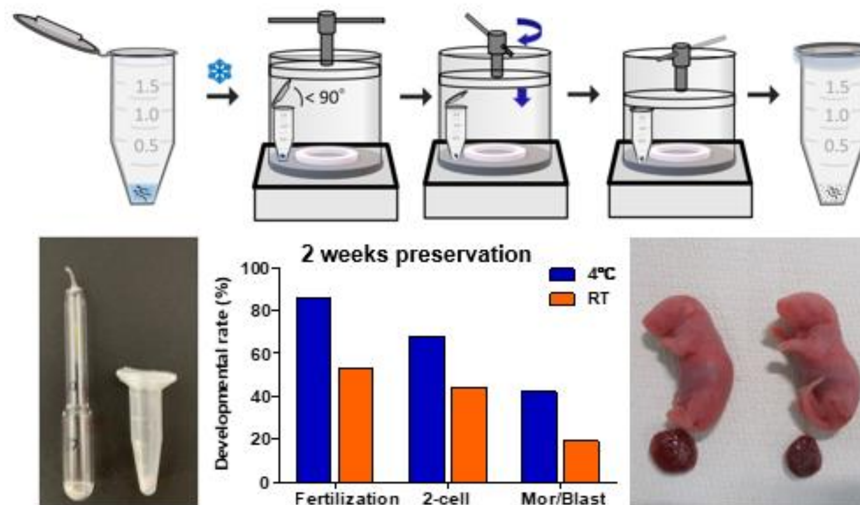
DOCTORAL DISSERTATION
(UNIVERSITY OF YAMANASHI)

DEVELOPMENT OF A LOW-COST AND SIMPLE DEVICE FOR FREEZE-DRYING AND PRESERVING MOUSE SPERM USING PLASTIC MICROTUBES

プラスチックチューブを用いた安価で簡便なマウス精子凍結乾燥保存法の開発



A Thesis

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By

Yang Li

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Cover page photographs:

(Up) Schematic preservation of the freeze-drying protocol for sperm using the microtube method.
(Down-left) Freeze-dried sperm preserved in glass ampoules and microtubes.
(Down-middle) In vitro development of embryos derived from freeze-dried sperm preserved at 4℃ or room temperature for 2 weeks.
(Down-right) Full-term development of offspring derived from microtube-FD sperm preserved at room temperature for 1 week.

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LIST OF ABBREVIATIONS

AI	artificial insemination
ART	assisted reproductive technologies
BSA	mouse serum albumin
COC	cumulus-oocyte complex
CPA	cryoprotective agent
DAPI	4’6-diamidino-2-phenylindole
ICM	inner cell mass
ICSI	Intracytoplasmic sperm injection
IVF	in vitro fertilization
LN2	liquid nitrogen
PN	pronucleus
PVP	polyvinyl pyrrolidone
TE	trophectoderm

Abbreviations used without definition

ANOVA	analysis of variance
DNA	deoxyribonucleic acid
SD	standard deviation

SUMMARY OF DISSERTATION

TITLE: ESTABLISHMENT OF EFFECTIVE AND SIMPLIFIED MOUSE PRESERVATION SYSTEM WITH FREEZE-DRIED SPERMATOZOA

NAME: YANG LI

The preservation of mammalian genetic resources is of great importance and is applied to a diverse range of scientific fields, including human medicine, farm animal production, laboratory animal record keeping and wildlife conservation. The genetic banking of spermatozoa provides a more efficient and cost-effective approach for preserving genetic resources compared to oocytes and embryos. However, the conventional spermatozoa cryopreservation requiring the use of liquid nitrogen in storage faces numerous challenges, such as the high maintenance cost, the restriction of transportation and the susceptibility to natural disasters and power outages.

Freeze-drying has been recently focused as a new tool for sperm preservation, which allows preservation at ambient or refrigerator temperature. On the other hand, application of intracytoplasmic sperm injection (ICSI) is needed for fertilization of freeze-dried (FD) sperm due to the loss of their motility.

The pH of the culture medium utilized in biochemistry and biology research is commonly adjusted to 7.4. While pH 7.4 is commonly considered suitable for in vitro fertilization (IVF) and is also utilized for ICSI. it is worth considering that pH 7.4 might not be optimal for ICSI due to the need for artificial breaking of the cell membrane. Therefore, it is crucial to expedite the investigation of optimal pH conditions specifically for ICSI.

In the first series of this study, optimal pH value of HEPES-CZB medium, which is the medium for ICSI, was identified for favorable ICSI outcomes. ICSI was performed using HEPES-CZB medium adjusted to the pH range of 4.0 to 12.0, the survival rate and blastocyst formation rate were evaluated under each pH condition. The most favorable outcomes in terms of ICSI survival rate, blastocyst rate and blastocyst rate per used eggs were observed under pH 7.6. In addition, an alkaline shift of the HEPES-CZB medium

during storage was observed. In practical scenario, long-term refrigerated storage is needed before use. Thus, initial pH adjustment of HEPES-CZB medium to a slightly alkaline level prior to storage was recommended for favorable ICSI outcomes.

Glass ampoules have been predominately used for FD sperm since healthy offspring were obtained from FD mouse spermatozoa about a quarter century ago. Despite the drawbacks of this method (e.g., high costs and risks) and the urgent need to overcome these issues, new methodologies, and substitutes for glass ampoules have rarely been reported.

In the second series of this study, a simple, economical method for FD sperm using commercially available plastic microtubes was developed. Mouse epididymal sperm suspensions were placed in 1.5 ml polypropylene tubes, frozen in liquid nitrogen and dried in an acrylic freeze-drying chamber, then the microtube caps were closed in a vacuum. FD sperm stored in microtubes at −30°C yielded healthy offspring without reducing the success rate even after 9 months of storage. Thus, microtubes are a practical and efficient substitute for glass ampoules when used to store FD sperm at −30°C.

While the freeze-drying method using plastic microtubes offers a solution to some of the drawbacks associated with glass ampoules, it also presents its own set of disadvantages. One such disadvantage is the requirement for high initial investment cost of specialized freeze-drying equipment prior to preserving spermatozoa. In nature, many anhydrobiotic organisms can tolerate severe desiccation. Trehalose is found to accumulate in high concentrations in many of these anhydrobiotic organisms during desiccation, and it is believed to play a major role in imparting desiccation tolerance to anhydrobiotic organisms. The objective of the last experiment was to explore the feasibility of preserving sperm in a glassified state using trehalose. The study investigated three drying methods: natural drying, heated drying and microwave drying. Sperm was recovered after rehydration in all groups, but natural drying was determined to be the most favorable option in terms of ease of use for ICSI and the subsequent developmental rate. Blastocysts were successfully derived from glassified sperm after 24h of RT storage. However, the obtained blastocyst rate was relatively low. Treatment with NaOH at concentrations of 10 mM and 20 mM significantly improved the 4-8 cell rate. Although further modification to the preparation protocol is required, this study demonstrated the

potential of glassified sperm for sperm preservation in regions where liquid nitrogen and freeze-drying facilities are limited.

These findings would contribute to the development of alternative, low-cost approaches to preserve and transport genetic resources.

論文内容の要旨

論文題目　プラスチックチューブを用いた安価で簡便なマウス精子凍結乾燥保存法の開発

氏　　名　YANG LI

哺乳類の遺伝資源の保存は非常に重要であり、ヒトの医学、家畜の生産、実験動物の記録管理、野生動物の保護など、様々な科学分野に応用されている。精子を用いたジーンバンクは、より効率的な遺伝資源の保存法を提供する。しかし、液体窒素を用いた従来の精子凍結保存は、維持費が高い、輸送に制約がある、自然災害や停電の影響を受けやすいなど、多くの課題を抱えている。

近年、精子保存の新たな手段として、常温または冷蔵庫で保存できる凍結乾燥精子が注目されている。一方、凍結乾燥精子は運動性を失うため、受精させるのに顕微授精が必要である。生化学や生物学の研究で利用される培養液の pH は、一般的に 7.4 に調整されている。pH7.4 は体外受精に適しているとされ、顕微授精にも利用されているが、顕微授精では人工的に細胞膜を破る必要があるため、pH 7.4 は最適でない可能性も考慮すべきである。 そのため、ICSI に特化した最適な pH 条件の検討を急ぐことが重要である。

本研究の最初の実験では、良好な顕微授精の結果を得るための HEPES-CZB 培地の最適な pH 値を明らかにした。pH4.0～12.0 に調整した HEPES-CZB 培地を用い顕微授精を実施し、各 pH 条件下での生存率、胚盤胞率を評価した。pH7.6 の条件下で生存率、胚盤胞率及び供試卵数に対する胚盤胞率が最もいい結果が得られた。また、HEPES-CZB 培地は保存中にアルカリ性になることが確認された。実際には、使用前に培地が長期間冷蔵保存される。従って、良好な顕微授精結果を得るために、保存前に HEPES-CZB 培地の pH を弱アルカリ性に調整するが推奨された。

約 25 年前にマウス精子の凍結乾燥精子から健康な子供が得られて以来、凍結乾燥精子の作製及び保存には主にガラスアンプルが使用されてきた。しかしながら、この方法には高コストや安全性のリスクなどの欠点が存在し、これらの問題を解決するためには新たな方法やガラスアンプルの代替物が必要である。しかし、現時点ではそれらに関する報告はごくわずかしかない。

次の実験では、市販のプラスチック製マイクロチューブを用いた簡便かつ経済的な凍結乾燥精子の作製及び保存法を開発した。マウスの精巣上体尾部精子の培養液を 1.5ml のマイクロチューブに入れ、液体窒素で凍結し、凍結乾燥機で乾燥された後、キャップを真空で密閉した。作製後の凍結乾燥精子を-30℃で保存した結果、9 か月間の保存期間中、凍結乾燥精子の発生能に有意な低下は観察されず、健康な産仔が得られた。これにより、前述の実験結果を踏まえ、マイクロ

チューブが凍結乾燥精子を−30℃で保存する際にガラスアンプルの代替として有用であり、実用的かつ効率的な方法であることが示された。

マイクロチューブを用いた凍結乾燥法は、ガラスアンプルの欠点を解決する一方で、独自のデメリットも存在する。その一つは、凍結乾燥機の導入に伴う初期費用が高いという点である。自然界では、多くの無水生物が極度の乾燥に耐えることができる。トレハロースは乾燥時に多くの無水生物に高濃度に蓄積されることが分かっており、無水生物に乾燥耐性を付与する上で大きな役割を果たすと考えられている。

最終実験の目的は、トレハロースを用い、精子をガラス化状態で保存することの実現可能性を調査することである。そのために、自然乾燥、加熱乾燥、マイクロ波乾燥の三つの乾燥方法を検討した。すべてのグループで加水後精子を回収したが、顕微授精の利便性とその後の発生率の観点から、自然乾燥が最も望ましい選択肢と判断された。ガラス化精子は 24 時間の室温保存後、胚盤胞まで発生できたが、胚盤胞率は低かった。10mM と 20mM の NaOH で精子を処理すると、4−8 細胞率が大幅に改善された。ガラス化精子の作製プロトコルのさらなる改良が必要であるが、本実験では、液体窒素や凍結乾燥設備が限られている地域で精子保存にガラス化精子の可能性を示した。

これらの知見は、精子を用いた遺伝資源の保存と輸送に代替的かつ経済的な手法の開発に貢献するものと考えられる。

CHAPTER 1

GENERAL INTRODUCTION

Introduction

In assisted reproductive technology (ART), in vitro fertilization (IVF) methods are commonly divided into two categories: conventional IVF and intracytoplasmic sperm injection (ICSI). The ability to generate blastocysts through IVF, where an oocyte is fertilized by a motile sperm in a culture medium in vitro, was first achieved in rabbit in 1959 (CHANG 1959). Subsequently, successful IVF procedures were performed in humans in1969 (Edwards, Bavister, and Steptoe 1969), coming with the birth of the world's first IVF baby in 1978 (Steptoe and Edwards 1978). On the other hand, the development of the ICSI technique paved the way for the utilization of sperm with limited or no motility (Uehara and Yanagimachi 1976). It then became possible to inseminate completely non-viable freeze-dried sperm and other dried sperm through ICSI, in contrast to traditional IVF techniques employing motile sperm. Consequently, ICSI has emerged as an effective and vital method not only in the field of reproductive engineering but also in the treatment of human infertility(Palermo et al. 1992). However, despite the notable difference between IVF and ICSI, the pH value of 7.4, determined for IVF, is currently applied as is in ICSI procedures without the existence of a specifically tailored medium pH for ICSI.

Biobanking is a rapidly growing industry, covering diverse fields such as human medicine, farm animal production, laboratory animal record keeping and wildlife conservation. Presently, biobanking is done almost exclusively by cryopreservation, followed by maintenance of the samples under liquid nitrogen. Spermatozoa are produced in much larger numbers compared with oocytes and embryos and therefore the banking of spermatozoa would be an efficient and cost-effective approach for the conservation of genes. Freeze-drying has been proposed as an alternative method for sperm preservation to overcome the disadvantages of the cryopreservation. However, the conventional sperm freeze-drying methods have many problems and the containers used for sperm freeze-drying have not progressed much since the development of this technology.

In this chapter, research history and background regarding ICSI of mouse oocytes and freeze-drying of mouse spermatozoa are overviewed, followed by the objective of this research thesis.

Development of the ICSI technique

In the late 1980s, different attempts were carried out by injecting the spermatozoon directly into the oocyte. The sea urchin and the Chinese hamster were the first species used to attempt these new procedures. Early on, a pregnancy was obtained in the rabbit followed by a birth in the bovine species. In 1987, this technique was applied in human oocytes as a proof-of-concept. In a small series aimed at achieving pregnancy in clinical settings, it was shown that most oocytes injected were damaged, and the few that were fertilized were somewhat hindered in their ability to develop, resulting in failed implantation. In spite of these initial disappointing results, with a further refinement of tools and equipment, this procedure evolved to a new technique, ICSI, which proved itself as the best way to treat male factor couples.

Since many ICSI experiments cannot be performed in humans for ethical reasons, animal models are desirable. Hamster oocytes are excellent for the study of oocyte activation and pronuclear development after ICSI; however, their use in ICSI studies is hampered because of difficulty in culturing manipulated oocytes to the transferable stage of development, even though the technique of culturing hamster eggs/embryos has been improving steadily. The mouse is ideal for ICSI experiments because of the ease of culturing eggs/embryos in vitro. However, ICSI in the mouse has been extremely difficult because the coherence (viscosity) of the ooplasm as well as the wound-healing ability of the oolemma is much lower in the mouse than in the hamster and human.

When a conventional sperm injection pipette was pushed against a mouse oocyte, the zona could be broken by a sharply pointed pipette, but the oolemma could not break even when the pipette almost reached the cortex of the opposite side of the oocyte. When a spermatozoon in the pipette was expelled at this position, the spermatozoon appeared as if it had been successfully deposited within the ooplasm. In reality, however, it was still outside the ooplasm, trapped in the oocyte, surrounded by the medium and the stretched

oolemma. It was possible to break the mouse oolemma with the conventional pipette but repeated stabbings were necessary to break the oolema, which may damage the oolemma irreversibly.

In contrast to the conventional pipette, the piezo-driven pipette harnesses the piezo-electric effect to transmit a small crystal lattice distortion to the tip of a pipette, driving it forward in a precise and controlled manner, which can break the oolemma readily and consistently.

ICSI in human infertility treatment

In the context of humans, the topic of infertility has been explored across a wide range of fields-medicine, social science, religion, and philosophy. Infertility has led to the appearance of what is known today as assisted reproductive technology (ART), with ICSI becoming the most widely used insemination method worldwide.

Indeed, ICSI is used today in about 66.5% of fertility centers. The reliability of ICSI to achieve fertilization in cases of severe male factor infertility has led to the drastic expansion of its use; it is now commonly utilized for low oocyte maturity, with cryopreserved oocytes, in conjunction with preimplantation genetic testing (PGT) and for patients of advanced maternal age (AMA).

