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A Comparison of Different Cryoprotectant Solutions and Thawing Methods for the Cryopreservation of Embryos of Mice and Rats

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Abstract—The proper choice of the cryoprotectant and thawing method affects the efficiency of cryopreservation. A freezing-thawing method aimed at the preservation of blastomere cells was evaluated in experiments with ICR mice. The cleavage-stage embryos of ICR mice, GC rats, and OXYS rats were collected on Day 3 of pregnancy and frozen in plastic straws according to the standard procedure. We compared the effect of permeating (ethylene glycol and glycerol) and nonpermeating (sucrose) cryoprotectants and their combinations on the survival rate of embryos after thawing. We also compared the effect of rapid (water bath, 10 s, 37°C) and slow (40 s, room temperature; then 40 s, 30°C) thawing methods. The viability of the embryos of mice and rats after cryopreservation was evaluated by their in vitro culturing after thawing. Our data prove that slow thawing is more suitable for mice embryos and provides a higher survival rate; the addition of sucrose to the basic cryoprotectant (ethylene glycol or glycerol) improves the parameters of the in vitro cultures of embryos after thawing, especially if glycerol is used as the basic cryoprotectant. This freezing-thawing method (glycerole and sucrose as the cryoprotectant solution and slow thawing) was used for cryopreservation of GC and OXYS rats. As a result, the survival rate of embryos after freezing was 68–83.3% and the rate of in vitro development after thawing was 64.7–66.6%.

Keywords: ICR mice, GC rats, OXYS rats, embryo cryopreservation, ethylene glycol, glycerol, sucrose
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INTRODUCTION

The number of lines of mice and rats has increased dramatically over the past decades (Abbott, 2004; Lasar et al., 2005). Due to the limitation of resources and high standards for quality of laboratory animals in the modern biological and medical studies, works on the rederivation and cryopreservation of embryos in mice and rats have been given special attention in many of genetic centers (Rall et al., 2000; Brusentsev et al., 2011; Amstislavsky et al., 2013).

The integrity of the egg coat (zona pellucida, ZP) is an important factor in rederivation (Brusentsev et al., 2011; Rozhkova et al., 2012; Amstislavsky et al., 2013). ZP has to be intact, since it is thought to be an efficient natural barrier that protects the egg against viral and bacterial infections (Van Soom et al., 2010). The program of rederivation is often based on the combination of the transfer and cryopreservation of the embryos (Morrell, 1999; Amstislavsky et al., 2013).

The purpose of this work is to find the freezing-thawing method that provides the maximal integrity of the embryos of mice and rats. For this purpose, the following steps were taken: (1) we compared the effect of glycerol and ethylene glycol on frozen embryos and estimated the effect of sucrose added to these cryoprotectants; (2) we compared the effect of two thawing methods (the rapid and the slow one) on the viability of mice embryos in vitro; (3) we used the most efficient procedure for the cryopreservation of the embryos of GC and OXYS rats.

MATERIALS AND METHODS

Experimental Animals

Mature female ICR mice (8–10 weeks; $n = 54$) and mature female GC (10–14 weeks, $n = 8$) and OXYS (10–14 weeks, $n = 4$) rats were used as the embryo donors. Female mice and rats were mated with males of the same strain of the same age to obtain embryos. The animals were kept under standard conditions in the conventional vivarium of the Institute of Cytology and Genetics (Novosibirsk, Russia).

All the experiments involving animals were approved by the Commission of Bioethics of the Institute of Cytology and Genetics (protocol no. 5, May 13, 2011) and performed in accordance with the European Convention for the Protection of Invertebrates.

The Collection of Preimplantation Embryos of Mice and Rats

Mice. We induced superovulation in female ICR mice according to the standard procedure (Amstislavsky, 2006). Each female with superovulation was

mated with a conspecific male. The day of the copulation plug to be observed was considered as the first day of pregnancy.

Rats. The estrous cycle in GC and OXYS rats was monitored by vaginal smears. Females at the stage of estrus were mated with the males of the same strain. The day when spermatozoa were found in the vaginal smear was considered as the first day of pregnancy.

