Acta fytotechn zootechn, 19, 2016(Special Issue), 96-98
http://www.acta.fapz.uniag.sk

Original Paper

Development of bovine and rabbit preimplantation embryos *in vitro* after cryostorage

Peter Chrenek[1,2*], Alexander V. Makarevich[1], Elena Kubovičová [1]

[1]*National Agriculture and Food Centre – Research Institute for Animal Production, Lužianky, Slovak Republic*
[2]*Slovak University of Agriculture, Faculty of Biotechnology and Food Sciences,Slovak Republic*

Article Details: Received: 2016-05-17 | Accepted: 2016-06-13 | Available online: 2016-09-01

http://dx.doi.org/10.15414/afz.2016.19.si.96-98

Evaluation of quality and viability of bovine and rabbit preimplantation embryos following cryopreservation was the objective of this study. Bovine embryos of Holstein breed (n = 88) at the morula-early blastocyst stage (on the 7th day after the first insemination) and rabbit embryos (n = 135) from New Zealand breed at the morula stage (90–92 hpc) were cryopreserved by the two-step vitrification procedure. Following thawing the embryos of both cattle and rabbit were cultured for 48 hours in order to reach expanded blastocyst stage, and then were analyzed for the developmental rate and viability (embryo cell number and incidence of the dead cells). The results demonstrate that cryopreservation can only slightly affect embryo viability and quality do not compromising their further development. Therefore, vitrification techniques tested in our study can be used for cryopreservation of embryos of national cattle and rabbit breeds for the purpose of long-term storage of embryos in the animal gene bank.

Keywords: cattle, rabbit, embryo, cryopreservation, viability

1 Introduction

Cryopreservation of preimplantation embryos is an important tool for maintaining animal genetic sources 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. Cryotolerance may be a useful indicator of blastocyst quality (Rizos et al., 2001). As a functional criteria for 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.

Rabbit embryos have been successfully cryopreserved either by conventional slow freezing (Naik et al., 2005), classical one-step or two-step vitrification (Kasai et al., 1992; Kauffman et al., 1998; Silvestre et al., 2003; Naik et al., 2005], open pulled straw (OPS) vitrification (Naik et al., 2005) or modified (sealed) OPS procedure (López-Béjar and López-Gatius, 2000). Cryopreservation of rabbit embryos at morula (Silvestre et al., 2003; Naik et al., 2005), blastocyst (López-Béjar and López-Gatius, 2000) and zona-free expanded or hatching blastocyst stages were reported (Cervera and Garcia-Ximenez, 2003), but higher rates of development were achieved when blastocyst (but not morula) stage embryos were 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. Since long-term storage of biological material at ultra-low temperatures is an effective tool for creating and maintaining of animal gene banks, the aim of this short study was to examine how vitrification procedure can affect further development of preimplantation cattle and rabbit embryos. We evaluated developmental potential and quality of the embryos following vitrification using morphological characteristics and fluorescent markers of the cell viability.

* **Corresponding Author:** Peter Chrenek, Slovak University of Agriculture, Faculty of Biotechnology and Food Sciences, Tr. A. Hlinku 2, 949 76 Nitra, Slovak Republic. E-mail: peter.chrenek@uniag.sk

2 Material and methods

Bovine embryos of Holstein breed (n= 88) at the morula-early blastocyst stage (on the 7th day after the first insemination) and rabbit embryos (n= 135) from New Zealand breed at the morula stage (90–92 hpc) were cryopreserved by the two-step vitrification procedure using EFS vitrification solution (Kasai et al., 1990; ethylene glycol – 40 % v/v, Ficoll 70–18 % w/v; 0.3 M sucrose in D-PBS + 20 % fetal calf serum and 5 µg/ml gentamycin), pulled into the open-pulled straws and slowly immersed into liquid nitrogen. As control groups, rabbit non-vitrified embryos (n= 135) and bovine non-vitrified embryos (n = 117) continued in the culture until 120 hours *post-coitum* and for subsequent 48 hours, respectively to reach expanded blastocyst stage. Following thawing the embryos of both cattle and rabbit were cultured for 48 hours in order to reach expanded blastocyst stage, afterwards these embryos were analyzed for the developmental rate, embryo cell number and incidence of the dead cells.

Total cell number was counted after staining of the embryos with DAPI fluorochrome. Number of ICM cells was counted after the differential staining of blastocysts for ICM and TE cells as described earlier (Chrenek et al., 2011). Apoptotic index was determined by TUNEL-reaction using a MEBSTAIN direct apoptosis kit (Immunotech, Marseille, France). Embryo diameters, excepting *zona pellucida*, were measured from the same images on the screen of the monitor using a scale bar micrometer (Leica, Germany). Total cell number, apoptotic index, and number of inner cell mass (ICM) cells were counted from the embryo images acquired on a Leica fluorescent microscope using appropriate wavelength fluorescent filters.

