Slovak J. Anim. Sci., 42, 2009 (4): 196 - 199

ISSN 1337-9984

Short communication

EFFECT OF THE LENGTH OF EMBRYO STORAGE IN LIQUID NITROGEN ON THEIR POST-WARMING SURVIVAL

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ABSTRACT

The purpose of this study was to investigate post-thaw survival of rabbit embryos vitrified at pre-compacted or morula stages after short-term (1 - 5 hours) or long-term (1 week – 1 year) storage in liquid nitrogen. We determined the influence of embryo stage at vitrification, as well as period of embryos storage in liquid nitrogen on their post-thaw survival. The development of vitrified/warmed rabbit embryos up to the hatched blastocyst stage *in vitro* was 38 % after short-term storage in liquid nitrogen. About 65 % of embryos, vitrified at the morula stage, reached the hatched blastocyst stage after the same time of storage in liquid nitrogen. Significant differences between both examinated groups (P< 0.001), as well as compared to control (intact, P< 0.001) were observed. The ratio of advanced blastocysts after a long-term storage and warming of morula stage embryos was 44%, which was statistically different (P< 0.001) from the intact group (75 %). We did not notice significant differences in the survival rate among embryos vitrified at the morula stage after different lenght of their storage in liquid nitrogen. However, stage of embryos at vitrification significantly influenced post-thaw survival of rabbit embryos.

Key words: rabbit embryos, vitrification, period of storage, open-pulled straw method,

INTRODUCTION

Cryopreservation represents a mean of long-term storage of biological material without loss of functional activity and without genetic alteration. The development of embryo freezing had a major impact on a world-wide breeding. Storage of embryos is important and useful for creation of gene banks of rare animals, but also for clinical practice in the sphere of human reproduction. First successful cryopreservation of mouse zygotes and embryos resulting in live births was done in 1972 by Whittingham and co-workers. Nowadays, various methods for embryo cryopreservation exist. Among these, vitrification has been widely used and is now regarded as a good alternative to transitional slow-rate freezing (Kong et al. 1999). Vitrification is a rapid cooling procedure designed to minimize damage caused by an ice crystal formation and growth (Shaw and Jones, 2003). Vajta et al. (1998) demonstrated that with OPS (open pulled straw) method of vitrification, the cooling and warming rates can be increased (over 20.000 °C/min) and the toxic and osmotic damage can be decreased. Several authors described the viability of embryos after their long-term storage (Glenister and Lyon; 1986; Kasai et al., 1992; Salvetti et al., 2007; Eum et al., 2009). Survival of rabbit embryos after devitrification, described in the study of Makarevich et al. (2008) was 58.8 %. Papis et al. (2005) published, that 59.1 % of rabbit embryos reached the advanced blastocyst stage after devitrification.

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Received: July 21, 2009
Accepted: September 10, 2009

The aim of our work was to investigate the effect of the lenght of rabbit embryo storage in liquid nitrogen, as well as the stage of embryos at vitrification on their post-warming survival rate *in vitro*.

MATERIAL AND METHODS

Biological material

Three days before mating, New Zeland White rabbit donors (SARC Nitra) 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. At 19 to 20h after mating, the pronuclear stage eggs were flushed from the oviducts of the rabbits with PBS. Selection of flushed eggs was done in CIM medium supplemented with 10 % fetal calf serum (FCS, Gibco BRL, USA). The embryos were cultured under *in vitro* conditions (5 % CO_2, 39°C, k-DMEM supplemented with 10 % FCS, Gibco BRL, USA), as was reported earlier (Chrenek et al., 2005).

Vitrification/warming procedure

Groups of 5 embryos at the pre-compacted or morula stage, were equilibrated in the solution containing CO_2 independent medium (Gibco BRL, USA) + 40% ethylene glycol (EG) + 18% Ficoll 70 + 0.3 M sucrose for 3-4 min at room temperature. After this period, the embryos were sucked up with minimal content of vitrification solution into the 0.25 ml plastic straw. Straws were kept in the vapours of liquid nitrogen for 1 min and then plunged into liquid nitrogen. The embryos were stored either for short-term (1 - 5 hours) or long-term (1 week - 1 year) period.

