow oocytes results in various damages: impaired ZP, fragmented cytoplasm, poorly distinguishable oolema and increased PVS **(Arav *et al.*, 1996)**. The oocyte cooling affected oolemma integrity **(Zeron *et al.*, 1999; Arav *et al.*, 1996)**. Decreased expression of tetraspanin CD9 at both mRNA and protein levels, as well as altered distribution of this molecule detected on ZP-free oocytes after vitrification have also been described **(Zhou *et al.*, 2013)**. It can be assumed that the damage to cryopreserved oocytes may be related to disruption of the tetraspanin web (CD9 and CD81 molecules) and alteration of its dynamics **(Jankovicova *et al.*, 2019)**. It could be used as another marker of the oocyte and embryo viability.

ROLE OF CRYOPRESERVATION TECHNIQUES

The choice of the most appropriate method of freezing gametes or embryos is determined by several factors such as embryo developmental stage, freezing and thawing rate (vitrification or slow freezing), freezing medium composition and concentration of individual cryoprotective agents. Vitrification is an alternative approach to slow freezing of oocytes and embryos. The advantage is that during the vitrification, ice crystals are not formed in both the intra- and extracellular space of the embryo **(Rall and Fahy, 1985)**. On the other hand, high concentrations of cryoprotectants, which may be toxic, represent a potential risk. For these reasons, research is focused on comparing the effects of various cryoprotectants and their combinations **(Otoi *et al.*, 1993; Rodrigues-Villamil *et al.*, 2014)** and optimizing the freezing process (vitrification).

Cryopreservation aims to maintain oocytes in a viable state for long periods of time. Cells can be cryopreserved using any of two approaches: conventional freezing or vitrification. In conventional freezing, sophisticated and expensive programmable freezers are required to assist the cooling procedure. Therefore, an alternative for cryopreservation in field conditions may be a vitrification. Although vitrification is currently a common method for cryopreservation of oocytes or embryos, it can have harmful effects. The most common form of damage is degeneration of the cytoplasm. The occurrence of damage also depends on the cryocarrier device used. For example, the Cryotop technique substantially reduced cow oocyte viability irrespective of the stage at which the oocyte was vitrified (GV, GVBD or metaphase II; **Sprícigo *et al.*, 2014**). **Zhou *et al.* (2010)** vitrified GV oocytes enclosed with cumulus cells and using a Cryotop obtained a higher blastocyst rate than with vitrification of MII oocytes. **Ortiz-Escribano *et al.* (2016)** using Cryotop vitrification found that cumulus cells reduced viability after vitrification of mature bovine oocytes. **Martino *et al.* (1996)** compared bovine oocyte vitrification in classic plastic 0.25 ml straws (rapid freezing) and electron microscopic meshes (EM grids; ultra-rapid freezing) and found that this technique represents a suitable method for oocyte freezing, because after thawing and IVF procedure a higher percentage of blastocysts was obtained than after freezing in conventional straws. In addition, the morphology of blastocysts obtained from such frozen oocytes was normal and did not differ from the non-vitrified control **(Martino *et al.*, 1996)**.

FACTORS INFLUENCING VITRIFICATION EFFICIENCY

Vitrification is a method generally used for successful cryopreservation of oocytes and early embryos of several animal species. Since the aim of vitrification is to avoid ice formation, it should in principle be a better alternative than slow freezing. However, this procedure requires extremely rapid cooling and replacement of water in the cell with permeable cryoprotective agents (cryoprotectants), which can produce a cytotoxic effect **(Lornage *et al.*, 2006)**. Various methods of reducing the concentration of cryoprotectants, such as a combination of relatively low concentrations of different cryoprotectants, are used to obtain a required vitrification concentration of the solutions while reducing specific toxicity **(Vajta and Nagy, 2006)**. Optionally, impermeable cryoprotectants, such as sera, sugars and polymers, are added. The high cooling and thawing rate, required for vitrification, can be achieved by conventional protocols, in which a small sample volume is immersed in a small volume of cryoprotectant and subsequently either directly immersed into liquid nitrogen or enclosed in a thin-walled straw with a minimum volume of a cryoprotectant **(Kuwayama, 2007)**.

