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## Investigations of motility and fertilization potential in thawed cryopreserved mouse sperm from cold-stored epididymides

Toru Takeo<sup>a</sup>, Kiyoko Fukumoto<sup>a</sup>, Tomoko Kondo<sup>a</sup>, Yukie Haruguchi<sup>a</sup>, Yumi Takeshita<sup>a</sup>, Yuko Nakamuta<sup>a</sup>, Shuuji Tsuchiyama<sup>a</sup>, Hidetaka Yoshimoto<sup>a</sup>, Norihiko Shimizu<sup>b</sup>, Ming-Wen Li<sup>c</sup>, Kristy Kinchen<sup>c</sup>, Jadine Vallelunga<sup>c</sup>, K C Kent Lloyd<sup>c</sup>, and Naomi Nakagata<sup>a</sup>

<sup>a</sup>Division of Reproductive Engineering, Center for Animal Resources and Development, Kumamoto University, 2-2-1 Honjo, Kumamoto 860-0811, Japan

<sup>b</sup>Animal Laboratory for Medical Research, Asahikawa Medical University, 2-1-1-1 Midorigaoka-Higashi, Asahikawa 078-8510, Japan

<sup>c</sup>Mouse Biology Program, University of California, Davis, CA 95618, USA

### Abstract

Cold transport of epididymides from genetically modified mice is an efficient alternative to the shipment of live animals between research facilities. Mouse sperm from epididymides cold-stored for short periods can maintain viability. We previously reported that cold storage of mouse epididymides in Lifer® perfusion medium prolonged sperm motility and fertilization potential and that the sperm efficiently fertilized oocytes when reduced glutathione was added to the fertilization medium. Cryopreservation usually results in decreased sperm viability; an optimized protocol for cold storage of epididymides plus sperm cryopreservation has yet to be established. Here, we examined the motility and fertilization potential of cryopreserved, thawed (frozen-thawed) sperm from previously cold-stored mouse epididymides. We also examined the protective effect of sphingosine-1-phosphate (S1P) on sperm viability when S1P was added to the preservation medium during cold storage. We assessed viability of frozen-thawed sperm from mouse epididymides that had been cold-transported domestically or internationally and investigated whether embryos fertilized *in vitro* with these sperm developed normally when implanted in pseudo-pregnant mice. Our results indicate that frozen-thawed sperm from epididymides cold-stored for up to 48 hours maintained high fertilization potential. Fertilization potential was reduced after cold storage for 72 hours, but not if S1P was included in the cold storage medium. Live pups were born normally to recipients after *in vitro* fertilization using frozen-thawed sperm from cold-transported epididymides. In summary, we demonstrate an improved protocol for cold-storage of epididymides that can facilitate transport of genetically engineered-mice and preserve sperm viability after cryopreservation.

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Corresponding author: Naomi Nakagata, Division of Reproductive Engineering, Center for Animal Resources and Development, Kumamoto University, 2-2-1 Honjo, Kumamoto 860-0811, Japan. Tel/Fax: 81-96-373-6570; nakagata@kumamoto-u.ac.jp.

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## Keywords

in vitro fertilization; cold storage; cold transport; sperm; epididymides; motility; sphingosine-1-phosphate

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## Introduction

Genetically engineered mice are frequently used in the fields of basic and life sciences. To facilitate mouse genome research, a global network of mouse resource repositories has been established to produce, archive, and distribute genetically modified mice for sharing in the scientific community [4; 5]. As a result, large numbers of genetically engineered mice are regularly transported between mouse banks and various research institutions.

Mouse transport efficiency is an important issue in experimental animal research. Transporting live animals is generally considered undesirable due to the possibility of the spread of infection or animal death/escape during transit, as well as animal welfare concerns [23]. In fact, some major air-cargo carriers have recently adopted policies that restrict the transport of live laboratory animals [38].

The cold transport of fresh mouse embryos or cauda epididymides is often done among research facilities [11; 12; 13; 15; 32; 33; 36]. It is typically a low-cost and low-risk alternative to live animal transport. Previously, we reported that sperm from mouse epididymides cold-stored for 96 hours in Lifor® perfusion solution (Lifor) maintained normal *in vitro* fertilization (IVF) rates when reduced glutathione (GSH) was added to the fertilization medium [36]. Additionally, viable embryos were obtained by IVF using these sperm, and the embryos developed normally into pups when implanted into recipients.