After different time intervals, the embryos were thawed by keeping straws in the air for 10s at room temperature and plunged into solution contained CIM (Gibco BRL, USA) + 20 % FCS + 0.3M sucrose for 7 min. After warming, the eggs were cultured under *in vitro* conditions (5 % CO_2, 39 °C, k-DMEM supplemented with 10 % FCS Gibco BRL, USA) up to the hatched blastocyst stage. Embryo development was evaluated under a Leica Microsystems inverted microscope (MIKRO spol. s r.o. Bratislava, Slovakia).

Statistical analysis

The χ^2- test was used to compare the developmental rate at advanced blastocyst stage in different groups.

RESULTS

In our work, the effect of different lenght of storage of embryos in liquid nitrogen and the effect of embryo stage at vitrification on their post-warming viability were examined. The viability of rabbit embryos after short-time lenght of storage of pre-compacted and morula stage embryos after warming is summarized in the table 1. Vitrification procedure affected blastocyst development in both examined groups, compared to the intact group ($P < 0.001$). Vitrification of the pre-compacted stage embryos resulted in a lowest yield of viable embryos, most embryos were arrested at cleavage after the warming, and development to the advanced blastocyst stage was the poorest, compared to vitrified embryos at the morula stage and also the intact group. The difference was statistically significant ($P < 0.001$). The results of short-term and long-term storage of the morula stage rabbit embryos are shown in table 2 and

Table 1: Post-thaw survival of rabbit embryos vitrified at pre-compacted or morula stages after short-term storage in liquid nitrogen

Embryo group	No. vitrif./devitrif.embryos	Advanced blastocysts n (%)	No. fragmented embryos n / (%)
Vitrified (8 -16- cell)	29	11 (37.93 %)^a	18 (62.07 %)
Vitrified (32- cell)	34	22 (64.71 %)^b	12 (35.29 %)
Control		35 (100 %)^c	0

Advanced blastocysts included expanded blastocyst and hatched blastocyst stage, short-term – 2 hours, Control – non-vitrified embryos (n = 35)
Values with different superscripts within columns are significantly different at $P < 0.001$ (Chi-square test)

Table 2: Post-thaw survival of rabbit embryos vitrified at the morula stage after long-term storage in liquid nitrogen

Embryo group	Lenght of storage in LN	No. vitrif./devitrif. embryos	Advanced blastocysts n (%)	No. fragmented embryos n / (%)
Vitrified (32-cell)	short-term	124	56 (46.16 %)^a	68 (53.84 %)
	long-term	236	104 (44.06 %)^a	132 (55.94 %)
Control			76 (74.51 %)^b	26 (25.49 %)

Advanced blastocysts included expanded blastocyst and hatched blastocyst stage, LN – liquid nitrogen, short-term storage – (1 – 5 hours), long-term storage – (1 week – 1 year). Control – non-vitrified embryos (n = 102)
Values with different superscripts within columns are significant different at $P < 0.001$ (Chi-square test)

