The 2nd International Scientific Conference „Animal Biotechnology“
Slovak J. Anim. Sci., 47, 2014 (4): 193-197
© 2014 NPPC
ISSN 1337-9984

NATIONAL AGRICULTURAL AND FOOD CENTRE
RESEARCH INSTITUTE FOR ANIMAL PRODUCTION NITRA

VIABILITY OF BOVINE PREIMPLANTATION EMBRYOS FOLLOWING CRYOPRESERVATION

A. V. MAKAREVICH[1]*, E. KUBOVIČOVÁ[1], P. CHRENEK[1,2]

[1]NAFC - Research Institute for Animal Production Nitra, Slovak Republic
[2]Slovak University of Agriculture in Nitra, Slovak Republic

ABSTRACT

In programs for the preservation of animal genetic resources a great significance belongs to a long-term storage of biological material (gametes, embryos) at extra-low temperatures. The aim of this study was to determine quality and viability of bovine embryos of Holstein breed cryopreserved by a vitrification procedure (n = 88) in comparison to intact (fresh) bovine embryos (n = 117). The embryos were recovered on 7th day after the first insemination by a flushing out of the uterine horns of superovulated cows with a Bioniche complete flush solution using a silicone two-way Foley catheter. The embryos of morula or early blastocyst stage were subjected to the two-step vitrification procedure using EFS vitrification solution (ethylene glycol - 40 % v/v, Ficoll 70 - 18 % w/v; 0.3 M sucrose in D-PBS + 20 % fetal calf serum and 5 $\mu g.ml^{-1}$ gentamycin), pulled into the open-pulled straws and slowly immersed into liquid nitrogen. Following thawing the embryos were cultured for 48 hours in order to reach advanced developmental stage (expanded blastocyst), afterwards these embryos were analyzed for the developmental rate, embryo cell number and incidence of the dead cells. About 83 % of all control embryos were developed to the expanded blastocyst stage, whilst in the frozen-thawed group only 60 % of embryos reached this stage ($p < 0.05$). Frozen-thawed embryos contained significantly less number of embryonal nuclei when compared with fresh embryos. Dead cell incidence (TUNEL-index) was more than twice higher in the frozen-thawed embryos (9.53 %) in comparison to the fresh embryos (4.32 %), but this value did not exceed 10 %, the critical value which may compromise the embryo viability. Our experiments confirm that cryopreservation may affect embryo viability. Therefore, monitoring of embryo quality following cryopreservation would provide a basis for understanding embryo sensitivity to freezing protocols and help to improve a cryopreservation technique.

Key words: embryo; blastocyst; morula; vitrification; apoptosis

INTRODUCTION

Cryopreservation of preimplantation embryos is an important tool for maintaining animal genetic resources by increasing the usage of reproductive potential of genetically valuable animals. However, cryopreservation procedures may negatively affect survival of gametes and embryos as a consequence of damages to the cell and even cell death (apoptosis or necrosis). Determination of these influences may have a great importance for the explanation of failures in reproductive processes. Research in this area enables us to follow processes running at the cell level and factors regulating these processes.

Reliable information about embryo viability can be provided either by ultrastructural analysis at the level of electron microscopy or by embryo transfer to recipient females, but these methods may not be practically available in every laboratory. Cryotolerance may be a useful indicator of blastocyst quality (Rizos *et al.*, 2001). As a functional criteria for

***Correspondence:** E-mail: makarevic@vuzv.sk
Alexander V. Makarevich, NAFC - Research Institute for Animal Production Nitra, Hlohovecká 2, 951 41 Lužianky, Slovak Republic
Tel.: +421 37 6546 334

Received: May 27, 2014
Accepted: October 14, 2014

the evaluation of embryo viability after cryopreservation, a post-thaw cleavage up to blastocyst stage (Popelkova *et al*., 2005; Makarevich *et al.* 2008), embryo cell number and embryo diameter (Popelkova *et al.*, 2009), proliferation (PCNA) index (Markkula *et al*., 2001) or number of apoptotic (TUNEL) cells (Marquez-Alvarado *et al.*, 2004; Makarevich *et al.*, 2008) and the state of actin cytoskeleton (Tharasanit *et al.* 2005; Makarevich *et al.*, 2008) have been used. Exact knowledge of processes leading to damages and embryonal loss may help to find out approaches for the improvement of embryo quality and survivability following cryopreservation.