Then, the females were euthanized by cervical dislocation on the third day of pregnancy. The uterus and oviducts were removed and washed with the EMCARE Complete Ultra Flushing Solution (ICPBio Reproduction, United States), as described earlier (Amstislavsky, 2013). The embryos were counted and examined with the use of a Leica S8 APO stereomicroscope with magnification of up to ×80 (Leica Microsystems, Germany). The quality of the embryos was estimated based on widely used parameters: the stage of embryonic development and the integrity of ZP (Rulicke and Autenried, 1995; Van Soom et al., 2010); and the number of viable cells (Emiliani et al., 2000). The low quality embryos were discarded; embryos with high quality were washed with three drops of the same medium, and then frozen according to the procedure described below or used right after that as the control.

Embryo Freezing

We used four combinations of cryoprotectants for the experiments with ICR mice embryos: 1.5 M ethylene glycol (EMCARE, ICPBio Reproduction, United States) with and without sucrose, and 10% v/v glycerol (EMCARE, ICPBio Reproduction, United States) with and without 0.1 M sucrose (Sigma, United States).

Only one of described combinations was used for the cryopreservation of GC and OXYS rat embryos: glycerol (EMCARE, ICPBio Reproduction, United States) with 0.1 M sucrose (Sigma, United States).

After adding cryoprotectant solution, 10–15 embryos were placed in 0.25 mL plastic straws (Cryo Bio System, France). Each straw contained three portions of cryoprotectant solution separated by two air vesicles; the central part of the straw contained the embryos.

Straws with embryos were frozen in a CL 8800 freezer (CryoLogic, Austria) according to the following procedure: from 18°C to –7°C at a rateof –1°C/min; 10 minutes at –7°C; seeding after 1 minute; from –7°C to –35°C at a rate of –0.3°C/min; 10 minutes at –35°C; then the straws were put in liquid nitrogen.

Embryo Thawing

We used two methods for thawing murine embryos: a slow one and a rapid one. For slow thawing, we removed the straws from liquid nitrogen, kept them at room temperature for 40 s, and then placed them in a water bath at a temperature of 30.0°C for 40 s. It was found earlier that the rate of thawing in this method is around 300°C/min (Renard and Babinet, 1984). We used this method for thawing embryos frozen in all four cryoprotectant solutions (ethylene glycol, glycerol, ethylene glycol with sucrose, and glycerol with sucrose).

For rapid thawing, we put the straws in water bath at a temperature of 37°C for 10 s. It was found earlier that the speed of thawing in this method was around 2500°C/min (Renard and Babinet, 1984). We used this method for thawing embryos frozen in two cryoprotectant solutions (ethylene glycol and glycerol).

We used only the first method for thawing rat embryos frozen in glycerol with sucrose.

Washing and Culture of Embryos

After thawing, all the liquid was removed from the straws and put onto 35 mm Petri dishes (Corning, United States). The cryoprotectant solutions were rinsed away from the embryos; the methods of washing were chosen based on the cryoprotectant used.

The embryos frozen in ethylene glycol were washed three times in fresh drops of the Holding Solution medium (EMCARE, ICPBio Reproduction, United States) at a temperature of 37°C (90 μL; 6–7 min in each drop).

For thawing the embryos frozen in glycerol, we used a special Thawing System (EMCARE, ICPBio Reproduction, United States). This system contains three solutions with descending concentrations of glycerol. The embryos were transferred between the solutions at a temperature of 37°C.

After thawing the embryos were frozen in different cryoprotectatnt solutions (ethylene glycol with sucrose or glycerol with sucrose), and the content of the straws was removed and kept in Petri dishes with these drops at room temperature for 15 minutes. Then the embryos were transferred to the fresh drops of a Holding Solution medium (EMCARE, ICPBio Reproduction, United States) and incubated at a temperature of 37°C for 15 minutes.

After removing the cryoprotectatnt solutions, the embryos (regardless of their quality) were rinsed in ten drops of a Holding Solution medium (200 μL; EMCARE, ICPBio Reproduction, United States); the glass capillary tubes were changed between the drops in order to provide a sterile transfer. Then the embryos were examined with the use of a M205 FA microscope (Leica Microsystems, ×230). Embryos with more than 25% of the cells damaged and/or an injured egg coat were discarded; the rest of the embryos were left for in vitro culture. Mice embryos were transferred to 50 μL of a M16 medium (Sigma, United States); the rat embryos were transferred to 50 μL of a R1ECM medium (Amstislavsky et al., 2015). Then the embryos were cultured under mineral oil (Sigma, United States) in 5% CO_2 at a temperature of

