

Research Article

# Dimethyl sulfoxide and quercetin prolong the survival, motility, and fertility of cold-stored mouse sperm for 10 days<sup>†</sup>

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<sup>†</sup>**Grant support:** This work was supported by a grant for Research on Development of New Drugs from the Japan Agency for Medical Research and Development.

Edited by Dr. Monika A. Ward, PhD, University of Hawaii John A. Burns School of Medicine.

Received 1 September 2017; Revised 12 October 2017; Accepted 7 November 2017

## Abstract

Technology for preserving sperm is useful for disseminating valuable male genetic traits. Cold storage is suitable for easily transporting sperm as an alternative to the shipment of live animals. However, there is a technical limitation in that the fertility of cold-stored sperm declines within 3 days. To overcome this problem, we examined the protective effects of quercetin and dimethyl sulfoxide (DMSO). DMSO and quercetin maintained the fertility and motility of cold-stored sperm for 10 days. In addition, quercetin attenuated the reduction of mitochondrial membrane potential of cold-stored sperm during sperm preincubation, allowing the induction of capacitation, and it localized to the midpiece of sperm. Furthermore, DMSO and quercetin enhanced the level of tyrosine phosphorylation of cold-stored sperm. DMSO and quercetin have life-prolonging effects on sperm during cold storage. Cold storage using DMSO and quercetin will provide a robust system for internationally transporting valuable sperm samples.

## Summary Sentence

Cold storage of mouse sperm with dimethyl sulfoxide and quercetin achieved the longest period to produce two-cell embryos by in vitro fertilization.

**Key words:** sperm, cold storage, quercetin, dimethyl sulfoxide, mitochondria, tyrosine phosphorylation.

## Introduction

Assisted reproductive technology is a useful tool in the fields of medicine, veterinary, agriculture, and biotechnology. Improving techniques for preserving reproductive cells is an important way of enhancing their quality and applicability. In 1949, Polge discovered the cryoprotective effect of glycerol (a permeable cryoprotectant) on sperm [1]. An important discovery of a compound that can protect sperm at low temperatures established the modern theory of sperm cryobiology.

Successful reports have been published regarding mouse sperm cryopreservation using an optimized cryoprotectant composed

of raffinose pentahydrate (a nonpermeable reagent) and skim milk with small amounts of L-glutamine or monothioglycerol [2–4]. Using sperm cryopreservation, mouse repositories have been established to enhance the availability of high-quality resources for genetically engineered mice for biomedical research in North America, Europe, Asia, and Oceania [5–7]. The archived resources are searchable via the website of International Mouse Strain Resource (<http://www.findmice.org/>) [5]. In the mouse repositories, the archived sperm of genetically modified mice can be efficiently re-animated via in vitro fertilization (IVF) and embryo transfer [8]. However, the transportation of cryopreserved sperm requires the

maintenance of an ultra-low temperature in a special container with dry ice or a dry shipper, and the quality of sperm samples must be ensured to successfully reanimate the mice. The technical limitations of material shipment represent a critical barrier to establishing seamless access to mouse resources.

To overcome these problems, transporting unfrozen sperm at refrigerated temperatures is a useful technique for transferring the genetics of genetically engineered mice. In general, mouse sperm is vulnerable to hypothermic stress, under which its fertility quickly declines within 24 h [9]. Recently, we reported that the motility and fertility of sperm could be maintained for 3 days via the cold storage of the mouse cauda epididymis [10–12]. The shipment of cold-stored sperm of genetically engineered mice is available for short-distance transport of mice. However, to establish a global network of mouse resources using cold-stored sperm, a longer-term storage method for mouse sperm is needed.

The preservation solution is a critical factor that determines the motility and fertility of sperm stored at refrigerated temperatures. Lifer perfusion medium (Lifer), which is a cold storage solution for human organs, was more effective for prolonging the storage period of mouse sperm than paraffin oil, M2 medium, or CPS-1 [10, 13]. To enhance the protective ability of preservation medium, we focused on the natural compound quercetin, which is present in the xylem parenchyma cells of boreal hardwood species, which can survive at subfreezing temperatures [14]. Previous studies indicated that quercetin alleviated oxidative stress in horse, human, and rabbit sperm [15–17]. However, the protective effect of quercetin on cold-stored sperm has not been examined.

In this study, we aimed to prolong the storage period of mouse sperm at refrigerated temperatures by adding a protective agent to the preservation medium. First, we examined the effects of quercetin and solvent (dimethyl sulfoxide [DMSO]) on the fertility of cold-stored sperm. Second, we measured the storage period of sperm cold-stored with DMSO and quercetin and evaluated their motility and fertility. Then, we analyzed sperm cold-stored with DMSO and quercetin regarding parameters of sperm activity (mitochondrial membrane potential) and sperm capacitation (protein tyrosine phosphorylation) after preincubation in a capacitating condition. Finally, we examined the developmental ability of embryos derived from IVF using sperm cold-stored with DMSO and quercetin.

## Materials and methods

### Animals

C57BL/6Jcl mice were purchased from CLEA Japan (Tokyo, Japan) for use as sperm and oocyte donors. Sperm were obtained from mature male mice (12–18 weeks old), and oocytes were obtained from mature female mice (8–12 weeks old). All animals were kept under a 12-h/12-h dark/light cycle (lights on: 07:00–19:00) at a constant temperature of  $22 \pm 1^\circ\text{C}$  with free access to food and water. All animal experiments were approved by the Animal Care and Use Committee at Kumamoto University.