The aim of this study was to determine quality and viability of bovine embryos cryopreserved by vitrification procedure in comparison to intact (fresh) bovine embryos. The embryos following vitrification-warming were cultured for 48 hours in order to reach advanced developmental stage (expanded blastocyst), afterwards these embryos were analyzed for the developmental rate, total cell number and dead cell index.

MATERIAL AND METHODS

Preparation of cows for flushing, embryo recovery and evaluation

The cows of Holstein breed were used as donors of embryos. Oestrus of the cows was synchronized by the injection of a PGF_{2alfa} analogue (Oestrophan, Bioveta a.s., Ivanovice na Hane, Czech Republic). The cows were superovulated by application of porcine pituitary gonadotropin (Pluset®- FSHp-LHp, Laboratorios Callier, Barcelona, Spain) twice daily during 5 days at 8.00 and 20.00 hours (given in a decreasing dosage rate; starting with the doses of 150 IU FSH +150 IU LH in the morning at the 11th day to 50 IU FSH+50 IU LH in the evening at the 15th day) of the oestrous cycle. Oestrophan was administered for luteolysis at the 13th day. Insemination was performed two times by the same AI technician with frozen-thawed AI doses from one sire only at 12 hrs intervals started at 12 hrs after the standing oestrous detection.

Embryo recovery was performed on 7th day after the first insemination by a standard non-surgical technique to flush out the uterine horns. Uterine flushing was conducted with a complete flush solution (Bioniche, Belleville, Ontario, Canada) using a silicone two-way Foley catheter (Minitüb GmbH, Tiefenbach, Germany). Flushed ova/embryos were transferred to the holding medium - phosphate buffered solution (PBS) with 20 % foetal calf serum (FCS, Gibco BRL) and assessed using a stereomicroscope. The embryos were classified according to their stage of development as of good quality (i.e., morulas, early blastocysts) and of poor quality (i.e., fragmented/degenerated embryos, unfertilized oocytes). Only good quality embryos were used for a cryopreservation by vitrification procedure.

Vitrification procedure

The two-step vitrification procedure was used in our experiments. In the first step the embryos were equilibrated by 3 min incubation in the holding medium (D-PBS with 20 % FCS). In the second step the embryos were placed into the vitrification solution EFS (described by Kasai *et al.*, 1990), consisted of ethylene glycol (40 % v/v), Ficoll 70 (18 % w/v) and 0.3 M sucrose dissolved in a holding medium (D-PBS + 20 % FCS and 5 $\mu g.ml^{-1}$ gentamycin). The embryos were pulled into the open-pulled straw by touching of the narrowed tip of the straw to a microdrop of EFS solution according to Vajta *et al.* (1998) and then the straw was slowly immersed into liquid nitrogen.

Warming of embryos and recovery

After two weeks of storage in liquid nitrogen, the embryos were warmed by holding the straw at room temperature for 10 sec. Afterward, the straw with embryos was plunged into the drop with holding medium consisted of D-PBS, 20 % FCS and 0.3 M sucrose for 3 min. The embryos expelled off the straw, then washed twice in culture medium (B2 INRA medium with 10 % FCS and 5 $\mu g.ml^{-1}$ gentamycin) and placed in a fresh culture medium B2 INRA medium for 48 h incubation, until advanced blastocyst stage.

