In vitro storage of functional sperm at room temperature in zebrafish and medaka

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Abstract

The longevity of sperm in teleost is short when isolated even in saline-balanced solution at a physiological temperature. In contrast, some internal fertilizers exhibit the long-term storage of sperm in the female reproductive tract. This evidence raises the question of whether sperm even from the external fertilizers possesses the ability to survive for a long time under appropriate conditions. Here, we show that the sperm of the external fertilizer, zebrafish, can survive and maintain fertility in L-15-based storage medium supplemented with bovine serum albumin, foetal bovine serum, glucose and lactic acid for 28 days at room temperature. The fertilized embryos developed to normal fertile adults. This storage medium was effective in another external fertilizer medaka sperm stored for 7 days at room temperature. These results reveal that sperm from external fertilizer teleost has the ability to survive in vitro for a long time. This sperm storage method allows to ship sperm in important lines by low-cost methods and to investigate key factors for motility and fertile ability in teleost sperm.

Background

Spermatozoa are one of the most differentiated cells that convey the paternal genome to oocytes. This cell has the unique feature of a small head comprising a nucleus and a flagellum and contains few cytoplasm, hence possessing low transcriptional and translational activities. There are reports that the longevity of sperm in teleost is short once it is isolated even in saline-balanced solution at a physiological temperature. For example, the motility of guppy (Poecilia reticulata) sperm is maintained for up to 120 minutes in Hank’s balanced salt solution at room temperature [1]. In contrast, female guppies can store sperm in the reproductive tract beyond lifetime of the donated male [2]. In addition to teleost, the long-term storage of sperm in the female reproductive tract and the spermatheca is found in many animals of internal fertilizers such as molluscs, insects, amphibians, reptiles, birds, and mammals [3–5]. This evidence suggests that the female reproductive tracts and the spermatheca of these organisms possess specialized structures and environments conducive to sperm survival, and also raises a question of whether the sperm itself, whether from an external fertilizer or an internal fertilizer, has the ability to survive for a long time under appropriate conditions, as a basic feature.

Zebrafish is an external fertilizer whose sperm fertility is lost within a few minutes after ejaculation into freshwater. However, the sperm in cold Hank’s solution continues to fertilize eggs efficiently for up to 90 minutes [6]. Furthermore, we have observed that in vitro differentiated sperm survive in each interval of medium change for 3–4 days under zebrafish spermatogonia culture [7, 8], and these cells start to swim strongly after being transferred to hypotonic water. This evidence implies that zebrafish sperm can survive for a long time in vitro in a near-physiological environment conditions. Therefore, we addressed the establishment of in vitro storage conditions of zebrafish sperm based on the medium that was used for the spermatogonia culture. By supplementation of bovine serum albumin (BSA), foetal bovine serum (FBS), glucose and lactic acid (LA) into L-15 medium, we succeeded in storing fertile zebrafish sperm for 28 days at room temperature. Furthermore, we found that this storage medium was effective in sperm of another external fertilizer medaka that is phylogenetically distant from zebrafish.
Materials And Methods

Collection, maintenance and artificial insemination of zebrafish sperm

Zebrafish of *India, AB*, or *vas::EGFP* transgenic lines [9] were used. A male fish was anaesthetized with 0.01% ethyl p-aminobenzoate (Wako, Osaka, Japan). After wiping with ethanol cotton, the sperm was sucked into a pipette tip from urogenital opening by gentle abdominal massage and suspended in the L-15-based storage medium. The storage medium was made from L-15 (Sigma) by the addition of the following stock solutions: 1/100 of 5000 Units/ml penicillin, 5000 µg/ml streptomycin (Gibco), 1/80 of 2M glucose (Wako, Japan), 3/100 of FBS (Biowest), 1/10 of 5% BSA in Milli-Q water (w/v, fraction V, Sigma), 1/10 of Milli-Q water, 1/100 of 1 M Hepes (pH 7.9 adjusted by NaOH, Wako), 1/10000 of 1 M LA (Sigma), 1/10000 of 1 M reduced glutathione (GSH, Sigma) to the total volume. All reagents were resolved in Milli-Q water, and the non-sterile solution was filtrated at 0.2µm. Finally, the storage medium contained 50 Units/ml penicillin, 50 µg/ml streptomycin, 25 mM glucose, 3% FBS, 0.5% (w/v) BSA, 10 mM Hepes (pH 7.9), 22% water, and 0.1 mM LA or 0.1 mM GSH. The number of sperm was counted using a haemocytometer and then stored at 37,000, 74,000 or 148,000 sperms/µl in 40 µl in 1.5 ml tubes at 23˚C or 4˚C.