It was assumed, that significant obstacle of vitrification success is insufficient cooling rate in used methods. For by-passing this problem several methods were suggested, which decreased the volume of freezing medium. Using these methods, few microliters of medium are filled into the glass or plastic straw and subsequently immersed into liquid nitrogen. These methods were successfully used for bovine oocyte and embryo cryopreservation. **Massip** with co-workers **(1995)** was first, who published successful vitrification of bovine embryos using 0.25 ml straw. Vajta with co-workers improved the method using pulled out and thinned plastic straw (open pulled straw, OPS; **Vajta *et al.*, 1998**). For the vitrification in order to minimize the vitrification solution volume by specific manner several carrying devices were used: cryoloop (**Lane *et al.*, 1999),** nylon loop **(Lane and Gardner, 2001)**, hemi-straw system (HS; **Vanderzwalmen *et al.*, 2003**), electron microscope grid **(Martino *et al.*, 1996; Olexiková *et al.*, 2020)**, French straws **(Hochi *et al.*, 1994)**, CryoTip **(Kuwayama, 2005)**, cryotop **(Hamawaki *et al.*, 1999; Kuwayama, 2007)** and microvolume air cooling method (MVAC; **Punyaway *et al.*, 2015**).

An alternative approach to cooling rate elevation was the method without carrying device. Direct dropping of vitrification solution with oocytes into liquid nitrogen **(Papis *et al.*, 2000)** prevented insulating effect of the straw wall during vitrification. Similar results were reached by placing the vitrification solution with oocyte onto the small loop **(Lane *et al.*, 1999)**. Immersing of warm object into liquid nitrogen caused local warming of nitrogen and boiling. Immediately after immersing, evaporated nitrogen creates an isolating barrier, which decelerating the cooling rate. Improvement in the heat conduction and elevation in the cooling rate is possible using metal surface cooled with liquid nitrogen. Solid surface vitrification method (SSV; **Dinnyés *et al.*, 2000**) is effective method for oocyte and embryo vitrification, which combined advantage of vitrification method without straw with accelerated heat conduction using supercooled metal surface. Additionally, clean metal surface facilitates sterility maintaining and prevents contamination from direct contact with liquid nitrogen.

SUBSTANCES INFLUENCING CRYORESISTANCE OF OOCYTES AND EMBRYOS

The aim of IVP embryo cryopreservation is their long-term storage for the purpose of later using in embryo transfer, therefore good quality of embryos is necessary. The quality of embryos after thawing is a prerequisite that such embryos after the transfer will have a chance to achieve high pregnancy rate in cows. Increasing of oocyte and embryo cryotolerance can be achieved by enriching culture and vitrification solutions by adding various substances: growth factors (IGF-I; EGF, FGF), antioxidants: melatonin (**Gao *et al.*, 2012**), glutathione, superoxide dismutase, glutathione peroxidase, catalase etc., delipidation agents (L-carnitine, fatty acids, phenazine ethosulfate and forskolin; **Braga *et al.*, 2019**), cytoskeleton stabilizers (cytochalasin B, cytochalasin D, colchicine, taxol (**Diez *et al.*, 2012**)); paclitaxel **(Fesahat *et al.*, 2016)**, substances sustaining high cAMP intraoocyte concentrations (cAMP modulators, dbcAMP, IBMX, DON – (**Lee *et al.*, 2020)** and others.

