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ANALYSIS OF TRANSGENIC RABBIT VITRIFIED EMBRYOS CARRYING EGFP GENE

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ABSTRACT

The aim of our study was to investigate influence of vitrification on developmental rate and quality (total cells number, number of ICM blastomeres, apoptotic index and embryo diameter) of rabbit transgenic embryos carrying exogenous EGFP gene. EGFP-positive rabbit embryos were produced under *in vitro* conditions by the microinjection of foreign gene into the pronucleus of fertilized eggs. EGFP expression was detected by fluorescent microscope (at 492 nm). Developmental rate of vitrified/thawed transgenic embryos reached hatching blastocyst stage (68.00 %) differed significantly ($p<0.001$) from those in control embryos (100.00 % resp.). The higher proportion of ICM cells (32.00 %) and embryo diameter (130.85 ± 10.90) were found in the control group compared to transgenic ones. The ratio of apoptotic cells was higher (2.90 %) in vitrified EGFP-positive embryos compared with the control group (2.50 %).
Our results demonstrate, that neither gene microinjection itself, nor exogenous (EGFP) gene expression do interfere with quality of rabbit embryos. However, combination of microinjection and vitrification significantly decreases ($p <0.001$) survival rate of rabbit embryos.

Key words: rabbit, embryos, transgenic, vitrification, quality

INTRODUCTION

The production of genetically modified (transgenic) embryos and animals is still actual task, what is confirmed by seeking of some new methods to increase efficiency of transgenesis (gene integration and expression) and decrease final cost. One of promising approach is to use the EGFP (enhanced green fluorescent protein) selection marker. Microinjection of foreign DNA into the pronucleus or into the both pronuclei of fertilized egg is one of techniques for the production of transgenic organism (Chrenek *et al*., 2005). Therefore, the main problem in this field of biotechnology is a securation of maximal embryo survival and viability. At the present time, the vitrification – quick freezing in liquid nitrogen is an important tool for preservation of mammalian embryos. At this temperature all biochemical activities, which can lead to cell death, are effectively stopted (Özkavukcu and Erdemli, 2002). Despite intensive research, procedures of cryopreservation still cause biochemical and morphological changes, which may result in loss of embryo viability and even induce cell death. Analysis of the viability and quality of vitrified/devitrified embryos is of great importance (Popelková et al., 2005). Documentation of the cell injury during or after cryopreservation provides convenient information for understanding sensitivity of cells toward vitrification. This monitoring can lead to improvement of vitrification protocols and to better understanding of embryology of domestic animals (Dobrinsky, 2002). The developmental potential of a single blastomere from mammalian embryo is usually determined by its ability to form blastocyst with

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a visible inner cell mass (ICM) and a distinct trophoblast (Chrenek *et al*., 2008). The proportion of apoptotic cells has been considered to be one of the most important parameters for evaluating embryo health or culture conditions (Hardy, 1999). The aim of this study was to assess quality of rabbit transgenic (carrying EGFP gene) embryos following vitrification in comparison to intact and non-vitrified transgenic embryos.

MATERIAL AND METHODS

Biological material

Three days before mating, New Zealand White rabbit donors (APRC Nitra, SK) were treated with PMSG (Werfaser, 20 IU/kg of body weight, Austria) followed by hCG (Werfachor, 40 IU/kg of body weight, Austria) 72h later (Chrenek *et al*., 2005). At 19 to 20 hours post coitum, the pronuclear stage eggs were flushed from the oviducts of the rabbit females with PBS (Sigma, USA). The selection of flushed eggs was done in CIM medium added with fetal bovine serum FBS (10 %; Gibco BRL, USA). The rabbit embryos were cultured *in vitro* under the conditions of 5 %CO_2, 39°C in k-DMEM medium with 10% FBS (Gibco BRL, USA) described earlier (Chrenek et al., 2005).

Gene construct

For the microinjection into fertilized rabbit eggs, the EGFP reporter gene after linearization of the plasmid (Clontech, USA) with *Cla* I was used (Chrenek *et al*., 2005).

Production of rabbit transgenic embryos *in vitro* **(EGFP exogenous gene)**

Following selection of the flushed eggs, the eggs with both pronuclei were subjected to gene microinjection in CIM medium suplemented with 10 % FBS using an Olympus microscope equipped with micromanipulation units (Alcatel, France) and microinjector (Eppendorf, Femto Jet, Germany), as reported earlier (Chrenek *et al*., 2005). The eggs were fixed by suction with a holding pipette, and 1-2 pl of the DNA (EGFP) solution (4µg/ml) were microinjected into the male pronucleus by single microinjection, (SM) using air pressure. Swelling of pronuclei by 10% of their size indicated successful microinjection. The eggs were cultured in k-DMEM medium supplemented with 10 % FBS at 5 % CO_2 and 39°C up to the blastocyst stage (96 hpc) (Chrenek and Makarevich, 2005). The embryos were analyzed for the transgene integration at morula stage using Leica fluorescent microscope (Leica, Germany).