### Media

Epididymides were collected and stored in Lifer (Lifeblood Medical Inc., NJ, USA) supplemented as previously described [18]. Specifically, DMSO was dissolved in Lifer at a final concentration of 1%–10%, whereas quercetin was dissolved in DMSO (1.0 mg/mL), producing a final concentration of 1–100  $\mu\text{g/mL}$ .

BSA-free Toyoda Yokoyama Hoshi medium (fTYH), a modified Krebs–Ringer bicarbonate solution, was used for sperm incubation

[19, 20]. BSA-free TYH containing 0.75 mM MBCD (Sigma) and 1.0 mg/mL polyvinyl alcohol (cold water soluble; Sigma) (cTYH) was used for sperm preincubation to induce capacitation [21]. Modified human tubal fluid (mHTF) medium containing 1.0 mM GSH was used as a medium for IVF [22–24]. Potassium simplex optimized medium (KSOM/AA) was used to culture two-cell embryos [25].

### Cold storage of cauda epididymides

Cold storage of mouse epididymides was performed as previously described [10]. For the cold storage experiments, male mice were euthanized via cervical dislocation, and their cauda epididymides were collected and transferred to 0.2-mL plastic tubes (three epididymides/tube) each containing 0.2 mL of preservation medium. The tubes were placed in a cardboard box with a tracker that also functions as a digital thermometer and data logger (Thermochron iButton; Maxim Integrated Products, Inc.), and a sheet of cotton wool. To reduce temperature fluctuations in the box caused by external temperatures, the box was placed in a vacuum bottle (JMK-500, Thermos Co., USA) with two cold packs (60 mm  $\times$  180 mm) that were precooled in a refrigerator at  $4^\circ\text{C}$ . The bottle was placed in a Styrofoam box (140 mm  $\times$  250 mm). The packed Styrofoam box was then stored in a refrigerator at  $4^\circ\text{C}$  for 0–11 days. The cold-transport kit is available via CosmoBio Co. Ltd (KYD-006-EX).

### In vitro fertilization

After cold storage, the cauda epididymides were removed from the storage medium, and the medium was gently wiped off with a filter paper. The cauda epididymides were washed in mHTF and transferred to a sperm incubation dish containing paraffin oil. In the paraffin oil, the duct of each cauda epididymides was cut using microdissecting scissors (Supplemental Figure S1). Clots of sperm released from the cauda epididymides were transferred into a 100- $\mu\text{L}$  drop of cTYH using a dissecting needle. To induce capacitation, sperm were incubated for 60 min at  $37^\circ\text{C}$  and 5%  $\text{CO}_2$ .

Mature female mice were superovulated via intraperitoneal injections of 7.5 IU of equine chorionic gonadotropin (ASKA Pharmaceutical Co. Ltd, Tokyo, Japan) and 7.5 IU of human chorionic gonadotropin (ASKA Pharmaceutical Co. Ltd) administered 48 h apart. Mice were sacrificed 15–17 h later via cervical dislocation, and oviducts were quickly removed and transferred into a fertilization dish containing paraffin oil. Four to six cumulus–oocyte complexes (COCs) were obtained from the ampullae of the fallopian tubes of two to three females. COCs were transferred to a dish containing a 200- $\mu\text{L}$  drop of IVF medium covered with paraffin oil.

An aliquot of sperm suspension was carefully collected from the edge of the sperm incubation drop, transferred to the IVF drop containing COCs, and then incubated at  $37^\circ\text{C}$  and 5%  $\text{CO}_2$ . The concentration of inseminated sperm was 1000–2000 sperm/ $\mu\text{L}$  in an IVF drop. Three hours later, the inseminated oocytes were washed three times in a 100- $\mu\text{L}$  drop of mHTF covered with paraffin oil and then cultured at  $37^\circ\text{C}$  with 5%  $\text{CO}_2$ . After 5–6 h of insemination, the pronuclear formation was observed to confirm the monospermic, polyspermic, or parthenogenic oocyte by phase-contrast microscopy. Polyspermic or parthenogenic oocyte was not observed in this experiment. 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.

### Assessment of sperm motility

The motility of cold-stored sperm preserved in preservation medium at  $4^\circ\text{C}$  was evaluated using a computer-assisted sperm analyzer

(CASA, IVOS Sperm Analyzer, Hamilton-Thorne Research, Beverly, MA, USA) [26]. Epididymal sperm were transferred and incubated in a 100- $\mu$ L drop of cTYH for 60 min. The sperm suspension was applied to a cell counting chamber (Hamilton-Thorne Research). Sperm motility was denoted by movement at a velocity of 5  $\mu$ m/s in any direction. Progressive motility denoted a path velocity >50  $\mu$ m/s and a straightness ratio >50%. In addition, the average path velocity, progressive velocity, track speed, lateral amplitude, and beat cross frequency were evaluated. In each experiment, 500–1000 sperm were analyzed. The experiments were independently conducted four to eight times.

### Analysis of quercetin levels in cold-stored sperm

To examine the localization of quercetin in sperm, we observed the localization of quercetin and measured the levels of quercetin by flow cytometry. Epididymal sperm were transferred into a 100- $\mu$ L drop of fTYH. The sperm were observed via fluorescence microscopy (Bioevo; Keyence Co., Japan). The level of quercetin in sperm was quantified via the fluorescence intensity of quercetin using a flow cytometer (Becton Dickinson, San Jose, CA). The relative mean fluorescence intensity was determined by dividing the fluorescence intensity of quercetin-stained cells by that of untreated quercetin-stained cells. More than 10,000 sperm were analyzed in each experiment. The experiments were independently conducted four times.

