NATIONAL AGRICULTURAL AND FOOD CENTRE
RESEARCH INSTITUTE FOR ANIMAL PRODUCTION NITRA

The 6th International Scientific Conference "Animal Biotechnology 2018"
Slovak J. Anim. Sci., 51, 2018 (4): 156–160
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ISSN 1337-9984

OOPLASM CRYOPRESERVATION: OVARIAN FRAGMENTS VERSUS OOCYTES ALONE

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ABSTRACT

The cryopreservation of the farm animal ooplasm is very useful in the conservation of female gametes. Since the use of histological assay alone is insufficient to assess the freezing-thawing process, the future oocyte developmental competence need to be validated by *in vitro* fertilization and embryo production. The aim of the study was to find optimal method of cryostorage of bovine oocytes with preserving their viability and fertility. In the first experimental series we vitrified ovarian fragments isolated from the cortical area of the cow ovary, which contained antral follicles, using two vitrification techniques: solid-surface vitrification (SSV) and liquid vitrification (LV). After warming we isolated the oocytes by follicle aspiration and placed them into the *in vitro* maturation (IVM). At maximum, only 8.3 % of vitrified/warmed (LV) oocytes were matured, whilst none of the oocytes were matured following SSV technique. In all frozen ovarian fragments, regardless of vitrification technique, serious damages in the oocytes were detected at the histological and ultrastructural levels, what prevented the oocyte development. In the second series of experiments, cumulus-oocyte complexes without surrounding ovarian tissue were vitrified following IVM procedure. Using this scheme we obtained more than 50 % embryo cleavage rate and 4.5 % of embryos reached the blastocyst stage, which proves that the cumulus-oocyte complexes after vitrification can retain their developmental ability. Our preliminary results show that cryopreservation of previously matured oocytes is more promising than the vitrification of ovarian tissue fragments.

Key words: bovine; ovary; oocyte; embryo; vitrification

INTRODUCTION

Cryopreservation of ovaries, their surface tissues or ovarian follicles represents a possible source of female gametes in future. In case of serious damage of the animal (limb fractures and others), when it is necessary to slaughter the animal, the ovarian tissue, ovarian follicle or the entire ovary can be collected and frozen. After thawing, the biological material may be transplanted or cultured *in vitro* as a source of oocytes for *in vitro* fertilization (IVF) and *in vitro* embryo production (IVP).

Cryopreservation of ovarian tissue offers many advantages over mature oocytes or embryos to preserve female germline of endangered animals. Firstly, the ovary contains a large pool of oocytes enclosed in follicles. Secondly, ovarian tissue can be collected from animals of almost all developmental age (adult, prepubertal and foetus) and status (alive or dead) (Cleary *et al*., 2001).

The cryopreservation of ovarian cortex containing the antral follicles would certainly be very useful in the conservation of female gametes. There are two possibilities: the excision of slices of the ovarian cortex after the puberty onset without disturbing the female reproductive system. Another way is to remove the whole ovaries after the animal's death, to be used later in techniques such as grafting or *in vitro* maturation. The attempt to *in vitro* mature oocytes from primordial follicles

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Received: September 25, 2018
Accepted: October 9, 2018

has not yielded satisfactory results (Lunardi *et al*., 2017). However, there are reports that small antral follicles frozen as part of vitrified ovarian tissues could be the source of oocytes for *in vitro* fertilization and successful embryo production (Faheem *et al*., 2011). Nevertheless, cryopreservation of ovarian tissue is still problematic and should be optimized to handle the diversity of cell types and tissue components (oocytes, granulosa cells, extracellular matrix) (Hovatta, 2005). The use of histology alone is insufficient to assess the freezing-thawing process as morphological analysis, and it is not often correlated with the ability or developmental competence of the oocytes (Celestino *et al*., 2008; Santos *et al*., 2007). The results need to be validated by *in vitro* oocyte development and by fertilization *in vitro*.

The aim of the study was to find optimal method of cryostorage of bovine oocytes with preserving their viability and fertility.

MATERIAL AND METHODS

Cryopreservation of ovarian fragments

Ovarian fragments (n = 451; approximate size of 4 x 4 mm), containing antral follicles (2-4 mm), were isolated from undefined cows at a local abattoir, and frozen by two methods previously used for freezing of ovarian primordial follicles.

For solid surface vitrification (SSV), ovarian fragments were exposed to 4 % ethylene glycol (EG) in DPBS + 10 % FBS for 15 min and then rinsed in a vitrification solution composed of 35 % EG and 0.4 M trehalose in DPBS + 10 % FBS. After 5 min equilibration in an ice bath, fragments were placed in a minimum volume of vitrification solution onto the surface of a metal plate pre-cooled by an immersion into a liquid nitrogen (LN).