The in vitro development rate of ICR mice embryos after freezing and thawing (M ± SEM)

Groups	The total number of fresh and cryopreserved embryos	The number of fresh/cryopreserved embryos	The number of blastocysts	Development rate, %
Fresh embryos (control)	3 (21)	7.0 ± 0.0	7.0 ± 0.0	100
Ethylene glycol, rapid thawing	5 (80)	16.0 ± 4.0	11.2 ± 2.6	71.2 ± 6.1*
Ethylene glycol, slow thawing	2 (28)	14.0 ± 1.0	10.0 ± 2.0	71.0 ± 9.0*
Ethylene glycol with sucrose, slow thawing	3 (20)	6.7 ± 0.3	6.0 ± 0.6	90.5 ± 9.5
Glycerol, rapid thawing	3 (38)	12.7 ± 1.5	3.3 ± 0.9	25.3 ± 3.9#
Glycerol, slow thawing	15 (273)	18.2 ± 2.3	14.7 ± 1.7	82.8 ± 3.4*
Glycerol with sucrose, slow thawing	3 (29)	9.7 ± 3.3	9.3 ± 0.3	96.7 ± 3.3

* $p < 0.05$, as compared with fresh embryos; # $p < 0.001$, as compared with embryos of other groups.

37°C and humidity of 90% in a BINDER 150-UL CO_2 incubator (Germany) for 48 h. Fresh embryos were received from donor mice and treated as described above, but without freezing and thawing. These embryos were used as the controls. The viability of the embryos was estimated based on their development rate in vitro.

Statistical Analysis

All the data, including the share of embryos developing in culture, are presented in the table in the form of Mean ± SEM. The effect of cryopreservation on embryo development was estimated based on the development rate in vitro after thawing by ANOVA and the subsequent post hoc comparison with the Newman-Keuls test and Student's *t*-test. We used the following factors for the analysis: the basic cryoprotectant (ethylene glycol/glycerol), thawing method (slow/rapid), and the addition of sucrose (with/without sucrose). The results were considered to be significant at $p < 0.05$. The data were analyzed with the use of the standard STATISTICA V 8.0 package (StatSoft, Inc).

RESULTS

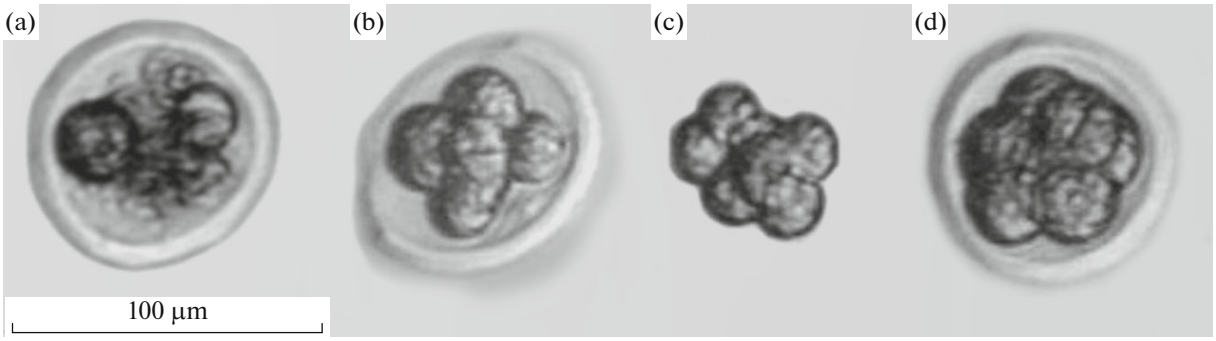
After thawing the murine embryos, we found with the method of light microscopy three types of typical damages; these are damaged embryonic cells (figure, a), damaged egg coat (figure, b), and the absence of an egg coat (figure, c). Only the embryos without these problems were considered as successfully cryopreserved (figure, d).

We studied the effect of the basic cryoprotectant (ethylene glycol/glycerol), the thawing method (slow/rapid), and the addition of sucrose (with/without sucrose) on mice embryos at the age of 4–8 days. The results are presented in the table. Statistical analysis by the method of ANOVA showed that the basic cryoprotectant ($F_{(1,25)} = 5.25$; $p < 0.05$) and the thawing method ($F_{(1,25)} = 18.56$; $p < 0.001$) had a significant effect on the development rate of the embryos after freezing and thawing. We also observed some interaction of these two factors ($F_{(1,25)} = 18.82$; $p < 0.001$). The addition of sucrose to the basic cryoprotectant provided higher development rates of the embryos in vitro after thawing ($F_{(1,25)} = 5.56$; $p < 0.05$).