### Evaluation of mitochondrial activity in cold-stored sperm

The mitochondrial activity of sperm cold-stored for 7 days was examined using a MitoProbe JC-1 Assay Kit (Thermo Fisher Scientific Inc., MA, USA). Sperm were incubated in a 100- $\mu$ L drop of cTYH for 0, 30, 60, 90, or 120 min. After incubation, aliquots of the sperm suspension were mixed with 2  $\times$  JC-1 in cTYH and incubated for 30 min at 37°C and 5% CO<sub>2</sub> in the dark. Sperm with live mitochondria were stained with JC-1 and visualized via JC-1 fluorescence (excitation, 514/529 nm; emission, 590 nm). The sperm were centrifuged and washed twice in fTYH. JC-1-stained sperm were observed and counted via fluorescence microscopy. Fifty sperm were randomly observed in each experiment, and this experiment was independently repeated four times. Mitochondrial activity (%) was calculated as the total number of JC-1-stained sperm divided by the total number of sperm  $\times$  100. The retention rate of mitochondrial activity (%) was calculated as the mitochondrial activity of each experimental group divided by the mitochondrial activity of non-cultured sperm  $\times$  100. In each experiment, 50 sperm were observed under fluorescence microscopy. The experiments were independently conducted four times.

### Detection of sperm incubation-associated tyrosine phosphorylation

Sperm were incubated in a 100- $\mu$ L drop of cTYH for 0 or 60 min. After incubation, the concentration of each sperm suspension was measured using a PiCOSCOPE® (USHIO, Japan) and equalized. Aliquots of the sperm suspension were collected by centrifugation at 14,000  $\times$  g for 5 min. Subsequent to washing with 1 mL of phosphate-buffered saline, the sperm pellet was resuspended in Laemmli sample buffer and 2-mercaptoethanol and boiled for 5 min. After disruption of sperm sample by ultrasonication for 1 min, SDS-PAGE was performed using Mini-PROTEAN® Tetra Vertical Electrophoresis Cell (Bio-Rad, USA), and proteins were transferred to a Trans-Blot® Turbo Mini PVDF Transfer Pack

(Bio-Rad). Western blot analysis was performed using the SNAP i.d.® 2.0 Protein Detection System (Merck Millipore, Germany). The protein-transferred membrane was blocked with PO Noise Cancelling Reagents for Phosphoprotein Detection using chemiluminescence or fluorescence techniques (Bløk; Merck Millipore) at room temperature. After removing the blocking solution, the membrane was immunoblotted with a monoclonal antibody against phosphotyrosine (4G10® Platinum, Anti-Phosphotyrosine Antibody; Merck Millipore) for 10 min at room temperature and horseradish peroxidase-conjugated secondary antibodies for 10 min at room temperature.  $\alpha$ -Tubulin was used as the internal control.

### Embryo transfer

After IVF, two-cell embryos were washed three times in a 100- $\mu$ L drop of KSOM/AA covered with paraffin oil and then transferred into the oviducts (6–12 embryos/oviduct) of pseudopregnant ICR (Institute of Cancer Research) female mice on the day a vaginal plug was found [27]. After 19 days, the number of offspring was recorded. Developmental ability was evaluated by the birth rate (number of live pups per number of transferred two-cell embryos  $\times$  100).

### Statistical analysis

The results are expressed as the mean  $\pm$ SD. The means for each treatment were compared by analysis of variance after arcsine transformation of the percentages. When more than one pair of means was to be compared, Tukey–Kramer's pairwise comparisons were performed on the means, with  $P < 0.05$  or  $P < 0.01$  indicating significance.

## Results

### Experiment 1: DMSO and quercetin enhanced the fertility of cold-stored sperm

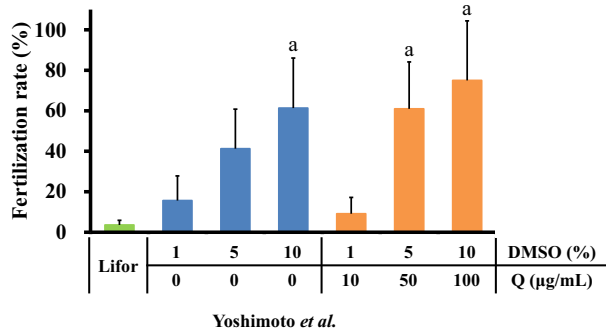
Both quercetin and DMSO increased the fertilization rates of sperm cold-stored at 4°C for 7 days (Figure 1). Co-treatment with 10% DMSO and 100  $\mu$ g/mL quercetin produced the highest fertilization rate. Based on this result, the subsequent experiments were performed using Lifor, Lifor containing 10% DMSO (Lifor-DMSO), or Lifor containing 10% DMSO and 100  $\mu$ g/mL quercetin (Lifor-DMSO-Q).

### Experiment 2: DMSO and quercetin exerted protective effects on cold-stored sperm for up to 10 days

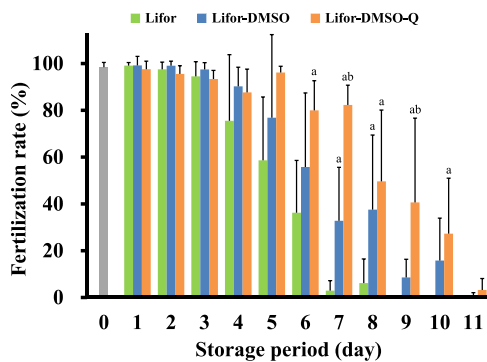
To examine the fertility of cold-stored sperm, we performed IVF using sperm in cold-stored Lifor, Lifor-DMSO, or Lifor-DMSO-Q for 0–11 days. Compared to the effects of Lifor alone, Lifor-DMSO maintained the fertility of cold-stored sperm for 8 days and Lifor-DMSO-Q for 10 days (Figure 2).