In 2003, the Society for Assisted Reproductive Technology (SART) reported a total of 112,988 annual ART cycles in the United states, which steadily grew to 248,086 cycles by 2017. Based on Centers for Disease Control and Prevention (CDC)'s 2020 Fertility Clinic Success Rates Report, there were 326,468 ART cycles performed at 449 reporting clinics in the United States during 2020.Along with this growth , patients are opting for additional ART procedures to enhance their chances of having a successful pregnancy, including oocytes cryopreservation to postpone conception, and PGT to assess the genetic profile of embryos and increase the likelihood of implantation. As the overall incidence rate of cancer patients in young adults (age 15-39) in the United States is increasing by 0.9% each year, many patients are also choosing oocyte cryopreservation to preserve their fertility. All of these factors have contributed to the growing popularity of ICSI.

The pH of HEPES-buffered CZB medium used in mouse ICSI

Despite the notable difference between IVF and ICSI, the pH value of 7.4, determined for IVF, is currently applied as is in ICSI procedures without the existence of a specifically tailored medium for ICSI. In IVF, sperm adhere and merge with the oocyte cell membrane, whereas in ICSI, the cell membrane is artificially disrupted, potentially leading to the introduction of culture medium into the cell. Studies have indicated that cumulus cells surrounding the zona, which are enclosed by gap junctions, provide the oocyte with the ability to regulate pH level (FitzHarris and Baltz 2006; FitzHarris, Siyanov, and Baltz 2007). In IVF, the cumulus-oocyte complex is employed, whereas in ICSI, naked oocytes without their surrounding cumulus cells are used, making the oocytes potentially more vulnerable to the effects of pH (Swain 2010). Therefore, the media conditions suitable for IVF may not be optimal for ICSI.

Moreover, it is important to recognize that nearly all biological processes are influenced by pH, and even slight alterations in pH can impact cell structure and function (Roos and Boron 1981). Therefore, maintaining a constant pH level in the medium is crucial, and this is achieved through the use of buffers. Selecting the appropriate buffer for the experimental system holds significance. Bicarbonate is a commonly employed buffer for stabilizing medium pH. It demonstrates buffering capacity in the presence of carbon dioxide, which necessitates the use of an incubator to maintain a carbon dioxide concentration of 5-10%. It is worth noting that bicarbonate buffer media tend to become alkaline when exposed to air for prolonged periods. On the other hand, HEPES buffer does not rely on carbon dioxide for its buffering effect and is therefore incorporated into the medium when cells are manipulated outside the incubator for extended durations. In the context of ICSI, HEPES-buffered CZB medium is used due to the inevitable manipulation of oocytes in open containers under atmospheric conditions. The pH of the medium can fluctuate depending on its composition, despite the excellent buffering capacity of HEPES buffers within the pH range of 7.2 to 7.4. Therefore, it is possible that the pH may fluctuate even when HEPES are added into the medium.

Furthermore, during the process of performing ICSI, the oocytes undergo stress because they must be removed from the body to perform ICSI. Understanding the in vivo

development becomes essential in minimizing stress on the oocytes during in vitro manipulation. In the ovary, oocyte development coincides with follicle development. The follicle progresses from a primordial follicle to a primary follicle, then to a secondary follicle, and finally to a vesicular ovarian follicle, or a mature follicle, which contains an internal reservoir of follicular fluid. Upon stimulation with gonadotropic hormone, the oocyte within the largest mature follicle undergoes maturation, leading to ovulation. It has been observed that the intracellular pH of mouse oocytes increases as they mature, with the intracellular pH of mature oocytes reaching approximately 7.4. (FitzHarris and Baltz 2006). Furthermore, no significant change in intracellular pH of oocytes before and after fertilization has been observed (Kline and Zagray 1995). Ovulated oocytes are transported through the fallopian tubes and eventually reach the uterus (Figure 2.1). The pH of human fallopian tube fluid has been reported to range between 7.2 and 7.3 (Edwards, n.d.; Shalgi, Kraicer, and Soferman 1972). The pH of fallopian tube fluid tends to be more alkaline while it is believed that the pH of the uterus is generally more acidic compared to the pH of the fallopian tubes (Iritani, Gomes, and Vandemark 1969; Maas, Storey, and Mastroianni 1977; Dale et al. 1998). Brian et al. reported that during the fertilization process, the oocyte or fertilized egg encounters three distinct sequential intracellular pH environments: follicular fluid, fallopian tube fluid, and intrauterine fluid. These findings suggest that the intracellular pH of oocyte remains stable, while changes in extracellular pH play a crucial role in normal development.

Drawbacks of cryopreservation method

To date, the most revolutionary and widely used method to preserve sperm of human and different animal species has been the cryopreservation, followed by maintenance of samples under liquid nitrogen (LN2) or freezer. Nevertheless, extended frozen sample storage introduced multiple challenges due to the utilization of LN2. Several notable drawbacks include high maintenance cost, transportation limits and safety concern. In addition, sample maintenance in cryopreservation requires continuous monitoring and uninterrupted LN2 supply, putting it out of reach for many developing countries and small business where LN2 and/or power supply are unreliable. Moreover, if power outages or

disasters occur and sufficiently low temperatures are not maintained, storage cannot be continued.

Several incidents have underscored the need for robust safeguards in the storage of valuable biological samples, particularly when utilizing cryopreservation method. One notable occurrence was the Harvard Brain Tissue Resource Center Incident in 2012, during which both the primary freezer system and its backup mechanism, designed to detect freezer malfunctions, failed simultaneously. Tragically, this event resulted in the loss of more than 147 human brains that had been earmarked for research, as temperature fluctuations within the storage units caused the samples to thaw and degrade. In 2019, a Los Angeles hospital encountered a similar setback when a freezer malfunction led to the loss of 56 stem cell samples obtained from pediatric cancer patients. Recent reports have brought to light another incident in Troy, New York, where a laboratory freezer was inadvertently powered off by a janitor in response to repeated "annoying alarms." Regrettably, this action resulted in the destruction of two decades' worth of research. These incidents collectively emphasize the importance of implementing reliable backup systems to avert the potential loss of invaluable biological samples due to freezer failures. Such incidents underscore the need for a more secure and dependable alternative to current preservation methods. Freeze-drying has emerged as a promising alternative for sperm preservation, offering potential solutions to mitigate the shortcomings associated with conventional cryopreservation method.

CHAPTER 2

AN ATTEMPT AT IMPROVING MOUSE BLASTOCYST YIELD BY OPTIMIZING PH OF HEPES-CZB MEDIA FOR ICSI

Abstract

The pH of the culture medium utilized in biochemistry and biology research is commonly adjusted to 7.4. However, it is worth considering that pH 7.4 might not be optimal for oocytes due to variations in the pH of the reproductive organs in female mammals. In contrast to in vitro fertilization, ICSI involves artificially breaking the cell membrane, necessitating the identification of optimal conditions for this technique. Furthermore, it is important to note that the culture medium generally tends to become alkaline during storage. Therefore, in this study, we investigated the pH variation during storage of HEPES-CZB medium, which was adjusted within the range of 4.0 to 12.0. Subsequently, ICSI was performed using HEPES-CZB medium adjusted to each pH range, and its impact on the survival rate and blastocyst formation rate was evaluated. The results showed no significant difference in survival rate when the pH was increased from 7.4 to 7.5 and 7.6 (75%, 73%,71%, respectively). However, there was an improvement in blastocyst rate as the pH increased (45%, 63%, 80%, respectively). Notably, the highest blastocyst formation rate per oocyte used was observed at pH7.6. To assess the quality of the obtained ICSI embryos, immunostaining was employed to examine cell differentiation into the internal cell mass and trophic ectoderm. Interestingly, when pH 9.0 medium was used, no significant difference in blastocyst quality was observed. Collectively, these findings indicate that pH 7.6 represents the optimal pH for HEPES-CZB medium in the context of ICSI.

Introduction

The medium utilized outside the controlled incubator environment contains specific components that mitigate pH variations resulting from changes in CO2 concentration. While certain treatments like oil covering can mitigate pH increases, even brief periods

outside the incubator can lead to pH elevation in conventional media, thereby posing challenges for procedures like ICSI (Barnett et al. 1997).

In the past, phosphate-buffered saline (PBS) was incorporated into the media used outside the incubator, and some laboratories continued to use this medium for oocyte retrieval. However, despite PBS exhibiting sufficient buffering capacity, it lacks crucial components such as bicarbonate and metabolic substrates. In addition to this inappropriate property, high phosphate concentrations may interfere with intracellular homeostasis. Indeed, even brief exposure to PBS has been shown to inhibit hamster and rabbit embryonic development and has been reported to result in abnormal gene expression in bovine embryos (Escribá et al. 2001).

Hence, in order to select a more appropriate buffer, it is necessary to utilize low concentrations of bicarbonate in combination with an organic synthetic buffer to create a medium that maintains the pH within a desirable range (Ferguson et al. 1980; N. E. Good and Izawa 1972). In the pursuit of suitable biochemical buffers, Good et al. identified various synthetic organic buffers characterized by their amphoteric ionic structures (Norman E. Good et al. 1966). These buffers, commonly known as Good buffers, exhibit buffering capacity across the physiological range, approximately pH 6.1 to 8.3. Specifically, HEPES-CZB medium is utilized for ICSI procedures.

Nevertheless, similar to many other media, HEPES-CZB medium also tends to become alkaline over extended periods of storage. Although it is recommended to utilize the medium within two weeks of preparation, practical circumstances often require additional time to use up the medium.

The mouse preimplantation embryo has the totipotency to become an embryo and placenta that nourishes the embryo until the 8-cell stage embryo. As development progresses, the embryo changes from totipotency to pluripotency, ultimately forming a blastocyst with the trophoblast (TE) on the outer layer, which will contribute to placental formation, and the inner cell mass (ICM) on the inside, which consists of pluripotent cells that will give rise to the definitive structure of the fetus. According to the prevailing theory, the transcription factor Nanog is expressed in the inner cell mass, while Cdx2 is expressed in the trophoblast.

Given this background, the objective of this study was to determine the appropriate pH ranges of HEPES-CZB medium for ICSI and assess the impact of pH on ICSI

performance. In this experiment, the pH of HEPES-CZB medium was adjusted within the range of 4.0-12.0. Firstly, the stability of the pH was evaluated during an 18-day storage period to simulate a practical scenario (long-term refrigerated storage before use). Subsequently, ICSI was conducted under each pH condition, and the survival rate of oocytes, the development into blastocysts, and the quality of the obtained blastocysts were evaluated by the differentiation of cells into the ICM and TE using anti-Nanog and anti-Cdx2 antibodies. The aim was to identify the optimal pH value for favorable ICSI outcomes.

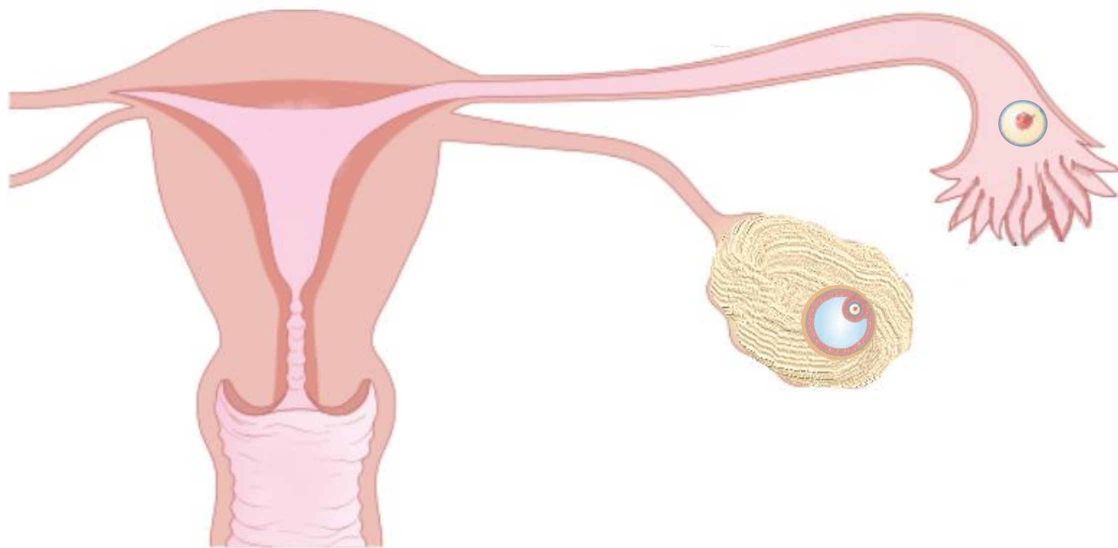


Figure 2.1. In mammalian fertilization, the oocyte is first released from the follicle, fertilized in the fallopian tube, and the fertilized egg migrates toward the uterus, finally implanting in the endometrium and growing.

Materials and methods

Animals

Female and male Institute of Cancer Research (ICR) mice aged 8 to 10 weeks were obtained from Japan SLC, Inc. (Shizuoka, Japan). Sperm was collected from the epididymides removed from male mice after sacrifice via cervical dislocation. The females were mated with vasectomized ICR males whose sterility had been previously demonstrated, then used as surrogates to receive embryos fertilized with FD sperm. The mice were euthanized via CO2 inhalation or cervical dislocation either on the day of the

experiment or upon completion of all experiments. All animal-based experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the Institutional Committee of Laboratory Animal Experimentation of the University of Yamanashi, which followed the ARRIVE guidelines (A4-10).

Media

Human tubal fluid (HTF) medium was used for capacitation and to freeze-dry the spermatozoa. CZB and HEPES-CZB media were used for oocyte/embryo incubation in 5% CO2 in air at 37°C and manipulation, respectively. Unless otherwise stated, all materials used were purchased from Sigma Aldrich (St Louis, MO, USA).

Table 2.1. Composition of HEPES-CZB medium

Reagent	mg/100 mL
NaCl [wako]	476.0
KCl [wako]	36.0
KH_2PO_4 [wako]	16.0
$MgSO_4$・$7H_2O$ [wako]	29.0
$NaHCO_3$ [wako]	42.0
EDTA・2Na [wako]	4.0
Phenolred [SIGMA-ALDRICH,Co]	Appropriate amount
Hepes・Na [SIGMA-ALDRICH,Co]	520.0
D-Glucose [NACALAI TESQUE]	100.0
Na-pyruvate [wako]	3.0
Na-lactate [SIGMA-ALDRICH, Co]	0.53 mL
Penicillin G [ICN Biomedicals Ins.]	5.0
L-Glutamine [SIGMA-ALDRICH,Co]	15.0
Streptomycin [ICN Biomedicals Ins.]	7.0
$CaCl_2$・$2H_2O$ [wako]	30.0
PVA [SIGMA-ALDRICH,Co]	10.0

Table 2.2. Composition of HTF medium (pH:7.4)

Reagent	**mg/100 mL**
(Stock solution)	
NaCl [wako]	581.7
KCl [wako]	35.0
KH_2PO_4 [wako]	5.0
$MgSO_4$・$7H_2O$ [wako]	5.0
$NaHCO_3$ [wako]	210.0
Phenolred [SIGMA-ALDRICH,Co]	Appropriate amount
(Energy source)	
D-Glucose [NACALAI TESQUE]	50.0
Na-puruvate [wako]	3.6
Na-lactate [SIGMA-ALDRICH,Co]	400 μL
Penicillin G [ICN Biomedicals Ins.]	10.0
Hypotaurine [SIGMA-ALDRICH,Co]	11.0
BSA [Millipore]	300.0
$CaCl_2$・$2H_2O$ [wako]	30.0

Table 2.3. Composition of CZB medium (pH:7.4)

Reagent	mg/100 mL
(Stock solution)	
NaCl [wako]	476.0
KCl [wako]	36.0
KH_2PO_4 [wako]	16.0
$MgSO_4$・$7H_2O$ [wako]	29.0
$NaHCO_3$ [wako]	211.0
EDTA・2Na [wako]	4.0
Phenolred [SIGMA-ALDRICH,Co]	Appropriate amount
(Energy source)	
D-Glucose [NACALAI TESQUE]	100.0
Na-puruvate [wako]	3.0
Na-lactate [SIGMA-ALDRICH,Co]	0.53 mL
L-Glutamine [SIGMA-ALDRICH,Co]	15.0
Penicillin G [ICN Biomedicals Ins.]	5.0
Streptomycin [ICN Biomedicals Ins.]	7.0
BSA [Millipore]	5 mg/mL
$CaCl_2$・$2H_2O$ [wako]	25.0

Adjustment of pH of HEPES-CZB medium

The stock solution was aseptically dispensed into 50 ml centrifuge tubes. Subsequently, 1N HCl or 1N NaOH solution was added to the tubes to adjust the pH within the desired range of 4.0 to 12.0. The pH adjustment process was monitored using a pH meter (LAQUAtwin pH-22B, HORIBA Advanced Techno, Japan). Once the medium was appropriately adjusted, it was stored at 4°C for further use (Figure 2.2).