Vitrification (quick freezing) and devitrification of rabbit embryos

Rabbit embryos at morula stage 48 hours after flushing were put into a vitrification solution containing CIM medium + 20 % FCS (Gibco BRL, USA) + 40 % ethylene glycol (Sigma, USA) + 18 % ficoll 70 (Sigma, USA) + 0.3M sucrose (Sigma, USA) for 3-4 minutes and then were plunged into liquid nitrogen (Papis, 2005). After three days the embryos were thawed. After removal from liquid nitrogen, the embryos were exposed to a devitrification solution containing CIM medium + 20 % FCS + 0.3 M sucrose for 7-8 minutes and then transfered into conditional solution, containing CIM medium + 20 % FCS. The embryos were then cultured under *in vitro* conditions (5 % CO_2, 39 °C, k-DMEM + 10 % FCS) up to the hatching blastocyt stage.

Differential staining

The embryos were incubated in freshly prepared solution of 0.2 % Triton X-100 in PBS containing 2 mg/ml BSA for 20 s, and immediately washed twice in PBS-BSA medium. The embryos were transferred into PBS-BSA containing 30 µg/ml of propidium iodide (PI) and incubated in the dark at 37 °C in warm chamber for 5 min, and then washed twice in PBS-BSA medium. Next, the embryos were incubated in 4 % paraformaldehyde (PFA) containing 10 µg/ml bisbenzimide (Hoechst 33342, Sigma, USA) for 30min at room temperature and then washed twice in PBS-BSA medium. The embryos were incubated in a freshly prepared ice-cold solution of 0.1 % Triton X-100 in 0.1% sodium citrate (v/v) for 5 min and then washed twice in PBS-BSA medium. Washed embryos were covered with a Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA), mounted onto glass slides with coverslip and examined under a Leica fluorescence microscope.

Analysis of apoptosis (TUNEL)

The embryos were removed from culture medium, washed 3x5 min in PBS supplemented with polyvinylpyrrolidone (PBS-PVP, 4mg/ml) and then fixed in 3.7 % neutrally buffered formalin for 5 min and in 70 % ethanol for 10 min. For membrane permeabilization, the embryos were incubated in 0.5 % Triton X-100 in PBS for 15 min. The embryos were processed for TUNEL using a MEBSTAIN Direct Apoptosis Detection Kit (Immunotech, Marseilles, France) according to the manufacturer´s instructions. Afterwards, the embryos were counterstained with propidium iodide (PI, 1µg/ml v PBS). After the washing, the embryos were covered with 5 µl of Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA) and attached to microslide using small columns of nailpolish (Makarevich *et al*., 2005).

Embryo diameter

Embryo diameter including *zona pellucida*, was measured from the images on the screen of the monitor using scale bar micrometer, which was previously calibrated on a 40x objective and 10x eyepiece. The diameter of the embryos was the mean of two measurements made perpendicularly to each other (Makarevich et al., 2005).

Statistical analysis

Influence of microinjection and vitrification on development rate and quality of rabbit transgenic and non-transgenic embryos were evaluated using Chí-square test.

RESULTS

Developmental rate and quality of intact, non-vitrified and vitrified transgenic rabbit embryos are represented in Table 1.

Table 1: Development rate and quality of rabbit vitrified transgenic embryos

Group of embryos	No embryos Vi/DeVi (N)	No. HBl n (%)	Total no. cells (N) $\bar{x}$ ± SD	No. Cells in ICM/embryo $\bar{x}$ ± SD (n / %)	No. apoptotic cells/embryo $\bar{x}$ ± SD (n / %)	Diameter of embryos (μm) $\bar{x}$ ± SD
Control	35	35 (100.00%)[a]	117.00 ± 36.00	37.00 ± 7.50 (32.00 %)	3.00 ± 1.50 (2.50 %)	130.85 ± 10.90
EGFP	25	25 (100%)[a]	125± 25.50	35.00 ± 5.50 (28.50 %)	3.00 ± 1.505 (2.40 %)	128.50 ± 5.50
Vi / deVi EGFP	25	17 (68.00%)[b]	135.00 ± 30.20	32.00 ± 6.50 (23.70 %)	4.00 ± 1.85 (2.90 %)	121.20 ± 7.20

Vi/deVi – vitrificated/devitrificated rabbit embryos;
HBl – hatching/hatched blastocysts
a vs. b significant difference at $p < 0.001$

Significant difference ($p<0.001$) in the developmental rate between control, non-vitrified transgenic embryos and vitrified EGFP-positive embryos (100.00 vs. 68.00 %) was observed. Proportion of the ICM cells to total cell counts in the control group was 32.00 %, whilst in transgenic/vitrified group lower percentages of cells in the ICM area (23.70 %) was found. No significant differences were observed in the number of apoptotic cells/embryo (Fig. 1) and in embryo diameter between control, non-vitrificated and transgenic vitrified embryos.