### Experiment 3: DMSO and quercetin improved the motility of cold-stored sperm

To examine the quality of sperm cold-stored in Lifor, Lifor-DMSO, or Lifor-DMSO-Q for 0, 3, 7, or 11 days, sperm motility was analyzed using a CASA. Lifor-DMSO-Q produced the highest rates of motile sperm and progressive motile sperm after 7 and 11 days of storage (Figure 3A and B, Supplemental Videos S1–S3, Figures 1–3).



**Figure 1.** Concentration-dependent effects of DMSO or quercetin on the fertility of cold-stored sperm. Cauda epididymides were taken from sacrificed male mice and stored in Lifor containing various concentrations of DMSO (1%, 5%, or 10%) or quercetin (10, 50, or 100 µg/mL) at 4°C for 7 days. After storage, sperm were isolated and incubated in BSA-free Toyoda Yokoyama Hoshi medium containing 0.75 mM MBCD and 1.0 mg/mL polyvinyl alcohol for 60 min. Oocytes were incubated in IVF medium (modified human tubal fluid containing 1.0 mM GSH) for 30–60 min. Subsequently, incubated sperm were introduced into the IVF medium. The fertilization rate was calculated as the number of two-cell embryos divided by the number of inseminated oocytes × 100. Values are given as the mean ± SD (n = 4). <sup>a</sup>P < 0.05 compared between Lifor and Lifor-DMSO or Lifor-DMSO-Q. DMSO, dimethyl sulfoxide. Q, quercetin.



**Figure 2.** Effect of the storage period on the fertility of cold-stored sperm. Cauda epididymides were taken from sacrificed male mice and stored in Lifor, Lifor-DMSO, or Lifor-DMSO-Q at 4°C for 0–11 days. After storage, sperm were isolated and incubated in BSA-free Toyoda Yokoyama Hoshi medium containing 0.75 mM MBCD and 1.0 mg/mL polyvinyl alcohol for 60 min. Oocytes were incubated in IVF medium (modified human tubal fluid containing 1.0 mM GSH) for 30 min. Subsequently, incubated sperm were introduced into the IVF medium. The fertilization rate was calculated as the number of two-cell embryos divided by the number of inseminated oocytes × 100. Values are given as the mean ± SD (n = 4–8). <sup>a</sup>P < 0.05 compared between Lifor and Lifor-DMSO or Lifor-DMSO-Q. <sup>b</sup>P < 0.05 compared between Lifor-DMSO and Lifor-DMSO-Q. DMSO, dimethyl sulfoxide. Q, quercetin.

#### Experiment 4: DMSO and quercetin maintained the mitochondrial activity of cold-stored sperm

Because the motility and fertility of sperm were highly maintained during cold storage in Lifor-DMSO-Q, the physiological activity of cold-stored sperm was monitored on the basis of mitochondrial membrane potential (mitochondrial activity) using JC-1 staining. The mitochondrial activity of sperm quickly decreased after 30 min of incubation (Figure 4E). Next, we examined the mitochondrial activity of sperm cold-stored in Lifor, Lifor-DMSO, and

Lifor-DMSO-Q, and Lifor-DMSO-Q was associated with higher mitochondrial activity than Lifor or Lifor-DMSO (Figure 4F).

#### Experiment 5: quercetin accumulated in the midpiece of sperm

To examine the localization and amounts of quercetin in sperm after cold storage in Lifor, Lifor-DMSO, or Lifor-DMSO-Q, the fluorescence of quercetin was observed by fluorescence microscopy, and the fluorescence intensity of quercetin was measured by flow cytometry. The results illustrated that quercetin was highly localized in the midpiece of sperm after cold storage in Lifor-DMSO-Q (Figure 5).

#### Experiment 6: DMSO and quercetin maintained the capacitating activity of sperm

To examine sperm capacitation after cold storage in Lifor, Lifor-DMSO, or Lifor-DMSO-Q, the occurrence of sperm capacitation was evaluated according to the status of protein tyrosine phosphorylation. Protein tyrosine phosphorylation increased after 60 min of incubation in Lifor, although the level was lower than that of fresh sperm (Figure 6A). Compared to the findings for cold storage in Lifor, protein tyrosine phosphorylation was elevated following storage in Lifor-DMSO and Lifor-DMSO-Q, as indicated by bands at 50 kDa and approximately 250 kDa.

