---Mini Review---

Mouse Sperm Cryopreservation and Effective Embryo Production Using Cryopreserved C57BL/6 Mouse Sperm

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Abstract: Mouse sperm cryopreservation has greatly developed and spread throughout the world since it was the first successfully performed in 1990. The technology of sperm cryopreservation is widely used for storage of genetically engineered mice produced by global large-scale knockout mice or mouse mutagenesis projects in mouse banks. There are several benefits to using sperm cryopreservation. These include the ability to archive a great number of genetically engineered mice at a low cost in a small space; the ease with which freezing and thawing procedures can be carried out; and the convenience of performing mouse reproduction via in vitro fertilization. Sperm cryopreservation provides the research community with permanent access to genetically engineered mice and as such is an essential part of research activities. Many researchers have actively been investigating the techniques surrounding mouse sperm cryopreservation and embryo production using cryopreserved sperm. In particular, the topic of improving the low fertility rate of cryopreserved sperm in the C57BL/6 strain of mouse, the most common inbred strain used as a base for genetically engineered mice, has been garnering much attention. This review focuses on recent work regarding various attractive technologies in the fields of sperm cryopreservation and efficient embryo production using cryopreserved C57BL/6 mouse sperm.

Key words: Sperm cryopreservation, Fertilization, Embryo production, C57BL/6 mouse

Introduction

Sperm cryopreservation is useful for the long-term storage of male germplasm ex vivo. The technology of sperm cryopreservation has been widely used in the various fields of human reproductive medicine, agriculture for animal husbandry and genome resource banking for endangered species and genetically engineered laboratory animals. In mice, sperm cryopreservation has been progressively improved to cope with the archive demands of the vast number of genetically engineered mice produced by large-scale knockout mice and mouse mutagenesis projects worldwide [1].

In the early 1950s, a major innovative work by Polge and Rowson reported the cryoprotective effect of glycerol on bull sperm [2]. Subsequently, there were many reports on the success of sperm cryopreservation in various mammalian species [3–5]. The technologies of sperm cryopreservation and embryo production using sperm have been developed and improved for each species.

The mouse is one of the most common mammalian species used for biological research. It is frequently used in research into innovative reproductive technologies. Mouse sperm cryopreservation was first successfully carried out in 1990 by three independent groups of Japanese investigators [6–8]. They reported that a cryoprotectant composed of raffinose pentahydrate combined with skim milk, or permeable organic compounds such as dimethyl sulfoxide or glycerol allowed the successful fertilization of eggs in in vitro fertilization (IVF) or artificial insemination using cryopreserved sperm. Subsequently, our group improved the method of sperm cryopreservation [9, 10]. This cryoprotectant composed of 18% raffinose pentahydrate and 3% skim milk (R18S3) or its modified solution has been widely used for sperm cryopreservation in mice [1, 9–16].
Production of live animals from cryopreserved sperm is performed by IVF and embryo transfer. IVF techniques using cryopreserved sperm are well established in mice \([1, 16, 17]\). In general, the cryopreserved sperm of many inbred or hybrid strains of mice show relatively high fertility rates and we can efficiently produce many embryos via conventional IVF \([1, 11, 14, 17, 18]\). However, cryopreserved sperm of the C57BL/6 strain of mice, which is a well-established inbred strain and mainly used as a base for genetically engineered mice, show very low fertility rates of about 0–20% in normal IVF.

To overcome this problem, many improvements to sperm cryopreservation and embryo production from cryopreserved C57BL/6 mouse sperm have been reported by various researchers. In this review, we introduce the efficacy and application of sperm cryopreservation in mouse banks along with the history of mouse sperm cryopreservation, and discuss the current findings regarding several technologies for sperm cryopreservation and the efficient production of embryos from cryopreserved sperm mainly for the C57BL/6 mouse.

**Mouse Bank**

Sperm cryopreservation is essential for effectively managing a research project using genetically engineered mice \([14]\). Recently, several large-scale knockout mouse projects or mouse mutagenesis programs have been started in North America, Europe and Asia \([19–21]\). In these projects, a vast number of genetically engineered mice are being continuously produced, while that number is predicted to increase exponentially in the future \([22, 23]\). For effective mouse management, the genetically engineered mice produced by these projects are being collected and archived in the form of cryopreserved embryos or sperm in animal resource facilities. These so-called mouse banks have been established in North America, Europe, Australia and Asia \([24, 25]\). The main function of a mouse bank is the collection, preservation and provision of valuable genetically engineered mice, to provide the research community with easier access to the mice.

The benefits of sperm cryopreservation for archiving many strains of mice in a mouse bank include being able to reduce the facilities and cost required to maintain breeding colonies of mice, and being able to easily preserve a great number of germplasm compared to embryo cryopreservation \([26]\). Moreover, cryopreserved sperm can easily be transferred between research facilities by packing them in dry ice and using a commercial transport company \([27]\). These practical reasons have meant that sperm cryopreservation has been widely adopted for storing genetically engineered mice in mouse banks worldwide \([28–32]\).