Measurement of pH

The pH was measured using a compact pH meter (LAQUAtwin pH-22B, HORIBA Advanced Techno, Japan) according to the instruction manual.

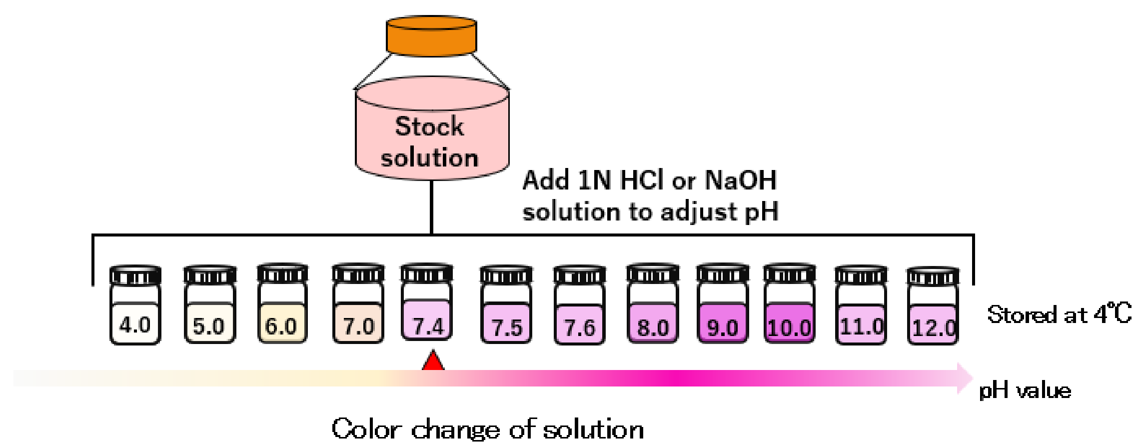


Figure 2.2. pH stability of Hepes-CZB medium during storage. From the same stock solution, the pH was adjusted to a range of 4 to 12 using hydrochloric acid or sodium hydroxide solution. The adjusted medium was stored at 4°C and pH fluctuations were measured for 18 days

Preparation of sperm

A 200 µl drop of HTF medium was dispensed onto a cell culture petri dish, covered with mineral oil. The dish was then equilibrated overnight in an incubator (37°C, 5% CO_2). Mature male mice (> three months old) were used for the experiment. After the male mouse were sacrificed by cervical dislocation, the cauda epididymides were collected. The fat and blood were wiped off with a laboratory wipe and the cauda epididymides were cut using scissors. The condensed sperm mass was then extruded using fingers and subsequently collected and deposited into the 200 µl of HTF medium with the assistance of tweezers. The sperm mass was pre-incubated for 30 minutes to 1 hour in an incubator (37°C, 5% CO_2).

Oocyte preparation

Female mice were superovulated via injection of 5 IU equine chorionic gonadotropin, followed by 5 IU human chorionic gonadotropin after 48 h. 14–16 hours after hCG injection, cumulus-oocyte complexes (COCs) were collected from the oviducts of the female mice and transferred to a Falcon dish containing HEPES-CZB medium. To disperse the cumulus, COCs were transferred to a 50 µl droplet of HEPES-CZB medium containing 0.1 % bovine testicular hyaluronidase for 3 min. Cumulus-free oocytes were washed twice and added to a 20 µl droplet of CZB for culture.

ICSI

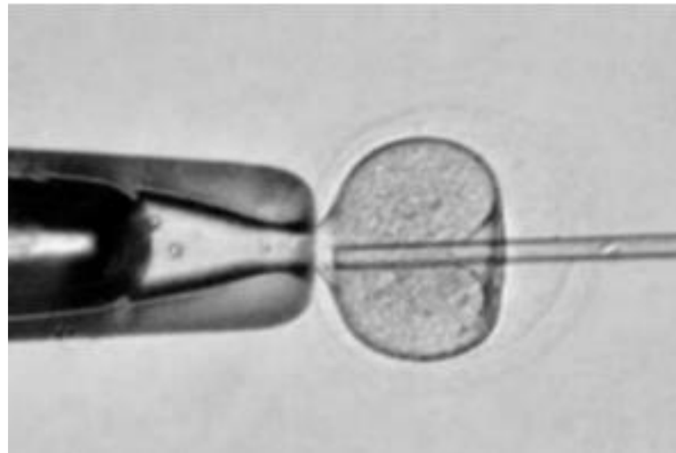


Figure 2.3. Intracytoplasmic sperm injection procedure in the mouse. Catching a motile spermatozoon by the injection pipette. Immobilization of the spermatozoon by several piezo pulses when the midpiece is touching to the tip of the injection pipette. Aspiration of immobilized sperm into the injection pipette and bringing it to the injection droplet containing the oocytes. Holding an oocyte with the first polar body located either at 6 or 12 o'clock and setting injection pipette at the center (Y and Z-axis) of the hold oocytes. Penetration of the zona pellucida by applying a few piezo pulses without any damage to plasma membrane. Removing of the zona core from injection pipette at the droplet. Re-positioning of the aspirated sperm to the tip of injection pipette. Pushing forward the injection pipette to the two thirds of the oocytes from right side and injecting the sperm with minimum amount of medium after applying a single piezo-pulse. Withdrawal of the injection pipette gently.

ICSI was performed as described previously (Figure 2.3). For the microinjection of the spermatozoa, 1-2 μl of the pre-incubated spermatozoa suspension was transferred directly to a droplet of 10% polyvinylpyrrolidone in the injection chamber. The sperm suspension was replaced every 30 min during ICSI. Several piezo pulses were applied to separate the sperm head from the tail, and the head was then injected into the oocyte. The oocytes that survived ICSI were incubated in CZB medium at 37 °C in 5% CO_2 in air. Pronucleus formation was verified 6 h after ICSI. The embryos were cultured for up to 4 days to evaluate their potential to develop into blastocysts.

Immunostaining

Blastocyst stage embryos obtained 120 hours after ICSI were fixed in a 4% paraformaldehyde solution for 20 minutes at room temperature. Subsequently, the embryos underwent overnight solubilization treatment at 4°C. On the following day, the fixed embryos were placed overnight in a 500-fold dilution of primary antibodies in blocking buffer. For this study, the primary antibodies used were anti-Nanog rabbit polyclonal antibody (Abcam) and anti-cdx2 antibody (BioGenex). The embryos were then transferred to a blocking buffer containing secondary antibodies diluted 500-fold, and incubated for 45 minutes at room temperature. Alexa Fluor 488-conjugated goat anti-mouse IgG (Molecular Probes) and Alexa Fluor 568-conjugated goat anti-rabbit IgG (Molecular Probes) were used as secondary antibodies. To visualize DNA, a solution of DAPI (Molecular Probes) at a concentration of 2 μg/ml was used for staining. (Figure 2.4)

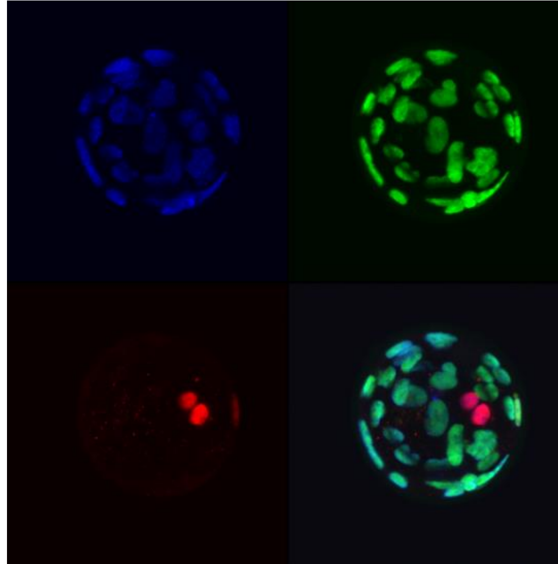


Figure 2.4. The quality of the resulting ICSI embryos was evaluated by cell differentiation into internal cell masses and trophic ectoderm using immunostaining techniques.

Statistical analysis

Data calculated as percentages were subjected to arcsine transformation in each replication before performing one-way ANOVA. The Tukey-Kramer test were used for multiple comparisons. Fertilization rates and *in vitro* development rates were analyzed using Mann-Whitney nonparametric *U* tests. Offspring rates were analyzed using Fisher's exact probability test (Prism, GraphPad Software, USA). Values of *P* less than 0.05 were considered to indicate statistical significance.

Results

Change in color of HEPES-CZB medium during pH adjustment

Phenol red exhibits a color change, turning red at approximately pH 7.0, yellow as the pH becomes more acidic, and reddish-purple as the pH becomes more alkaline. To monitor pH changes, phenol red was incorporated into the initial solution as a pH indicator. As the pH was modified, the color of the medium was observed to progressively darken to a reddish-purple within the range of 4.0 to 10.0. However, as the pH reached the range of 10.0 to 12.0, the color of medium became lighter (Figure 2.2).

Fluctuations in pH of HEPES-CZB medium stored at 4•C for 18 days

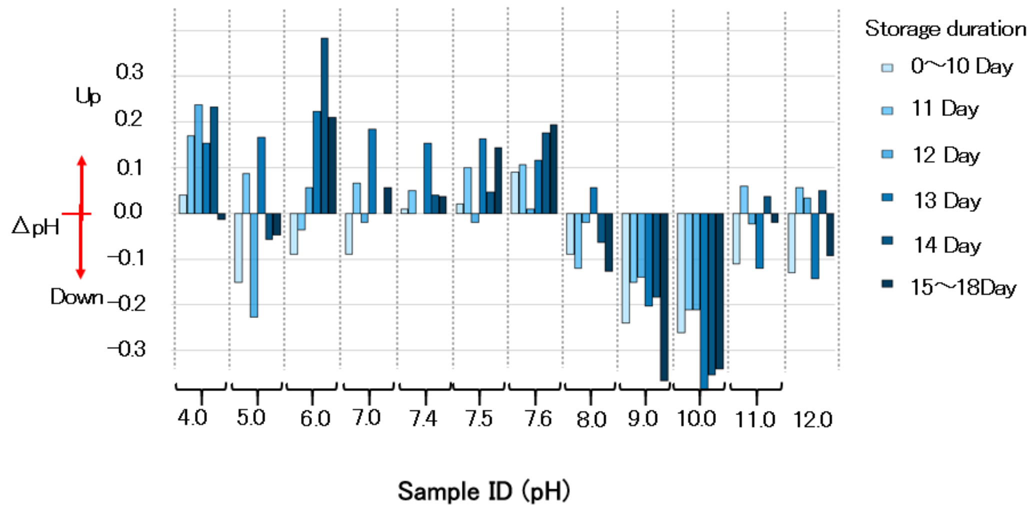


Figure 2.5. The HEPES-CZB medium adjusted to pH 7.0-8.0 and pH 11.0-12.0 exhibited a relatively small range of variation in pH during the storage period. In contrast, the medium that was adjusted to pH 4.0-6.0 and 9.0-10.0 showed a larger range of pH variation over the same storage period.

The HEPES-CZB medium adjusted to pH 7.0-8.0 and pH 11.0-12.0 exhibited a relatively small range of variation in pH during the storage period. In contrast, the medium that was adjusted to pH 4.0-6.0 and 9.0-10.0 showed a larger range of pH variation over the same storage period (pH 7.0-8.0: ±0.2, pH 11.0-12.0: ±0.2, pH 4.0-6.0: ±0.3, pH 9.0-10.0: ±0.3). (Figure 2.6; Table 2.4) These findings suggested that the pH stability of the HEPES-CZB medium differs depending on the pH range to which it was adjusted.

Table 2.4. Fluctuations in pH during storage of Hepes-CZB medium

Target pH	pH on the day of adjustment	ΔpH over the storage period (pH value on the day of measurement - pH value on the day of adjustment)						
		0 Day	10 Days	11Days	12Days	13Days	14Days	18Days
pH4	4.07	0.00	0.04	0.17	0.24	0.15	0.23	-0.01
pH5	5.07	0.00	-0.15	0.09	-0.23	0.17	-0.06	-0.05
pH6	6.05	0.00	-0.09	-0.04	0.06	0.22	0.38	0.21
pH7	6.99	0.00	-0.09	0.07	-0.02	0.18	0.00	0.06
pH7.4	7.40	0.00	0.01	0.05	0.00	0.15	0.04	0.04
pH7.5	7.52	0.00	0.02	0.10	-0.02	0.16	0.05	0.14
pH7.6	7.63	0.00	0.09	0.11	0.01	0.12	0.18	0.19
pH8	7.99	0.00	-0.09	-0.12	-0.02	0.06	-0.06	-0.13
pH9	9.00	0.00	-0.24	-0.15	-0.14	-0.20	-0.18	-0.37
pH10	10.01	0.00	-0.26	-0.21	-0.21	-0.39	-0.35	-0.34
pH11	11.04	0.00	-0.11	0.06	-0.02	-0.12	0.04	-0.02
pH12	12.00	0.00	-0.13	0.06	0.03	-0.14	0.05	-0.09

Detection of effect of pH of HEPES-CZB medium on ICSI performance

When using the HEPES-CZB medium at various pH levels, the survival rate of oocytes following ICSI exhibited differences among experimental groups. Specifically, the survival rate was significantly lower in groups at pH 6.0 or lower or 10.0 or higher compared to pH 7.4 (35% vs. 75% and 13% vs. 75%, respectively) (Table 2.5). Furthermore, the blastocyst rates, considering both fertilized and used eggs, were significantly reduced in the experimental groups with pH below 6.0 or above 10.0 compared to pH 7.4. Notably, at pH levels of 7.4, 7.5 and 7.6, no differences were observed in survival rates (75%, 73% and 71%, respectively), but there was a noticeable trend towards improved blastocyst rates (45%, 63% and 80%, respectively) (Figure 2.9). As the pH level increased beyond 7.6, there was a noticeable trend towards a decrease in both the survival and blastocyst rates. Notably, when the pH reached 10.0, a small number of oocytes managed to survive after ICSI, but failed to develop into blastocysts (Figure 2.6; Figure 2.7; Table 2.5). It is worth mentioning that mouse oocytes exposed to extreme pH environments were observed to undergo cell death before the ICSI procedure could be performed (Figure 2.8). Under highly acidic conditions (pH 4.0 and 5.0), the oocyte cytoplasm exhibited aggregation, turned black, along with the disappearance of zona pellucida. On the other hand, under highly alkaline conditions (pH 11.0 and 12.0), the oocyte membrane and zona pellucida underwent dissolution.