Development of embryos up to blastocyst stage was analysed using the Chi-square test. Differences between groups in total cell number, ICM cell number and TUNEL index were analyzed using analysis of variance (ANOVA).

3 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. About 83% of all non-vitrified bovine 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 %). Nevertheless, this value did not exceed 10 %, the critical value which may compromise the embryo viability.

Totally 270 rabbit embryos were used, of them 135 were vitrified at the morula stage and the other 135 were cultured further until the blastocyst stage serving as a control. Following thawing, almost 73 % of the vitrified embryos were survived and developed to advanced blastocyst stage versus 96 % in the intact control. Total cell number in the vitrified embryos (117 ±36.0) was significantly lower than in the intact control (135 ±30.2). However, there were no significant differences between the vitrified and intact embryos in the proportion of ICM cells to the total cell number and in the embryo diameter. On the other hand, the incidence of dead cells was almost twice higher in the vitrified embryos (4.21 ±1.85) compared to control (2.08 ±0.50).

Our observation indicates that preimplantation embryos after freezing have altered their viability and quality, though not all studied parameters were affected by cryopreservation procedure. The more affected parameters were the total cell number of blastocysts and the dead cell index. This difference between vitrified and intact embryos can be explained by their different dynamics of the development: embryos following devitrification need some time to recover from the deep freezing and, therefore, have the delayed proliferation and the lower cell number compared to the intact embryos.

4 Conclusions

Our experiments confirm that cryopreservation may affect embryo viability increasing the incidence of dead cells. However, this value is still in the physiological range, therefore, the embryos after freezing have only slightly altered viability and quality, what does not compromise their further development. Therefore, vitrification techniques tested in our study can be used for cryopreservation of embryos of national cattle and rabbit breeds for the purpose of long-term storage of embryos in the animal gene bank.

Acknowledgments

This study was supported by the Agency for the Support of Science and Technology (APVV) in the Slovak Republic: the grants APVV-14-0043 and APVV-15-0196.

References

CERVERA, R.P. and GARCIA-XIMENEZ, F. (2003) Vitrification of zona-free rabbit expanded or hatching blastocysts: a possible model for human blastocysts. *Hum. Reprod*., 18, 2003, p. 2151–2156.

CHRENEK, P., BAUER, M. and MAKAREVICH, A. (2011) Quality of transgenic rabbit embryos with different EGFP gene constructs*. Zygote*, vol. 9, no. 1, pp. 85–90.

KAUFFMAN, R.D. et al. (1998) Superovulation of rabbits with FSH alters in vivo development of vitrified morulae. *Theriogenol*., vol. 50, pp.1081–1092.

KASAI, M. et al. (1992) High survival of rabbit morulae after vitrification in an ethylene glycol-based solution by a simple method. *Biol. Reprod*., vol. 46, pp. 1042–1046.

KASAI, M. et al. (1990) A simple method for mouse embryo cryopreservation in a low toxicity vitrification solution, without appreciable loss of viability. *J. Reprod. Fert*., vol. 89, pp.91–97.

LÓPEZ-BÉJAR, M. and LÓPEZ-GATIUS, F. (2000) *In vitro* and *in vivo* survival of vitrified rabbit embryos (abstract). *Theriogenol*., vol. 53, p. 259.

MAKAREVICH, A.V. et al. (2008) Post-thaw survival, cell death and actin cytoskeleton in gene-microinjected rabbit embryos after vitrification. *Theriogenol*., vol. 70, pp. 675–681.

MARKKULA, M. et al. (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. *Biol. Reprod*.,vol. 65, **p**p. 52–59.

MARQUEZ-ALVARADO, Y.C. et al. (2004) Evidence of damage in cryopreserved and fresh bovine embryos using the Tunel technique. *Reprod. Dom. Anim*., vol. 39, pp. 141–145.

NAIK, B.R. – RAO, B.S. – VAGDEVI, R. – GNANPRAKASH, M. – AMARNATH, D. – RAO, V.H. 2005. Conventional slow freezing, vitrification and open pulled straw (OPS) vitrification of rabbit embryos. Anim. Reprod. Sci., 86, 2005, p.329–338.

POPELKOVA, M. et al. (2005) Survival and ultrastructure of gene-microinjected rabbit embryos after vitrification. *Zygote*, vol. 13, pp. 283–293.

POPELKOVA, M. et al. (2009) Effect of vitrification technique and assisted hatching on rabbit embryo developmental rate. *Zygote*, vol. 17, pp.57–61.

RIZOS, D. et al. (2001) Effect of culture system on the yield and quality of bovine blastocysts as assessed by survival after vitrification. *Theriogenol*., vol. 56, **p**p. 1–16.

SILVESTRE, M.A. et al. (2003) Vitrification of *in vitro* cultured rabbit morulae. *Anim. Reprod. Sci*., vol. 76, pp.113–124.

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