For liquid vitrification (LV), ovarian fragments were equilibrated in a vitrification medium containing 40 % EG, 30 % Ficoll 70, 1M sucrose and 4 $mg.ml^{-1}$ of BSA at room temperature for 5 min. Then the tissues in 1.8 ml cryovials were placed into LN. After thawing the fragments were processed for histology (toluidine blue, haematoxylin-eosin) and ultrastructure (transmission electron microscopy) analyses.

Part of the oocytes were tested for the ability to mature *in vitro* (IVM). After 24 h of IVM in maturation medium (TCM 199 (Gibco), sodium pyruvate (0.25 $mmol.l^{-1}$), gentamycin (0.05 $mg.ml^{-1}$), foetal bovine serum (FBS) 10 % and FSH/LH (1/1 I.U., Pluset) at 38.5 °C and 5 % CO_2, the oocytes were fixed in formalin, stained with a DAPI dye and fluorescently evaluated.

Cryopreservation of oocytes

For cryopreservation of matured oocytes alone, ultra-rapid cooling technique in minimum volume was used. Matured oocytes with cumulus cell layers were placed into equilibration solution (ES: 7.5 % ethylene glycol +7.5 % DMSO in M199-HEPES, supplemented 20 % foetal calf serum and 50 $ng.ml^{-1}$ gentamycin) for 5 min. Following equilibration the oocytes were transferred to vitrification solution (15 % EG + 15 % DMSO + 0.5M sucrose in M199-HEPES) at room temperature for 45-60 sec. The oocytes (10-15) in a small drop were placed with a glass micropipette onto 300 mesh nickel grids (electron microscopy grade), 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 (1M sucrose in M199-Hepes, at 37 °C) for 1 min. The warmed oocytes were transferred to the diluent solution (0.5M sucrose in M199-HEPES) for 3 min, and then washed twice in M199-HEPES with FCS for 5 min. Oocyte survival was evaluated on the basis of the integrity of the oocyte membrane and the zona pellucida after 20 h culture post-thawing.

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

Warmed oocytes were washed in IVF-TALP medium (TALP solution, 10 $\mu g.mL^{-1}$ heparin, 50 $ng.ml^{-1}$ 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 penicylamine, 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 45 s to remove residual cumulus cells. Denuded zygotes were transferred to the dish with the granulosa cell (about 10 %) layer for 24 h. Afterwards, the embryos were transferred to a new dish with the granulosa cells (about 40 % of confluence) in B2 medium with 10 % FBS.

On the Day 2 since insemination, the cleavage, and on Day 7, 8 and 9 – the number of blastocysts were counted.

RESULTS

When ovarian fragments were cut off and cryopreserved by an SSV vitrification technique, none of the recovered oocytes (n = 47) were matured *in vitro*. When an LV technique was applied, only 8.4 % of oocytes showed the signs of nuclear maturation in contrast to 60.6 % in the control (IVM) group. Most of degenerated oocytes were found in the SSV group after thawing or thawing/IVM (Table 1.).

In all frozen ovarian fragments, regardless of vitrification technique, serious damages of oocytes at the light or electron microscopy levels were detected like extensive vacuolization and disintegration of the ooplasm and organelle dislocation. Visible *zona pellucida* cracks and deformities of oocytes were caused likely by the mechanical action of ice crystals formed in the follicle cavity. The granulosa cell nuclei were largely pyknotic. Germinal vesicles showed disintegrated nuclear envelope. Microvilli of cytoplasmic membrane were disrupted. The damaged *zona pellucida* acquired layer-like structure, and cells of *corona radiata* showed extensive damages. In conclusion, our experiments did not confirm that the oocytes, frozen in small antral follicles from ovarian tissue fragments using SSV or LV, are able to mature *in vitro*, due to extensive cellular damages revealed by histological and ultrastructural analyses.

In the second series of experiments we chose another approach where we tried to freeze cumulus-oocyte complexes without surrounding ovarian tissue. The oocytes were matured by the incubation in maturation medium and afterwards immediately frozen by a liquid vitrification. The results of oocyte vitrification and *in vitro* fertilization are presented in Table 2.