Establishment of a successful process for mouse sperm cryopreservation after cold transport of epididymides can contribute to a simple and efficient system of transportation, production, and preservation of genetically engineered mice. Following cold transport/storage of mouse epididymides, epididymal sperm can be cryopreserved and later thawed (frozen-thawed) for use in IVF. The use of frozen-thawed sperm for IVF can increase the convenience and efficiency of animal production; however, low fertilization success of frozen-thawed sperm derived from cold-stored mouse epididymides is a major obstacle in IVF. It is essential to optimize the cold storage conditions to maintain high sperm motility and viability prior to cryopreservation.

Sphingosine-1-phosphate (S1P) is phosphorylated sphingosine, which is derived from ceramide formed from cell membrane sphingomyelin [26; 27]. S1P is a bioactive sphingolipid metabolite that regulates cell growth and suppresses programmed cell death [3; 25]. There have been numerous reports on S1P's protective effects against stress-induced apoptosis in male germ cells [22; 29; 30]; we hypothesized that S1P could exert these effects in mouse epididymides during cold storage.

In this study, we assessed motility and fertilization potential of frozen-thawed sperm collected from cold-stored epididymides of C57BL/6 mice both with and without the

addition of S1P to the cold storage medium. Additionally, using the cold storage system and a preservation medium containing S1P, we transported mouse epididymides both domestically (from Asahikawa Medical University to our laboratory) and internationally (from the University of California-Davis [UC Davis] to our laboratory) and assessed the viability of the cold-transported frozen-thawed sperm.

## Materials and Methods

### Animals

C57BL/6J male and female mice were purchased from CLEA Japan Inc. (Tokyo, Japan) and from the Jackson laboratory (Bar Harbor, ME, United States) for use as sperm and oocyte donors. Sperm was obtained from mice aged 12–16 weeks, and oocytes were obtained from mice aged 8–10 weeks. Pseudo-pregnant ICR mice (8–16 weeks old, CLEA Japan Inc.) served as recipients for the transfer of two-cell embryos. All animals were kept under a 12-hour dark-light cycle (lights on 07:00 to 19:00) at a constant temperature of  $22\pm 1$  °C with free access to food and water. All animal experiments were approved by the Animal Care and Use Committee at Kumamoto University (Japan) and by the Institutional Animal Care and Use Committee at UC Davis (United States).

### Media

Cauda epididymides were collected from male mice and stored in Lifer (Lifeblood Medical Inc., Freehold, NJ, USA) with or without S1P. For sperm cryopreservation, a modified 18% raffinose pentahydrate and 3% skim milk solution (Difco, Beckton Dickinson and Co., Franklin Lakes, NJ, USA) containing 100 mM L-glutamine (mR18S3 solution) was prepared as previously described [34]. A modified Krebs-Ringer bicarbonate solution (TYH medium) containing 1.0 mg/mL polyvinyl alcohol and 0.75 mM methyl- $\beta$ -cyclodextrin (MBCD, Sigma-Aldrich Co.) was used for sperm preincubation [2; 31]. Human tubal fluid medium (HTF) modified to contain 1.0 mM GSH (mHTF) was used for IVF [18; 35]. Potassium simplex optimization medium (KSOM) was used to culture two-cell embryos to the blastocyst stage [7].

### Cold storage and transport of mouse epididymides

Cold storage and transport of mouse epididymides were performed as previously described [36]. For the cold storage experiments, male mice were euthanized via cervical dislocation, and cauda epididymides were collected and transferred to 0.2-mL plastic tubes (three epididymides/tube) each containing 0.2 mL Lifer. The tubes were placed underneath a sheet of cotton wool in a cardboard box with a tracking monitor that also functions as a digital thermometer and data logger (Thermochron® iButton; Maxim Integrated, Inc., San Jose, CA, USA). To minimize any effects of external temperature fluctuations, the box was placed in a vacuum bottle (JMK-500, Thermos LLC, Schaumburg, IL, USA) with two cold packs (60×180 mm each) that had been pre-cooled in a refrigerator at 4 °C for overnight. The bottle was placed in a styrofoam box (205×125×130 mm) and covered with four cold packs (140×250 mm each). The packed styrofoam box was then stored in a refrigerator at 4 °C for 0, 24, 48, or 72 h. To investigate the effects of the addition of S1P to the cold storage

medium, the above procedure was repeated with the following exception: some of the collected epididymides were placed in tubes containing 0.2 mL Lifer plus 10 $\mu$ M S1P.