Oocytes (unfertilized eggs) were prepared from wild-type females according to the method of Westerfield [6]. In all experiments, 100–200 oocytes were used for each fertilization. A single female was squeezed on two or three dishes successively, and the same batch of oocytes was used for each pair or series of experimental conditions. The sperm suspension was added, and the dish was shaken gently for 2 minutes to mix. PBS (100 µl) was added gradually, with shaking. After an additional 2 minutes, 5 ml of fish water was gradually added. Successful fertilization was assessed at 5–6 hours postfertilization.

Collection, Maintenance And Artificial Insemination Of Medaka Sperm

Medaka of *OKcab* was used. A male medaka fish was anaesthetized with 0.01% ethyl p-aminobenzoate. A testis was removed and treated with balanced salt solution (BSS) containing 0.5% bleach for 30 seconds and washed twice with BBS. Sperm were collected by tearing the testis with fine forceps in 66 µl of sperm storage medium. The zebrafish sperm storage medium and two modified media were used to store medaka sperm. Medaka medium-1 was the same component of the zebrafish medium, except with 0.2 mM lactic acid. Medaka medium-2 was made by mixing an equal amount of L-15 and inorganic balanced salt solution, the same as L-15 (0.185 g of CaCl₂·H₂O (1.26 mM), 0.2 g of MgCl₂·6H₂O (1 mM), 0.09767 g of MgSO₄ (0.8 mM), 0.4 g of KCl (5.4 mM), 0.06 g of KH₂PO₄ (0.44 mM), 8 g of NaCl (137 mM), and 0.19 g of Na₂HPO₄ (1.3 mM) per litre, filtrated at 0.2µm), and adding same supplements of the zebrafish medium, 50 Units/ml penicillin, 50 µg/ml streptomycin, 25 mM glucose, 3% FBS, 0.5% (w/v) BSA, 10 mM Hepes (pH 7.9), 22% water, and 0.1 mM LA at final concentration. The number of sperm was
counted using a haemocytometer and then stored at 450,000 sperms/ml in 100 µl in 1.5 ml tubes at 23°C or 4°C. Oocytes were collected from 8–12 females and pooled. The cells were divided into six groups (10–20 oocytes in each) and used for each series of experimental conditions in duplicate. Artificial insemination was performed as described previously [10].

**Genotyping**

PCR was performed with genomic DNAs extracted from sperm and embryos at 7 days post fertilization (dpf). PCR was performed with GoTaq Green Master Mix (Promega) using specific primers to the EGFP sequence (up: accacatgaagcagcacgact, dn: cttctcgttggtctttgc) and the genomic spo11 locus [11]. PCR products were examined by agarose gel electrophoresis.

**Statistical analysis.** Data are presented as the mean ± standard deviation of at least four independent experiments. Statistical differences between the two groups were determined using Student’s t-test. \( P < 0.05 \) was considered statistically significant.

**Results**

**L-15 medium supplemented with FBS, BSA and glucose prolonged the fertilization ability of zebrafish sperm**

L-15 medium is established for culture in free gas exchange with the atmosphere [12], and was used the culture of zebrafish spermatogonia to functional sperm [7]. Therefore, we used L-15 medium as a basal medium for zebrafish sperm storage. Since zebrafish sperm has been maintained in ice-cold condition in Hank’s solution [6], we stored sperm in 4° in this experiment. After examination of different concentration of sperm at 1.5 \times 10^6, 3.0 \times 10^6 and 6.0 \times 10^6 sperms/40 µl (Supplemental Fig. S1), we chose a concentration of 3.0 \times 10^6 sperms/40 µl. The addition of 50 Units/ml penicillin and 50 µg/ml streptomycin to L15-medium did not decrease fertility after storage for 4 days (Supplemental Fig. S2).

At first, we examined FBS and BSA that are used for the differentiation of zebrafish spermatogonia to fertile sperm [7, 8]. The addition of 3% FBS increased the fertility of stored sperm six times higher (51.5 ± 15.6%) than did the medium without FBS for 4 days at 4°C (Fig. 1A). BSA at 0.5% (w/v) also increased sperm fertility significantly (from 38.0 ± 7.7% to 55.5 ± 13.8%) after 4 days of storage at 4°C (Fig. 1B). In addition, glycolysis plays a major role in adenosine triphosphate supplementation in mouse sperm flagellar movement [13]. The addition of 25 mM glucose resulted in a slight increase in the fertility of stored sperm at 4°C but this effect was not significant (Fig. 1C).