Recently, inter- and intranational transport of cryopreserved sperm of genetically engineered mice between mouse banks and laboratories has become a frequent procedure in research \([25]\). In general, the transport of cryopreserved embryos or sperm is preferable to that of live animals. User-friendly transport system facilitates worldwide access to mouse resources. We are now trying to develop a novel transport system for unfrozen embryos derived from cryopreserved sperm \([33, 34]\). In this system, embryos are transferred to a 0.5 ml tube filled with M2 medium and packed in a cool box. The box containing the embryos is transported to a recipient laboratory by a commercial transport company at 4ºC. This system makes it possible for the recipient laboratory to easily withdraw archived valuable mice in the form of cryopreserved sperm, without needing the special instruments and techniques involved in embryo freezing and thawing. In the future, a combination of various reproductive technologies will contribute to the establishment of a simple system for a global network of genetically engineered mice.

**Mouse Sperm Cryopreservation**

Mouse sperm are quite vulnerable to freezing and thawing procedures, so it is difficult to maintain their motility and fertility after cryopreservation. In the early 1990s, three groups of researchers, Okuyama *et al.*, Tada *et al.*, and Yokoyama *et al.* reported the successful cryopreservation of mouse sperm \([6–8]\). They found that raffinose pentahydrate, which is a trisaccharide composed of galactose, fructose and glucose, effectively protected mouse sperm against freezing and thawing stress. We subsequently improved their methods for practical use and created a modified cryoprotectant composed of 18% raffinose pentahydrate/3% skim milk solution (R18S3) \([9–12]\). At the present time, our protocol for sperm cryopreservation is widely used in many laboratories. It is known as the Nakagata method \([1]\).

Many groups have reported the factors affecting the successful cryopreservation of mouse sperm. The cryoprotectant is one of the important factors affecting sperm viability after freezing and thawing. Many papers have reported the various permeating (glycerol and...
dimethyl sulfoxide) and nonpermeating agents (sugars or polymers) as well as biological materials (skim milk or egg yolk) that are used for mouse sperm cryopreservation [35–46]. These cryoprotectants play roles in preventing the formation of intracellular ice, supporting membrane stability and regulating cell volume against osmolarity change. Mazur et al. have focused on oxidative stress in mouse sperm after freezing and thawing [47–49]. The production of reactive oxygen species (ROS) in mouse sperm depends on the concentration of O2 in the medium [50, 51]. Mazur et al. incorporated an Escherichia coli membrane preparation (Oxyrase) to achieve low oxygen tensions in the medium and decrease the motility loss resulting from the production of ROS. ROS cause lipid peroxidation of plasma membrane, leading to sperm malfunction such as the loss of membrane integrity, mitochondria injuries and DNA fragmentation [52]. The efficacy of novel cryoprotectants such as alpha-monothioglycerol or amino acids, especially L-glutamine, have also been reported and are mentioned below [16, 53, 54].

### Improvement of Sperm Cryopreservation and IVF for C57BL/6 Mouse

The C57BL/6 strain of mouse is the most well-established inbred strain of mouse and is commonly used for producing genetically engineered mice for biological research. Therefore, it is important to establish the technology of sperm cryopreservation and embryo production for the C57BL/6 mouse in order to support reliable archiving and reproduction in mouse banks. For a long time, it has been difficult to efficiently produce live animals due to the low fertility rate of frozen-thawed C57BL/6 mouse sperm in vitro [11, 14, 17, 18]. Many researchers have tackled this problem in order to improve the low fertility rate of cryopreserved C57BL/6 mouse sperm using various approaches. The strategies for effectively producing embryos from cryopreserved sperm can be divided into four types, namely the modification of sperm cryopreservation, regulation of sperm activation in preincubation media, selection of viable sperm, and mechanical support for fertilization events.

Previously, we demonstrated that large populations of frozen-thawed C57BL/6 mouse sperm cause severe cellular injuries in the acrosomal region or tail, which are related to the low fertility rate [55]. Recently, we overcame this problem and succeeded in obtaining a stable and high rate of fertilization with C57BL/6 mouse sperm cryopreserved using modified R18S3 containing L-glutamine (Table 1) for cryopreservation and modified Krebs-Ringer bicarbonate solution (TYH) containing methyl-β-cyclodextrin (MBCD, Table 2) for preincubation (Fig. 1) [16, 56]. L-glutamine is the most abundant free amino acid in the plasma and tissues. There are many reports about the protective effects of L-glutamine in the sperm cryopreservation of several mammalian species [57–62]. L-glutamine has the potential to protect the lipid membrane by interaction with the positively-charged amine group of amino acids and the negatively-charged phospholipids [63]. Liu et al. recently revealed the efficacy of essential amino acid in

<table>
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<th>Table 1. Composition of sperm cryopreservation medium</th>
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<td><strong>Reagent</strong></td>
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<tr>
<td>Raffinose pentahydrate</td>
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<td>Skim milk</td>
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<td>L-glutamine</td>
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Instructions for preparing the sperm cryopreservation medium are as follows: 1) Dissolve raffinose pentahydrate, skim milk and L-glutamine in 10 ml of distilled water at 60°C in a 15-ml disposable tube. 2) Incubate the solution in a water bath at 60°C for 90 min. 3) Divide the solution into 1.0 ml aliquots and transfer to 1.5-ml micro-centrifuge tubes. 4) Centrifuge the samples at 10,000 × g for 60 min. 5) Carefully collect 0.7 ml supernatant of each sample from the central region of the tube. 6) Filter the supernatant using a disposable filter unit (pore size: 0.22 μm). 7) Store the filtrated samples in 1.0-ml glass ampoules at room temperature.