For the domestic transport experiment, a packed styrofoam box of three 0.2-mL tubes containing epididymides (in Lifer plus S1P) was prepared as described above and transported from Asahikawa Medical University to our laboratory (Kumamoto University). The box arrived within 72 hours via parcel delivery. The temperature in the box during transport was maintained at 4–8°C. For the international transport experiment, four 0.2-mL tubes containing epididymides (in Lifer plus S1P) were packaged in a box within a vacuum bottle as described above and placed in a cold reserving box (410×370×420 mm) obtained from Office Oota and Tanpopo Co. Ltd (Osaka, Japan) along with five cold packs precooled at 4°C (140×250 mm each) and four hard bottle-type ice packs precooled at –20°C (140×250 mm each). The box was shipped from UC Davis to our laboratory under temperature-controlled conditions and arrived within 72 h. The temperature in the box during transport was maintained at 4–10 °C. The internal temperature of the box can be kept at 4–8°C for 48 hours under the ambient temperature at 22°C.

### Sperm cryopreservation

A previously-described modified sperm cryopreservation procedure was used [17; 34]. A 180- $\mu$ L aliquot of mR18S3 solution (60  $\mu$ L/epididymis) was placed in a 35-mm culture dish and covered with paraffin oil. After the cold storage or transport of cauda epididymides, the epididymides were removed from the storage medium and gently wiped with filter paper. The collected epididymides were cleaned of all fat and blood under a microscope before transfer to the culture dish (3 epididymides/dish) where each was cut into five or six pieces with microsurgical scissors. The dish was maintained at 37°C for 3 min and gently shaken each minute to separate the sperm from the tissue. During sperm preparation, 15 freezing straws (0.25-mL plastic straw; IMV Technologies, Paris, France) were loaded with 100  $\mu$ L HTF and 15 mm air using a 1-mL syringe. The sperm suspension was then divided into 15 aliquots (10  $\mu$ L each) on the culture dish. Each aliquot of sperm suspension, along with 15 mm air, was loaded into each of the 15 freezing straws and both ends of the straw were heat-sealed. The sealed straws were transferred to a freezing canister made from a 50-mL syringe and cooled in the gas layer in the neck of a liquid nitrogen tank (MVE XC 47/11-10, CHART Industries, Garfield Heights, OH, USA) for 10 min. Once equilibrium was achieved, the freezing canister containing the straws was plunged directly into liquid nitrogen, and the straws were transferred from the canister to a pre-cooled triangular cassette in the liquid nitrogen tank and stored for 1–3 months until use. The level of liquid nitrogen was checked before sperm cryopreservation and maintained at 25 to 30 cm from the bottom of the tank.

Prior to sperm motility assessments and IVF, the straws were removed from the liquid nitrogen and thawed in a water bath at 37°C for 10 min.

### Assessment of sperm motility

Motility of the frozen-thawed sperm collected from cold-stored epididymides (4°C) for 0, 24, 48, or 72 hours in Lifer was determined using an IVOS Sperm Analyzer (Hamilton-

Thorne Research, Beverly, MA, USA). A 10- $\mu$ L aliquot of sperm suspension was transferred and cultured in a 90- $\mu$ L drop of HTF for 30 minutes. Incubated sperm was applied to a cell counting chamber (Conception Technologies, San Diego, CA, USA). This experiment was independently performed 4 times. The average number of cells counted per sample was approximately 300. The motility of cold-stored sperm in Lifor with or without SIP was examined by the same procedure described above.

### **In vitro fertilization (IVF)**

IVF was performed as described previously [35]. Mature female mice were superovulated via intraperitoneal injections of 7.5 IU equine chorionic gonadotropin (ASKA Pharmaceutical Co. Ltd, Tokyo, Japan) and 7.5 IU human chorionic gonadotropin (ASKA Pharmaceutical Co. Ltd) 48 hours apart. Mice were sacrificed 15–17 hours later via cervical dislocation, and oviducts were quickly removed and transferred into a fertilization dish with paraffin oil. Four to six cumulus-oocyte complexes (COCs) were obtained from the ampullae of the fallopian tubes of two to three females, respectively, using a needle and forceps under a dissecting microscope. The COCs were transferred to a dish containing a 90- $\mu$ L drop of mHTF medium covered with paraffin oil.

