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Evolution of embryonic diapause in mammals and applying reproductive technologies to diapausing embryos

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Motivation and Aim: Arrests in the embryo development are characteristic to species belonging to various taxa of the animal kingdom [1]. Among mammals, obligate embryonic diapause (ED), i. e. suspension of embryo development at the blastocyst stage and delayed implantation, present in each development cycle, occurs in no less than 60 species belonging to 12 families [2]. Because of ED the periods of mating and the offspring birth may be attributed to the most favorable seasons [1, 2]. The evolutional aspects of ED in mammals are discussed in the literature, e. g. whether ED is a trait acquired during evolution *de novo* or is it characteristic of the ancestral forms and the loss of this trait occurs in some species [3–5]. Modern reproductive technologies, such as embryo *in vitro* culture (IVC), cryopreservation, and embryo transfer may be helpful in addressing issues related to the evolution of diapause and provide an insight to disclose the mechanism of this reproductive phenomenon [5–8]. In particular, by transferring ovine embryos from sheep into the reproductive tract of the mouse, it was possible to induce a reversible state of diapause, which sheep embryos normally lacking; this leads the authors to formulate the hypothesis that genes associated with diapause are evolutionarily conserved [5]. Experimental modeling of ED in farm and laboratory animals is valuable not only to address the questions related to the evolution of this trait, but also helpful for the Genome Resource Banking concept applying to diapausing mammalian species. As some rare and threatened mammals exhibit ED, there is motivation to develop reproductive technologies especially targeting diapausing embryos [9]. The goal of this work was to develop reproductive technologies for diapausing embryos using the mouse as an experimental model.

Methods and Algorithms: The studies were carried out on CD1 mice kept under standard conditions of SPF-Vivarium and approved by the Bioethics Committee of the Institute of Cytology and Genetics SB RAS (No. 144, 29.03.2023). Diapausing embryos were collected on the 6th day *post coitum* (dpc), after fertile mating of CD1 mice, treated with equine chorionic gonadotropin (eCG) and human chorionic gonadotropin (hCG). The mice were ovariectomized on the 4th dpc and received progesterone injections on the 4th and 5th dpc (diapause group) [6, 7]. Cryopreservation was performed using a CL-8800i programmable controlled rate freezer (CryoLogic, Australia) with 1.8 M ethylene glycol and 0.1 M sucrose as cryoprotectant agents [6, 7]. The viability of frozen-thawed diapausing embryos was evaluated *in vitro* by their culturing during 24 h and 47 h in the

CO_2-incubator New Brunswick™ Galaxy 48R CO_2-incubator at 37 °C, 5 % CO_2, and 90 % humidity in 20-μL drops of the KSOM (Merck, Germany) supplemented with 1 % essential amino acids, 0.5 % non-essential amino acids, 0.5 % vitamins, and 5 % fetal calf serum (Thermo Fisher Scientific, USA), covered with mineral oil (FertiPro, Belgium). The embryonic development was monitored by visual examination under an S8 APO microscope (Leica Microsystems, Germany). The development rate after 24 h IVC was estimated as the percentage of blastocysts with expanded blastocoel cavity from the total number of thawed blastocysts [6–8]. Randomly chosen frozen-thawed diapausing embryos were fixed immediately after thawing as well as after 24 h and 47 h of IVC and processed for DAPI/TUNEL staining to evaluate interphase cell numbers as well as apoptotic and fragmentation indexes as described earlier [6]. The results were analyzed using the STATISTICA v. 12.0 software (StatSoft, Inc., USA). Data were analyzed using analysis of variance (one-way ANOVA) followed by *post-hoc* comparison (Fisher LSD-test).

Results: The yield of embryos per diapausing mouse female was 9.6 ± 1.5 [6] and 10.4 ± 1.5 [7]. All the diapausing embryos collected were at blastocyst stage. Total number of diapausing embryos cryopreserved in two independent studies was 160 (11 replicates) [6, 7]. Diapausing murine blastocysts have already lost *zona pellucida* and are characterized by the large size as the volume of the blastocoel increases during the period of implantation delay (Fig. 1). Some other mammalian species are characterized by even larger blastocysts during diapause [10]. This makes cryopreservation of diapausing embryos challenging. However, the percentage of blastocysts with expanded blastocoel cavity after freezing-thawing and subsequent *in vitro* culture was relatively high: 82.5 % [6] and 85.0 % [7]. Moreover, the viability of frozen-thawed blastocysts was confirmed by different methods. Cryopreservation did not cause a decrease of protein metabolism in diapausing embryos [6]. In addition, cryopreservation did not cause a decrease of *Odc1* and *RhoA* gene expression in diapausing embryos [7].

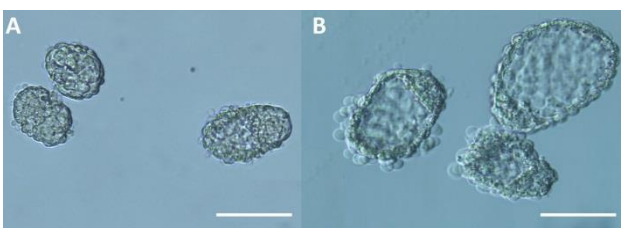

Fig. 1. Diapausing murine embryos after cryopreservation. A, diapausing frozen-thawed blastocysts before IVC, collapsed blastocoel; B, diapausing blastocysts with re-expansion of blastocoel, 24 h IVC after thawing. Scale bar 100 μm

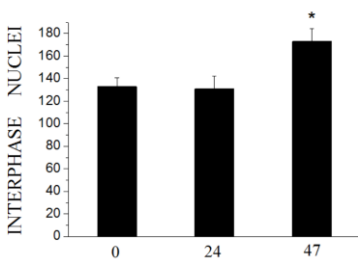

Fig. 2. Cell numbers (M ± SEM) in diapausing murine embryos after freezing-thawing and subsequent IVC in a medium supplemented with 100 μM putrescine. * $P < 0.05$

The increase of cells in the frozen-thawed embryos after IVC in a medium containing putrescine is another evidence of their viability (Fig. 2). The analysis using ANOVA revealed a significant effect of the culturing time on the number of interphase nuclei per embryo [$F(2,21) = 4.9$, $P < 0.05$].

Conclusion: Embryonic diapause disentangles breeding season and the offspring birth, thus evolutionally perpetuate the species. We studied the possibility of diapausing embryos program freezing using the mouse as a model species. The post-thaw embryo viability was assessed by different criteria; all these criteria indicated that the diapausing murine embryos survived cryopreservation. Meanwhile, our study revealed that diapausing embryos need re-activation time to resume cell division after freezing-thawing procedures. To expand embryo cryopreservation technology to other mammalian species, which possess delayed implantation, the evolutional difference in diapause phenotype between species of mammals should be taken into account.

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