Table 2.5. Oocyte survival rate and blastocyst rate after ICSI using Hepes-CZB medium at various pH

Sample pH	No. of oocytes injected	Survival(%)	Blast	Blast (/2PN%)	Blast (/No.of oocytes injected%)
4	28	0 (0%)	0	0%	0%
5	28	0 (0%)	0	0%	0%
6	43	15 (35%)	2	13%	5%
7	55	36 (65%)	15	42%	27%
7.4	121	91 (75%)	41	45%	34%
7.5	26	19 (73%)	12	63%	46%
7.6	28	20 (71%)	16	80%	57%
8	41	24 (59%)	19	79%	46%
9	38	18 (47%)	14	78%	37%
10	32	4 (13%)	0	0%	0%
11	32	0 (0%)	0	0%	0%
12	30	0 (0%)	0	0%	0%

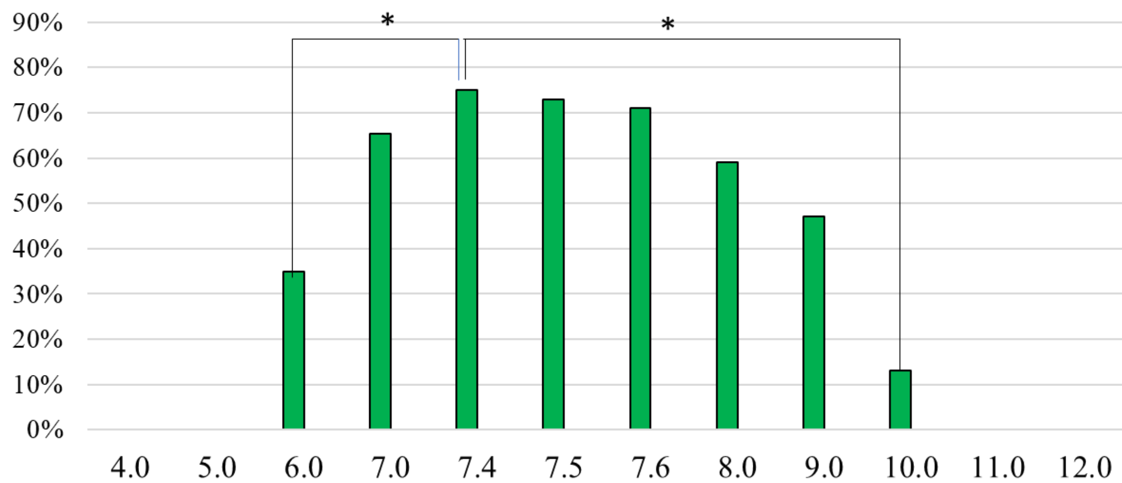


Figure 2.6. Survival rate of oocytes after ICSI using HEPES-CZB medium for each pH level. ICSI was performed using HEPES-CZB medium adjusted to each pH. Asterisks indicate significant differences between experimental and control group (pH 7.4) ($P<0.05$).

Detection of effect of pH of HEPES-CZB medium on blastocyst quality by immunostaining

At pH7.0 and 8.0, there was no significant difference in the number of cells expressing Nanog and cdx2 in the obtained blastocysts. However, a slight trend was observed towards higher numbers of ICM and TE at pH 8.0. On the other hand, at pH 9.0, there was a tendency for a decrease in the number of cells expressing Nanog and cdx2. (Figure 2.10)

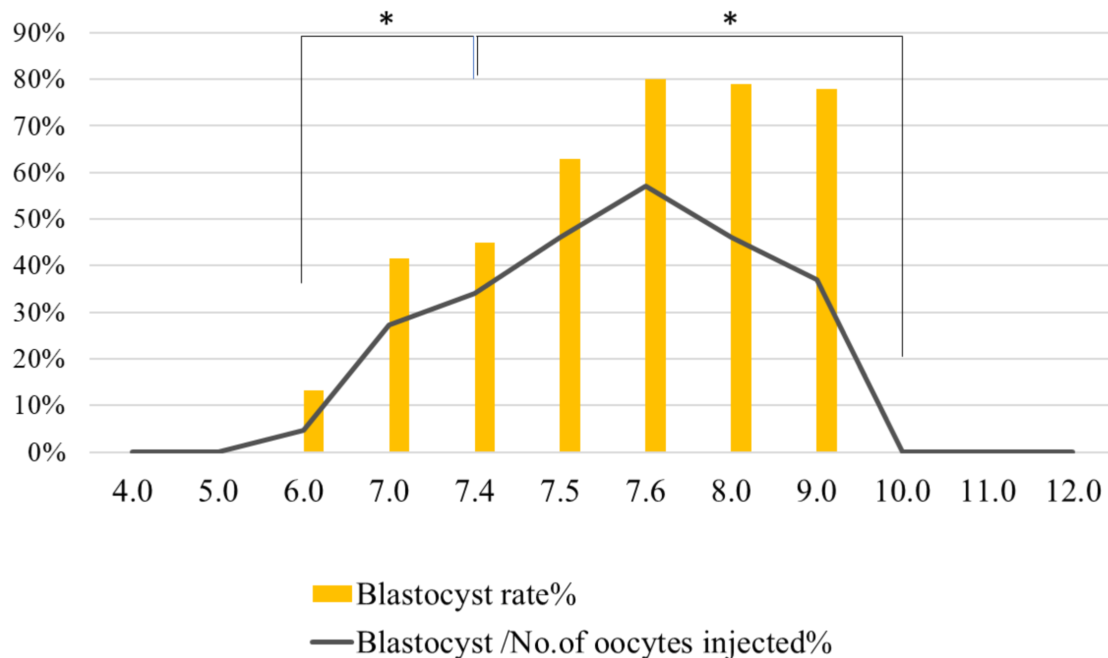


Figure 2.7. Blastocyst rate of ICSI embryos using HEPES-CZB medium for each pH level. ICSI was performed using HEPES-CZB medium adjusted to each pH. Asterisks indicate significant differences between experimental and control group (pH 7.4) ($P<0.05$).

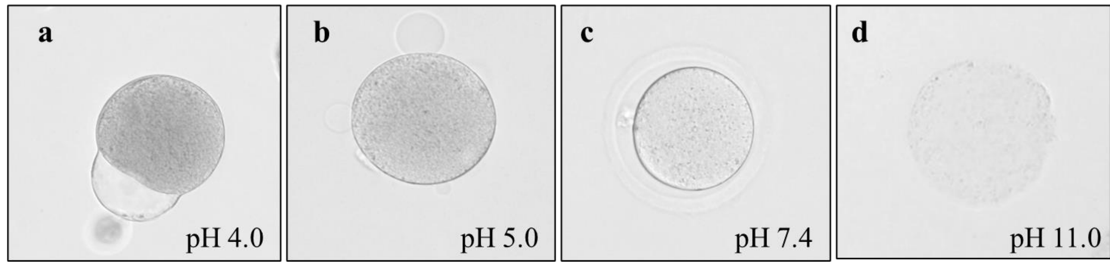


Figure 2.8. Mouse oocytes in HEPES-CZB nedium under extreme pH conditions. After being placed in a HEPES-CZB drop for 1 minute, oocytes displayed normal morphology at pH 7.4 (c). At highly acidic pH levels (pH 4.0 and 5.0), oocytes underwent agglutination, turned black and lost zona pellucida (b, c). Under strong basic conditions (pH 11.0 and 12.0), the oolemma disappeared together with the zona pellucida (d).

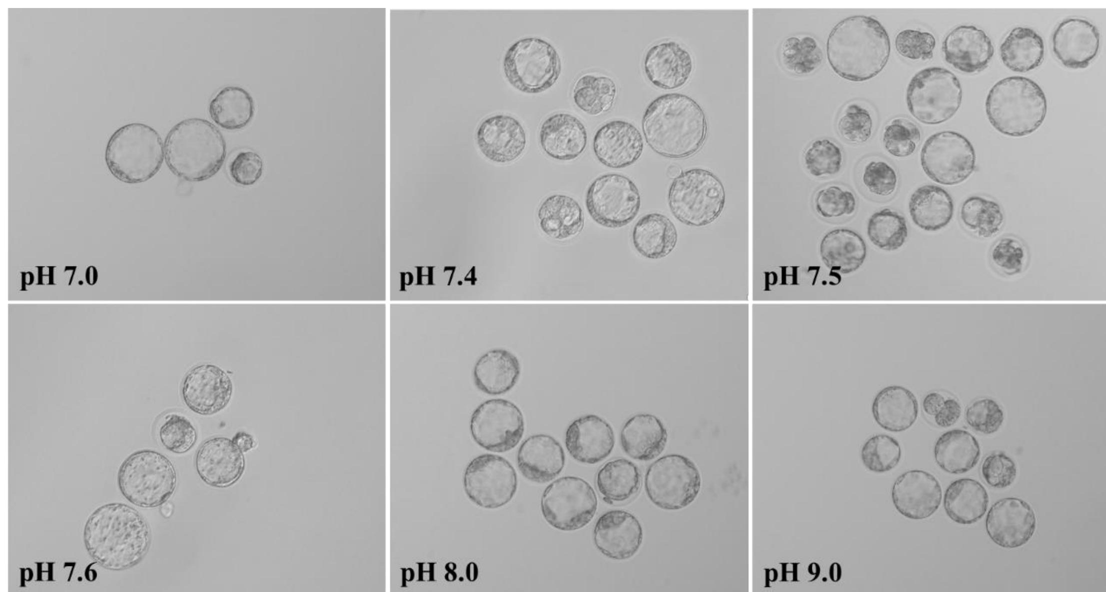


Figure 2.9. Blastocysts from HEPES-CZB medium of different pH level. ICSI was performed using HEPES-CZB medium adjusted to each pH level. Blastocysts obtained using HEPES-CZB medium adjusted to pH 7.0, 7.4, 7.5, 7.6, 8.0 and 9.0 after ICSI.

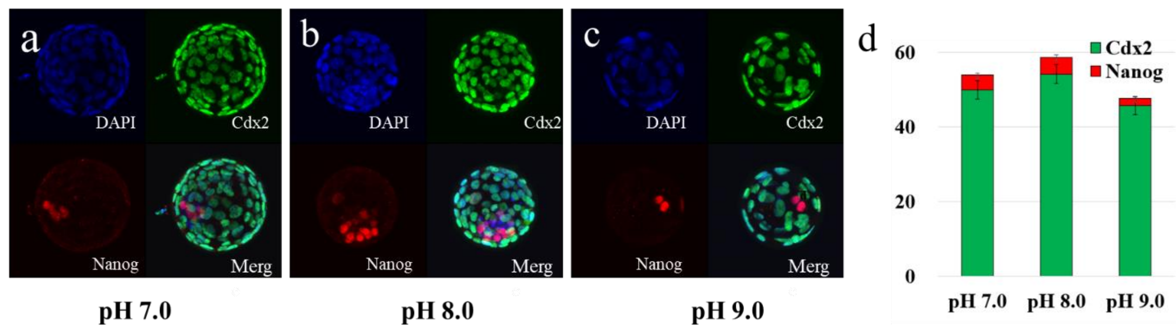


Figure 2.10. Detection of cell differentiation into inner cell mass and trophectoderm in blastocysts by Nanog and cdx2 immunostaining. Blastocysts at the 120-hour post-ICSI stage were subjected to immunostaining using antibodies against Nanog and cdx2. The images captured the entire blastocyst stained with DAPI, in inner cell mass exhibiting red fluorescence due to Nanog antibody labeling, and the trophectoderm labeled with green fluorescence. Merged images were generated to visualize the cell differentiation. There was no significant difference in the number of Nanog- and cdx2-expressing cells among the obtained blastocysts at pH 7.0 , 8.0 and 9.0 (a-d).

Discussion

It is known that the majority of culture media typically possess a composition that maintains neutrality when exposed to 5% CO2 conditions. However, as the CO2 concentration decreases during the incubation process, the media tends to become alkaline. Moreover, when the media is placed excessively outside the controlled incubator environment, it also exhibits an alkaline shift. In the present study, we made an intriguing observation regarding HEPES-CZB medium. Despite being stored in a sealed condition and adjusted to a pH range of 7.0-7.6, we observed an alkaline shift during the measurement period. This is likely due to CO2 deviation from the medium, resulting from contact with ambient air during the pH measurement procedure.

The pH range suitable for effective buffering of buffers is commonly defined as the compound's pKa ± 1. For HEPES, with a pKa of 7.55 at 20°C, it is considered suitable for preparing buffers within the pH range of 6.8-8.2. In this study, we made the observation that HEPES-CZB medium with pH ranging from 7.0 to 8.0 exhibited minimal fluctuations throughout the measurement period. On the other hand, the medium with pH ranging from 4.0 to 6.0 and 9.0 to 10.0 showed considerable fluctuations in pH. This suggests that the buffering effect of the medium is likely possible for the stability observed within the pH range of 7.0 to 8.0. Furthermore, it is recommended to use a HEPES concentration of 10-25 mM to ensure sufficient buffering capacity. Interestingly, in our experiment, the medium with pH 11.0-12.0 exhibited a distinct pattern of variation. It can be inferred that the addition of a large volume of NaOH solution to adjust the medium to the target pH caused dilution of HEPES, resulting in the loss of buffering capacity in this specific case.

Previous studies have reported that mammalian oocytes exhibit a similar initial response when exposed to extreme extracellular acidic and alkaline environments (Dale et al. 1998). In the current experiment, the immediate reactions of mouse oocytes to extreme pH conditions were found to be nearly equal, regardless of whether the pH was acidic (pH 4.0, 5.0) or alkaline (pH 11.0, 12.0).

It has been reported that in the reproductive tract of the mammalian female, oocytes encounter three distinct pH environments as they migrate from the ovary to the fallopian

tubes and to the uterus (Dale et al. 1998). In humans, the pH of the follicular fluid is typically around 7.2-7.3, while the fallopian tube fluid is more alkaline, ranging from 7.6 to 7.9. Comparatively, the uterus is more acidic than the fallopian tubes. In this experiment, the most favorable outcomes in terms of ICSI survival rate, blastocyst rate and blastocyst rate per used eggs were observed under pH 7.6, rather than pH 7.4. This finding suggests that performing ICSI under conditions that resemble the alkaline environment of the fallopian tubes might yield improved results.

In mammalian oocytes and preimplantation embryos, there is a deficiency in the ability to correct acidosis because protons can easily pass through the cell membrane. However, during development from the morula stage to blastocyst stage, these embryos acquire the ability to regulate protons. On the other hand, alkalosis, similar to other somatic cells, is regulated by HCO3-/Cl- transporters, which are responsible for regulating the increase in intracellular pH . In the current experiment, it was observed that the survival rate after ICSI at pH 8.0 and 9.0 was reduced. However, the fertilized eggs obtained from these conditions developed into blastocysts, and a high rate of blastocyst formation was achieved. On the other hand, at pH 6.0 and 7.0, the surviving oocytes showed a lower rate of development to blastocysts. It is speculated that under more alkaline conditions, the surviving oocytes exhibited a corrective function for alkalosis, which may explain the higher blastocyst formation rate observed after ICSI.

Previous studies have indicated that pH levels on the acidic or basic side can have an impact on the interaction between sperm and zona pellucida, leading to suboptimal IVF outcomes. In the current study, we hypothesize that an environment with a pH higher than the physiological range caused alterations in the structure of zona pellucida. This hypothesis is based on our observation of increased stickiness of the injection pipette when using pH 8.0 and more alkaline HEPES-CZB medium.

When ICSI was conducted under pH 9.0, the surviving oocytes were capable of developing into blastocysts. However, there was a tendency for a decrease in the total number of cells, as well as in the inner cell mass and trophectoderm. These findings indicate that although the fertilized oocytes managed to recover from the damage inflicted by the ICSI procedure, there are still noticeable effects of alkaline shock on embryonic development. It is possible that these embryos may exhibit variations in their ability to develop into viable offspring following implantation.