To enhance capacitation a 10- $\mu$ L aliquot of frozen-thawed sperm was added to a 90- $\mu$ L drop of TYH medium containing 1.0 mg/mL polyvinyl alcohol and 0.75 mM MBCD, and sperm were pre-incubated with 5% carbon dioxide (CO<sub>2</sub>) at 37 °C for 30 min. After pre-incubation, an aliquot of sperm suspension was carefully collected from the edge of the sperm pre-incubation drop, transferred to the IVF drop containing mHTF medium and COCs (final motile sperm concentration: 500–1000/ $\mu$ L), and incubated at 37 °C with 5% CO<sub>2</sub>. Three hours later, the inseminated oocytes were washed 3 times in 80- $\mu$ L drops of mHTF, covered with paraffin oil, and cultured at 37°C with 5% CO<sub>2</sub>. At this time, pronuclear formation was observed by phase-contrast microscopy. Twenty-four hours after insemination, fertilization rates were calculated as the total number of two-cell embryos divided by the total number of inseminated oocytes  $\times$  100. Sperm from mouse epididymides cold-stored both with and without SIP and for 72-hour cold storage durations were assessed.

### **Embryo culture and transfer**

Two-cell embryos were either cultured in KSOM to the blastocyst stage or transferred into the oviducts (10 embryos/oviduct) of pseudo-pregnant ICR female mice on the day a vaginal plug was found (day 1 of pseudopregnancy) [16]. After 19 d, the number of offspring was recorded at birth. For the embryos that has been cultured in KSOM, the numbers of four-cell embryos, morulae, and blastocysts were recorded also after 19 d. Blastocyst developmental rate (number of blastocysts per number of two-cell embryos $\times$ 100) as well as birth rate (number of live pups per number of transferred two-cell embryos $\times$ 100) were calculated.

### **Statistical analysis**

Statistical analysis was performed using Prism v. 5.0 (GraphPad Software Inc., San Diego, CA, USA). Results are expressed as means  $\pm$  standard deviations (SDs). The means for each treatment were compared by analysis of variance after arcsine transformation of the percentages. Differences between mean values were considered significant at  $p < 0.05$ .

## Results

### Effects of mouse epididymides cold storage on sperm motility and IVF

After cold storage of cauda epididymides for 0, 24, 48, or 72 h, both overall and progressive sperm motility decreased as a function of increasing cold storage duration (Figure 1) and were lower ( $p < 0.05$ ) after 72 hours of cold storage compared to 0 hours. The IVF rate was high after 24 hours of cold storage ( $84.3 \pm 9.8\%$ ), but decreased with increasing cold storage duration. The decreases at 48 and 72 hours were statistically significant ( $p < 0.05$ ) compared with 0 hours (Table 1).

### Effects of addition of S1P to the mouse epididymides cold storage medium on sperm motility and IVF

To determine whether addition of S1P to Lifer helps to maintain sperm viability during cold storage of cauda epididymides, motility and IVF assessments were performed using frozen-thawed sperm after cold storage of the epididymides in Lifer with or without S1P. Both overall and progressive sperm motility were unaffected by the addition of S1P to the cold storage medium (Figure 2). On the other hand, for the 72-hour cold storage period, Lifer plus S1P significantly improved fertilization rate compared with Lifer alone (Table 2).

### *In vivo* and *in vitro* embryo development following IVF using frozen-thawed sperm from cold-transported mouse epididymides

Mouse epididymides stored in Lifer plus S1P were transported from Asahikawa Medical University to our laboratory (domestic transport within Japan) or from UC Davis to our laboratory (international transport from USA to Japan). When samples arrived (within 72 hours in both cases), sperm were collected from the received epididymides, cryopreserved, and later thawed prior to experiments. *In vitro* fertilization was carried out using the frozen-thawed sperm, followed by *in vitro* embryo culture or embryo transfer to pseudo-pregnant mice. Sperm from both domestically- and internationally-transported epididymides had comparable fertilization rates, percentages of blastocysts, and percentages of embryos that developed normally into pups (Table 3).

## Discussion

In the present study, we show that cold storage of mouse epididymides for 24 hours had no significant effect on frozen-thawed epididymal sperm IVF rates. Cold storage of epididymides for more than 48 hours, however, resulted in markedly decreased IVF rates compared with 0 hours of cold storage. The addition of S1P to the cold storage medium significantly increased fertilization rate of frozen-thawed sperm derived from cold-stored epididymides for 72 hours. Using a cold-storage system, we have demonstrated that sperm cryopreserved after cold-storage and transportation can be used for IVF. Further, IVF-generated embryos develop normally to live pups. There were no significant differences in fertilization or birth rates between the laboratories in Japan and the United States collaborating on this project.



