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DEVELOPMENTAL POTENTIAL OF VITRIFIED RABBIT EMBRYOS

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ABSTRACT

The aim of this study was to examine how vitrification procedure can affect further development of preimplantation rabbit embryos. We evaluated post-thaw development and quality of the embryos following vitrification using morphological characteristics and fluorescent markers of the cell viability. The pronuclear stage eggs were flushed from the oviducts of the New Zealand rabbit females at 19-20 h *post coitum* (hpc) and randomly divided into two groups: first - intended for vitrification (n = 135) and second - non-vitrified (intact) control (n = 135) eggs. Then zygotes were cultured for 72 h, afterwards the embryos of the first group, which reached morula stage, were frozen by vitrification in EFS medium (ethylene glycol, Ficoll70, sucrose), whilst the embryos (morula) of the second (control) group continued in the culture until 120 h (expanded blastocyst stage). Then all the embryos including intact control (n = 270) were processed for total cell number, differential staining and cell death (apoptosis). Following thawing almost 73 % of embryos survived and developed to advanced blastocyst stage versus 96 % in the intact control. In regards to the embryo quality, in the vitrified embryos total cell number (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 (inner cell mass cells) to the total cell number and in the embryo diameter. On the other hand, the incidence of dead cells (apoptotic index) was almost twice higher in the embryos subjected to cryopreservation (4.21 ± 1.85) compared to control (2.08 ± 0.50). However, this incidence of dead cells in vitrified embryos is still in the physiological range, and does not compromise further development. Our observation indicates that rabbit embryos after freezing have only slightly altered viability and quality. Therefore, vitrification techniques tested in our study can be used for cryopreservation of embryos of national rabbit breeds for the purpose of long-term storage of embryo samples in the animal gene bank.

Key words: rabbit; embryo; vitrification; survival; apoptosis

INTRODUCTION

Cryopreservation is an important tool for creation of embryo banks for future use in animal breeding, veterinary and human medicine. This technique also enables to protect germ cells of rare or endangered species and strains of farm and wild animals. In certain cases optimization of cryopreservation protocols for more sensitive embryos is relevant. Vitrification technique, introduced for rabbit embryo cryopreservation by Smorag *et al.* (1989) and Kobayashi *et al*. (1990), improved general efficiency of embryo survival. The efficiency of rabbit embryo vitrification evaluated *in vitro* (Papis *et al*., 1993) and/or in vivo (Kasai *et al.*, 1992) depends partly on resistant morula stage embryos obtained from outbreed strains of rabbits.

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

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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.

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 rabbit embryos. We evaluated developmental potential and quality of the embryos following vitrification using morphological characteristics and fluorescent markers of the cell viability.

MATERIAL AND METHODS

Egg collection

New Zealand White rabbit does (4-6 month old), kept on the local farm were superovulated using a single *i.m.* injection of 20 IU.kg^{-1} PMSG (Werfaser, Alvetra und WERFFT, Wien, Austria). The females were given 40 IU.kg^{-1} hCG (Werfachor, Alvetra und WERFFT) *i.m.* 72 h after PMSG administration and does were mated with fertile bucks belonging to the same breed. The pronuclear stage eggs were flushed from the oviducts of the slaughtered animals with PBS supplemented with 5 % fetal calf serum (FCS) both from Gibco BRL (Auckland, New Zealand) 19 to 20 h *post coitum* (hpc). Recovered eggs were evaluated morphologically and the eggs with pronuclei, two polar bodies and compact cytoplasm were selected and randomly divided into two groups: 1) intended for vitrification and 2) non-vitrified (intact) control eggs. Then zygotes were cultured in the CO_2 incubator, and after 72 h the embryos of the first group, which reached morula stage, were frozen by vitrification, whilst the embryos (morula) of the second (control) group continued in the culture until 120 h (expanded blastocyst stage).

Procedure of vitrification-devitrification

For the vitrification an EFS medium based on a CO_2-independent medium (CIM, Gibco BRL) supplemented with 20 % FCS and composed of 40 % EG, 18 % Ficoll and 0.3M sucrose was used. The morula stage embryos (5-8) were equilibrated in EFS medium for 3-4 min at room temperature and then sucked up into 0.25 ml straw in EFS medium. Straws were melted on the tip, exposed for 60 sec to vapour of liquid nitrogen and then plunged into liquid nitrogen.

The embryos were devitrified by keeping the straw on the air for 10 sec and then plunging to water bath at 20 ºC for 7 sec. Then the content of the straw was released into 0.5 M sucrose and after 7-8 min embryos were transferred to CIM medium added with FCS in order to be washed out of the cryoprotectant. After warming the embryos were cultured up to expanded blastocyst (48 h) stage when a developmental rate (at 120 hpc) was determined. All embryos including intact control were processed for total cell number measurement, differential staining of cell compartments (ICM, TE) and cell death (apoptosis) detection.

Assays of embryo viability

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), which was previously calibrated on a ×10 or ×20 objective and ×10 eyepiece. 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.

Statistics

Development of rabbit embryos up to blastocyst stage was analyzed 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).

RESULTS AND DISCUSSION

In our study 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 (Table 1). Earlier reports (Fabian *et al.*, 2005; Popelkova *et al.*, 2005) documented that post-thaw embryo survival can be a valid tool to evaluate the efficiency of cryopreservation technique. After cryostorage and devitrification almost 73 % of embryos survived and developed to advanced blastocyst stage versus 96 % in the intact control.

In regards to the embryo quality, in the vitrified embryos total cell number was significantly lower than in the intact control. However, there were no significant differences between the vitrified and intact

Table 1: Survival and quality of vitrified or intact rabbit embryos

Group of embryos	Total no. embryos, N	Post-thaw development to expanded blastocyst, n (%)	Embryo quality			
			Total cell. number (n) x ± SD	Number of ICM cells/ embryo, N, x ± SD (%)	Embryo diameter (μm), x ± SD	Apoptotic index, % x ± SD
Control	135	130 (96.0)[a]	135 ± 30.2[d]	37 ± 7.5 (32.0)	129.85 ± 10.9	2.08 ± 0.50[e]
Vitrified	135	98 (72.6)[b]	117 ± 36.0[c]	32 ± 6.5 (23.7)	123.2 ± 7.2	4.21 ± 1.85[f]

Values with different superscripts within columns are significantly different ($p < 0.05$).

embryos in the proportion of ICM (inner cell mass cells) to the total cell number and in the embryo diameter. On the other hand, the incidence of dead cells (apoptotic index) was almost twice higher in the embryos subjected to cryopreservation (Table 1) compared to control.

Our observation indicates that rabbit 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.

The number of cells and the extension of apoptosis are important parameters of embryo development and health (Brison and Schultz, 1998). 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, 1999). In our study, increased apoptotic index in vitrified embryos evidences that some cells were damaged following the process of devitrification, however, this percentage of dead cells in these embryos (4.21 %) is still in the physiological range. This ratio of dead cells is lower compared to the previous study (Makarevich *et al.*, 2008), where, depending on vitrification protocol used, the proportion of dead cell was 7.5 % and 10.3 %, respectively.

In conclusion, the vitrification procedure using in our study (EG+ Ficoll70+ Sucrose) only slightly altered post-thaw survival, so that embryo viability and quality was not affected seriously. Therefore, this vitrification technique can be used for cryopreservation of embryos of national rabbit breeds (Zoborsky and Nitriansky) for the purpose of long-term storage of embryo samples in animal gene bank.

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