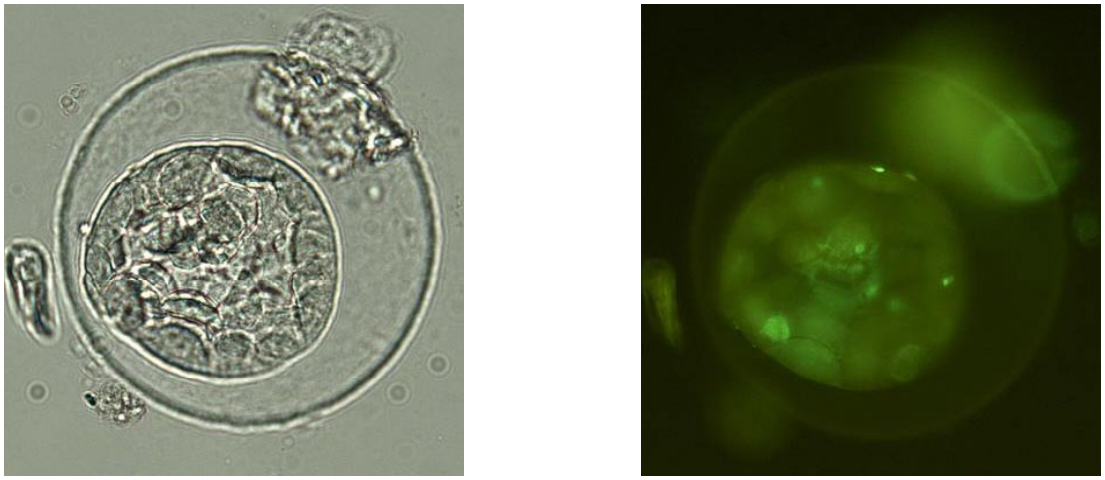

Figure 1: Rabbit transgenic vitrified embryos with apoptotic cell (green signal) detections

DISCUSSION

In our previous study (Chrenek *et al*., 2005) we showed that double microinjection technique increased transgene integration rate in rabbit embryos with a significant difference in blastocyst survival rate between double and single microinjection. Generally, microinjection, transgene integration and vitrification are factors that can affect the quality of manipulated embryos. In our experiments we investigated also influence of vitrification on the rabbit transgenic (EGFP) embryo quality. Rabbit transgenic embryos, which have undergone vitrification process, had significantly reduced viability after thawing (68.00 %) compared to the control group (100.00 %). Decrease in embryo viability can be caused by the type and concentration of the cryoprotectant, animal species, the genotype, developmental stage of the embryo and the time of exposure to cryoprotectant substances (Fabian *et al*., 2005). Popelková *et al*. (2005), using DMSO as a cryoprotectant, did not find significant difference in the developmental rate of rabbit embryos, because 57.00 % of vitrified and 56.00 % of intact rabbit embryos reached the hatching blastocyst stage. Papis *et al*., (2005) reported comparable data with our results. Makarevich *et al*. (2008) monitored the effect of vitrification method on the developmental rate of rabbit embryos, using the vitrification medium with ethyleneglycol and ficoll 70. They showed significantly lower ability ($p < 0.05$) of hatching blastocyst formation when compared to intact (97.00 %) or vitrified rabbit embryos (63.00 %). Embryo diameter and cell number are non-invasive markers of embryo quality. Vitrification procedure in our study caused decrease in total cell numbers of vitrified hFVIII- positive embryos compared to the control group (117.00 ± 36.00 vs. 141.00 ± 34.80).

The occurrence of apoptosis indicates suboptimal culture conditions or the effects of experimental treatments (Makarevich et al., 2005). The higher proportion of apoptotic cells was observed in the EGFP - positive vitrified embryos. One of the reasons can be a finding, that GFP, derived from *Aequorea victoria*, induces apoptosis (Hsiao-Sheng *et al*., 1999). Fabian *et al*., (2007) reported, that apoptosis in intact embryos does not occur earlier than the 16-cell stage embryos. In the case of early rabbit blastocysts, proportion of apoptotic cells was 1.38 %, but mouse embryos showed higher proportion of apoptotic cells (6.60 %) at this developmental stage.

In conclusion, our results demonstrate, that neither microinjection of foreign gene, nor exogenous (EGFP) gene expression do interfere with developmental rate and quality of rabbit embryos. However, combination of microinjection and vitrification significantly decreases ($p<0.001$) developmental rate and increases proportion of apoptotic cells in rabbit embryos.

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