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EFFECT OF *IN VITRO* CULTURE CONDITIONS ON THE QUALITY OF RABBIT EMBRYOS

B. KULÍKOVÁ[3], J. SLAMEČKA JR.[1,2], A.V. MAKAREVICH[1], P. CHRENEK[1,2]*

[1]Animal Production Research Centre Nitra, Slovak Republic
[2]Slovak University of Agriculture, Nitra, Slovak Republic
[3]Constantine the Philosopher University, Nitra, Slovak Republic

ABSTRACT

The aim of our study was to examine effect of *in vitro* culture on preimplantation rabbit embryo quality (total cell number, number of blastomeres in the inner cell mass (ICM) area, apoptotic index and embryo diameter).
Rabbit embryos of the New Zeland White breed were flushed from the oviducts 19 – 20 hours *post coitum* and cultured up to the blastocyst stage (the experimental group). As a control group, the embryos developed *in vivo* were used, which were flushed from the uterus at the expanded blastocyst stage 94 hours *post coitum*. Embryos were stained by propidium iodide and Hoechst 33342 to detect total cell number and number of cells in the ICM area. For detection of apoptotic cells, TUNEL-reaction was used.
Significantly higher number of blastomeres in ICM ($p<0.05$) was found out in the control group (44.18±2.50) when compared to the experimental group (36.22±2.45). However, total cell number of embryos was not significantly different between these two groups (155.12±15.00 vs. 127.23±14.50), respectively. Significant difference between control and experimental groups in the number of apoptotic cells (0.35 vs. 1.25) and embryo diameter (133.95±8.95 vs. 123.40±7.25 μm) was not proved too.
Our results demonstrate slightly worse quality of rabbit embryos cultured *in vitro*, compared to embryos developed *in vivo* what is reflected in the number of cells in embryoblast.

Key words: rabbit; embryos; *in vitro* cultivation; embryo quality

INTRODUCTION

The most useful criterium of quality of preimplantation embryos is the evaluation of developmental stage of the embryos basing on visual inspection of their morphology under a light microscope. However, such evaluation is often confused and subjectively influenced.

In the earlier report the embryo cell number was proposed as a valid indicator of embryo quality (Papaioannou and Ebert, 1988). Cell number of bovine *in vitro* produced blastocysts varied depending on the morphological grade, and later developing blastocysts were of poor quality as proved by the cell number (Jiang *et al*., 1992). It was observed that embryos developing quickly to the blastocyst stage had a higher total cell number than embryos developing slower (Iwasaki *et al*., 1990).

The developmental potential of a single blastomere from mammalian embryo is usually determined by its ability to form blastocyst with a visible inner cell mass (ICM) and a distinct trophoblast (Chrenek *et al*., 2008). For a better estimation of embryo quality and allocation of cells to the embryoblast and the trophoblast, differential staining can be used. The possibility of using this technique enables a good comparison of ICM development under different culture conditions and to control for deviations in development due to micromanipulations or environmental influences (Van Soom *et al*., 2001). The proportion of apoptotic cells has been considered one of the most important

***Correspondence:** E-mail: chrenekp@yahoo.com
Peter Chrenek, Animal Production Research Centre Nitra,
Hlohovecká 2, 951 41 Lužianky, Slovak Republic
Tel.: +421 37 6546285 Fax: +421 37 6546189

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parameters for evaluating embryo viability or culture conditions (Chrenek and Makarevich, 2005; Makarevich *et al*., 2006).

Another non-invasive parameter for the testing of embryo viability seems to be an embryo diameter. Mori *et al*. (2002) reported high positive correlations between the cell number and the diameter of bovine embryos collected on days 7-9 after *in vitro* fertilization. The measurement of embryo diameter and cell number using nuclear staining with vital permeant dyes (Hoechst 33342, DAPI) enables the non-invasive selection of the embryos with best developmental potential without its destruction (Makarevich *et al*., 2005).

The aim of this study was to evaluate quality of the preimplatation rabbit embryos fertilized *in vivo* and developed either under conditions of the maternal tract or in vitro conditions, examining the total cell number, ICM cell number and the diameter of rabbit embryos as well as number of apoptotic cells.

MATERIAL AND METHODS

Biological material

Three days before mating, New Zealand White rabbit donors (APRC Nitra, SR) were treated with PMSG (Werfaser, 20 IU/kg of body weight, Austria) followed by hCG (Werfachor, 40 IU/kg of body weight, Austria) 72 h later (Chrenek *et al*., 2002). At 19 to 20 hours *post coitum* (h*pc*), the pronuclear stage eggs (the experimental group) 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).

Presumptive zygotes were cultured *in vitro* in k-DMEM medium with 10 % of fetal bovine serum (FBS; Gibco BRL, USA) up to the expanded blastocyst (ExBl) stage for 94 hours at 39°C in the atmosphere of 5 % CO_2 in air.

Rabbit embryos flushed from the uterus of does 94 h*pc* at the expanded blastocyst stage were used as a control group.

