—Mini Review—

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

*Toru Takeo and Naomi Nakagata**

Division of Reproductive Engineering, Center for Animal Resources and Development, Kumamoto University, Kumamoto 860-0811, Japan

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 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].

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*To whom correspondence should be addressed.
e-mail: nakagata@kumamoto-u.ac.jp

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

Table 1. Composition of sperm cryopreservation medium

Reagent	mg/10 ml
Raffinose pentahydrate	1,800
Skim milk	300
L-glutamine	146

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 60ºC in a 15-ml disposable tube. 2) Incubate the solution in a water bath at 60 ºC 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.

Table 2. Composition of sperm preincubation medium

Reagent	mg/100 ml
NaCl	697.6
KCl	35.6
$MgSO_4/7H_2O$	29.3
KH_2PO_4	16.2
$NaHCO_3$	210.6
Sodium pyruvate	5.5
Glucose (D+)	100.0
$CaCl_2/2H_2O$	25.1
Methyl-β-cyclodextrin	98.3
Penicillin G potassium salt	7.5
Streptomycin sulfate	5.0
Polyvinylalcohol	100.0

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 O_2 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

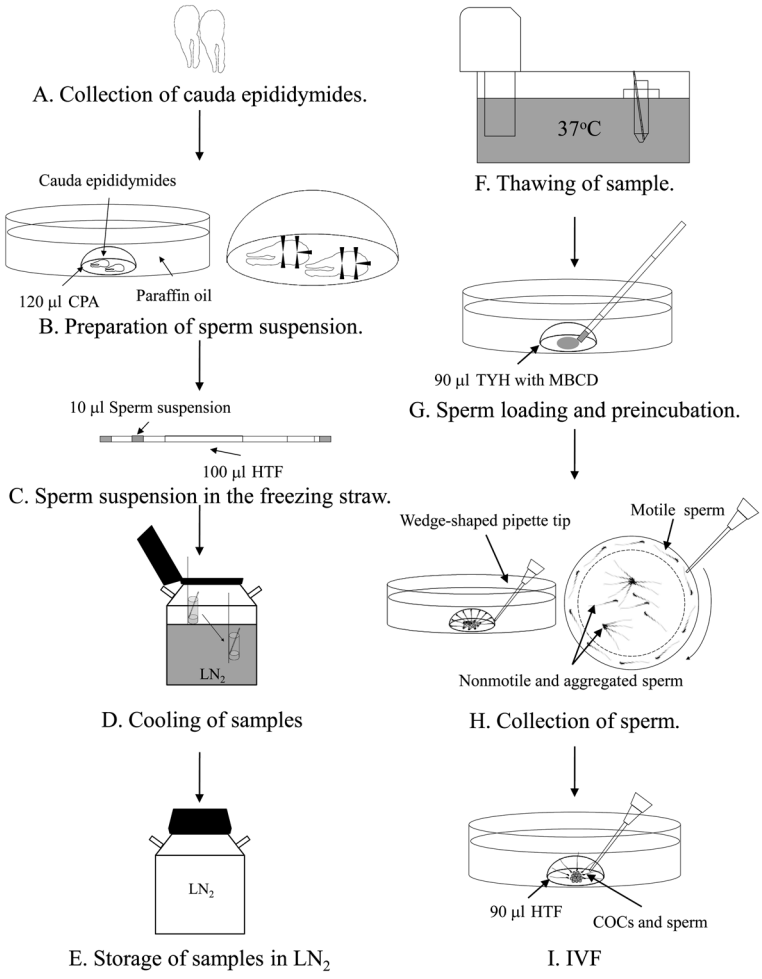

Fig. 1. Schematic descriptions of mouse sperm cryopreservation and IVF protocol. Sperm cryopreservation was performed following procedures (A-E). A) One pair of cauda epididymides was collected from a male mouse. B) The cauda epididymides were transferred into a 120-μl drop of CPA composed of raffinose pentahydrate, skim milk and L-glutamine (Table 1), and cut into five portions as indicated by the arrowheads. C) The prepared sperm suspension was divided into 10 aliquots and each aliquot was packed into a 0.25-ml plastic straw. D) The plastic straws containing the 10-μl sperm suspension were cooled in liquid nitrogen (LN_2) vapor for 10 min, then plunged directly into LN_2. E) These samples were stored in the liquid nitrogen until use. Thereafter, *in vitro* fertilization using frozen-thawed sperm was performed following steps (F-I). F) The stored samples were retrieved from the LN_2 and immediately soaked and warmed in a water bath at 37ºC for 10 min. G) Aliquots of 10 μl of sperm suspension were loaded and preincubated in a 90-μl drop of TYH with MBCD (Table 2) for 30 min at 37ºC. H) An aliquot of 10-μl of sperm suspension was collected from the peripheral part of the drop containing motile sperm using a wedge-shaped pipette tip in the direction shown by the arrow. I) The sperm suspension was added to 90-μl of human tubal fluid (HTF) containing cumulus oocytes complexes (COCs) and coincubated for 5–6 hours. Twenty-four hours after insemination, 2-cell embryos were obtained at 37ºC with 5% CO_2 in the air.

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

center [65]. MBCD strongly interacts with the components of plasma membrane and extracts some lipids, especially cholesterol [66–68]. It is well known that cholesterol is key molecule in mammalian sperm capacitation [69]. The efflux of cholesterol initiates a signal transduction pathway that promotes capacitation [70]. Choi and Toyoda reported that MBCD effectively induces capacitation by removing cholesterol from mouse sperm and can be utilized as a bovine serum albumin substitute for IVF using fresh sperm [64]. Our groups was the first to demonstrate the efficacy of MBCD in IVF using frozen-thawed C57BL/6 mouse sperm [56]. Subsequently, other groups were able to reproduce the results and additionally showed that the technique is applicable to IVF using cryopreserved sperm of the C57BL/6 and other strains of mice [54, 71].

The presence of Ca^{2+} in a medium for sperm preincubation is also likely to negatively influence the fertility of frozen-thawed sperm. There are a few reports about sperm preincubation in media without Ca^{2+} which prevented acrosomal loss and improved the fertility of frozen-thawed sperm [72, 73]. The dynamic changes in the intracellular concentration of Ca^{2+} through the ion channels on the plasma membrane are engaged in regulating functions such as sperm motility, capacitation and the acrosome reaction in mammalian sperm [74, 75]. Fraser elucidated the requirements of extracellular Ca^{2+} for capacitation and the acrosome reaction in mouse sperm, and noted the absence of Ca^{2+} from sperm preincubation medium suppressed acrosome loss during preincubation [76]. The suppression of spontaneous acrosome loss during preincubation may contribute to improvement in the fertility of frozen-thawed C57BL/6 mouse sperm.

The generation of ROS derived from sperm after freezing and thawing is a possible factor in fertility decrease [77]. Ostermeier *et al.* reported that a reducing agent of alpha-monothioglycerol added to R18S3 greatly maintained the fertility of C57BL/6 mouse sperm after freezing and thawing [53]. In their paper, they pointed out the impairment of fertilizing functions and cellular injuries caused by the overproduction of ROS in frozen-thawed sperm. In addition, Bath showed that applying a treatment of glutathione to frozen-thawed sperm during fertilization remarkably enhanced the fertility. This effect has been observed in various strains of mice such as C57BL/6J, 129S1 and FVB/NJ [78]. Bath additionally reported that the ROS derived from damaged or moribund sperm perturb the function of the thiol-sensitive proteins involved in sperm capacitation [78].

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 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

[80]. 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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