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Cryopreservation of mouse morulae through different methods: slow-freezing, vitrification and quick-freezing

Criopreservação de mórulas de camundongos por diferentes métodos: lento, vitrificação e rápido

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SUMMARY

The *in vitro* and *in vivo* development of mouse morulae after cryopreservation through different methods was examined. The slow-freezing involved an equilibration in 1.5M ethylene glycol (EG) and cooled at 0.5; 0.7; 1.0 or 1.2ºC/minute. The vitrification involved a 3 minutes equilibration in 20% EG and 60 seconds in solution containing 40% EG, 18% ficoll and 10.26% sucrose. The quick-freezing involved an equilibration in 3M EG + 0.3M sucrose for 5 minutes and 2 minutes in nitrogen vapor. In all three methods the straws were thawed in air for 10 seconds and in water at 25ºC for 20 seconds and the embryos cultured *in vitro* for 72 hours to estimate blastocyst rate. To assess viability *in vivo*, frozen morulae as well as fresh embryos were transferred into recipients. The *in vitro* development rates with 0.5, 0.7; 1.0 and 1.2ºC/minute were, respectively, 72.3; 79.6; 76.5 and 84.8%. There was no significant difference among the cooling rates of 0.7; 1.0 and 1.2ºC/minute ($p > 0.01$). The *in vitro* survival rates of vitrification and quick-freezing (84.5 and 74.3%, respectively) were similar to the slow-freezing. *In vivo*, the implantation rate and number of fetuses from embryos frozen through slow-freezing at 1.2ºC/minute, vitrification and quick-freezing were not significantly different.

UNITERMS: Cryopreservation; Embryo; Mouse.

INTRODUCTION

The first report of successful embryo cryopreservation was published in 1972[18]. The original technique employed slow cooling (0.5ºC/minute) to –60ºC or below before transferring into liquid nitrogen. Since then, several studies have been performed using mouse embryos as experimental models and many methods have been described to cryopreserve embryos[4,8,10]. Basic and applied research carried out during these two decades provided improvements in nearly all steps of the cryopreservation process, resulting in considerable simplification of the original slow freezing procedure. The freezing rate employed is critical in order to assure success and three general procedures are recognized: i) controlled slow freezing, ii) vitrification, and iii) quick-freezing in nitrogen vapor. Of these methods, quick-freezing and vitrification offer a simple, practical and economical technique for the cryopreservation of embryos of several animal species[2]. Vitrification is a thermodynamic process in which the viscosity of a fluid is increased by many orders of magnitude, giving the fluid the mechanical properties of solid matter[2]. According to Rall and Wood[11], the vitrification procedure, which uses high cryoprotectant concentration and fast cooling and warming rates, offers two important advantages over conventional slow-freezing approaches. First, potential injury associated with the formation of ice is eliminated because no ice forms in vitrified suspensions during freezing. In second place, vitrification permits a substantial reduction in the time and the required equipment for cryopreservation.

Successful cryopreservation of mouse embryos by quick freezing (direct plunging into liquid nitrogen vapor) was first achieved with glicerol and sucrose[13]. Quick freezing procedures also provide a simple and time-saving approach to embryos freezing. In this method, embryos are equilibrated in moderate (3M) concentration solutions of extracelular and intracelular cryoprotectants. High levels of survival have been reported for mouse embryos using this procedure[1,3].

The present study on mouse embryo cryopreservation was conducted to evaluate the efficiency *in vitro* and *in vivo* of different freezing protocols (slow-freezing, vitrification and quick-freezing).

MATERIAL AND METHOD

Reagents

All reagents were obtained from Sigma (Sigma Aldrich CO.) unless stated otherwise.

Superovulation and recovery of mouse embryos

Outbreed 6 to 13 week-old Swiss female mice on a 14 light: 10 dark cycles were superovulated with an intraperitoneal injection of 5 UI of eCG (Equine Chorionic Gonadotrophin - Intergonan® – Intervet) 48 hours later followed by 5 UI of hCG (Human Chorionic Gonadotrophin - Ovogest® – Intervet). After the hCG injection, the donors were paired with males of the same strain overnight and checked on the following day for the presence of vaginal plug (Day 1). Upon 75-78 hours after the hCG injection

(Day 3), the donors were sacrificed by cervical dislocation and embryos were flushed from the oviducts and uteri using Dulbecco's phosphate buffered saline (DPBS) supplemented with 1% of fetal calf serum (FCS). For cryopreservation, only compact excellent quality morulae were selected.

Freezing procedures

Three different freezing procedures were tested: slow-freezing, vitrification and quick-freezing.