Differential staining

The embryos were incubated in freshly prepared 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 30 min 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 (Fouladi-Nashta *et al*., 2005). Afterwards, the embryos were covered with 5 µl of Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA), attached to microslide using small columns of nail polish (Makarevich *et al*., 2005) and examined under a Zeiss confocal microscope.

Analysis of apoptosis (TUNEL)

The embryos were removed from culture medium, washed 3x5 min in PBS supplemented with polyvinylpyrrolidone (PBS-PVP, 4 mg/ml) and then fixed in 3.7 % 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, 1 µg/ml in PBS).

After the three-time washing of embryos in PBS-PVP, they were covered with 5 µl of Vectashield mounting

Table 1: Quality of rabbit embryos after in vitro culture

Group	No. of ExBl n	Diameter of embryos (mm) $\bar{x}$ ± SD	Total cell no. (N) $\bar{x}$ ± SD	No. of cells in ICM $\bar{x}$ ± SD	ICM/total (%)	No. of apoptotic cells/embryo $\bar{x}$ ± SD (%)
control	36	133.95±8.95[a]	155.12±15.00[a]	44.18±2.50[a]	(28.48)	0.35±0.25[a] (0.25 %)
in vitro cultured	45	123.40±7.25[a]	127.23±14.50[a]	36.22±2.45[b]	(28.46)	1.25±1.50[a] (1.00 %)

[a]vs[b] significant different at $p<0.05$

medium (Vector Laboratories, Burlingame, CA, USA) and attached to the microslide using small columns of nail polish (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 40 x objective and 10 x eyepiece. The diameter of the embryos was the mean of two measurements made perpendicularly to each other (Makarevich *et al*., 2006).

Statistical analysis

The differences between groups were evaluated statistically by the unpaired Student´s t-test.

RESULTS AND DISCUSSION

Significantly higher ICM cell number ($p<0.05$) was found out in the control group (44.18±2.50) compared to the experimental group (36.22±2.45) (Table 1).

Higher total cell number was found in the control embryos. However, difference between control and experimental group was not statistically significant ($p<0.05$). Blastocysts obtained *in vivo* had higher diameter as well than *in vitro* cultured embryos, but significant difference was not confirmed. We also did not find significant difference in apoptotic cell number, however higher number of these cells was recorded in the experimental group. The proportion of the ICM cells to total cell counts was similar both in the control and the experimental group (28.48 vs. 28. 46 %), respectively (Table 1).

In our experiments we investigated influence of culture conditions on the rabbit embryo quality. One of the most important features determining embryo quality is number of blastomeres in ICM (Iwasaki, 1990; Uhm, 2009). We found out that cultured rabbit embryos had significantly reduced ICM cell number (36.22±2.45) compared to the control group (44.18±2.50), but the proportion of ICM to total cell counts did not differ between these two groups. The same results have been reported by Macháty *et al.* (1998), who checked number of cells in porcine embryos and by Giles and Foote (1995) in rabbit blastocysts, where the ratio ICM/total number of cells was similar to our results (21 – 30 %).

Olexíková *et al*., (2006) reported similar total cell number in rabbit blastocyst (124±3.24) analysed by DAPI after *in vitro* incubation for 100 h at 37.5°C. This was also confirmed in the other paper of Olexíková *et al*. (2010), where they found similar total cell number (121.1±4.41) in rabbit embryos at blastocyst stage derived from superovulated female at 19-20 h*pc* and cultured under *in vitro* conditions (k-DMEM with 10 % FCS at 37°C, 5 % CO_2) up to 100 h. Higher total cell number (up to 379.5±22.2) in rabbit blastocyst was reported by Tao and Niemann (1999).

Saenz-de-Juano *et al.* (2010) found different rabbit embryo diameters depended on day of gravidity (4^{th}, 5^{th} and 6^{th} day) in range from 244-375 µm up to 2255 – 2822 µm including mucosa layer. In our experiment we observed lower embryo diameters (133.95±8.95), however we evaluated them without mucosa layer which eventual diameter conspicuously enlarge.

The occurrence of apoptosis in preimplantation embryos indicates suboptimal culture conditions or the effects of experimental treatments (Makarevich *et al.*, 2005). This founding corresponds with our results, where the higher proportion of apoptotic cells was observed in *in vitro* cultured rabbit embryos compared to *in vivo* ones, although the significant difference was not confirmed. Fabian *et al.* (2007) reported that apoptosis in intact embryos does not occur earlier than at the 16-cell stage of embryos. In case of early rabbit blastocysts, the proportion of apoptotic cells was 1.38 %, but mouse embryos showed higher proportion of apoptotic cells (6.60 %) at this developmental stage. Makarevich *et al.*, (2008) observed the influence of microinjection and vitrification itself on the presence of apoptotic cells in rabbit embryos. They recorded lower proportion of apoptotic cells (5.70 %) in the embryos subjected only to microinjection compared to embryos subjected to combination of microinjection and vitrification (7.54 %).

CONCLUSION

In conclusion, our results demonstrate significant difference in ICM cell number between *in vivo* and *in vitro* cultured rabbit embryos at blastocyst stage. No significant difference in total cell number, apoptotic index and embryo diameter was found, although control group had higher total cell number and embryo diameter and lower apoptotic cell number.

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