In the further experiments with rats, only one of the studied freezing and thawing methods was used (glycerol with sucrose as a cryoprotectant solution and slow thawing). We used 2–4-cell GC rat embryos for cryopreservation; the viability of embryos was estimated by optical microscopy; 51 of the 75 embryos (68%) were found to be viable and then cultured in vitro. After 48 h of culture, 30 embryos (58.8%) reached the stage of morula, and 3 embryos (5.9%) reached the stage of blastocyst. Thus, 64.7% of the embryos developed successfully in vitro. We also used 2–4-cell OXYS rat embryos for cryopreservation; the viability of the embryos was estimated by light microscopy; 15 of the 18 embryos (83.3%) were found to be viable and then cultured in vitro. After 48 h of culture, 8 embryos (53.3%) reached the stage of morula, and 2 embryos (13.3%) reached the stage of blastocyst. Thus, 66.6% of OXYS rat embryos developed successfully in vitro.

DISCUSSION

The results of in vitro culture cultivation of murine embryos show that the supplementation with sucrose both ethylene glycol and glycerol solutions used as the basic cryoprotectant and the use of the method of slow thawing provide the same development rate of embryos as that in the control group. Intact embryonic cells and the integrity of ZP are important factors for the successful rederivation (Van Soom et al., 2010; Brusentsev et al., 2011; Rozhkova et al., 2012). It was found that the use of both permeating (basic) and nonpermeating (supplementary) cryoprotectants and slow thawing of mice embryos provide a better survival rate of the embryonic cells and the whole embryos. The results prove that this protocol should be chosen first and foremost in cases when freezing and thawing is part of the rederivation process. Moreover, it was



Preimplantation embryos pf ICR mice after freezing in glycerol (10%) and thawing at a temperature of 37°C for 10 s. a—dead embryos; b—with damaged ZP; c—without ZP; d—survived after cryopreservation. Scale bar: 100 μm.

found that when glycerol is used as a cryoprotectant, the viability of the embryos after cryopreservation depends greatly on the thawing method. Our observations prove that the development rate of embryos frozen in glycerol in culture after rapid thawing is the lowest. In contrast, the development rate of embryos after slow thawing is higher.

The experiments with GC and OXYS rats showed that the use of the slow thawing method and the addition of sucrose to the basic cryoprotectant (for the cryopreservation of rat embryos only glycerol was used) provided a survival rate of at least 66% of the embryos; at least 64% of them developed in vitro. There are few data available on the procedures of freezing rat embryos similar to those used in our studies (Rall et al., 2000; Pfaff et al., 2000). The survival rate of GC and OXYS rat embryos after cryopreservation according to the optimal procedure, which was chosen based on the results of our experiments with mice embryos (glycerol with sucrose and slow thawing), was comparable with the survival rate of the embryos in the cited studies.

Glycerol and ethylene glycol are often used as cryoprotectants for freezing mice and rat embryos (Morrell, 1999; Emiliani et al., 2000; Pfaff et al., 2000; Rall et al., 2000); however, both of these compounds have some unique properties. It is remarkable that the rate of the permeation of glycerol into the embryonic cells is lower than that of ethylene glycol (Pedro et al., 2005). The results of the in vitro culture cultivation of the mice embryos show that the efficiency of ethylene glycol and glycerol used as cryoprotectants is almost the same, but only if the slow thawing method is used. These data are in a good agreement with the data obtained on other rodent species (Renard, Babinet, 1984; Ridha, Dukelow, 1985), which prove that the use of ethylene glycol and glycerol cryoprotectants requires slower thawing procedures.

Sucrose behaves as an osmotic buffer, thus reducing the osmotic shock forin the embryonic cells after thawing (McWilliams et al., 1995). Therefore, the highest development rate of the embryos in culture was only observed when sucrose was added to the basic cryoprotectant.

The R1ECM medium was created especially for the culture cultivation of rat embryos (Miyoshi et al., 1995); now it is widely used for other rodent species (Amstislavsky et al., 2015). In our study, it was first used for the cultivation of GC and OXYS rat embryos after freezing and thawing.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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