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<th>Table 2. Composition of sperm preincubation medium</th>
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<td><strong>Reagent</strong></td>
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<tr>
<td>NaCl</td>
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<tr>
<td>KCl</td>
</tr>
<tr>
<td>MgSO4/7H2O</td>
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<tr>
<td>KH2PO4</td>
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<tr>
<td>NaHCO3</td>
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<tr>
<td>Sodium pyruvate</td>
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<td>Glucose (D+)</td>
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<tr>
<td>CaCl2/2H2O</td>
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<tr>
<td>Methyl-β-cyclodextrin</td>
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<td>Penicillin G potassium salt</td>
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<td>Streptomycin sulfate</td>
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<td>Polyvinylalcohol</td>
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R18S3 in maintaining the fertility of cryopreserved sperm in three substrains of C57BL/6 mice [54]. The combination of amino acids, especially L-glutamine, in R18S3 is suitable for mouse sperm cryopreservation.

MBCD is also well known as a potent cholesterol acceptor and inducer of sperm capacitation [56, 64]. MBCD is a methylated cyclic heptasaccharide with a hydrophilic outer surface and a lipophilic cavity at its
After freezing and thawing, putative inhibitory factors which are derived from damaged sperm may negatively affect and decrease the ability of normally motile sperm to fertilize an oocyte [79]. Therefore, it is important to select motile sperm or remove inhibitory factors in order to achieve a high rate of fertilization. There are many reports about selection methods for motile sperm or the removal of inhibitory factors before insemination. Szczygiel et al. first showed that separation of motile sperm with a sephadex column significantly enhanced fertilization rates in various inbred strains of mice [79]. This original concept of selecting motile sperm has been incorporated into various protocols for IVF using cryopreserved sperm [16, 56, 71, 72, 78].

In a previous paper, we demonstrated a simple method of collecting motile sperm after preincubation from the periphery of a drop covered with paraffin oil using a wedge-shaped pipette tip [16, 56]. There is a large population of motile sperm in the peripheral area of the drop and also of nonmotile or aggregated sperm in the center of the drop. It is important to observe the area mainly containing motile sperm during sperm collection for insemination. In another paper, Taguma et al. performed sperm preincubation using an oil-covered medium in a 4-well dish [71]. They collected sperm suspension from the center of the well where motile sperm were localized. Recently, Bath described a novel IVF system using a cell culture insert (Transwell) which was coincubated with sperm and oocytes in the fertilization medium and progressively transferred the gametes to the well containing fresh fertilization medium [78]. This method may dilute and remove inhibitory factors derived from frozen-thawed sperm during fertilization.

Mechanical Aids for Fertilization with Cryopreserved Sperm

Sperm penetration through the zona pellucida is the main barrier to fertilization of an egg. Our group revealed that frozen-thawed sperm of the C57BL/6 mouse demonstrated severe dysfunction in penetrating the zona pellucida [55]. There are many reports describing zona pellucida penetration assisted by various mechanical operations which aim to increase the fertilization rates of frozen-thawed C57BL/6 mouse sperm.

Previously, our group reported that partial zona pellucida dissection (PZD) by an injection needle before insemination dramatically increased the rate of fertilization with frozen-thawed C57BL/6 mouse sperm.
various mammalian species. Furthermore, we applied a laser micro-dissection system (Saturn 3 laser system, Research Instruments Ltd, UK) to incise the zona pellucida, which also improved the fertility of frozen-thawed C57BL/6 mouse sperm [81, 82]. In addition, Kawase et al. created an incision in the zona pellucida using a piezo-micromanipulator (ZIP). This too was as effective as PZD at increasing the fertilization rate [83, 84].

Furthermore, intracytoplasmic sperm injection (ICSI) technology is a well-established mice reproduction technique [85, 86]. ICSI can be applied for severe fertility problems with sperm with low or no motility. Szczygiel et al. achieved a high fertilization rate by ICSI compared to IVF using frozen-thawed C57BL/6 mouse sperm [87]. These mechanical supports are very useful for remedying accidentally-occurring cases of low sperm motility after freezing and thawing.

Conclusions

Mouse sperm cryopreservation is a practical and useful technology for efficiently preserving valuable genetic resources of genetically engineered mice. In the 20 years since the first successful cryopreservation of sperm, many innovative findings have been incorporated into the original procedure of sperm cryopreservation and embryo production. These technologies are perpetually advancing towards an easy and efficient method based on the well-integrated fields of molecular biology and bioengineering science. We strongly believe that the advancements in technology for mice will contribute to a breakthrough which will improve the sperm cryopreservation of various mammalian species.

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References


74) Felix, R. (2005): Molecular physiology and pathology of