Analysis of embryos for total cell number and dead cell index (TUNEL)

Following 48 h culture the embryos were evaluated for the developmental stage and selected for analysis of cell number and dead cell index. The embryos were washed 3-times for 5 min in PBS-PVP washing solution (PBS with 4 $mg.ml^{-1}$ polyvinylpyrrolidone; Sigma-Aldrich Chemie, Steinheim, Germany). Then the embryos were fixed in 3.7 % neutrally buffered formalin (Fluka, Buchs, Switzerland) for 5 min and in 70 % ethanol for 10 min. Permeabilization was done by 15 min incubation of embryos in 0.5 % Triton X-100 in PBS. The embryos were processed for TUNEL using MEBSTAIN Direct Apoptosis Detection Kit (IM3171, Immunotech, Marseille, France) according to the product manual. Briefly, fixed and permeabilized embryos were incubated at 37 °C in TdT-labelling mixture (TdT buffer, FITC - dUTP a TdT) for 1 h. Following this incubation TUNEL-reaction was stopped by three-time washing of embryos in PBS-PVP solution. Thereafter the embryos were

transferred onto coverslip and covered with 5 µl of Vectashield anti-fade mounting medium, containing DAPI stain (Vector Laboratories, Burlingame, CA, USA). The coverslip was attached to microslide using nail polish. All treatments were performed at ambient temperature. The samples were stored at - 20 °C until fluorescence analysis.

TUNEL-indexes were determined on the basis of proportion of TUNEL-positive nuclei (green fluorescence) to total embryo cell number (DAPI-stained nuclei), which have been counted under the Leica fluorescent microscope (Leica Microsystems, Germany) using specific wave-length filters.

Statistics

The experiment was performed in 4 replications. A one-way ANOVA and Tukey-test were used to analyze differences in dead-cell index between groups. Since variable "TUNEL-index" was normally distributed, a log-transformation of original values has not been done. The hypothesis of normality was rejected for "Total cell number". For these two variables the log-transformation of original values was used. All calculations were performed using the SAS software package.

RESULTS AND DISCUSSION

Totally 205 embryos were used in experiments. Both fresh and frozen-thawed embryos were cultured since the morula stage up to 48 hours, when it was expected that most of embryos can reach either blastocyst or expanded blastocyst stage. As it is shown in Table 1, about 83 % of all fresh embryos (control) were developed to the advanced blastocyst stage, whilst in the frozen-thawed group only 60 % of embryos reached this stage, and the difference between groups was statistically significant ($p < 0.05$). Frozen-thawed embryos contained significantly less number of embryonal nuclei when compared with fresh embryos. Dead-cell index calculated on the basis of the number of TUNEL-positive cells was more than twice higher in the frozen-thawed embryos in comparison to the fresh embryos (Table 1), but this value did not exceed 10 %, the critical value which may compromise the embryo viability.

Despite intensive research efforts various cryopreservation procedures still cause significant morphological and biochemical alterations, which may lead to cell death and loss of embryo viability. The loss of viability can be influenced by the type and concentration of cryoprotectant, the freezing protocol, animal species, developmental stage of the embryo and system of embryo production (Fabian *et al.*, 2005). In our study we used the embryos of cattle which were *in vivo* recovered from the hormonally superovulated cows at the stage of compact morula or early blastocyst. Such embryos were frozen by vitrification, where the vitrification solution contained, beside ethylene glycol, also ficoll 70 as cryoprotectant, which was previously validated in several reports. In particular, Ficoll 70 was previously successfully used as a cryoprotectant in many studies on cryopreservation of embryos of different animal species including rabbit (Kasai *et al*., 1992; Papis *et al*., 2005; Makarevich *et al.*, 2008) and cattle (Darvelid *et al.*, 1994; Mahmoudzadeh *et al.*, 1995; Nguyen *et al.*, 2000).

Developmental stage and morphological quality of frozen-thawed embryos are the primary parameters affecting their survival rate. Morula stage embryos following vitrification and warming were in our experiments post-cultured for two days; it is the time required to reach advanced blastocyst stage. About 60 % of vitrified embryos were developed to expanded blastocysts stage after thawing, what is less than the blastocyst rate in fresh control (83 %), but this value is in the standard range of embryo survival

Table 1: Influence of vitrification-warming on the embryo viability

Groups	No. embryos, N	Developed to the advanced Bl stage, n (%)	Total cell number, n x ± S.E.M.	Dead cell (TUNEL) index, % x ± S.E.M.
Fresh (control)	117	97 (82.91)[a]	137 ± 7.50[a]	4.32 ± 0.52[a]
Frozen-thawed	88	53 (60.22)[b]	122.7 ± 3.98[b]	9.53 ± 1.49[b]

[a] versus [b] – significant differences between groups at $p < 0.05$

rate after cryopreservation.