Worldwide transportation of genetically engineered mice has increased over the years. Live animal transport, however, can be problematic because of concerns about animal welfare, infection control, animal escape or death, and cost. Transportation of cauda epididymides from genetically engineered mice represents an alternative with many advantages. Sperm from transported epididymides can be cryopreserved and stored in liquid nitrogen until needed. The cryopreserved sperm can be used for IVF to quickly produce many embryos and serve as a readily available back-up of the mice.

We previously reported on an IVF process using frozen-thawed or cold-stored C57BL/6 mouse sperm pre-incubated with MBCD and treatment of GSH to oocytes which achieved high fertilization rates [35; 36]. MBCD facilitated cholesterol efflux from the plasma membranes of frozen-thawed C57BL/6 mouse sperm, enhancing fertilization capability [31]. Reduced glutathione broke disulfide bonds of the zona pellucida, loosening its structure and facilitating sperm penetration [35]. Bath (2010) also reported that incubation with GSH increased IVF of frozen-thawed mouse sperm, and that reactive oxygen species (ROS) generated by some incompetent frozen-thawed sperm during fertilization may impair fertilization by other capable mouse sperm [1]. Recently, Gray et al. (2013) showed that enhancing cholesterol efflux with MBCD restored capacitation-dependent function of frozen-thawed mouse sperm and that GSH decreased oxidative stress caused by mitochondrial hydrogen peroxide production and improved IVF rates by reducing peroxidative acrosomal damage [6]. In our present study, with the exception of frozen-thawed sperm from epididymides that had been cold-stored for 48 or 72 hours, IVF rates for cold-stored/cold-transported frozen-thawed sperm were high (>83%) with the addition of MBCD and GSH during IVF. These results suggest that MBCD-induced cholesterol efflux in sperm, ROS scavenging by GSH in sperm, and disulfide bond breakage in the zona pellucida by GSH contribute to the maintenance of high IVF rates using cold-stored, frozen-thawed sperm.

Numerous studies have reported that sperm storage (without loss of viability) is only possible for short periods (e.g., 48 hours) at ambient or refrigeration temperatures [9; 10; 12; 15; 24]. These reports document the difficulties associated with maintaining normal mouse sperm motility and viability in vitro for more than 48 hours. We previously reported that sperm from epididymides cold-stored in Lifer for 96 hours maintained high IVF rates; however, sperm frozen-thawed following >48 hours of cold storage had greatly decreased IVF rates in spite of the addition of MBCD and GSH [36]. In the present study, the addition of S1P to the cold storage medium clearly improved the IVF rates of frozen-thawed sperm from the cold-stored epididymides. S1P is a bioactive sphingolipid metabolite involved in various biological processes including calcium mobilization, cytoskeletal organization, and cell growth, differentiation, survival, and motility [26; 27]. One of the most prominent roles of S1P is the suppression of apoptosis [3]. In male germ cells, S1P suppressed apoptosis initiated by radiation-induced stress [22; 29]. The DNA-binding activity of nuclear factor  $\kappa$ B (NF- $\kappa$ B) and expression of phosphorylated Akt which are regulating apoptosis were suppressed by S1P in germ cells. Sphingosine kinase-1, which catalyzes sphingosine phosphorylation to form S1P, is localized in the acrosome region of mature mouse sperm; additionally, the presence of S1P receptors was confirmed in sperm [14]. This suggests that S1P may be involved in the sperm acrosome reaction, a key element of fertilization that

facilitates egg penetration [28]. During cold storage of epididymides, S1P may exert protective effects on sperm via binding to various S1P receptors on the sperm cell surface.