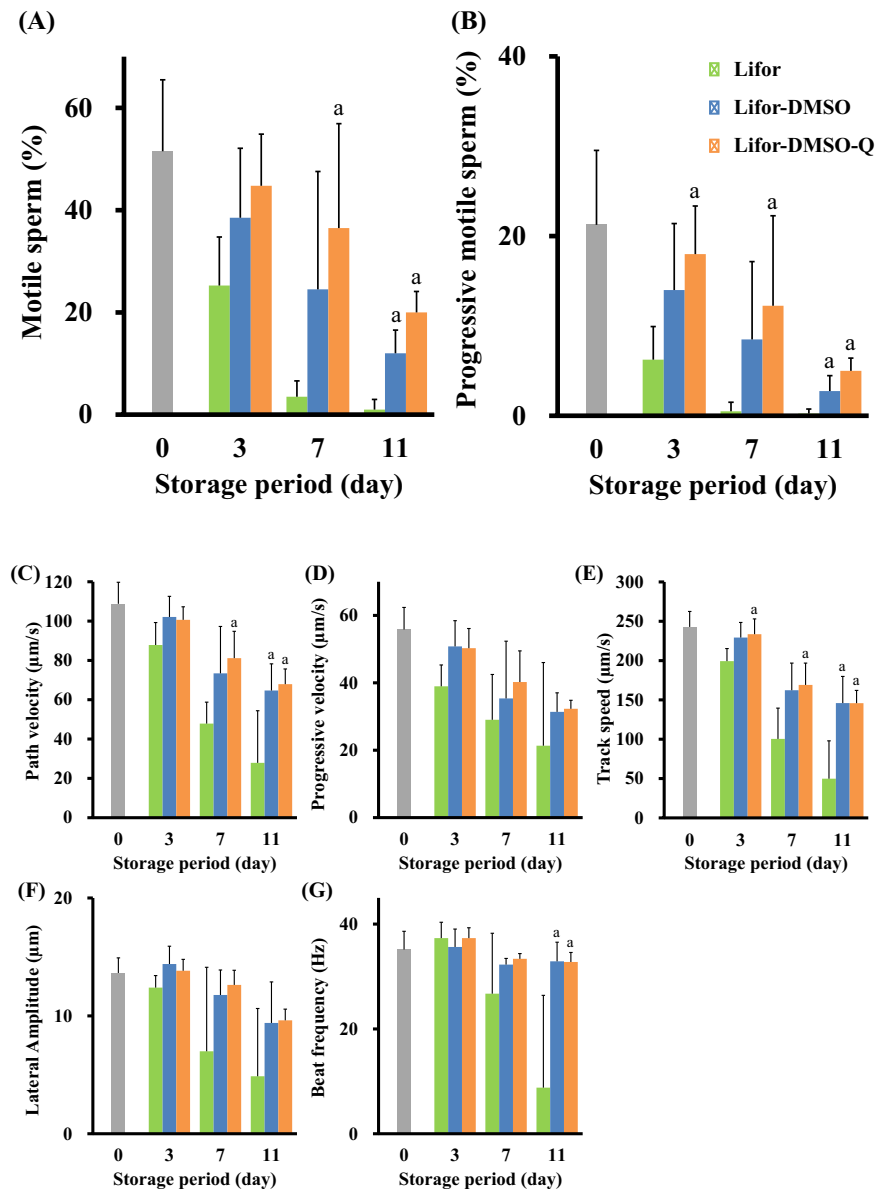
#### Experiment 7: development of two-cell embryos derived from sperm cold-stored with DMSO and quercetin into live pups

To examine the developmental ability of two-cell embryos produced by IVF using fresh sperm or sperm cold-stored with DMSO and quercetin, birth rates were evaluated by embryo transfer. Birth rates were similar between fresh sperm and sperm cold-stored in Lifor-DMSO-Q (Table 1).

## Discussion

In this study, we demonstrated that DMSO and quercetin could prolong the motility and fertility of cold-stored sperm. In addition, the reduction of mitochondrial activity associated with cold storage was alleviated by exposure to quercetin, and the agent localized to the midpiece of sperm. Furthermore, protein tyrosine phosphorylation, as a marker of capacitation, was observed in sperm cold-stored with DMSO and quercetin. These results suggest that DMSO and quercetin can prolong the vitality of sperm stored at refrigerated temperatures.

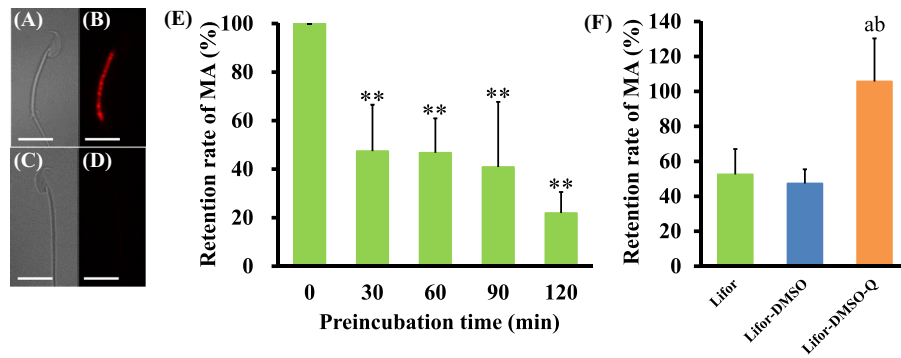
Genetically engineered mice are frequently shipped worldwide. However, shipping live mice has potential risks of expanding pathogens, losing animals by escape or death, and unintentionally violating regulations or rules of genetically modified organisms. In addition, based on the animal welfare policy, a suitable alternative transport system is required. Previously, many researchers addressed to the transport technique by preserving mouse sperm in various environments and producing embryos using the sperm. Cryopreserved, freeze-dried, or evaporative-dried technologies are possible means to store and transport mouse sperm. Sperm cryopreservation is the best way to preserve mouse resource because the sperm can permanently maintain motility and fertility and applicable to IVF after thawing [8]. More than 22,000 strains of cryopreserved sperm were archived in mouse repositories, and the sperm can be transported at −196°C in dryshipper or −79°C using dry ice [5, 28, 29]. However, the motility of frozen-thawed mouse sperm declined compared