CHAPTER 3

A NOVEL, SIMPLIFIED METHOD TO PREPARE AND PRESERVE FREEZE-DRIED MOUSE SPERM IN PLASTIC MICROTUBES

Abstract

Although freeze-drying of the sperm can save space, reduce maintenance costs, and facilitate the transportation of genetic samples, the current freeze-dried (FD) method requires the use of breakable, custom-made and expensive glass ampoules. Here, we developed a simple, economical method for FD sperm using commercially available plastic microtubes. Mouse epididymal sperm suspensions were placed in 1.5 ml polypropylene tubes, frozen in liquid nitrogen and dried in an acrylic freeze-drying chamber, then the microtube caps were closed in a vacuum. The drying duration did not differ between the microtube method and glass ampoule method (control), but the sperm recovery rate was higher using the microtube method. The physical damage to the sperm after rehydration was also reduced. Intracytoplasmic sperm injection (ICSI) using freeze-dried sperm stored in microtubes at −30°C yielded healthy offspring without reducing the success rate, even after 9 months of storage. Air infiltration into all microtubes stored at room temperature (RT) within 2 weeks of storage caused a drastic decrease in fertilization rate for the FD sperm. Underwater storage did not prevent air infiltration. The RT storage of FD sperm in the microtubes for 1 week did result in healthy offspring after ICSI (5% to 18%), but the addition of silica gel or $CaCl_2$ did not improve the success rate. We propose that our novel microtube method is the simplest and most effective as currently available for FD sperm. This method contributes to the development of alternative, low-cost approaches to preserve, and transport genetic resources.

Introduction

The preservation of mammalian genetic resources is of great importance and is applied to a diverse range of scientific fields, including human medicine, farm animal production, laboratory animal record keeping, and wildlife conservation(Saragusty et al. 2020).

Preserving genetic resources in oocytes and embryos is unrealistic due to the difficulty in obtaining a sufficient number of oocytes and/or embryos from each individual, even when they are in good health. In contrast, spermatozoa are obtainable in large numbers from a single male animal. Thus, the genetic banking of spermatozoa provides an efficient and cost-effective approach for the preservation of genetic resources(Mochida et al. 2021; Anzalone et al. 2018).

Presently, spermatozoa are preserved almost exclusively via cryopreservation(Olaciregui and Gil 2017; Yánez-Ortiz et al. 2022). Long-term, frozen sperm storage is inconvenient because of the need for a steady supply of liquid nitrogen (LN2) and considerable storage space, as well as its inherent risk of cross contamination(Bielanski et al. 2003; Clarke 1999). The maintenance of frozen stock is also energy-dependent and requires continuous monitoring and an uninterrupted power supply, which makes biobanks susceptible to natural disasters and power outages. These characteristics make such biobanks unrealistic and often unobtainable in developing countries and small businesses where LN2 and/or the power supply are unreliable or impractical. LN2 is also dangerous because of its exceptionally low temperature and risk of oxygen deficiency. In addition, the transportation of cryopreserved sperm necessitates the use of a LN2 container or dry shipper that conforms to international standards, which is associated with additional disadvantages in associated labor and costs(Bielanski 2005).

Freeze-drying is an alternative method for sperm preservation that can overcome the disadvantages of cryopreservation. The freeze-drying process causes spermatozoa to lose their motility and viability but preserves their DNA and activation potential; therefore, FD spermatozoa injected into oocytes via intracytoplasmic sperm injection (ICSI) can successfully form viable embryos(Kusakabe et al. 2001; T. Wakayama and Yanagimachi 1998; Takehito Kaneko and Serikawa 2012). Live birth reports of viable offspring obtained from FD sperm have occurred in mice(Kusakabe et al. 2001), rabbits(Liu et al., 2004), rats(T. Kaneko, Kimura, and Nakagata 2007; Hirabayashi et al. 2005), and horses(Choi et al. 2011). In addition, sperm from livestock(Keskintepe and Eroglu 2015; Keskintepe et al. 2002), rare animal species (Takehito Kaneko et al. 2014)and humans (Gianaroli et al. 2012)have also been freeze-dried, as have spermatids (S. Wakayama, Ito, Ooga, et al. 2022) and somatic cells(S. Wakayama, Ito, Hayashi, et al. 2022). FD sperm can be stored and transported for short periods of time at RT without the use of LN2 or

dry ice as cooling agents (T. Wakayama and Yanagimachi 1998; Kamada et al. 2018; Ito et al. 2021). Furthermore, the nuclei of FD mouse sperm are highly resistant to extreme conditions, including frequent temperature changes, extremely high and low temperatures (S. Wakayama et al. 2019), and even space radiation, as evidenced by the long-term storage of FD mouse sperm on the International Space Station (S. Wakayama et al. 2017; 2021). Unfortunately, FD sperm results in a lower live birth success rate than fresh sperm when ICSI is used; however, this drawback is being minimized by improved drying methods (Ushigome et al., n.d.)and the addition of trehalose as a freeze-drying protectant (Ito et al. 2019).

The current freeze-drying method requires the use of breakable glass ampoules. The ampoules need to be able to resist low temperatures and vacuum treatments, which considerably increases their cost. Additionally, the glass ampoules require a heat-sealing process that carries the risk of explosion; thus, putting the experimenters in potential danger. Glass ampoule specifications (e.g., volume and inner diameter) also vary, which makes it harder to share results between laboratories. Attempts to place dehumidifying agents and/or oxygen absorbents into the glass ampoules to improve the live birth success rate have been unsuccessful due to the too-small opening of the ampoule bottles and the resulting inability to insert protective agents (Kamada et al. 2018).

Plastic microtubes, commonly referred to as microcentrifuge polypropylene tubes, could provide a potential solution to these drawbacks if they could be used to store FD sperm. Microtubes are easy to handle and are commercially available in specified sizes on a global scale. They have a wide entrance that enables the insertion of various protective agents into the microtube and can be closed by simply pushing down the cap, which eliminates the need for heat-sealing. Despite these potential benefits, no studies have evaluated the possibility of using microtubes for FD sperm. Here, we aimed to develop a method of preparing and preserving mouse FD sperm in microtubes to create a safer and more cost-effective method using commercially available 1.5 ml microtubes. This method would make it possible to safely manage a large number of mouse sperm using easily accessible, low-cost materials without any special skills.

Materials and methods

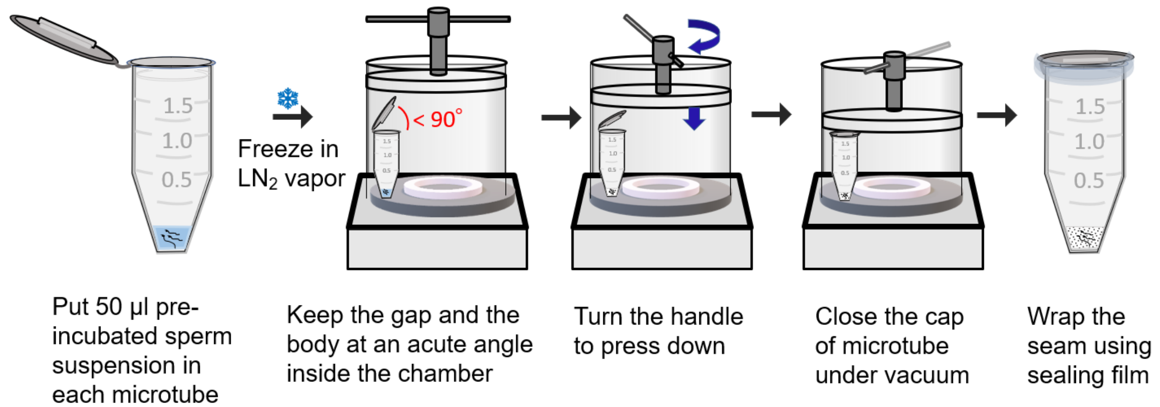
Animals

Female and male Institute of Cancer Research (ICR) mice aged 8 to 10 weeks were obtained from Japan SLC, Inc. (Shizuoka, Japan). Sperm was collected from the epididymides removed from male mice after sacrifice via cervical dislocation. The females were mated with vasectomized ICR males whose sterility had been previously demonstrated, then used as surrogates to receive embryos fertilized with FD sperm. The mice were euthanized via CO_2 inhalation or cervical dislocation either on the day of the experiment or upon completion of all experiments. All animal-based experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the Institutional Committee of Laboratory Animal Experimentation of the University of Yamanashi, which followed the ARRIVE guidelines (A4-10).

Media

Human tubal fluid (HTF) medium (Quinn et al. 1995) was used for capacitation and to freeze-dry the spermatozoa. CZB (Chatot et al. 1990) and HEPES-CZB (Kimura and Yanagimachi 1995) media were used for oocyte/embryo incubation in 5% CO_2 in air at 37°C and manipulation, respectively. Unless otherwise stated, all materials used were purchased from Sigma Aldrich (St Louis, MO, USA).

Preparation of FD sperm



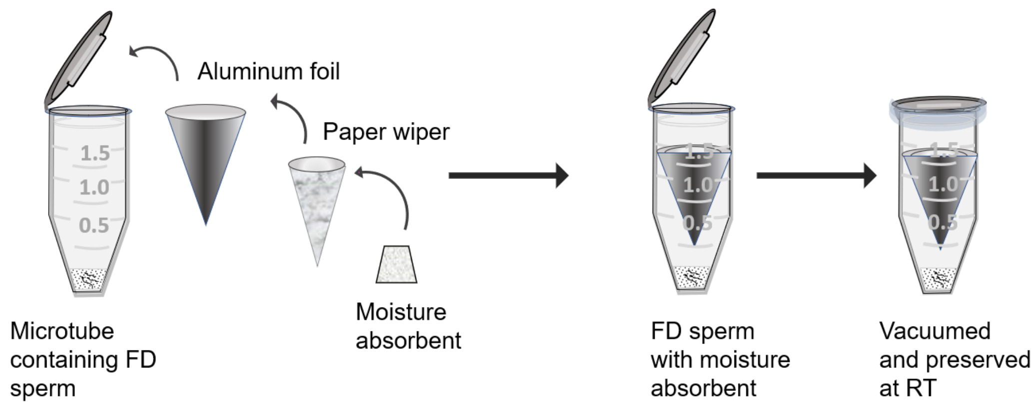


Figure 3.1. Schematic presentation of the freeze-drying protocol for sperm using the microtube method. (A) Sperm freeze-drying procedure. (B) Preservation of Freeze-dried sperm using moisture absorbents via the microtube method.

We preserved FD sperm using the standard glass ampoule method (S. Wakayama et al. 2019; Ushigome et al., n.d.) and 1.5 ml microtube (polypropylene, colorless) (Eppendorf Inc. Hamburg, Germany; microtube-FD method; Figure 3.1) method. To prepare the samples, semen was suspended in 1 mL HTF medium and incubated for 30 min at 37°C in 5% CO_2 in air. After sperm preculture, 50 μl aliquots of the sperm suspension were dispensed into glass ampoules or microtubes. In glass ampoule method, ampoules were flash-frozen in LN2 and freeze-dried using a FDU-2200 freeze dryer (EYELA, Tokyo, Japan), as described previously (Kamada et al. 2018). In microtube method, the microtubes were put in a microtube rack and frozen in nitrogen vapor above the surface of LN2 for 10 min, then dried in a vacuum dryer at −50°C (FreeZone2.5, Labconco, MO, USA). The pressure was maintained at 0.100 mBar throughout the entire drying process. After drying, the microtubes manually were sealed by closing the microtube cap. The sealing procedure was performed inside the acrylic chamber of the freeze drier so that vacuumed microtubes were obtained. The sperm-filled microtubes were removed from the chamber and subjected to a Tesla coil leak detector (Tesla lamp checker HF-20, Shinko Electric & Instrumentation, Osaka, Japan). Only those tubes determined to be highly-vacuumed were selected for further use (Fig. 2A). All samples were stored at −30°C or room temperature (RT; 15 to 25°C) until further use.

Assessment of drying duration

We compared the drying rates of frozen spermatozoa using three different methods: the standard method using glass ampoules and a manifold freeze dryer; the microtube method using 1.5 ml plastic microtubes and the acrylic FD chamber; or a method using glass ampoules and an acrylic FD chamber (Figure 3.1). The microtubes containing frozen sperm were dried for 3, 6, 9, 12, or 24 h before ICSI to determine the effects of drying time on the quality of FD sperm. The weight of the microtube with 50 μl fresh sperm solution was measured before and after the freezing treatment and measured every 3 h during the drying process up to 24 h. For each drying duration experiment, the samples were rehydrated immediately after the completion of the drying duration, and then used for ICSI or stored at −80°C for later use.

Effect of storage duration and temperature on fertilization

We examined the effect of storage duration by storing FD sperm at −30°C for 3 days, 1–2 months, and 9 months, with ICSI immediately after rehydration. We also studied the effect of storage temperature on the fertilization ability of microtube-FD spermatozoa by storing additional sperm samples at 4°C or RT for 1 or 2 weeks, respectively.

Counting of spermatozoa and measurement of head-tail separation rate

Glass ampoules or microtubes with 50 μl frozen sperm were attached to the FD apparatus (standard method) or put in an acrylic freeze-drying chamber (microtube-FD sperm method), respectively, and dried for 6–8 h, which is the routine drying duration for FD sperm used in previous research (S. Wakayama et al. 2019). Once drying was complete, the samples were rehydrated with distilled water, then 5 μl of the sperm suspension was transferred to a microtube containing 45 μl ultrapure water to obtain a dilution of 1:10. The samples were immediately analyzed to determine the retrieval rate of the sperm and the mechanical damage rate as judged by sperm without a tail (Figure 3.2). The spermatozoa were counted using a Haemocytometer (Burker-turk line, ERMA, Tokyo, Japan) counting chamber at a magnification of 100, with 80 out of 400 squares counted for each determination. The same counting chamber and cover glass were used throughout the investigation. The retrieval rate was measured by dividing the total number of sperm after rehydration by the total number of fresh sperm prior to FD. The head-tail separation rate was measured by dividing the number of sperm without a tail by the total number of sperm.

Detection of trapped air in microtubes using a Tesla coil leak detector

Microtubes containing air were identified using a Tesla coil leak detector as per the manufacturer's instructions. In brief, microtubes were exposed in the dark to the Tesla detector. Microtubes containing only a small amount of residual air will become bright, due to the ionization of the low-pressure gas inside the microtube when the Tesla coil tip was brought close to the microtube. Such microtubes were considered positive and vacuumed. When a significant amount of air is trapped inside the microtube, it cannot be ionized. Such microtubes remained dark and were considered Tesla negative and non-

vacuumed. The vacuumed microtubes were stored for 2, 3, or 4 weeks under water or in a desk drawer at RT, then the amount of air trapped in the microtubes was measured.

Measurement of air trapped in microtubes stored at RT

To measure the amount of air trapped in the microtubes, each microtube was opened under water. The bubble of air released from the microtube was captured using a 1 ml syringe and the volume was measured. Unless otherwise stated, RT storage refers to specimens kept in a drawer. Some specimens kept in water at RT were placed in water-filled bags from which the air was expelled until no air bubbles were visible to the naked eye.

Absorption of residual moisture in microtubes

We sought to examine whether the quality of microtube-FD sperm could be improved by adding silica gel or calcium chloride ($CaCl_2$) to the microtubes to absorb moisture. A simple device was designed to prevent the desiccant from coming into direct contact with the FD sperm. Briefly, aluminum foil was folded into a cone and embedded with a paper wipe to cover its inner wall and form a 2-layer, cone-shaped container (Figure 3.1). Silica gel or calcium chloride was added to this container and inserted into the microtube just before the end of the FD process. The microtubes were then vacuumed for at least two hours and sealed. For the control experiment, FD sperm were prepared as previously described using microtubes without any additional devices or desiccant. The microtubes were stored at RT for 1 week or 2 weeks, the fertilization rate was determined and 2-cell embryos were transferred into the recipient females to further determine whether the increase in the rate of fertilized oocytes and 2-cell embryos by moisture absorbent treatment was associated with an increased rate of offspring production.

Oocyte preparation

Female mice were superovulated via injection of 5 IU equine chorionic gonadotropin, followed by 5 IU human chorionic gonadotropin after 48 h. 14–16 hours after hCG injection, cumulus-oocyte complexes (COCs) were collected from the oviducts of the female mice and transferred to a Falcon dish containing HEPES-CZB medium. To disperse the cumulus, COCs were transferred to a 50 µl droplet of HEPES-CZB medium

containing 0.1 % bovine testicular hyaluronidase for 3 min. Cumulus-free oocytes were washed twice and added to a 20 µl droplet of CZB for culture.