The number of cells and the extension of apoptosis are important parameters of embryo development and embryo health (Brison and Schultz, 1998). Total cell number in our experimental embryos (following freezing-thawing) was significantly less than that of the intact (control) embryos. This indicates that cryopreservation procedure inhibits proliferation of the embryo cells (127 cells per embryo versus 137 cells in control), however this inhibition is not crucial for further development of the embryo.

Apoptosis at the blastocyst stage eliminates cells that are damaged, excessive or no longer required or developmentally incompetent. Therefore, apoptosis can be considered to be a normal process in preimplantation embryos to eliminate deviating cells, but a high incidence of apoptotic cells is correlated with abnormal morphology of the embryo (Hardy *et al.*, 1999). In our experiments dead (TUNEL-positive) cells were observed both in cryopreserved and in intact (control embryos), however the incidence of dead cells (TUNEL-index) in embryos was different. In particular, cryopreserved embryos contained more than twice higher proportion of dead/apoptotic cells (9.5 %), than intact control (4.32 %). This difference can be explained by the fact that vitrification and/or thawing procedure can compromise the quality of the embryo by damaging their membranes and organelles and thereby by triggering protective mechanisms to eliminate damaged cells. The proportion of dead cells in the embryos following vitrification in our study does not exceed 10 %, what may suggest that this parameter is still within the physiological range and the embryos following vitrification are principally able to survive and develop further.

Similar results about the quality of vitrified embryos were obtained in the experiments with rabbit embryos (Chrenek *et al*., 2014) using the same cryopreservation medium (EFS). In particular, rabbit embryo development (expanded blastocyst rate) following vitrification was deteriorated from 96 % in the intact control to 72.6 % in the frozen-thawed embryos, even though those authors used, oppositely to bovine embryos, *in vivo* fertilized rabbit embryos (Chrenek *et al*., 2014). This report and our present experiments confirm that cryopreservation may affect embryo viability, as a consequence of the toxic action of cryoprotectants during vitrification or thawing procedure itself.

In conclusion, monitoring of embryo quality following cryopreservation would provide a basis for understanding embryo sensitivity to freezing protocol and will lead to improved cryopreservation technique. At the process of the creation of animal gene banks we should expect possible deteriorating effect of a cryopreservation procedure on the embryo survivability.

ACKNOWLEDGEMENT

This study was supported by the Agency for the Support of Science and Technology (APVV) in the Slovak Republic: projects APVV-0556-11 and APVV-0137-10. This article was written during realization of the project “LAGEZ 26220120051” supported by the Operational Programme Research and Development funded from the European Regional Development Fund. The research leading to these results has received funding also from the European Community under project no 26220220180: Building Research Centre „AgroBioTech“ also.

REFERENCES

BRISON, D. R. – SCHULTZ, R. M. 1998. Increased incidence of apoptosis in transforming growth factor alpha-deficient mouse blastocysts. *Biology of Reproduction*, vol. 59, 1998, p. 136-144.

DARVELID, U. – GUSTAFSSON, H. – SHAMSUDDIN, M. – LARSSON, B. – RODRIGUEZ-MARTINEZ, H. 1994. Survival rate and ultrastructure of vitrified bovine *in vitro* and *in vivo* developed embryos. *Acta Veterinaria Scandinavica*, vol. 35, 1994, p. 417-426.

FABIAN, D. – GJORETT, J. O. – BERTHELOT, F. - MARTINAT-BOTTE, F. – MADDOX-HYTTEL, P. 2005. Ultrastructure and cell death of *in vivo* derived and vitrified porcine blastocysts. *Molecular Reproduction and Development*, vol. 70, 2005, p. 155-165.

HARDY, K. 1999. Apoptosis in the human embryo. *Reviews of Reproduction*, vol. 4, 1999, p. 125-134.