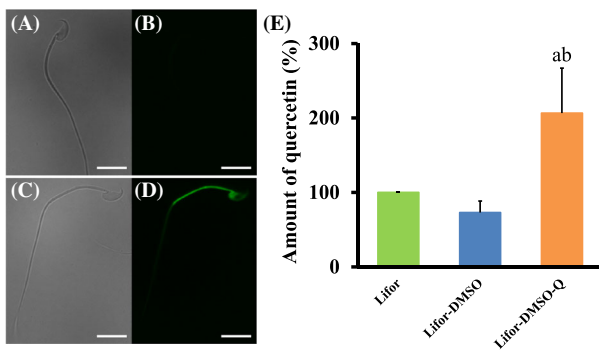
**Figure 3.** Effect of the storage period on the motility of cold-stored sperm. Cauda epididymides were taken from sacrificed male mice and stored in Lifer, Lifer-DMSO, or Lifer-DMSO-Q at 4°C for 0, 3, 7, or 11 days. After storage, sperm were isolated and incubated in BSA-free Toyoda Yokoyama Hoshi medium containing 0.75 mM MBCD and 1.0 mg/mL polyvinyl alcohol for 60 min. Subsequently, the incubated sperm were diluted with modified human tubal fluid, and their motility was analyzed using an HTM-IVOS. The following motility parameters were measured: percentage of motile sperm (A), percentage of progressive motile sperm (B), path velocity (C), progressive velocity (D), track speed (E), lateral amplitude (F), and beat frequency (G). Values are given as the mean  $\pm$  SD ( $n = 4-8$ ).  $^*P < 0.05$  compared between Lifer and Lifer-DMSO or Lifer-DMSO-Q. DMSO, dimethyl sulfoxide. Q, quercetin.

with that of fresh sperm, and the quality of cryopreserved sperm depends on protocol, skill, and mouse strains. Cold-stored sperm indicate similar motility with fresh sperm and can be helpful to reduce the variation of sperm quality due to simple procedure. In addition, in the case of keeping live mice, the cold transport of cauda epididymis collected from anesthetized male mice can skip the process of sperm cryopreservation for the shipment of mouse line. On the contrary, freeze-dried or evaporative-dried mouse sperm is possible to store for 2 years or more at refrigerated or ambient temperatures, which needs intracytoplasmic sperm injection (ICSI) technique to produce embryos from the sperm [30, 31]. The high portability of the freeze-dried or evaporative-dried sperm is very useful to handle the

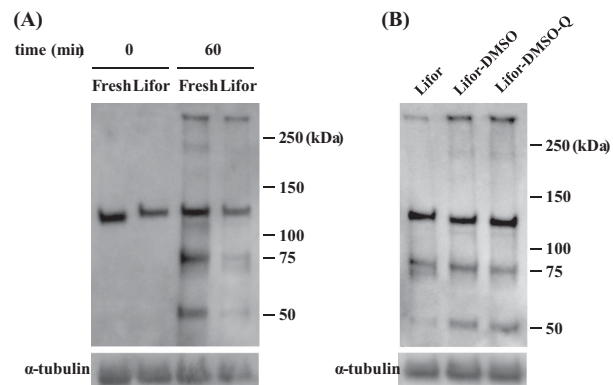
sample without special device for shipment and storage. Recently, freeze-dried mouse sperm traveled to space and yielded healthy mice after 9 months storage in space [32]. The application of the cold storage of sperm will transport the mouse lines between research institutes, whereas the freeze-dried and evaporative-dried techniques will be the choice to send the sample to the frontier place. There are some reports about the storage of sperm in whole body at refrigeration or freezing temperatures. In cold-stored male mice after euthanization, sperm of outbred and hybrid strains of ICR and B6D2F1 mice could fertilize oocytes by IVF using zona-free or partial zona dissected oocytes for 7 days or ICSI for 20 days [33, 34]. Ogonuki et al produced live pups using sperm and spermatid retrieved from



**Figure 4.** Effect of preincubation on the mitochondrial activity of cold-stored sperm. Cauda epididymides were taken from sacrificed male mice and stored in Lifor, Lifor-DMSO, or Lifor-DMSO-Q at 4°C for 7 days. After storage, sperm were isolated and incubated in BSA-free Toyoda Yokoyama Hoshi medium containing 0.75 mM MBDC and 1.0 mg/mL polyvinyl alcohol (cTYH). After incubation, sperm with live mitochondria were stained with JC-1 and visualized via JC-1 fluorescence. (A, B) Sperm with live mitochondria. (C, D) Sperm with dead mitochondria. The mitochondrial activity (MA) (%) was calculated as the total number of JC-1-stained sperm divided by the total number of sperm  $\times 100$ . The retention rate of MA (%) was calculated as the MA of each experimental group divided by the MA of noncultured sperm  $\times 100$ . (E) Cauda epididymides were stored in Lifor at 4°C for 7 days. After storage, sperm were incubated in cTYH for 0, 30, 60, 90, or 120 min. Values are given as the mean  $\pm$ SD ( $n = 4$ ). An asterisk indicates a significant difference ( $P < 0.01$ ) compared with the control (0 min). (F) Cauda epididymides were stored in Lifor, Lifor-DMSO, or Lifor-DMSO-Q at 4°C for 7 days. After storage, sperm were incubated in cTYH for 0 or 60 min. Values are given as the mean  $\pm$ SD ( $n = 4$ ). \*\* $P < 0.01$  compared with 0 min. <sup>a</sup> $P < 0.05$  compared between Lifor and Lifor-DMSO or Lifor-DMSO-Q. <sup>b</sup> $P < 0.05$  compared between Lifor-DMSO and Lifor-DMSO-Q. The bars indicate 10  $\mu$ m. DMSO, dimethyl sulfoxide. Q, quercetin.



