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QUALITY OF RABBIT TRANSGENIC EMBRYOS

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ABSTRACT

The objective of this study was to compare quality of transgenic (TR) and non-transgenic rabbit embryos in regard to a total cells number, number of cells in ICM, percentage of apoptotic cells and average diameter of the embryo. The TR embryos were produced by microinjection of the EGFP gene into male pronucleus of fertilized rabbit egg, or they were obtained by mating of transgenic rabbits, carrying the gene for hFVIII. No significant differences were observed in the total cell number, ICM cell number and the embryo diameter between the groups. The higher total cell number (142 ±35.42), ICM cell number (36 ± 4.09) and embryo diameter (133.97 ± 10.93) were found in non - transgenic rabbit embryos. On the other hand, rate of apoptotic cells was significantly higher ($p \leq 0.05$) in transgenic embryos (6.6 %), compared to *in vivo* developed transgenic embryos (3.2 %). Our results indicate that transgenic rabbit embryos (with EGFP or hFVIII gene construct) do not differ in quality from non-transgenic ones.

Key words: transgenic rabbit, microinjection, hFVIII gene, EGFP gene

INTRODUCTION

In vitro manipulations on embryos have been widely used to produce genetically modified animals (transgenic, chimeric, cloned). However, each additional in vitro manipulation diminishes the viability of the resulting embryo. Microinjection of gene construct into the pronucleus of fertilized egg is one of techniques for transgenic animal production. The main problem using this approach is a decreased quality of obtained embryos, which is resulted in higher percentage of degenerated or developmentally arrested ova (Makarevich et al., 2005, Olexikova et al., 2007). This may be caused by several factors including microinjection using single or double technique (Chrenek et al., 2005), culture conditions (Chrenek et al., 1998), transgene localization, number of integrated copies into the genome of the embryo, total and ICM cell number (Chrenek and Makarevich, 2005, Makarevich et al., 2006). The objective of this study was to compare quality of transgenic embryos produced both *in vitro* (by the microinjection of EGFP gene into pronucleus of fertilized egg) or derived *in vivo* (by mating of transgenic rabbits with hFVIII gene) with non - transgenic rabbit embryos in regard to a total cell number of embryos, number of blastomers in ICM area, percentage of apoptotic cells and the diameter of the embryo.

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MATERIAL AND METHODS

Biological material

Three days before mating, New Zealand White rabbit donors (SARC 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., 2002). At 19 to 20h after mating, the pronuclear stage eggs were flushed from the oviducts of the animals with PBS. The selection of flushed eggs was done in CIM medium added with foetal bovine serum FBS (10 % ; Gibco BRL). The embryos were cultured *in vitro* under the conditions (5%CO_2, 39°C, k-DMEM + 10% FCS, Gibco BRL, USA) described earlier (Chrenek et al., 2005).

Gene constructs

The EGFP reporter gene (plasmid pEGFP-N1, Clontech, USA) was used for the microinjection into fertilized rabbit eggs after linearization of the plasmid with *Cla* I. The second gene construct consisted of a 2.5 kb murine whey acidic protein promoter (mWAP), 7.2 kb cDNA of the human clotting factor VIII (hFVIII), and 4.6 kb of 3' flanking sequences of the mWAP gene. This gene was provided by Dr. H. Lubon, American Red Cross, MD, USA (Chrenek et.al, 2005a). The plasmid was digested with *Not* I to make the 14.3 kb insert and purified in a Qiaex II gel extraction kit (Qiagen, USA).

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

Following selection of the flushed ova, the eggs with both pronuclei were subjected to gene microinjection in CIM medium suplemented with 10% FBS (both from Gibco BRL, USA) using the Olympus microscope equipped with micromanipulation units (Alcatel, France) and microinjector (Eppendorf, Femto Jet, Germany), as was 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 male pronucleus by single microinjection, (SM) using air pressure. Swelling of pronuclei by 10% indicated successful microinjection. The eggs were cultured in k-DMEM medium supplemented with 10% FBS (Gibco BRL, USA) at 5% CO_2 and 39°C up to the blastocyst stage (96hpc). Blastocysts were analyzed for the transgene integration using fluorescent microscope (Chrenek and Makarevich, 2005).

Production of rabbit transgenic embryos *in vivo* (hFVIII gene)

Transgenic rabbits (F4 generation) carrying the human factor VIII gene (gene construct mWAP-hFVIII, Chrenek et al., 2005) were mated and rabbit transgenic embryos were then flushed out from oviducts of transgenic donors at 1-cell stage (20hpc).

Differential staining

The embryos were incubated in freshly prepared 0.2% Triton X-100 in PBS containing 2mg/ml BSA for 20s, and immediately washed twice in PBS-BSA medium (Chrenek and Makarevich, 2005). The embryos were transferred into PBS-BSA containing 30µg/ml of propidium iodide (PI) and incubated in the dark at 37°C in warm chamber for 5min, and then washed twice in PBS-BSA medium. Next, the embryos were incubated in 4% paraformaldehyde (PFA) containing 10µg/ml bisbenzimide (Hoechst 33342, Sigma, USA) for 30min at RT 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 Vectashield mounting medium, mounted on to glass slides with coverslip and examined under a Leica fluorescence microscope (Chrenek and Makarevich, 2005).