ICSI and embryo transfer

ICSI was performed as described previously (Kimura and Yanagimachi 1995). Immediately before ICSI, the microtube caps were opened and 50 µl sterile distilled water was added, and then the solution was mixed using a pipette. For the microinjection of the spermatozoa, 1-2 µl of the spermatozoa suspension was transferred directly to a droplet of 10% polyvinylpyrrolidone in the injection chamber. The sperm suspension was replaced every 30 min during ICSI (S. Wakayama et al. 2019; Ushigome et al., n.d.). Several piezo pulses were applied to separate the sperm head from the tail, and the head was then injected into the oocyte. The oocytes that survived ICSI were incubated in CZB medium at 37 °C in 5% CO_2 in air. Pronucleus formation was verified 6 h after ICSI. Embryos at the two-cell stage were transferred to day-0.5 pseudo-pregnant ICR female mice that had been mated with a vasectomized male the night before the transfer (Hayashi et al. 2022) . Five to fifteen embryos were transferred into each oviduct. On day 18.5 of gestation, the offspring were delivered via cesarean section and allowed to mature. The remaining unused embryos were cultured for up to 4 days to evaluate their potential to develop into blastocysts.

Statistical analysis

Data calculated as percentages were subjected to arcsine transformation before performing one-way ANOVA. The Tukey-Kramer test were used for multiple comparisons. Fertilization rates and *in vitro* development rates were analyzed using Mann-Whitney nonparametric *U* tests. Offspring rates were analyzed using Fisher's exact probability test (Prism, GraphPad Software, USA). Values of *P* less than 0.05 were considered to indicate statistical significance.

Results

Drying rates of spermatozoa using microtubes

The standard method glass ampoules reached a stable state after 3 h of drying (Figure 3.2). The acrylic FD chamber resulted in a stable weight after 3 h, regardless of whether microtubes, or glass ampoules were used.

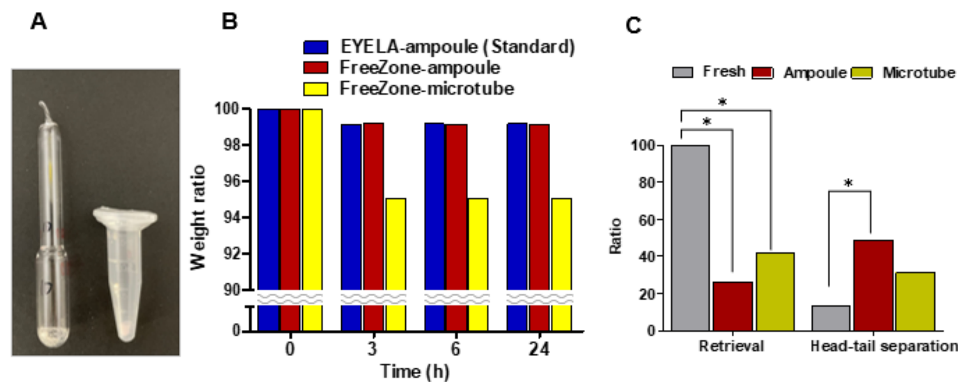


Figure 3.2. Sample drying time and retrieval rates of freeze-dried sperm. (A) Freeze-dried (FD) sperm preserved in glass ampoules (left) or microtubes (right). (B) Sample drying time. The horizontal axis indicates the drying time and the vertical axis shows the weight ratio of a container filled with 50 µl of frozen sperm suspension at various times during the FD process compared to the initial weight before vacuum drying (n=10 replicates/samples per each group). (C) Retrieval rates and head-tail separation rates of FD sperm using the conventional ampoule method and the microtube method (n=5 replicates/samples per each group). The asterisks indicate significant differences between groups ($P < 0.05$).

Retrieval rate and mechanical damage of spermatozoa after FD using microtubes

When FD sperm were stored in glass ampoules, the retrieval rate of spermatozoa was lower than the initial concentration, and the head-tail separation rate, which suggests the physical damage to the spermatozoa increased significantly (P <0.05, respectively). In contrast, when FD sperm were stored in microtubes, the retrieval rate and the head-tail separation rate of spermatozoa were improved compared to the glass ampoules, although the differences were not significant.

Drying duration optimal for the microtube-FD sperm method

We sought to optimize the drying duration for the microtube-FD sperm method by assessing whether the duration of drying could have influenced the development capabilities of the sperm after ICSI. Differences in drying duration did not result in significantly different rates of development to the blastocyst stage; however, more desirable results were obtained when the sperm was dried for 6, 9, or 12 h (56%, 60%, and 58%, respectively; Table 3.1). Based on these results, we decided that 6 to 12 h was the ideal drying duration to enable better and easier preparation of the microtube-FD sperm.

Integrity of FD sperm stored for long-term in microtubes at −30°C, 4°C or RT

High fertilization rates of 92%, 99%, and 85% were obtained for FD sperm stored in microtubes for 3 days, 1–2 months, and 9 months, respectively. The 2-cell embryos obtained from each group had comparable rates of 2-cell stage embryos and births, even when microtube-FD sperm were stored at −30°C for 9 months (29%; Table 3.2). The reproductive potential of randomly selected offspring derived from microtube-FD sperm stored for 1–2 months was examined after they grew to adulthood to examine whether they had normal reproductive potential. All examined offspring (male n=4, female n=9) demonstrated normal reproduction potential and delivered the next generation 3 to 4 months later. Thus, FD spermatozoa prepared with microtubes and stored at −30°C for 9 months had adequate fertilization ability to produce offspring at practical efficiencies for preserving genetic strains.

The mean fertilization rates of FD sperm stored for 1 week at 4°C or RT were 92% and 93%, respectively (Figure 3.3). Most of the fertilized oocytes developed to the 2-cell stage, with 51% and 39% of zygotes developing into morulae and blastocysts, respectively (Figure 3.3). When FD sperm were stored for 2 weeks, the fertilization rates decreased significantly for sperm stored at RT compared to those stored at 4°C, with only 19 % of the zygotes developing into morulae and blastocysts from the RT-stored FD sperm.

Exploration of the cause of impaired development in microtube-FD sperm under RT storage

We quantified the amount of air inside the microtubes after RT storage. The vacuumed tubes contained around 50 µl of air prior to storage (Figure 3.3). After 2 weeks of storage at RT, the amount of air in the microtubes increased to 420 µl when stored under water and 560 µl when stored in a desk drawer (Figure 3.3). In both instances, negative results were confirmed when subjected to the Tesla detector. Weight increases were also confirmed after storage.

Effect of moisture absorbent on RT-preserved microtube-FD sperm on full -term development

When microtube-FD sperm were stored for 2 weeks at RT, the control spermatozoa did not dissolve upon rehydration and compact aggregates were observed. Thus, only a few spermatozoa were collected and used for ICSI, and the mean fertilization rate and 2-cell rate was low. However, spermatozoa with normal morphology could be easily retrieved when silica gel or $CaCl_2$ was inserted into the microtubes. The 2-cell rates significantly increased in FD sperm stored with $CaCl_2$ compared to the control. When the embryos were cultured for 4 days after ICSI, only a few embryos developed into blastocysts, regardless of which moisture absorbent was used.

The mean birth rate from the control sperm stored for 3 days at RT was 18 % and only 5% when stored for 1 week. Contrary to our prediction, a lower birth rate of offspring (1% or 2%) was noted in both moisture absorbent groups (Table 3.3). When FD sperm were stored for 2 weeks at RT, no offspring were obtained, regardless of which moisture absorbent was used.

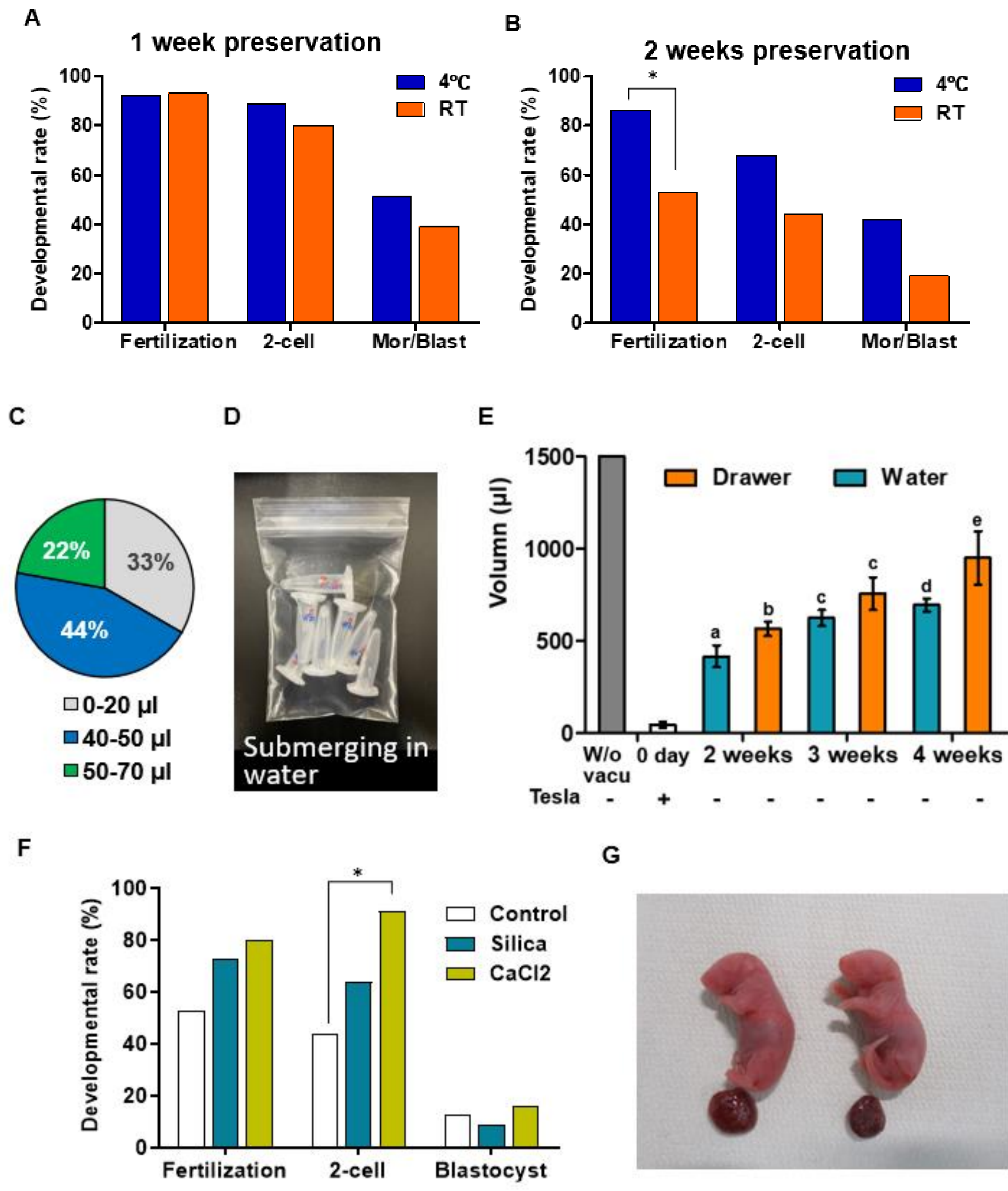


Figure 3.3. Developmental potential of embryos fertilized with FD sperm preserved at RT in microtubes. (Refer to the next page for a detailed legend)

In vitro development of embryos derived from freeze-dried (FD) sperm preserved at 4°C or room temperature (RT) for 1 week (4°C, n=193; RT, n=177) (A) or 2 weeks (4°C, n=78; RT, n=92) (B). Mor/blast: morulae/blastocyst. (C) Relative proportions of air in the Tesla-positive vacuumed microtubes immediately after measurement (n=9). (D) Microtube-FD sperms were preserved in water. (E) Measurement of air trapped in microtubes containing FD sperm underwater and in a drawer at RT (n=5 replicates per group). Data are expressed as the mean ± standard deviation (SD). W/o vacu: Without vacuum. Significant differences are indicated by superscript letters ($P < 0.05$). Note: Only Tesla-positive microtubes were used for storage. (F) *In vitro* development of embryos derived from microtube-FD sperm with or without moisture absorbents stored for 2 weeks at RT (control, n=46; silica, n=59; $CaCl_2$, n=65). Asterisks indicate statistically significant differences compared to the standard microtube method without absorbent agents ($P < 0.05$). (G) Full-term development of offspring derived from microtube-FD sperm preserved at RT for 1 week.

Table 3.1. Effect of different drying time on fertilization rate and in vitro development of freeze-dried sperm by microtube method

Drying time (h)	No. of oocytes surviving after ICSI	No. (%) of fertilized embryos	No. (%) of embryos developed to			
			2-cell	4- 8-cell	Morula	Blastocyst
3	55	50 (91)	47 (94)	42 (84)	36 (72)	25 (50)
6	116	93 (80)	87 (94)	75 (81)	64 (69)	52 (56)
9	99	85 (86)	84 (99)	75 (88)	67 (79)	51 (60)
12	82	76 (93)	71 (93)	64 (84)	55 (72)	44 (58)
24	96	83 (86)	76 (92)	50 (60)	44 (53)	43 (52)

There were no significant differences in the fertilization or blastocyst rates between the groups by chi-squared test.

Table 3.2. Full-term development of embryos derived from freeze-dried sperm preserved at -30°C for up to 9-12 months by microtube method

Storage period	No. of oocytes surviving after ICSI	No. (%) of fertilized zygotes	No. (%) of 2cell embryos	No. of transferred embryos (No. of recipients)	No. (%) [min-max] of offspring*	Mean body weight(g)
3 day	50	46 (92)^{ab}	45 (98)	45 (4)	5 (11) [1-22]	2.07
1-2 mo	68	67 (99)^{a}	61 (91)	61 (5)	15 (25) [0-54]	1.82
9 mo	92	78 (85)^{b}	74 (95)	68 (6)	20 (29) [15-80]	1.73

*Offspring rate: number of offspring/transferred embryos.

Fertilization, two-cell rates were evaluated using chi-squared test. Offspring rates were evaluated using Fisher's exact test. Significant difference indicated by different superscript letters. (^{a,b} $P<0.01$)

Table 3.3. Full-term development of embryos derived from freeze-dried sperm preserved at RT for 1 week without any agent (Control), with silica gel or calcium chloride.

Moisture absorbent	No. of oocytes surviving after ICSI	No. (%) of fertilized zygotes	No. (%) of 2cell embryos	No. of transferred embryos (No. of recipients)	No. (%) of [min-max] offspring*	Mean body weight (g)
Non (3day)	44	41 (93)	34 (83)	33 (3)	6 (18)^{a} [17-20]	1.93
Non (1w)	203	184 (91)	168 (91)	168 (9)	8 (5)^{b} [0-14]	1.99
Silica gel	232	216 (93)	198 (92)	198 (11)	1 (1)^{bc} [0-5]	2.02
CaCl2	205	198 (97)	187 (94)	187 (12)	3 (2)^{c} [0-11]	2.15

*Offspring rate: number of offspring/transferred embryos.

There were no significant differences in fertilization and two-cell rates by chi-squared test. Significant difference in offspring rates using Fisher's exact test were indicated by different superscript letters. ($P<0.05$)

Discussion

Glass ampoules have been predominately used for FD sperm since healthy offspring were obtained from FD mouse spermatozoa about a quarter century ago. Despite the drawbacks of this method (e.g., high costs and risks) and the urgent need to overcome these issues, new methodologies, and substitutes for glass ampoules have rarely been reported. In a previous study, we successfully preserved FD sperm in a thin plastic sheet, which was cheaper, more resistant to glass breakage, and generally less bulky than the glass ampoule method (Ito et al. 2021; Ito and Wakayama 2021); however, the method has many challenges that still need to be addressed, such as its RT storage limitation of less than 3 days. In addition, this method requires the removal of air from the gap between the plastic sheets; thus, inserting additives without negating the space-saving features of this method is difficult. In the present study, we succeeded in preserving FD sperm in commercially available microtubes with an extended storage duration of 7 days at RT, which indicates great potential for international delivery. This new method is a simpler, more effective, and secure alternative for the preservation of genetic resources compared to the current plastic sheet-based method.