**Figure 5.** The localization of quercetin in cold-stored sperm. Cauda epididymides were taken from sacrificed male mice and stored in Lifor or Lifor-DMSO-Q at 4°C for 7 days. After storage, sperm were transferred in BSA-free Toyoda Yokoyama Hoshi medium and observed via fluorescence microscopy. Nonstained (A, B) or quercetin-stained sperm (C, D) were quantified on the basis of the fluorescence intensity of quercetin by fluorescence-activated cell sorting. The amount of quercetin (%) was calculated as follows: (mean fluorescent intensity [MFI] of each experimental group/MFI of sperm cold-stored in Lifor)  $\times 100$ . Values are given as the mean  $\pm$ SD ( $n = 4$ ). <sup>a</sup> $P < 0.05$  compared between Lifor and Lifor-DMSO or Lifor-DMSO-Q. <sup>b</sup> $P < 0.05$  compared between Lifor-DMSO and Lifor-DMSO-Q. The bars indicate 10  $\mu$ m. DMSO, dimethyl sulfoxide. Q, quercetin.



**Figure 6.** Effect of DMSO and quercetin on the protein tyrosine phosphorylation of cold-stored sperm during capacitation. Cauda epididymides were taken from sacrificed male mice and stored in Lifor, Lifor-DMSO, or Lifor-DMSO-Q at 4°C for 7 days. After storage, sperm were isolated and incubated in BSA-free Toyoda Yokoyama Hoshi medium containing 0.75 mM MBDC and 1.0 mg/mL polyvinyl alcohol. The protein tyrosine phosphorylation of cold-stored sperm was assessed by western blot analysis.  $\alpha$ -Tubulin was used as the loading control. (A) Sperm stored in Lifor were incubated for 0 or 60 min. Fresh sperm was used as the control. (B) Sperm stored in Lifor, Lifor-DMSO, or Lifor-DMSO-Q were incubated for 60 min. DMSO, dimethyl sulfoxide. Q, quercetin.

frozen reproductive organs or whole body by ICSI or round spermatid injection (ROSI) [35]. The storage of organs or whole body at refrigeration or freezing temperatures is simple means to emergently store male germ cells of valuable mouse lines when the mice suddenly died. However, the advanced reproductive techniques of zona dissection, ICSI, and ROSI required producing embryos using the low-motile or immotile sperm. In this case, the cold storage of cauda epididymides collected from dead mice can rescue the mouse line by IVF using the sperm with high motility.

Cold storage of sperm is a reliable means of transferring haploid male genetics to share useful resources, manage efficient breeding, and avoid the transmission of pathogens. The technique of shipping cold-stored sperm is potentially applicable for transporting human

**Table 1.** Birth rate derived from in vitro fertilization using cold-stored sperm.

Storage period (day)	Sperm preservation medium	No. of transferred two-cell embryos (no. of recipients)	No. of pups (%)
0	-	112 (6)	34 (30.4 $\pm$ 35.6)
7	Lifor-DMSO-Q	124 (6)	39 (31.5 $\pm$ 22.5)

Results are expressed as the mean  $\pm$  SD ( $n = 6$ ).  
DMSO, dimethyl sulfoxide; Q, quercetin.

sperm to assess its quality at diagnostic andrology laboratories and disseminate boar sperm for breeding livestock of excellent quality at farms [36, 37]. Practically, the cold-transport technique can be utilized to share and archive useful strains of genetically engineered mice. To establish a transport system for cold-stored mouse sperm, we have continuously improved the shipment box, preservation medium, sperm preincubation medium, and fertilization medium to maintain motility during cold storage and enhance fertility in IVF [10–12]. The shipment technique was applicable to the short-distance transport of mouse sperm (within 3 days), which is acceptable for domestic transportation. However, this duration is not sufficient to ensure international transportation. In this study, we prolonged the transportable period of cold-stored sperm to 7 days. This duration would permit the transport of sperm to research institutes around the world.

Previously, we identified Lifor as a suitable preservation medium for storing mouse sperm at refrigerated temperatures for 3 days [10]. In this study, we found that the addition of DMSO and quercetin to Lifor can prolong the survival period to 10 days. Specifically, the addition of high concentrations of DMSO (10%) and quercetin (100  $\mu\text{g}/\text{mL}$ ) to Lifor alleviated the reduction of motility associated with cold storage at 4°C. Similarly, a low concentration of DMSO (1%) in preservation medium (0.2% KCl and 0.7% NaCl) prolonged the survival period of rainbow trout sperm stored at 4°C [38]. DMSO is a polar aprotic solvent that has antioxidant activity based on its ability to react with the harmful reactive oxygen species hydroxyl radicals [39]. The excess production of hydroxyl radicals results in damage to plasma membranes and mitochondria, leading to reduced sperm motility [40]. In the cold storage of mouse sperm, DMSO may scavenge hydroxyl radicals produced at refrigerated temperatures.

Quercetin increased the protective effect of DMSO and produced the highest rates of fertility, motility, and mitochondrial activity (Figures 2, 3A, and 4F). Quercetin is a flavonoid produced in cold plants with cryoprotective ability, and it has strong ability to scavenge free radicals by inhibiting xanthine oxidase [14, 41, 42]. Treatment with quercetin protected against oxidative stress in precryopreserved human (50  $\mu\text{M}$ , 15.1  $\mu\text{g}/\text{mL}$ ) and horse semen (150  $\mu\text{M}$ , 45.3  $\mu\text{g}/\text{mL}$ ) and chilled rabbit semen (100–200  $\mu\text{M}$ , 30.2–60.4  $\mu\text{g}/\text{mL}$ ) [15–17, 43]. The motility of quercetin-treated sperm was increased via its ability to suppress the production of intracellular  $\text{H}_2\text{O}_2$  and lipid peroxidation. However, the ability of quercetin to maintain sperm motility and fertility was not examined using reproductive technology, and the detailed mechanism has not been fully investigated.