Analysis of apoptosis (TUNEL)

Embryos were removed from culture medium, washed 3x5min in PBS supplemented with polyvinylpyrrolidone (PBS-PVP, 4mg/ml) and then fixed in 3.7% formaline for 5min and in 70% ethanol for 10min. For membrane permeabilization, the embryos were incubated in 0.5% Triton X-100 in PBS for 15min (Makarevich et al, 2005). 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, 1 µg/ml v PBS). After the washing, embryos were covered with 5µl of Vectashield mounting medium (Vector Laboratories, Burlingame, USA) and attached to microslide using small blocks of nailpolish.

Embryo diameter

Embryo diameters including *zona pellucida*, were 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. 2006).

Statistic analysis

Differences between groups in total number of cells, ICM cells, number of apoptotic nuclei and diameter

of embryos were estimated using one-way ANOVA by SAS software (SAS Institute, 2002).

RESULTS AND DISCUSSION

In our experiment a quality of two types of transgenic (hFVIII or EGFP) and non-transgenic rabbit embryos was determined. No significant differences in the total cell number, ICM cell number and diameter of the embryo between the groups were found (Table 1). The embryo diameter and cell number are non-invasive markers of embryo quality, as their determination does not require destruction of the embryo, when vital dye staining is used (Makarevich et.al., 2006). Although embryo diameter is assumed to be potential marker for the viability testing of bovine expanded blastocyst (Mori et al., 2002), this statement has not been confirmed in rabbit transgenic embryos. We observed, that proportion of ICM cells to total cell number was similar in transgenic (28 % vs. 26 %) and non-transgenic (25 %) groups of rabbit embryos. In the study of Shu-Zhen Liu et al. (2005) the total cell number in rabbit cloned embryos was significant lower (145.4 ± 20.2 resp. 126.1 ± 21.8) than in *in vivo* derived rabbit embryos (179.6 ± 18.1). On the other hand the ICM cell number in cloned embryos (76.9 ± 11.9, resp. 73.2 ± 13.7) was significantly higher than that in *in vivo* derived embryos (69.9 ± 10.2).

Significant difference in number of apoptotic cells between transgenic hFVIII rabbit embryos (3.2 %) and transgenic EGFP rabbit embryos (6.6 %) was observed. Apoptosis (programmed cell death) is an active physiological process and result of this process is elimination of abundant, damaged or harmful cells. This process is genetically controlled (Schwarzman and Cidlowski, 1993). The presence of various molecular components of the apoptotic cascade has been proved in mouse, human and bovine preimplantation embryos (Warner et al., 1998, Jurisicova and Acton, 2004, Gutierrez – Adan et al., 2004). Our results confirmed result of Makarevich et al. (2005), that apoptosis is not always the primary cause of the decrease in embryo cell number. Apoptotic processes at earlier stages of preimplantation development showed obvious dissimilarities from apoptotic processes in the blastocyst. In this case, the occurrence of apoptosis was sporadic and its presence was noted only after reaching particular developmental stages. Percentage of apoptotic cells in mouse embryos was usually higher than in rabbit embryos (Fabian et al., 2007). Occurrence of apoptosis *in vitro* signalized about suboptimal culture conditions or influence of experimental procedures (Schwarzman and Cidlowski, 1993, Makarevich et al., 2005). Significant differences between both transgenic groups found in our study can be explained by the fact that transgenic EGFP rabbit embryos were produced by microinjection into pronucleus of eggs, whilst hFVIII transgenic embryos were recovered from transgenic females. Therefore the higher proportion of apoptotic cells in transgenic EGFP embryos can be caused by many factors associated with the microinjection itself, for example, mechanical damage by microinjection pipette, exposure of the zygote to a microscope light of a higher intensity, or their combination (Chrenek et al., 2005, Makarevich et al., 2005).

CONCLUSION

Present preliminary results, obtained on the rabbit transgenic embryos with two different gene constructs demonstrated that total cell number, ICM area cell number and average diameter of the embryo were comparable to those in the non-transgenic embryos. The microinjection procedure in our study tended to increase apoptotic cell number, but embryo quality was not significantly differed from non-transgenic one.

Table 1: Quality of transgenic rabbit embryos *in vitro*

Embryos	No. of embryos in HBl (N)	No. of total cells $\bar{x}$ ± SD	No. of cells in ICM $\bar{x}$ ± SD (%)	No. of apoptotic cells $\bar{x}$ ± SD (%)	Diameter of embryos (μm) $\bar{x}$ ± SD
Non-transgenic	31	142 ± 35.42	36 ± 4.09 (25 %)	7 ± 2.6 (5 %)	133.97 ± 10.93
Transgenic *in vitro* EGFP	64	136 ± 23.29	35 ± 5.07 (26 %)	9 ± 4.96 (6.6 %)a	124.42 ± 7.26
Transgenic *in vivo* hFVIII	85	122 ± 19.34	34 ± 4.05 (28 %)	4 ± 2.08 (3.2 %)b	130.55 ± 8.50

a - vs; b - significant diference at $p < 0.05$

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