Slow-freezing: The compact morulae were equilibrated in 200 µl droplet of medium composed of DPBS + 0.4% BSA + 1.5M of Ethylene Glycol (EG – J. T. Baker®) at room temperature (22 to 25ºC), for 10 minutes. Ten to thirty embryos were loaded into the 0.25 ml straws using two columns of DPBS separated by air bubbles from a central column containing the embryos as shown in Figure 1. After equilibration, the straws were placed horizontally into an alcohol bath chamber of a programmable freezer (HAAKE SK 91) at –7°C and after 5 minutes manual seeding was carried out. Ten minutes after seeding, the straws were cooled at 0.5; 0.7; 1.0 or 1.2°C/minute. When temperature reached -25°C, the straws were held for 10 minutes, and then plunged and stored in liquid nitrogen at -196°C.

Vitrification: The vitrification solution used in this experiment has been described previously[6]. It consisted of 40% EG, 18% Ficoll and 10.26% sucrose diluted in DPBS supplemented with 0.4% BSA (EFS solution). Before vitrification, the morulae were equilibrated in 200 µl droplet of 20% EG in DPBS for 3 minutes at room temperature. After exposure for 3 minutes, the embryos were transferred to a 200 µl droplet of EFS solution for 60 seconds. Ten to thirty embryos were loaded into a 0.25 ml straw as in the slow-freezing procedure. For vitrification, the first part of straw filled with DPBS was slowly immersed into liquid nitrogen, the remaining part of the straw was plunged in quickly.

Quick-freezing. The procedure for quick-freezing, used in this experiment, has been described previously[1]. The morulae were equilibrated in 200 µl droplet of 3M EG + 0.3M sucrose diluted in DPBS supplemented with 0.4 BSA for 5 minutes at room temperature. Ten to thirty embryos were loaded into a 0.25 ml straw for slow-freezing procedure. After equilibration, the straws were frozen by placing them horizontally on a Styrofoam boat with dimensions 14.5 x 12 x 0.8 cm in the vapor phase of a nitrogen bath at approximately - 170°C. After 2 minutes, the straws were plunged into liquid nitrogen.

Thawing of frozen embryos

The embryos from three freezing methods were thawed by gentle agitation for 10 seconds in air and 20 seconds in a 25°C water bath. Immediately after thawing, embryos were expelled and diluted in petri dishes using the liquid contents of each straw (method one-step of dilution). After 5 minutes in this solution, the embryos were transferred to holding (DPBS + 0.4% BSA) for an average of 5 minutes and then cultured *in vitro* or transferred into recipients.

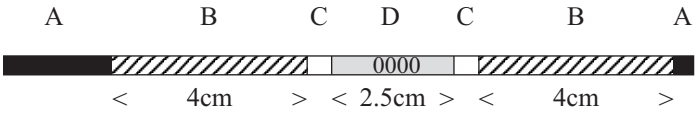

Figure 1

Diagram of a 0.25 ml straw for cryopreservation: (A) plug; (B) 0.4% BSA in Dulbecco's phosphate buffered saline; (C) air bubble; (D) cryoprotectant solution + mouse morulae.

***In vitro* survival**

Frozen-thawed morulae, regardless of their quality, were washed in Whitten's medium[17] and cultured in 50 µl of Whitten's medium microdroplet at 37ºC, 5% CO_2 and high humidity for 72 hours. Survival of embryos was assessed by their ability to develop into expanded, hatching or hatched blastocyst stage.

***In vivo* survival**

Frozen-thawed morulae, regardless of their quality, were transferred into recipients to examine their viability *in vivo*. The freezing protocols were those described above. After thawing and cryoprotectant dilution, the morulae were transferred to the uterine horns of a day-3 pseudopregnant recipient (day of vaginal plug = day 1) that had been mated to a vasectomized male using the procedure previously described[5]. Recipients were anesthetized with xylazine + ketamine (1:1), which was diluted (17%) with physiological saline. All recipients received 5 to 7 frozen-thawed morulae per horn (right horn) while 5 to 7 nonfrozen morulae were transferred in the contralateral horn (left horn) of the same foster mother as control. This allowed to reduce the effect of the foster mother on the pregnancy rates. Eleven days after transferring, the recipients were sacrificed in order to determine the viability *in vivo* of embryos. The number of normal fetuses, resorbed fetuses and implantation sites were recorded.

Experimental design

In all experiments, the development of frozen-thawed embryos was compared to control (nonfrozen embryos), which had been kept under the same conditions of frozen embryos. Each experiment was replicated 2-3 times.

Experiment 1. The viability of mouse embryos cryopreserved through slow-freezing method was determined *in vitro*. Four cooling rates were compared: 0.5; 0.7; 1.0 and 1.2°C/minute (respectively, groups 1, 2, 3 and 4). A total of 105 morulae were frozen in each group.

Experiment 2. The viability of mouse embryos cryopreserved through vitrification and quick-freezing methods was determined *in vitro*. A total of 105 morulae were frozen in each method.