The acrosome reaction appears essential for sperm penetration of the zona pellucida and fusion with the oocyte. Jin et al. (2011) reported that most fertilizing sperm underwent the acrosome reaction prior to contact with the zona pellucida [8]. The addition of S1P to the medium for cold storage of epididymides may facilitate the induction of the acrosome reaction in sperm from cold-stored epididymides. In our experiment, pre-incubation with MBCD greatly enhanced IVF rates of sperm from cold-stored epididymides (data not shown). Treatment of sperm with MBCD efficiently induces capacitation by promoting cholesterol efflux from the plasma membrane [2; 37]. In addition, MBCD-treated sperm underwent the acrosome reaction at high frequencies, with rearranging of plasma membrane lipid rafts and release of glycosylphosphatidylinositol-anchored proteins in the sperm head [39]. These findings suggest that MBCD plays a role in induction of the acrosome reaction and therefore can potentially facilitate IVF. Likewise, the effects of S1P on the acrosome reaction indicate that S1P can enhance IVF by sperm from cold-stored epididymides. However, further studies are needed in order to elucidate the mechanisms of S1P in affecting the viability of cold-stored and frozen-thawed sperm.

After cold-storage of epididymides, sperm motility decreased time-dependently. Ogonuki et al. reported live pups were obtained derived from non-motile sperm derived from transport of frozen mouse epididymides [20; 21]. In case of disappearing sperm motility after cold transport of epididymides, intracytoplasmic sperm injection (ICSI) may be useful to produce embryos using the sperm collected from epididymides.

Cold storage of mouse epididymides can enable easy transport of genetically modified mice between research institutes. In the present study, addition of S1P to the cold storage medium Lifer improved IVF by frozen-thawed sperm after cold storage of epididymides for 72 hours. In addition, live pups were born after IVF using frozen-thawed sperm from mouse epididymides that had been cold-transported either domestically or internationally. Moreover, we recently established an IVF process for cryopreserved mouse oocytes and fresh, cold-stored, or cryopreserved sperm [19]. It is important to explore alternatives to the transport of live mice. Our system for cold transport of epididymides and IVF using frozen-thawed epididymal sperm is one such alternative. It enables sharing of genetically engineered mice in the scientific community, and can foster collaboration and facilitate mouse genome research.

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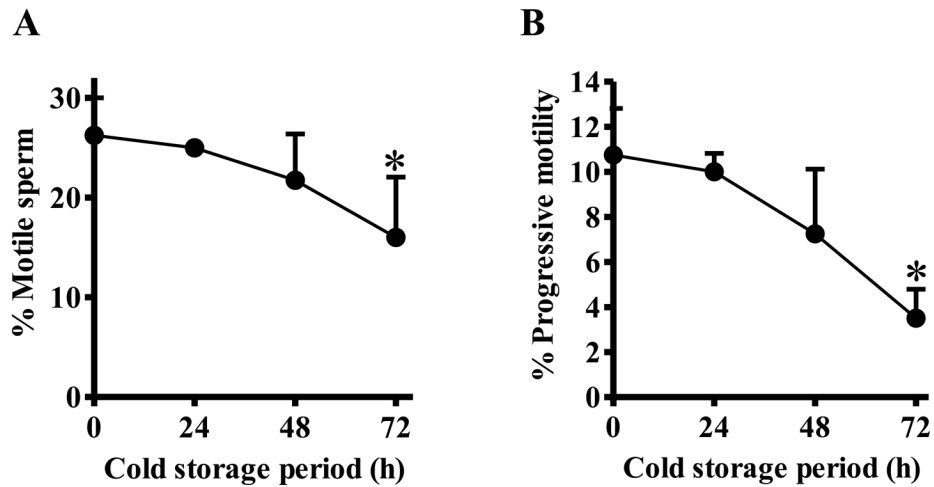


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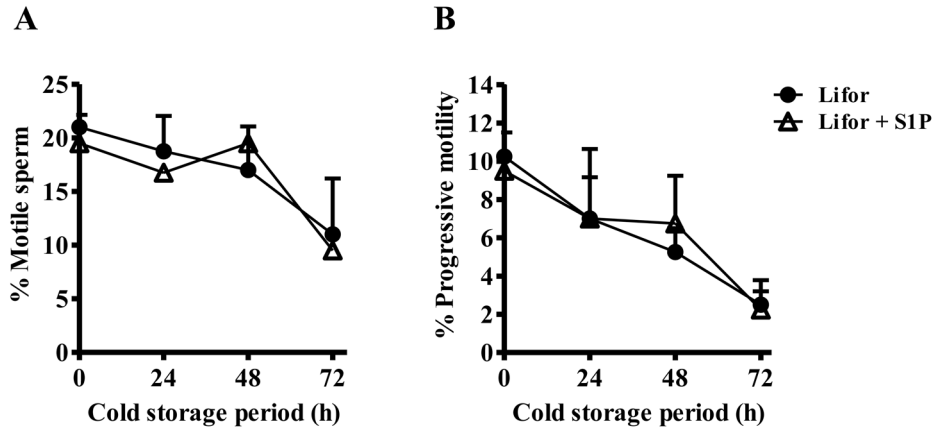
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**Figure 1. Effects of cold storage of mouse epididymides on sperm motility**