From a total of 184 oocytes vitrified oocytes, 116 were warmed and afterwards only 66 oocytes were selected for IVF procedure. As a control group, 175 freshly isolated oocytes were matured

Table 1. Developmental status of oocytes after cryopreservation/*in vitro* maturation (IVM)

Groups	Total no. oocytes (N)	GV-stage, n (%)	Metaphase-stage, n (%)	Degenerated oocytes, n (%)
Control (fresh)	98	65 (66.21)	32 (32.7)	1 (1)
Control (IVM)	71	27 (38.0)	43 (60.6)	1 (1.4)
LV–thawed	29	21 (72.4)	4 (13.8)	4 (13.8)
LV–thawed/IVM	48	38 (79.2)	4 (8.3)	6 (12.5)
SSV–thawed	32	22 (68.8)	2 (6.3)	8 (25.0)
SSV–thawed/IVM	47	31 (66.0)	0 (0)	16 (34)
Totally oocytes	325	204	85	36

Table 2. Development of fresh or vitrified-warmed oocytes after IVF

Groups	Oocytes totally	Oocytes vitrified	Oocytes warmed	Oocytes in IVF	Embryo cleavage	Blastocyst rate
Vitrified	197	184	116	66	34 (51.50 %)[a]	3 (4.55 %)[a]
Control	175	-	-	175	141 (80.57 %)[b]	34 (19.43 %)[b]

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

in vitro and immediately subjected to the IVF procedure. Cleavage rate of vitrified oocytes was significantly lower (51.5 %; $p < 0.05$) than that of a fresh control oocytes (80.57 %). In the vitrified group only 3 expanded blastocysts were developed (4.55 %) in comparison with 19.43 % in the fresh control group oocytes ($p < 0.05$).

DISCUSSION

The oocyte has always been the most challenging specimen to cryopreserve because of its high sensitivity and intolerance to cryopreservation due to large cytoplasmic volume and intricate cellular structure (Kim, 2006).

An alternative approach to harvesting and vitrifying oocytes would be to cryopreserve ovarian tissues instead of follicles. Dissection of the ovary into ovarian fragments followed by puncture of the follicles is considered as an effective technique to get a high number of oocytes in excellent conditions (Faheem *et al*., 2011). Therefore, attention must be given to preserve this yield of good oocytes. Faheem *et al*. (2011) isolated bovine oocytes for *in vitro* culture using the procedure of dissection of ovarian cortex into small fragments followed by puncture of the follicle from frozen-thawed ovarian tissue. They recovered a great number of good quality oocytes, which were successfully *in vitro* matured (maturation rate 73-80 %), fertilized and developed into *in vitro* produced embryos of morula and blastocyst stage.

In our study we performed two steps of the experiments. In the first step we tried to vitrify ovarian fragments isolated from the cortical area of the ovary. Following vitrification/warming we isolated the oocytes from antral follicles by a punction or aspiration and then placed them into *in vitro* maturation (IVM). Two vitrification techniques (solid-surface vitrification – SSV and liquid vitrification – LV) were applied for cryopreservation of ovarian fragments. At maximum, only 8.3 % of vitrified (LV) oocytes were matured during IVM, whilst none of the oocytes were matured following SSV technique. This poor oocyte development was caused by substantial injuries in the oocyte structure, which was also confirmed by histological and ultrastructural analyses. These injuries were probably arisen as a consequence of thawing process. Therefore, our first step experiments did not confirm that the oocytes, frozen in small antral follicles from ovarian tissue fragments using SSV or LV, are able to mature *in vitro*, due to the loss of developmental competence.

In the second step, the cumulus-oocyte complexes were isolated from the follicles and put into the maturation *in vitro*. Afterward, matured oocytes enclosed by cumulus cells, were vitrified by ultra-rapid cooling technique in minimal volume and stored in liquid nitrogen for several weeks. After warming the oocytes were *in vitro* fertilized and put into embryo culture medium to develop until higher embryonal stages. Using this scheme we obtained more than 50 % cleavage rate and 4.5 % reached the expanded blastocyst stage, which proves that the cumulus-oocyte complexes after vitrification can retain their developmental ability. Similar results were published also by Chian *et al*. 2004 (7.4 %) and Zhou *et al*. 2010 (5.4 %).

A success in bovine oocytes cryopreservation was achieved firstly when an approach of minimizing the vitrified sample was used in order to obtain a much faster cooling rate. Martino *et al*. (1996), using the minimum volume technique, reported development to the blastocyst stage or high cleavage rate of vitrified *in vitro* matured bovine oocytes. Better results (more than 10 % of blastocyst) after bovine oocytes cryopreservation were achieved by Vajta *et al*. (1998), Papis *et al*. (2001) and Ishi *et al*. (2018).