Although the weight of FD sperm decreased to a stable state after 3 h of vacuum treatment in this study, a higher blastocyst rate was found in the samples dried for up to 24 h. This result indicates that vacuum treatment and dehydration within the microtube does not attenuate the developmental ability of embryos fertilized with FD sperm. The dry state is essential for the long-term storage of FD sperm at RT, but we were unable to determine the effect of drying duration on the sperm. Similarly, precise analytical methods have not yet been used to quantify the amount of residual humidity in FD sperm. In this study, air accumulated in all microtubes stored at RT in a desk drawer. Less air accumulated in the microtubes when they were stored in water, but it was not possible to stop the air accumulation completely. This finding may be due to the ability of gases (e.g., O_2 and water vapor) to penetrate the materials that form the microtube, since this is an

intrinsic characteristic of propylene (Siracusa et al. 2012). Submerging in water may slow down the rate of gas penetration, but it does not provide an effective barrier. Wrapping microtubes with gas-impermeable metalized films may help maintain a vacuum state and preserve the quality of sperm.

Degradation of sperm chromosomes is related to membrane damage (Palenno et al. 1996; Kuretake et al. 1996; Tateno, Kimura, and Yanagimachi 2000), and the membranes of sperm heads have been shown to be severely damaged by freeze-drying treatment (T. Wakayama and Yanagimachi 1998). In addition, it is known that the separation of the sperm tail from the head is commonly found in FD sperm (Takehito Kaneko, Whittingham, and Yanagimachi 2003), thus, separation rate of the sperm tail from head would be a clear indicator of the extent of damage caused by freeze-drying treatment. Despite this finding, the embryos fertilized with microtube FD sperm that came into contact with air in the microtubes after vacuum treatment had a poorer development potential to the blastocyst stage compared to the embryos fertilized with FD sperm from the vacuumed glass ampoules. This finding indicates that the penetration of air into the microtubes lowered the quality of the FD sperm when stored for a short period at RT.

The developmental rates of FD sperm to the 2-cell stage significantly increased when moisture absorbent agents were included in the microtubes; however, this effect was limited and development to the blastocyst stage decreased slightly (Fig. 3F). In fact, no offspring were obtained from embryos fertilized with these sperm (Table 3). It is very likely that both FD sperm and moisture absorbent agents exhibited hygroscopic behavior in the system; thus, competing randomly to absorb any moisture that entered the microtube. If the FD sperm absorbed the moisture first, the moisture absorbent agents would have reacted by absorbing the water from the FD sperm. This would result in the FD sperm repeatedly undergoing hydration and dehydration, which would increase sperm damage with each alteration in moisture. Another possible explanation for this phenomenon is that the moisture absorbent agents dehydrated the sperm long-term, thus absorbing the water bound to the FD sperm. Since sperm need to maintain a fraction of bound water that is covalently bonded to its various biological macromolecules (e.g., proteins, lipids, and nucleic acids (May et al. 1992; Privalov and Crane-Robinson 2017)) to develop successfully, removing these water molecules would be detrimental to development. This is especially true for DNA, which needs water molecules to support

its characteristic double-helix form and the proper decoding of its instructions (Auffinger and Westhof 2001).

Lyophilized proteins and DNA are liable to moisture-induced, solid-state aggregation during storage (Sharma and Klibanov 2007; Klibanov and Schefiliti 2004). During this process, these molecules undergo structural changes in the dry state due to their hydrogen-bonded cross-links that are dependent on their level of hydration, which is controlled by the relative humidity in the environment. Furthermore, the denaturing process of proteins and DNA is accompanied by a loss of aqueous solubility. In this study, FD sperm stored at RT for 2 weeks formed insoluble aggregates upon rehydration. Adding moisture absorbent agents to the microtubes seemed to prevent this aggregation, but the possibility that the aggregation continues to occur at the molecular level cannot be ruled out.

Plastic has a lower air resistance than other materials (e.g., glass or metals), which is the main cause for the limited storage duration of FD sperm at RT in this study. We showed that this drawback can be mitigated using a regular freezer, by successfully producing offspring from FD sperm stored for 9 months at −30°C at a rather high birth rate similar to that of glass ampoules. In addition, we also found that the microtubes could remain Tesla-positive longer when stored at −30°C than when stored at RT. Thus, microtubes are a practical and efficient substitute for glass ampoules when used to store FD sperm at −30°C.

In conclusion, we developed a protocol for FD sperm using conventional microtubes. Microtubes are easy to handle and provide the simplest, most effective method for FD mouse sperm compared to the currently used protocols. Additional experiments are needed to develop more suitable storage conditions for FD sperm, especially since we found a decline in the developmental ability of the FD sperm when stored at RT. Our study contributes to the development of alternative, low-cost approaches for the preservation and transportation of genetic resources.

However, although freeze-drying method can overcome the several disadvantages of cryopreservation, this method also has many disadvantages. For example, it requires freeze-drying equipment before preservation of spermatozoa, and the production of offspring is considered to require a certain cost because a series of microscopic manipulators as well as the training of technicians are required. Therefore, it would be best to choose the most convenient method or a combination of both, depending on

purpose of use, mouse strains used and environment of the places (countries) where they are used.

CHAPTER 4

PRESERVATION OF MOUSE SPERM IN "GLASS-STATE" AT ROOM TEMPERATURE

Abstract

Even though freeze-dried and dried sperm is not viable, it can be used for fertilization through intracytoplasmic sperm injection (ICSI) as long as its DNA remains intact. In this study, we investigated the preparation temperature and developmental potential of "glassified sperm", as well as methods to enhance their viability. Sperm were treated with 1M trehalose solution and subsequently dried on cover glass at various temperatures to assess "glass-state" status. The developmental potential and storage stability of the vitrified sperm under different preparation conditions were evaluated. Furthermore, strategies to increase sugar incorporation into sperm and alkaline treatment of the sperm membrane were explored to enhance developmental potential. Results demonstrated that naturally dried "glassified sperm" exhibited favorable morphology upon rehydration and were suitable for ICSI. Moreover, these glassified sperm, when stored at room temperature for 24 hours, were capable of developing into blastocysts, albeit at a relatively low rate. Membrane treatment with sodium hydroxide (NaOH) solution prior to drying improved the developmental performance of rehydrated sperm. Overall, this study highlights the potential of "glass-state" sperm as a cost-effective method for sperm preservation, offering promising prospects for future applications in diverse fields such as laboratory animals record keeping and wildlife conservation.

Introduction

Biobanking is a rapidly growing industry, covering diverse fields such as human medicine, farm animal production, laboratory animals record keeping, and wildlife conservation. The banking of spermatozoa alone would be an efficient and cost-effective approach for the storage of transgenic and mutant stocks.

Presently, biobanking is done almost exclusively by cryopreservation. With the cryopreservation methods, it is thought that sperm can be stored semi-permanently. However, the constant supply of LN2 and electricity lead to high costs, and in these conditions, sample handling becomes difficult because of the very low temperature and the risk of oxygen deficiency. Moreover, the samples may not be preserved continuously at a cold enough temperature when accidents or natural disasters occur.

In a recent incident at Rensselaer Polytechnic Institute located in Troy, New York, an unfortunate event occured where a lab freezer containing highly delicate cell cultures and samples was powered off by a janitor. This incident resulted in what has been described as "catastrophic damage", leading to the irreversible destruction of over two decades's worth of valuable research. In addition, the Harvard Brain Tissue Resource Center Incident was also a significant event in which a malfunction in the freezer system of the center resulted in the devastating loss of more than 150 human brains designated for research. The incident occurred due to a failure in the freezer system, causing a fluctuation in temperature that led to the unintended thawing and subsequent degradation of the valuable brain samples. These incidents highlight the importance of maintaining proper storage conditions and implementing robust backup systems to safeguard irreplaceable research materials to prevent the loss of valuable biological samples due to freezer or liquid nitrogen failures.

Freeze-dried sperm offers the advantage of room temperature storage, making it more resilient against natural disasters and power outages compared to traditional cryopreservation methods. Despite being stored in a vacuum container, the freeze-dried sperm still comes into contact with air, albeit in small quantities. By keeping sperm in a "glass-state", the air exposure is likely to be blocked to some extent. Preserving sperm in a "glass-state" would not only reduce costs significantly by eliminating the need for expensive freeze dryers but also allow for more space-efficient storage. Consequently, "glass-state" sperm is considered a promising option for serving as a backup for preserving valuable species and strains.

Many organisms in nature can tolerate severe desiccation. Trehalose is found to accumulate in high concentrations in a wide variety of organisms capable of surviving almost complete dehydration, including bacteria, yeast, tardigrades, and nematodes. Trehalose has been shown to protect membranes, proteins and nucleic acids during

freezing and desiccation. The mechanism of trehalose protection is an active area of research. Recent studies have used the non-reducing disaccharide trehalose to protect mammalian cells during drying. The major challenge in using trehalose for mammalian cells is to overcome the impermeability of the plasma membranes to the sugar and to introduce this disaccharide at a sufficiently high intracellular concentration. A research showed that trehalose can be loaded into RBCs through a combination of osmotic imbalance and the phospholipid phase transition. (Satpathy et al. 2004) RBCs can be loaded with trehalose from the extracellular medium through a combination of osmotic imbalance and the phospholipid phase transition. In the case of RBCs, using a trehalose concentration of 800 mM, the efficiency of uptake increased considerably with increasing concentrations of trehalose in the extracellular solution. In a temperature study involving incubations at 4, 23, 37, 40°C, uptake remained very low at 4°C while at 37°C the uptake concentration was higher.

In this study, we investigated the preparation temperature and developmental potential of "glassified sperm" using trehalose. In addition, strategies to increase sugar incorporation into sperm and alkaline treatment of the sperm membrane were explored to enhance developmental potential.

Materials and methods

Animals

Female and male Institute of Cancer Research (ICR) mice aged 8 to 10 weeks were obtained from Japan SLC, Inc. (Shizuoka, Japan). Sperm was collected from the epididymides removed from male mice after sacrifice via cervical dislocation. The females were mated with vasectomized ICR males whose sterility had been previously demonstrated, then used as surrogates to receive embryos fertilized with FD sperm. The mice were euthanized via CO_2 inhalation or cervical dislocation either on the day of the experiment or upon completion of all experiments. All animal-based experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the Institutional Committee of Laboratory Animal Experimentation of the University of Yamanashi, which followed the ARRIVE guidelines (A4-10).

Media

Human tubal fluid (HTF) medium (Quinn et al. 1995) was used for capacitation and to freeze-dry the spermatozoa. CZB (Chatot et al. 1990) and HEPES-CZB (Kimura and Yanagimachi 1995) media were used for oocyte/embryo incubation in 5% CO_2 in air at 37°C and manipulation, respectively. Unless otherwise stated, all materials used were purchased from Sigma Aldrich (St Louis, MO, USA).

Oocyte preparation

Female mice were superovulated via injection of 5 IU equine chorionic gonadotropin, followed by 5 IU human chorionic gonadotropin after 48 h. 14–16 hours after hCG injection, cumulus-oocyte complexes (COCs) were collected from the oviducts of the female mice and transferred to a Falcon dish containing HEPES-CZB medium. To disperse the cumulus, COCs were transferred to a 50 µl droplet of HEPES-CZB medium containing 0.1 % bovine testicular hyaluronidase for 3 min. Cumulus-free oocytes were washed twice and added to a 20 µl droplet of CZB for culture.

Preparation of sperm in "glass-state"

Disperse the sperm from one mouse in 500 µl of 1 M trehalose solution and incubate for 10 minutes at 37°C. Make droplets by putting the sperm solution onto a cover glass; make 3 droplets at 10 µl. Dry the droplets at room temperature, on a hot plate or in a microwave (Figure 4.2).

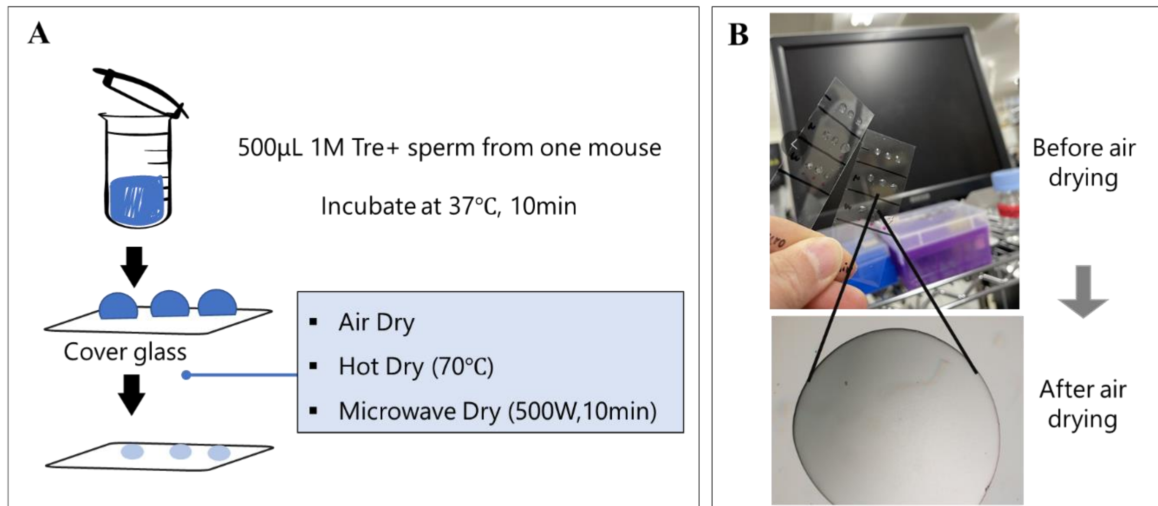


Figure 4.2. Preparation of "glass-state" sperm. (A) Glassified sperm preparation procedure. (B) Droplets of sperm before and after air drying.

ICSI

ICSI was performed as described previously (Kimura and Yanagimachi 1995). Immediately before ICSI, 3 drops of glassified sperm was rehydrated by adding 10 μl sterile distilled water, and then the solution was mixed using a pipette. For the microinjection of the spermatozoa, 1-2 μl of the spermatozoa suspension was transferred directly to a droplet of 10% polyvinylpyrrolidone in the injection chamber. The sperm suspension was replaced every 30 min during ICSI (S. Wakayama et al. 2019; Ushigome et al., n.d.). Several piezo pulses were applied to separate the sperm head from the tail, and the head was then injected into the oocyte. The oocytes that survived ICSI were incubated in CZB medium at 37 °C in 5% CO_2 in air. Pronucleus formation was verified 6 h after ICSI.

Statistical analysis

Data calculated as percentages were subjected to arcsine transformation in each replication before performing one-way ANOVA. The Tukey-Kramer test were used for multiple comparisons. Fertilization rates and *in vitro* development rates were analyzed using Mann-Whitney nonparametric *U* tests. Offspring rates were analyzed using Fisher's

exact probability test (Prism, GraphPad Software, USA). Values of P less than 0.05 were considered to indicate statistical significance.

Results

Effects of drying conditions on the appearance of droplets and sperm morphology after rehydration

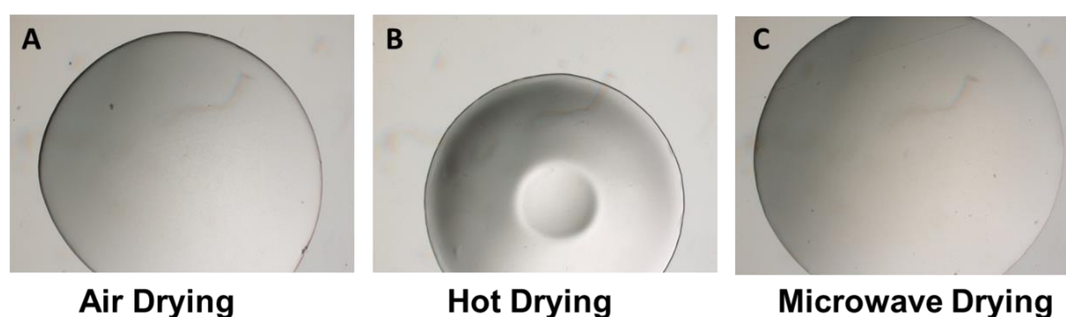


Figure 4.3. Droplets of sperm in a glass-like state after different drying methods. (A)Air drying. (B) Hot drying. (C) Microwave drying.