Antioxidant (AO) molecules can protect the embryos against the harmful effect of increased oxidative stress induced by cryopreservation **(Corrêa *et al.* 2008)**. However, most studies have evaluated the use of AOs during *in vitro* culture (IVC) and only rarely these substances were added to vitrification and/or thawing solutions **(Giaretta et al., 2013)**. In a recent study **(Gaviria *et al.*, 2019)**, the effect of resveratrol added not only to culture but also to vitrification media was tested, and it was found that resveratrol applied improved the quality of the embryos after thawing to the level of their initial quality before cryopreservation. Although the number of studies using some antioxidants has increased in recent years, it is not yet clear at what cultivation step it is optimal to add AOs to improve cryotolerance. The cytoskeleton is one of the cellular components of the oocyte which often is damaged during vitrification. Several substances – stabilizers of the cytoskeleton, such as cytochalasin B, cytochalasin D, colchicine and taxol (a microtubule stabilizer; **Morató *et al.* 2008**) may improve the cryotolerance of both mature and immature oocyte, when adding to culture media prior to cryopreservation **(Diez *et al.*, 2012)**. **Fesahat *et al.* (2016)** reported that low concentration of paclitaxel - a microtubule stabilizer may improve the mouse oocyte survival rate after freezing/ thawing.

EVALUATION OF EMBRYO CRYORESISTANCE

Several methods of cryopreservation of embryos and oocytes have been tested over the past decades, but only a few approaches of assessing embryo cryotolerance have been described. The use of morphological evaluation of samples after freezing alone is not sufficient, as morphological analysis is not always correlated with oocyte viability or developmental competence **(Santos *et al.*, 2007)**. The expression and distribution of tetraspanins CD63, CD82 and CD151 in vitrified and fresh oocytes was studied by **Jankovičová *et al.* (2023)**. These authors suggested that the changes in the expression and distribution of individual tetraspanins can be considered as another marker of oocyte viability. When assessing oocyte cryotolerance, the best and most reliable indicator is the development after *in vitro* fertilization to the blastocyst stage.

Cryopreserved embryos can be evaluated on the basis of developmental potency after thawing, which is a unique criterion evaluated in preimplantation embryos and is very closely related to their quality. The commonly used criterion for assessing embryo survival is re-expansion of blastocoel within 3 to 48 hours after thawing **(Leão *et al.*, 2017; Lopera-Vasquez *et al.*, 2017)**. This method is inexpensive and simple, but sometimes the evaluation is difficult because blastocysts after re-expansion can repeatedly collapse. Therefore, it is appropriate to use more accurate and reproducible evaluation methods, for example, fluorescent staining of embryos for viability testing. Evaluation of cryotolerance of cow embryos after vitrification using fluorescent staining with Calcein-AM and Ethidium homodimer-1 (Eh-1) was first described by **Banliat *et al.* (2019)**. These authors classified embryos after thawing as viable when they had 50% or more viable cells. Surprisingly, however, from the number of blastocysts that showed at least 50% viable cells after staining, not all embryos had re-expanded blastocoel (only 57% of them; **Banliat *et al.*, 2019**). It indicates that it is optimal to use two complementary methods for assessing embryo cryotolerance. Other methods for verifying embryo quality will include assessing the total number of cells in blastocysts, the incidence of apoptosis in blastomeres, mitochondrial and lysosomal activity, the overall quality of the actin cytoskeleton, and changes at the ultrastructural level using electron microscopy **(Olexiková *et al.*, 2020; Olexiková *et al.*, 2022)**.

CONCLUSION

Procedure of cryopreservation has undergone many modifications since its discovery in order to improve the cryotolerance of oocytes and embryos. Viability of warmed oocytes/embryos is not only affected by the cryopreservation itself, but also by *in vitro* culture conditions, used cryoprotectants and addition of

biologically active substances capable of supporting normal embryonic development. An appropriate combination of these factors may contribute to improved cryotolerance of oocytes and embryos. Therefore, further search for a new cryopreservation protocol and continuous improvement in existing cryoprocedures is still necessary to ensure the better cryotolerance of oocytes and embryos.

Acknowledgements: This study was supported by the Operational Programme Integrated Infrastructure within the project: Creation of nucleated herds of dairy cows requiring high health status through the use of genomic selection, innovative biotechnological methods and optimal breeding management (NUKLEUS 313011V387).

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