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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INTRODUCTION

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 1.2°C/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 25°C 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 1.2°C/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 1.2°C/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 1.2°C/minute, vitrification and quick-freezing were not significantly different.

SUMMARY

Successful cryopreservation of mouse embryos by quick freezing (direct plunging into liquid nitrogen vapor) was first achieved with glicerol and sucrose13. 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 extracellular and intracellular cryoprotectants. High levels of survival have been reported for mouse embryos using this procedure1–3.

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 IU of eCG (Equine Chorionic Gonadotrophin - Intergonan® – Intervet) 48 hours later followed by 5 IU 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
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 vivo 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 Mazur8 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 Mazzni et al.1 and Cseh et al.3, using comparable

| Table 1 |

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cryopreserved embryos (replicates)</th>
<th>Cultured embryos (recovery rate)</th>
<th>Number of embryos cultured that developed into expanded, hatching or hatched blastocysts (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slow-freezing 0.5°C/min</td>
<td>105 (2)</td>
<td>101 (96%)</td>
<td>73 (72.3)</td>
</tr>
<tr>
<td>Slow-freezing 0.7°C/min</td>
<td>105 (2)</td>
<td>103 (98%)</td>
<td>82 (79.6)</td>
</tr>
<tr>
<td>Slow-freezing 1.0°C/min</td>
<td>105 (3)</td>
<td>102 (97%)</td>
<td>78 (76.5)</td>
</tr>
<tr>
<td>Slow-freezing 1.2°C/min</td>
<td>105 (2)</td>
<td>105 (100%)</td>
<td>89 (84.8)</td>
</tr>
<tr>
<td>Vitrification</td>
<td>105 (3)</td>
<td>97 (92%)</td>
<td>82 (84.5)</td>
</tr>
<tr>
<td>Quick-freezing</td>
<td>105 (3)</td>
<td>105 (100%)</td>
<td>78 (74.3)</td>
</tr>
<tr>
<td>Nonfrozen control</td>
<td>109</td>
<td>100 (100%)</td>
<td>100 (91.7)</td>
</tr>
</tbody>
</table>

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

| Table 2 |

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Embryos transferred (recipients)</th>
<th>Implantation sites (%)</th>
<th>Resorptions (%)</th>
<th>Fetuses (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slow-freezing at 1.2°C/min</td>
<td>103 (16)</td>
<td>47 (45.6)</td>
<td>21 (20.4)</td>
<td>26 (25.2)</td>
</tr>
<tr>
<td>Vitrification</td>
<td>107 (16)</td>
<td>42 (39.3)</td>
<td>13 (12.2)</td>
<td>29 (27.1)</td>
</tr>
<tr>
<td>Quick-freezing</td>
<td>105 (16)</td>
<td>54 (51.4)</td>
<td>36 (34.3)</td>
<td>18 (17.1)</td>
</tr>
<tr>
<td>Nonfrozen control</td>
<td>277 (48)</td>
<td>158 (57.0)</td>
<td>48 (17.3)</td>
<td>110 (39.7)</td>
</tr>
</tbody>
</table>

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.13 and Mukaida et al.14. 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 reports13. 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 McLaren16, Williams and Johnson17, Abas Mazni et al.1 and Valdez et al.18 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 etíleno glicol (EG) sendo os embriões resfriados a 0,5; 0,7; 1,0 e 1,2°C/minute. 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 os três métodos foram descongelados pela exposição das palhetas ao ar por 10 segundos e imersão em água a 25°C 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 1,2°C/minute foi, respectivamente, 72,3; 79,6; 76,5 e 84,8%. Não houve diferença estatística entre as velocidades de 0,7; 1,0 e 1,2°C/minute (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 estaticisticamente entre os grupos lento a 1,2°C/minute, vitrificação e rápido.

UNITERMOS: Criopreservação; Embrião; Camundongo.

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