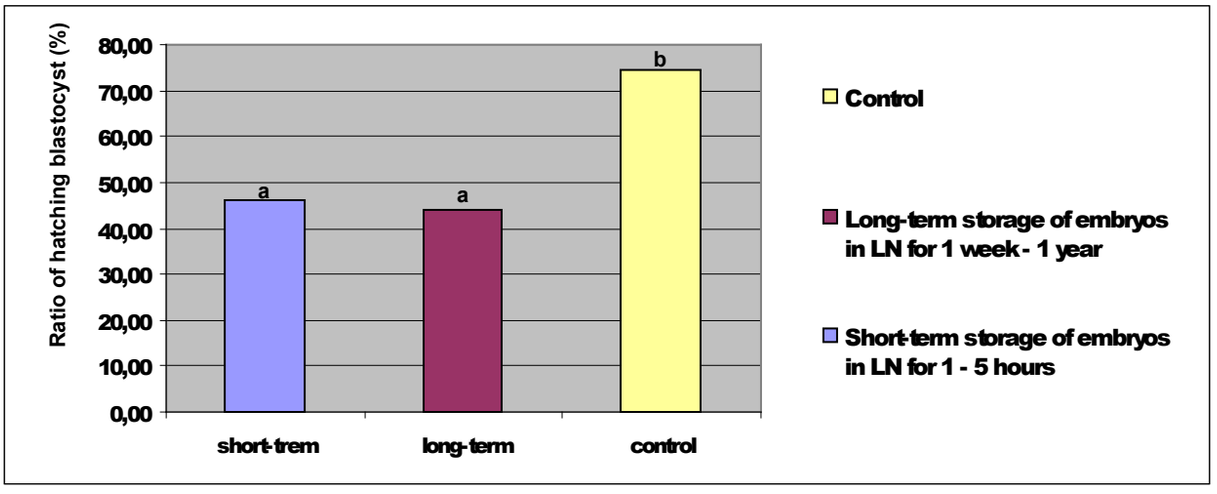

Fig. 1: Developmental rate of embryos after vitrification/warming

figure 1. The viability of the embryos after short-term storage in LN was 46.16 % and after long-term storage it was 44.06 %. The percentage of intact embryos reached hatching blastocyst stage was 74.51 %. The difference was significant, compared to both examined groups ($P < 0.001$). However, no significant differences in the development up to the blastocyst stage of the embryos after short-term and long-term storage were found.

DISCUSSION

Viability of vitrified/warmed embryos can be influenced by many factors. One of them is the quality of embryos flushed from the oviducts of donor rabbits, that are originated from different lines (Vicente et al., 2003). Problems with viability of embryos after vitrification can be also caused by permeable cryoprotectants presented in vitrification solution (Shaw and Jones, 2003). Lower ability of growing embryos in our study *in vitro* after warming of the pre-compacted embryos can be caused by a transition of the embryos from maternal to embryonal genome. Vitrification at this stage is stressful and can cause cleavage arrest. Also the metabolism of each blastomere within the limits of embryo is different. Our results, in case of vitrified morula, are comparable to the results of Makarevich et al. (2008), who used the same vitrification protocol as we do. They showed, that 58.8 % of embryos reached hatching blastocyst stage. Kasai et al. (1992) vitrified rabbit embryos using vitrification procedure with Ficoll 70. Their experiments demonstrated, that 87 % of intact embryos reached hatched blastocyst stage. Papis et al. (2005) presented 59.1 % success in a cleavage rate of embryos after devitrification. Eum et al. (2009) investigated the effect of short- and long-term storage of mouse embryos in liquid nitrogen. There were no significant differences in the blastocyst rate after different time of storage. Salvetti et al. (2007) in their study reported a survival of rabbit embryos after 15 years of storage in liquid nitrogen. They used slow-cooling method for cryopreservation of 134 rabbit morulas. Fifteen years later, they were defrosted and transfered into 14 synchronized recipients, and 69 live newborn (51.5 %) were obtained. This is the longest known period of storage of rabbit embryos. This storage system thus represents a useful method for safe and effective long-term storage of rabbit embryos.

Our results indicate, that the longevity of embryo storage in liquid nitrogen did not affect the development of rabbit morulas *in vitro* up to advanced blastocyst stage. However, stage of embryos at vitrification significantly influenced the post-thaw survival of rabbit embryos.

ACKNOWLEDGEMENT

This work was supported by the Slovak Research and Development Agency under the contract No. LPP-0126-06. The authors are grateful to the Slovakian branch of Leica (MIKRO spol. s.r.o. Bratislava, Slovak Republic) for their contribution to this study.

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