KASAI, M. – HAMAGUCHI, Y. – ZHU, S. E. – MIYAKE, T. – SAKURAI, T. – MACHIDA, T. 1992. High survival of rabbit morulae after vitrification in an ethylene glycol-based solution by a simple method. *Biology of Reproduction*, vol. 46, 1992, p. 1042-1046.

KASAI, M. – KOMI, J. H. – TAKAKAMA, A. – TSUDERA, H. – SAKURAI, T. – MACHIDA, T. 1990. A simple method for mouse embryo cryopreservation in a low toxicity vitrification solution, without appreciable loss of viability. *Journal of Reproduction and Fertility*, vol. 89, 1990, p. 91-97.

MAHMOUDZADEH, A. R. – VAN SOOM, A. – BOLS, P. – YSEBAERT, M. T. – DE KRUIF,

A. 1995. Optimization of a simple vitrification procedure for bovine embryos produced *in vitro*: effect of developmental stage, two-step addition of cryoprotectant and sucrose dilution on embryonic survival. *Journal of Reproduction and Fertility*, vol. 103, 1995, p. 33-39.

MAKAREVICH, A. V. – CHRENEK, P. – OLEXIKOVA, L. – POPELKOVA, M. – TURANOVA, Z. – OSTRO, A. – PIVKO, J. 2008. Post-thaw survival, cell death and actin cytoskeleton in gene-microinjected rabbit embryos after vitrification. *Theriogenology*, vol. 70, 2008, p. 675-681.

MARKKULA, M. – RATY, M. – JAUHIAINEN, L. – PARANKO, J. – RAULA, J. – MAKAREVICH, A. V. 2001. Ratio of proliferating cell nuclear antigen-positive nuclei to total cell number is higher in Day 7 than in Day 8 vitrified *in vitro*-produced bovine embryos. *Biology of Reproduction*, vol. 65, 2001, p. 52-59.

MARQUEZ-ALVARADO, Y. C. – GALINA, C. S. – CASTILLA, B. – LEON, H. – MORENA-MENDOZA, N. 2004. Evidence of damage in cryopreserved and fresh bovine embryos using the Tunel technique. *Reproduction in Domestic Animals*, vol. 39, 2004, p. 141-145.

NGUYEN, B. X. – SOTOMARU, Y. – TANI, T. – KATO, Y. – TSUNODA Y. 2000. Efficient cryopreservation of bovine blastocysts derived from nuclear transfer with somatic cells using partial dehydration and vitrification. *Theriogenology*, vol. 53, 2000, p. 1439-1448.

PAPIS, K. – SYPECKA, J. – KORWIN-KOSSAKOWSKI, M. – WENTA-MUCHALSKA, E. – BILSKA, B. Banking of embryos of mutated, paralytic tremor rabbit by means of vitrification. *Laboratory Animals*, vol. 39, 2005, p. 284-289.

POPELKOVA, M. – CHRENEK, P. – PIVKO, J. – MAKAREVIČ, A. V. – KUBOVIČOVÁ, E. –KAČMÁRIK, J. 2005. Survival and ultrastructure of gene-microinjected rabbit embryos after vitrification. *Zygote*, vol. 13, 2005, p. 283-293.

POPELKOVA, M. – TURANOVA, Z. – KOPRDOVA, L. – OSTRO, A. – TOPORCEROVA, S. – MAKAREVICH, A.V. – CHRENEK, P. 2009. Effect of vitrification technique and assisted hatching on rabbit embryo developmental rate. *Zygote*, vol. 17, 2009, p. 57-61.

RIZOS, D. – WARD, F. – BOLAND, M. P. – LONERGAN, P. 2001. Effect of culture system on the yield and quality of bovine blastocysts as assessed by survival after vitrification. *Theriogenology*, vol. 56, 2001, p. 1-16.

THARASANIT, T. – COLENBRANDER, B. – STOUT, T. A. E. 2005. Effect of cryopreservation on the cellular integrity of equine embryos. *Reproduction*, vol. 129, 2005, p.789-798.

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.