Our preliminary experiments show that cryopreservation of matured cumulus-oocyte complexes is more promising than the vitrification of ovarian tissue fragments. Although, in our experiments, we obtained only few blastocysts (4.5 %) following IVF of vitrified oocytes, blastocyst development exceeding 10 % is really possible. Due to the high variability of results, a standard method for bovine oocyte cryopreservation remains to be optimized.

ACKNOWLEDGEMENTS

This study was supported by the Slovak Research and Development Agency (grant no. APVV-15-0196).

REFERENCES

CELESTINO, J.J. – dos SANTOS, R.R. – LOPES, C.A. – MARTINS, F.S. – MATOS, M.H. – MELO, M.A. – BAO, S.N. – RODRIGUES, A.P. – SILVA, J.R. – de FIGUEIREDO, J.R. 2008. Preservation of bovine preantral follicle viability and ultrastructure after cooling and freezing of ovarian tissue. *Animal Reproduction Science*, vol. 108, 2008, p. 309–318.

CHIAN, R.C. – KUWAYAMA, M. – TAN, L. – TAN, J. – KATO, O. – NAGAI, T. 2004. High survival rate of bovine oocytes matured *in vitro* following vitrification. *Journal of Reproduction and Development*, vol. 50, 2004, p. 685-696.

CLEARY, M. – SNOW, M. – PARIS, M. – SHAW, J. – COX, S.L. – JENKIN, G. 2001. Cryopreservation of mouse ovarian tissue following prolonged exposure to an ischemic environment. *Cryobiology*, vol. 42, 2001, p. 121–133.

FAHEEM, M.S. – CARVALHAIS, I. – CHAVEIRO, A. – MOREIRA DA SILVA, F. 2011. *In vitro* oocyte fertilization and subsequent embryonic development after cryopreservation of bovine ovarian tissue, using an effective approach for oocyte collection. *Animal Reproduction Science*, vol. 125, 2011, p. 49–55.

HOVATTA, O. 2005. Methods for cryopreservation of human ovarian tissue. *Reproductive BioMedicine Online*, vol. 10, 2005, p. 729–734.

ISHII, T. – TOMITA, K. – SAKAKIBARA, H. – OHKURA, S. 2018. Embryogenesis of vitrified mature bovine oocytes is improved in the presence of multi-layered cumulus cells. *Journal of Reproduction and Development*, vol. 64, 2018, p. 95-99.

KIM, S.S. 2006. Fertility preservation in female cancer patients: current developments and future directions. *Fertility and Sterility*, vol. 85, 2006, p. 1–11.

LUNARDI, F.O. – DE AGUIAR, F.L.N. – APOLLONI, L.B. – DUARTE, A.B.G. – DE SÁ, N.A.R. – LEAL, É.S.S. – SALES, A.D. – LOBO, C.H. – CAMPELLO, C.C. – SMITZ, J. – APGAR, G.A. – DE FIGUEIREDO, J.R. – RODRIGUES, A.P.R. 2017. Sheep isolated secondary follicles are able to produce metaphase ii oocytes after vitrification and long-term *in vitro* growth. *Biopreservation and Biobanking*, vol. 15, 2017, p. 321-331.

MARTINO, A. – SONGSASEN, N. – LEIBO, S.P. 1996. Development into blastocysts of bovine oocytes cryopreserved by ultra-rapid cooling. *Biology of Reproduction*, vol. 54, 1996, p. 1059–1069.

PAPIS, K. – SHIMIZU, M. – IZAIKE, Y. 2000. Factors affecting the survivability of bovine oocytes vitrifled in droplets. *Theriogenology*, vol. 54, 2000, 651-658.

SANTOS, R.R. – VAN DEN HURK, R. – RODRIGUES, A.P. – COSTA, S.H. – MARTINS, F.S. – MATOS, M.H. – CELESTINO, J.J. – FIGUEIREDO, J.R. 2007. Effect of cryopreservation on viability, activation and growth of in situ and isolated ovine early-stage follicles. *Animal Reproduction Science*, vol. 99, 2007, p. 53–64.

VAJTA, G. – HOLM, P. – KUWAYAMA, M. – BOOTH, P.J. – JACOBSEN, H. – GREVE, T. – CALLESEN, H. 1998. Open pulled straw (OPS) vitrification: a new way to reduce cryoinjuries of bovine ova and embryos. *Molecular Reproduction and Development*, vol. 51, 1998, p. 53–58.

ZHOU, X.L. – AL NAIB, A. – SUN, D.W. – LONERGAN, P. 2010. Bovine oocyte vitrification using the Cryotop method: Effect of cumulus cells and vitrification protocol on survival and subsequent development. *Cryobiology*, vol. 61, 2010, p. 66–72.