Table 4.1. A rough evaluation of the recovery rates of sperm in glass-like state under different drying conditions and the applicability of ICSI.

Drying Methods	Glass-like State	Recovery rates after rehydration	Ease of use in ICSI
Air Drying	+	++++	++++
Hot (70℃) Drying	+	++++	++++
Microwave Drying (500W, 10min)	+	++++	-

In terms of appearance, air drying, hot drying and microwave drying methods all resulted in the formation of droplets in a glass-like state (Figure 4.3). Sperm recovery is possible from all three groups after rehydration. However, microwave-dried sperm exhibited a distinctive feature of having a rigid or hardened tail, which made them unsuitable for utilization in ICSI.

Assessment of developmental ability of glass-like sperm under different drying conditions

Table 4.2. Assessment of developmental potential of sperm in glass-like state prepared under different drying conditions after RT storage for 24h by ICSI.

Group	No. of oocytes injected	Survival (%)	2PN (%)	2cell (%)	4-8cell (%)	Morula (%)	Blast (%)
Air Dry	21	15 (71)	11 (73)	11 (100)	3 (27)	3 (27)	2 (18)
70°C Hot Dry	25	16 (64)	10 (63)	8 (80)	0 (0)	0 (0)	0 (0)
Trehalose solution	20	14 (70)	13 (93)	7 (54)	2 (15)	1 (8)	1 (8)

Table 4.3. Assessment of developmental potential of sperm in glass-like state prepared under different drying conditions and different storage duration.

Storage	Group	No. of oocytes injected	Survival (%)	2PN (%)	2cell (%)	4-8cell (%)	Morula (%)	Blastocyst (%)
0h	Trehalose solution	28	11 (39)	7 (64)	6 (86)	2 (29)	2 (29)	1 (14)
0h	Air dry	31	16 (52)	11 (69)	10 (91)	2 (18)	2 (18)	2 (18)
RT 24h	Trehalose solution	20	14 (70)	13 (93)	7 (54)	2 (15)	1 (8)	1 (8)
RT 24h	Air Dry	21	15 (71)	11 (73)	11 (100)	3 (27)	3 (27)	2 (18)

After storage at RT for 24h, sperm preserved in air-dried state and in 1M trehalose solution preserved their developmental ability to some extent and yielded blastocysts. All embryos derived from sperm dried at 70°C exhibited developmental arrest at 2-cell stage. Therefore, the air-drying method was used for making glassified sperm.

In addition, sperm that were suspended in trehalose solution, irrespective if whether they were subjected to drying or not, displayed a low developmental rate immediately following the preparation process.

Exploring the cause of reduced developmental capacity of glassified sperm

Based on the observation that the developmental rated in the solution and air-drying groups were comparable after preparation procedure, we assumed that air drying did not contribute to the reduction in developmental ability. Instead, we hypothesized that the

treatment with trehalose solution was responsible for the observed decrease. To further validate this assumption, we conducted an additional experiment where the sperm were treated with a different sugar solution, sucrose solution, for a brief period of 5 mins at RT. All groups in this experiment remained in a solution state without drying. Similar to the glassified sperm, the sperm treated with the sugar solution exhibited a reduction in developmental rate, specifically from the 4-8 cell stage, confirming our previous assumption.

Table 4.4. Assessment of developmental potential of sperm without drying after treatment with different high concentrations of sugar solutions.

Storage	Group	No. of oocytes injected	Survival (%)	2PN (%)	2cell (%)	4-8cell (%)	Morula (%)	Blastocyst (%)
0 h	Ctr	22	13 (59)	10 (77)	10 (100)	8 (80)	7 (70)	7 (70)
0 h	Tre	20	16 (80)	15 (94)	10 (67)	4 (27)	3 (20)	3 (20)
0 h	Suc	20	16 (80)	15 (94)	9 (60)	4 (27)	4 (27)	4 (27)

Changes in developmental capacity of glassified sperm during RT storage

Storage for a duration of 24 hours at RT had minimal impact on the developmental ability of glassified sperm. However, when stored for a longer period of one month, a noticeable decrease in developmental rate from 4-8 cell stage was observed. These findings suggest that the duration of RT storage plays a crucial role in determining the viability and developmental capacity of glassified sperm.

Table 4.5. Developmental potential of air-dried glassified sperm during RT storage.

Storage	No. of oocytes injected	Survival (%)	2PN (%)	2cell (%)	4-8cell (%)	Morula (%)	Blastocyst (%)
RT, 0 h	31	16 (52)	11 (69)	10 (91)	2 (18)	2 (18)	2 (18)
RT, 24 h	21	15 (71)	11 (73)	11 (100)	3 (27)	3 (27)	2 (18)
RT, 1 month	71	41 (58)	31 (76)	28 (90)	2 (6)	0 (0)	0 (0)

Attempt to enhance the developmental ability of glassified sperm using 1M extracellular trehalose at 37℃

No improvement was observed with extended trehalose loading time.

Table 4.6. Attempt to improve the developmental ability of glassified sperm by extending trehalose loading time

Storage	Dry method	Trehalose loading time	No. of oocytes injected	Survival (%)	2PN (%)	2cell (%)	4-8cell (%)	Morula (%)	Blastocyst (%)
RT 24h	Air Dry	20min	21	15 (71)	11 (73)	11 (100)	3 (27)	3 (27)	2 (18)
		9h	23	16 (70)	12 (75)	7 (58)	2 (17)	2 (17)	0 (0)

Improved developmental ability of glassified sperm by NaOH treatment before air drying

Treatment with both 10 mM and 20 mM NaOH demonstrated a significant improvement in the rate of 4-8 cell stage embryos. Interestingly, regarding embryonic development to the Morula stage, the treatment with 10mM NaOH was found to be more effective compared to the 20mM concentration.

Table 4.7. Developmental ability of glassified sperm after NaOH treatment before air drying

NaOH Concentration	Storage	No. of oocytes injected	Survival (%)	2PN (%)	2cell (%)	4-8cell (%)	Morula (%)	Blastocyst (%)
0mM	RT, 24 h	21	15 (71)	11 (73)	11 (100)	3 (27)	3 (27)	2 (18)
10mM	RT, 24 h	28	26 (93)	15 (58)	15 (100)	14 (93)	11 (73)	4 (27)
	RT, 72 h	32	22 (69)	10 (45)	10 (100)	5 (50)	5 (50)	2 (20)
	-30°C,1w	61	47 (77)	32 (68)	29 (91)	18 (56)	13 (41)	10 (31)
20mM	RT, 24 h	26	23 (88)	17 (74)	15 (88)	13 (76)	12 (71)	7 (41)
	RT, 72 h	26	16 (62)	1 (6)	1 (100)	1 (100)	0 (0)	0 (0)
	-30°C, 1w	57	25 (44)	3 (12)	3 (100)	2 (67)	1 (33)	0 (0)

The effect of NaOH on glassified sperm prior to drying

Table 4.8. Developmental ability of sperm in sugar solution with or without 10mM NaOH treatment prior to drying

Group	No. of oocytes injected	Survival (%)	2PN (%)	2cell (%)	4-8cell (%)	Morula (%)	Blastocyst (%)
1M Tre	34	22 (65)	20 (91)	19 (95)	17 (85)	13 (65)	7 (35)
NaOH-1M Tre	32	24 (75)	19 (79)	19 (100)	13 (68)	10 (53)	8 (42)
1M Suc	16	11 (69)	11 (100)	10 (91)	6 (55)	6 (55)	5 (45)
NaOH-1M Suc	16	9 (56)	8 (89)	7 (88)	4 (50)	4 (50)	4 (50)

To determine at which stage NaOH treatment makes a difference, further investigations were conducted. The developmental ability of sperm in the sugar solution state with or without NaOH treatment prior to drying was evaluated. Results showed that sperm in the sugar solution state before drying revealed minimal differences in ICSI outcomes resulting from NaOH treatment. These findings suggest that NaOH treatment does not negatively impact the developmental ability of sperm in the sugar solution state prior to drying.

The observation of a higher developmental ability in NaOH-treated samples after transforming into a glassy state and storage implicated that the beneficial effects of NaOH treatment manifest during storage phase rather than the initial preparation stage. This suggests that NaOH treatment may contribute to the preparation and enhancement of sperm quality during storage, thereby improving the overall developmental ability of glassified samples.

Discussion

In our study, the treatment with high concentrations of sugar solutions exhibited adverse effect on the developmental potential of fresh sperm. This outcome aligns with previous research, which has established that abrupt fluctuations in osmotic pressure can disrupt the lipid membrane structure and membrane proteins of spermatozoa, thereby causing leakage of membrane ions, which leads to morphological abnormalities. (Holt and North 1986; Hezavehei et al. 2018) We previously demonstrated that when epididymides were placed directly into salt powder and rapidly dehydrated, spermatozoa could be stored for only 1 day , whereas mouse spermatozoa could be stored for 2 months

when slowly dehydrated in a hyperosmotic solution. These results indicate that rapid osmotic pressure changes may severely damage the sperm DNA, resulting in a lower success rate of offspring formation after fertilization.

Conversely, Thuan et al. found that increasing the osmolarity of the preservation medium resulted in decreased sperm motility, but sperm stored in high osmolarity media retained a greater ability to activate oocytes and higher levels of developmental competence (Van Thuan et al. 2005). In this study, optimal conditions of 800 mOsmol KSOM containing 4 mg/ml BSA and a holding temperature of 4 ℃ for preservation of mouse sperm was reported. From the overall viewpoint, we hypothesis that the ingredients used to adjust the osmolarity of the preservation solution and the addition of BSA play an important role in retaining sperm viability.

Overall, membrane treatment with NaOH, the ingredients used to modulate the osmolarity, the addition of BSA and other protectants implicate factors to improve this glassified sperm technique, contributing to establish a new promising sperm preservation method.

CHAPTER 5: CONCLUSIVE SUMMARY

The preservation of mammalian genetic resources is of great importance and is applied to a diverse range of scientific fields, including human medicine, farm animal production, laboratory animal record keeping and wildlife conservation. The genetic banking of spermatozoa provides a more efficient and cost-effective approach for preserving genetic resources compared to oocytes and embryos. However, the conventional spermatozoa cryopreservation requiring the use of liquid nitrogen in storage faces numerous challenges, such as the high maintenance cost, the restriction of transportation and the susceptibility to natural disasters and power outages.

Freeze-drying has been recently focused as a new tool for sperm preservation, which allows preservation at ambient or refrigerator temperature. On the other hand, application of intracytoplasmic sperm injection (ICSI) is needed for fertilization of freeze-dried (FD) sperm due to the loss of their motility.

The pH of the culture medium utilized in biochemistry and biology research is commonly adjusted to 7.4. While pH 7.4 is commonly considered suitable for in vitro fertilization (IVF) and is also utilized for ICSI. it is worth considering that pH 7.4 might not be optimal for ICSI due to the need for artificial breaking of the cell membrane. Therefore, it is crucial to expedite the investigation of optimal pH conditions specifically for ICSI.

In the first series of this study, optimal pH value of HEPES-CZB medium, which is the medium for ICSI, was identified for favorable ICSI outcomes. ICSI was performed using HEPES-CZB medium adjusted to the pH range of 4.0 to 12.0, the survival rate and blastocyst formation rate were evaluated under each pH condition. The most favorable outcomes in terms of ICSI survival rate, blastocyst rate and blastocyst rate per used eggs were observed under pH 7.6. In addition, an alkaline shift of the HEPES-CZB medium during storage was observed. In practical scenario, long-term refrigerated storage is needed before use. Thus, initial pH adjustment of HEPES-CZB medium to a slightly alkaline level prior to storage was recommended for favorable ICSI outcomes.

Glass ampoules have been predominately used for FD sperm since healthy offspring were obtained from FD mouse spermatozoa about a quarter century ago. Despite the drawbacks of this method (e.g., high costs and risks) and the urgent need to overcome

these issues, new methodologies, and substitutes for glass ampoules have rarely been reported.

In the second series of this study, a simple, economical method for FD sperm using commercially available plastic microtubes was developed. Mouse epididymal sperm suspensions were placed in 1.5 ml polypropylene tubes, frozen in liquid nitrogen and dried in an acrylic freeze-drying chamber, then the microtube caps were closed in a vacuum. FD sperm stored in microtubes at −30°C yielded healthy offspring without reducing the success rate even after 9 months of storage. Thus, microtubes are a practical and efficient substitute for glass ampoules when used to store FD sperm at −30°C.

While the freeze-drying method using plastic microtubes offers a solution to some of the drawbacks associated with glass ampoules, it also presents its own set of disadvantages. One such disadvantage is the requirement for high initial investment cost of specialized freeze-drying equipment prior to preserving spermatozoa. In nature, many anhydrobiotic organisms can tolerate severe desiccation. Trehalose is found to accumulate in high concentrations in many of these anhydrobiotic organisms during desiccation, and it is believed to play a major role in imparting desiccation tolerance to anhydrobiotic organisms. The objective of the last experiment was to explore the feasibility of preserving sperm in a glassified state using trehalose. The study investigated three drying methods: natural drying, heated drying and microwave drying. Sperm was recovered after rehydration in all groups, but natural drying was determined to be the most favorable option in terms of ease of use for ICSI and the subsequent developmental rate. Blastocysts were successfully derived from glassified sperm after 24h of RT storage. However, the obtained blastocyst rate was relatively low. Treatment with NaOH at concentrations of 10 mM and 20 mM significantly improved the 4-8 cell rate. Although further modification to the preparation protocol is required, this study demonstrated the potential of glassified sperm for sperm preservation in regions where liquid nitrogen and freeze-drying facilities are limited.

These findings would contribute to the development of alternative, low-cost approaches to preserve and transport genetic resources.

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LIST OF PUBLICATIONS

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論文目録

1　学位論文題目

1. **Yang LL**, Ito D, Ushigome N, Wakayama S, Ooga M, Wakayama T. A novel, simplified method to prepare and preserve freeze-dried mouse sperm in plastic microtubes. J Reprod Dev. 2023;69(4):198-205. doi:10.1262/jrd.2023-034

2　関係論文

1. Wakayama S, Ito D, Kamada Y, Shimazu T, Suzuki T, Nagamatsu A, Araki R, Ishikawa T, Kamimura S, Hirose N, Kazama K, **Yang L**, Inoue R, Kikuchi Y, Hayashi E, Emura R, Watanabe R, Nagatomo H, Suzuki H, Yamamori T, Tada MN, Osada I, Umehara M, Sano H, Kasahara H, Higashibata A, Yano S, Abe M, Kishigami S, Kohda T, Ooga M, Wakayama T. Evaluating the long-term effect of space radiation on the reproductive normality of mammalian sperm preserved on the International Space Station. Sci Adv. 2021 Jun 11;7(24):eabg5554. doi: 10.1126/sciadv.abg5554. PMID: 34117068; PMCID: PMC8195474.
2. Sakamoto M, Ito D, Inoue R, Wakayama S, Kikuchi Y, **Yang L**, Hayashi E, Emura R, Shiura H, Kohda T, Namekawa SH, Ishiuchi T, Wakayama T, Ooga M. Paternally inherited H3K27me3 affects chromatin accessibility in mouse embryos produced by round spermatid injection. Development. 2022 Sep 15;149(18):dev200696. doi: 10.1242/dev.200696. Epub 2022 Sep 15. PMID: 35993297.