One side of cauda epididymides collected from 3 males were stored in Lifer at 4 °C for 0, 24, 48, or 72 h, cryopreserved, and then thawed. Motility of the frozen-thawed sperm was assessed using an IVOS Sperm Analyzer; an average of 300 sperm per sample were assessed. Four sperm samples were assessed for each of the 4 cold storage treatments. Graph A shows overall motility (i.e., percentage of sperm moving their tails, but not necessarily advancing forward) and graph B shows progressive motility (i.e., percentage of sperm advancing from one place to another in a relatively straight line). Percentages are means, and error bars represent on SD (n=4). Asterisk denotes  $p < 0.05$  compared with 0 h.



**Figure 2. Effects of cold storage of mouse epididymides in medium containing S1P on sperm motility**

Three pairs of cauda epididymides collected from 3 males were divided and transfer into Lifor with or without 10  $\mu$ M S1P. The cauda epididymides were cold-stored at 4 °C for 0, 24, 48, or 72 h. Following cold storage, sperm were collected, cryopreserved, and later thawed. Motility of the frozen-thawed sperm was assessed using an IVOS Sperm Analyzer. An average of 300 sperm per sample were assessed. Graph A shows overall motility (i.e., the percentage of sperm moving their tails, but not necessarily advancing forward) and graph B shows progressive motility (i.e., the percentage of sperm advancing from one place to another in a relatively straight line). Percentages are means, and error bars represent one SD (n=4).

**Table 1**

Results of IVF using frozen-thawed sperm from cold-stored mouse epididymides

Cold storage period (h)	No. of inseminated oocytes	No. of 2-cell embryos (%)
0	444	426 (95.9±3.8)
24	626	528 (84.3±9.8)
48	357	251 (70.3±17.2)*
72	318	122 (38.4±18.4)*

Percentages are means±SDs. The number of sperm samples tested per cold storage duration (n) was 5–8 (15–24 males per group). Three to five females were used for oocyte donor for each experiment. Asterisks denote  $p < 0.05$  compared with 0 hours.



**Table 2**

Results of IVF using frozen-thawed sperm from mouse epididymides that were cold-stored for 72 hours either with or without 10 $\mu$ M S1P.

Cold storage medium	No. of inseminated oocytes	No. of 2-cell embryos (%)
Lifor®	526	197 (37.5 $\pm$ 8.9)
Lifor® + S1P	581	491 (84.5 $\pm$ 4.9)*

One side of 3 epididymides collected from 3 males were transferred into a tube containing Lifor and another side of 3 epididymides were transferred into a tube containing Lifor + S1P (24 males were used in both groups). Oocytes collected from three females were transferred into the drop of mHTF containing GHS (24 females and 8 drops per group). Percentages of two-cell embryos are means $\pm$ SDs (n=8). Asterisk denotes  $p < 0.05$ .

**Table 3**  
Fertilization and developmental ability of frozen-thawed sperm from cold-transported mouse epididymides

Shipping facility	No. of inseminated	No. of 2-cell embryos (%)	<i>in vitro</i>		<i>in vivo</i>	
			No. of cultured 2-cell embryos	No. of blastocysts (%)	No. of transferred 2-cell	No. of live pups (%)
Asahikawa Med. Univ.	301	254 (84.4±16.3)	134	92 (68.7±20.9)	120	39 (32.5±4.3)
UC Davis	536	447 (83.4±10.5)	287	225 (78.4±14.2)	160	54 (33.8±13.0)

IVF (No. of droplet for IVF; Asahikawa Med. Univ: 6, UC Davis: 8) was performed using frozen-thawed sperm from different batches of transported epididymides in tubes (three tubes [three epididymides/tube] for the domestic transport and four tubes [three epididymides/tube] for the international transport). The cold storage medium in these experiments consisted of Lifer plus SIP. Twenty embryos were transferred into the oviducts (No. of transferred embryos: 10 embryos/oviduct) of each pseudo-pregnant mouse (No of recipient mice: Asahikawa Med Univ; 6, UC Davis; 8). Percentages are means±SDs.