Experiment 3. The viability of embryos cryopreserved through different methods was determined *in vivo*. However, regarding the slow-freezing method, the group which resulted in the highest *in vitro* survival rate was chosen for *in vivo* evaluation.

Statistical analysis

Data on the survival of embryos *in vitro* and *in vivo* were analyzed by chi-square test, considering $p < 0.05$ as significantly different. When the expected frequency was < 5, Fisher's exact probability test was used.

RESULTS

The results obtained *in vitro* (experiments 1 and 2) after slow-freezing, vitrification and quick-freezing of mouse compact morulae are presented in Table 1.

Experiment 1. Viability of slow-frozen embryos was determined *in vitro* and is summarized in Table 1. In total, 97.8% (411 out of 420) of embryos were recovered after thawing. The proportion of embryos that developed *in vitro* was influenced by the cooling rates ($p < 0.05$). The viability of embryos cooled at 1.2°C/minute was significantly higher than 0.5°C/minute (Table 1). There was no significant difference in survival rates among 0.7; 1.0 and 1.2°C/minute (respectively, 79.6%; 76.5% and 84.8%). Compared to control, the survival rates of slow-frozen embryos were significantly lower except for embryos cooled at 1.2°C/minute.

Experiment 2. In total, 92% (97 out of 105) of embryos vitrified and 100% of embryos quick frozen were recovered after thawing. The survival rates of morulae cryopreserved through vitrification and quick-freezing methods were 84.5% and 74.3% respectively, and there was no significant difference between them. However, the proportion of embryos frozen through the quick-freezing method that developed in culture was significantly lower than control (nonfrozen embryos).

Experiment 3. The viability *in vivo* of embryos frozen through different methods is shown in Table 2. The percentage of embryos developing into normal fetuses was 25.2%, 17.1% and 27.1%, respectively, for the slow-freezing, quick-freezing and vitrification methods. There was no significant difference among them. However, normal fetal development in all three groups were significantly lower than in the unfrozen group ($p < 0.05$). The resorption rates of embryos frozen through slow-freezing, quick-freezing and vitrification methods were 20.4%, 34.3% and 12.1%, respectively. The resorption rate of embryos frozen through quick-freezing was significantly higher ($p < 0.05$) than the other two freezing groups and the control. Finally, there was no significant difference among the implantation sites of the slow-freezing, quick-freezing and vitrification methods (respectively, 45.6%; 51.4% and 39.2%).

DISCUSSION

Slow freezing is an expensive and time-consuming technique for embryo cryopreservation as compared to vitrification and quick freezing. The primary advantages of the vitrification and quick freezing methods for the cryopreservation of mammalian embryos are that the freezing equipment is not necessary and the time required for cooling is reduced. In this study, mouse morulae were cryopreserved using slow freezing, vitrification and quick freezing in order to compare the viability *in vitro* and *in vivo*.

The study shows that the mouse morulae can be slow frozen at rates of 0.5; 0.7; 1.0 or 1.2°C/minute without considerable reduction of viability. These results are similar to Leibo and Mazur[8] who demonstrated that appropriate freezing rates for mammalian embryos are approximately 0.2 to 2°C/minute.

The data show that 74% of the compact morulae developed in culture after quick-freezing in 3M of Ethylene Glycol with 0.3M sucrose. Abas Mazni et al.[1] and Cseh et al.[3], using comparable

Table 1

In vitro survival of mouse embryos after cryopreservation using the different methods. São Paulo, 1999.

Treatment	Cryopreserved embryos (replicates)	Cultured embryos (recovery rate)	Number of embryos cultured that developed into expanded, hatching or hatched blastocysts (%)
Slow-freezing 0.5ºC/min	105 (2)	101 (96%)	73 (72.3)^{a}
Slow-freezing 0.7ºC/min	105 (2)	103 (98%)	82 (79.6)^{ab}
Slow-freezing 1.0ºC/min	105 (3)	102 (97%)	78 (76.5)^{ab}
Slow-freezing 1.2°C/min	105 (2)	105 (100%)	89 (84.8)^{bc}
Vitrification	105 (3)	97 (92%)	82 (84.5)^{abc}
Quick-freezing	105 (3)	105 (100%)	78 (74.3)^{ab}
Nonfrozen control		109	100 (91.7)^{c}

Values with different superscripts are significantly different ($p < 0.05$, Chi-Square Test).

Table 2

In vivo survival of mouse embryos after cryopreservation through different methods. São Paulo, 1999.