We first revealed that quercetin maintained the mitochondrial activity of cold-stored sperm during sperm preincubation, allowing them to undergo capacitation (Figure 4F). Additionally, quercetin localized to the midpiece of sperm (Figure 5D). We assume that quercetin protected the function of mitochondria in sperm during cold storage and sperm preincubation. The integrity of mitochondrial membrane potential is important for maintaining sperm motility and fertility [44, 45]. In addition to its antioxidant effects, quercetin was recently identified as a phytochemical that can modulate mitochondrial function [46]. In human cells, treatment with quercetin (0.3–30  $\mu\text{M}$ , 0.9–9.1  $\mu\text{g}/\text{mL}$ ) restored mitochondrial membrane potential by improving ATP levels and complex I activity, suppressing caspase-3 activity, or reducing DNA fragmentation [47–50]. Further investigation is needed to elucidate the novel protective effects of quercetin on sperm.

Protein tyrosine phosphorylation is a robust marker of sperm capacitation [51, 52]. Our study illustrated that sperm cold-stored in Lifor displayed decreased protein tyrosine phosphorylation com-

pared with the findings in fresh sperm (Figure 6A). Exposure to Lifor-DMSO and Lifor-DMSO-Q partially restored protein tyrosine phosphorylation in cold-stored sperm (Figure 6B). Proteins observed to undergo tyrosine phosphorylation are candidate moieties involved in the restoration of the capacitating ability of cold-stored sperm following exposure to DMSO and quercetin.

We confirmed the birth of offspring derived via IVF using sperm cold-stored with DMSO and quercetin for 7 days (Table 1). The data established that cold-stored sperm can be used to produce embryos and live pups in mice. Practically, we have already introduced a shipment system for cold-stored sperm in the mouse repository at the Center for Animal Resources and Development (CARD) in Kumamoto University, Japan [53]. Recently, almost all depositors have been sending us cauda epididymides to archive genetically modified mice. At CARD, we have been producing and archiving two-cell embryos by IVF using cold-stored sperm or cryopreserved sperm after cold storage [11]. Cryopreserved sperm can be permanently stored in liquid nitrogen, and they are available for colony expansion and rederivation. In some cases, we have performed IVF using cold-stored sperm and vitrified-warmed oocytes [54]. Cold-transport technology for mouse sperm can seamlessly connect geographically separated institutes and permit long-range projects, thus providing an efficient global platform for mouse research. We strongly believe that the rapid expansion of improved reproductive technology will accelerate innovative discoveries in medical science using genetically engineered mice.

This study demonstrated the life-prolonging effects of DMSO and quercetin on cold-stored sperm. The cold storage technology using DMSO and quercetin will be helpful for improving the transport system of clinical samples to assess male infertility, livestock and marine resources to breed efficiently, and valuable genetic material to preserve endangered species.

## Supplementary data

Supplementary data are available at *BIOLRE* online.

**Supplemental Figure S1.** Sperm preparation for cold storage and IVF. Cauda epididymides were taken from sacrificed male mice and stored in storage medium at 4°C. After cold storage, the cauda epididymides were removed and were transferred to a sperm incubation dish containing paraffin oil. In the paraffin oil, the duct of each cauda epididymides was cut using microdissecting scissors. Clots of sperm released from the cauda epididymides were transferred into the preincubation medium using a dissecting needle. After incubation, an aliquot of sperm suspension was carefully collected from the edge of the sperm incubation drop and transferred to the IVF drop containing COCs.

**Supplemental Video S1.** Sperm stored in Lifor. Cauda epididymides were taken from sacrificed male mice and stored in Lifor at 4°C for 7 days. After storage, sperm were isolated and incubated in BSA-free Toyoda Yokoyama Hoshi medium containing 0.75 mM MBCD and 1.0 mg/mL polyvinyl alcohol for 60 min.

**Supplemental Video S2.** Sperm stored in Lifor-DMSO. Cauda epididymides were taken from sacrificed male mice and stored in Lifor-DMSO at 4°C for 7 days. After storage, sperm were isolated and incubated in BSA-free Toyoda Yokoyama Hoshi medium containing 0.75 mM MBCD and 1.0 mg/mL polyvinyl alcohol for 60 min. DMSO, dimethyl sulfoxide.

**Supplemental Video S3.** Sperm stored in Lifor-DMSO-Q. Cauda epididymides were taken from sacrificed male mice and stored in

Lifor-DMSO-Q at 4°C for 7 days. After storage, sperm were isolated and incubated in BSA-free Toyoda Yokoyama Hoshi medium containing 0.75 mM MBCD and 1.0 mg/mL polyvinyl alcohol for 60 min. DMSO, dimethyl sulfoxide. Q, quercetin.

## Acknowledgments

We are grateful to Prof. Seizo Fujikawa, Division of Bioresources and Product Science, Research Group of Forest Resource Science, Graduate School of Agriculture Research Faculty of Agriculture, Hokkaido University, for his useful advice and comments. We thank Ms Shiori Takeuji, Ms Koharu Kirikihira, and Dr Shota Tsuchida of our laboratory for their technical assistance and useful discussion with the experiments.

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