Treatment	Embryos transferred (recipients)	Implantation sites (%)	Resorptions (%)	Fetuses (%)
Slow-freezing at 1.2ºC/min	103 (16)	47 (45.6)^{ab}	21 (20.4)^{a}	26 (25.2)^{a}
Vitrification	107 (16)	42 (39.3)^{a}	13 (12.2)^{a}	29 (27.1)^{a}
Quick-freezing	105 (16)	54 (51.4)^{ab}	36 (34.3)^{b}	18 (17.1)^{a}
Nonfrozen control	277 (48)	158 (57.0)^{b}	48 (17.3)^{a}	110 (39.7)^{b}

Values with different superscripts are significantly different ($p < 0.05$, Chi-Square Test).

freezing protocol, reported in vitro survival rate for frozen-thawed morulae of 91.8% and 80%, respectively. The embryonic development rate obtained in this study was a little lower than the data of Abas Mazni et al.[1] and Cseh et al.[3]. This may be due to the different equilibration period employed in their experiments. Rayos et al.[12] also obtained a high survival rate of 77.7% when a mouse eight-cell embryos were equilibrated in 3M ethylene glycol with 0.25M sucrose for 10 minutes before plunging into nitrogen vapor.

The high in vitro survival rate of morulae after vitrification in this study is comparable to the results of Kasai et al.[7] and Mukaida et al.[9]. This result shows that in spite of highly concentrated solution employed in this study, the EFS solution provided considerable protection against freezing damage to mouse morulae. Theoretically, the high concentration of cryoprotectants in the vitrification solution would require multistep dilution, high volume of diluents, and high concentration of sucrose to counterbalance the osmotic shock. However, Vajta et al.[15], in recent review, revealed that in certain vitrification methods, in-straw dilution or even in-straw direct rehydration (dilution of the cryoprotectants in the holding medium without sucrose) is possible. Thus, this study confirms that the direct transfer can also be applied in the case of vitrified mouse embryos with high survival rates.

In this study, the survival rate obtained with the quick freezing method was comparable to those obtained by conventional slow freezing and vitrification. These results are similar to other reports[1,3].

The *in vivo* development rates, i.e., implantation sites and fetuses, among the freezing groups were not significantly different. However, the number of resorptions after transferring quick frozen embryos was significantly higher than the other two groups. The significant number of resorptions obtained with quick freezing was probably due to reduction in cell numbers or damage to some of the blastomeres during freezing and thawing that was not detected by microscope. It might be possible to decrease this proportion of resorptions by modifying some details in the protocol, such as reduction of equilibration period or concentration of cryoprotectants. In the fresh control group, the number of fetuses (39.7%) was significantly higher than the freezing groups. This rate is relatively low when compared to other reports. Tsunoda and McLaren[14], Willians and Johnson[19], Abas Mazni et al.[1] and Valdez et al.[16] have reported *in vivo* survival rates of 45%, 54%, 73.8% and 60.0%, respectively, for fresh mouse morulae that developed into normal fetuses after transfer into recipients. The lower *in vivo* survival rate for nonfrozen embryos obtained in our study was probably due to the different technician's skills at the moment of embryo transfer and also due to quality of recipients.

The results of this study suggest that mouse embryos at the morulae stage can be cryopreserved by vitrification or quick freezing with the same efficiency as a conventional slow freezing procedure.

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RESUMO

Este trabalho avaliou o desenvolvimento *in vitro* e *in vivo* de mórulas de camundongos congeladas por diferentes métodos. A congelação lenta foi realizada em 1,5M de etileno glicol (EG) sendo os embriões resfriados a 0,5; 0,7; 1,0 e 1,2ºC/minuto. Na vitrificação, as mórulas foram equilibradas por 3 minutos em 20% de EG e vitrificadas em solução contendo 40% de EG, 18% de ficol e 10,26% de sacarose após 60 segundos de exposição. A congelação rápida em vapor de nitrogênio foi realizada em solução contendo 3M de EG + 0,3M de sacarose após 2 ou 5 minutos de exposição. Os embriões dos três métodos foram descongelados pela exposição das palhetas ao ar por 10 segundos e imersão em água a 25ºC por 20 segundos. Todos os embriões descongelados foram cultivados *in vitro* por 72 horas para avaliação da sobrevivência *in vitro*. Para avaliação da sobrevivência *in vivo*, mórulas congeladas e não congeladas (controle) foram transferidas para receptoras. O desenvolvimento *in vitro* nas velocidades de 0,5; 0,7; 1,0 e 1,2ºC/minuto foi, respectivamente, 72,3; 79,6; 76,5 e 84,8%. Não houve diferença estatística entre as velocidades de 0,7; 1,0 e 1,2ºC/minuto ($p > 0,01$). O desenvolvimento *in vitro* das mórulas congeladas por vitrificação e pelo método rápido (84,5 e 74,3%, respectivamente) foi semelhante ao método lento. *In vivo*, a taxa de implantação e o número de fetos vivos não diferiram estatisticamente entre os grupos lento a 1,2ºC/minute, vitrificação e rápido.

UNITERMOS: Criopreservação; Embrião; Camundongo.

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