**Effects Of Antioxidants, La And Gsh, On The Maintenance Of Zebrafish Sperm**
Since lactic acid is reported as a sperm motility inactivation factor in the sperm storage tubules in birds [14], its effect on zebrafish sperm storage was examined. The addition of 0.1 mM LA decreased the fertility after 4 days of storage at 4°C (Supplemental Fig. S3). Although the sperm survived, we observed that the motility of sperm stored in the LA-containing medium was weaker than that in medium without LA after the induction of motility by the addition of fresh water. We speculated that the combination of LA and low temperature at 4°C leads to the over-suppression of sperm motility. Thus, we examined different storage temperatures. When sperm was stored at 23°C, the fertility was significantly increased compared to that of sperm stored at 4°C, in LA-containing medium (Fig. 2A). Interestingly, when comparing sperm stored at 4°C without LA to those stored at 23°C with LA, the latter sperm showed higher fertilization ability at 92.5 ± 4.8% and 92.2 ± 3.3% than the former sperm 66.9 ± 8.2% and 19.3 ± 4.5% after 4 and 7 days of storage, respectively (Fig. 2B). These results indicate that LA containing medium at room temperature is suitable for the long-term storage of zebrafish sperm.

LA has a reductive function. In zebrafish, the fertility of sperm produced by in vitro culture at a low O2 concentration increase compared with that produced at a normal O2 concentration [8]. In addition, antioxidant GSH enhances the fertility of frozen/thawed mouse sperm [15]. Therefore, we compared the effect of LA and GSH. We added either 0.1 mM LA or 0.1 mM GSH into the storage medium. Sperm stored in the LA-containing medium showed slightly better fertility at 70.0 ± 23.2% than did those stored in GSH at 28.3 ± 37.3% after 18 days (Fig. 2C). In addition, the results of GSH varied widely, suggesting that LA is more suitable than GSH for the storage of zebrafish sperm. GSH may be effective but be more toxic to sperm than LA. Sperm stored at 23°C with LA exhibited fertility 32.8 ± 5.0% after 28 days of storage (Fig. 2D). The fertilized embryos developed normally and spawn the next generation after they grew up. When sperm of the vas::EGFP transgenic line [9] was fertilized after 28 days of storage, the gene was confirmed to be transmitted in a Mendelian fashion (Fig. 3).

These results suggest that zebrafish sperm has a property to survive for long-term in vitro under appropriate medium conditions, such as L-15, which contains mammalian serum components, glucose and some reducing agents.

**Sperm Storage Medium Prolongs The Fertilization Ability Of Medaka Sperm**

The medium based on L-15 and supplemented with FBS, BSA, glucose, and LA as described above maintained the fertility of zebrafish sperm for long-term. Thus, we examined whether this storage medium can be applied to the sperm of another external fertilizing fish, medaka, due to the large phylogenetic distance between zebrafish and medaka in teleost [16].

When medaka sperm was stored in the zebrafish sperm storage medium for 7 days at 23°C and for 8 days at 4°C, fertilized eggs were successfully obtained by the artificial insemination at 45.5 ± 29.2% and 97.5 ± 2.5%, respectively (Fig. 4). The fertility of sperm stored in LA at 4°C was not decreased compared with that of those stored in LA at 23°C, unlike zebrafish sperm. This result is presumably because medaka
is a temperate zone fish. We sometimes observed spoiled storage medium as showing a yellow colour (a pH decrease) at 23°C, and sperm stored in the spoiled storage medium resulted in low fertility. Since medaka sperm were collected by tearing the testis with fine forceps in the sperm storage medium, we speculated that testicular somatic cells that had been carried with sperm grew excessively. To avoid this, we modified the zebrafish medium by the following two procedures: increase lactic acid twice (medaka medium-1) and reduce the amino acids and vitamins of L-15 to 1/2 by the addition of an equal amount of inorganic balanced salt solution as that of L-15 (medaka medium-2). The medium-1 maintained the fertility of medaka sperm at 43.0 ± 11.0% for 7 days at 23°C and 97.5 ± 2.5% for 8 days at 4°C, which was almost equivalent to the fertility of sperm stored in zebrafish sperm storage medium, but the variation decreased (Fig. 4). The medium-2 also maintained fertility at 50.3 ± 23.6% for 7 days at 23°C and 75.8 ± 16.6% for 8 days at 4°C, although results varied widely. We did not observe a spoiled yellow colour in either medium. These results indicate that medaka sperm can be stored in vitro in medium similar to zebrafish sperm storage medium at room temperature for at least 1 week, although storage at low temperature is likely more suitable for long-term storage.

**Discussion**

The present study shows that the sperm of phylogenetic distant external fertilizers zebrafish and medaka can be stored in vitro under a novel composition of the storage medium at room temperature. Therefore, it is revealed that sperm even from external fertilizer teleost can survive for a long period of time at physiological temperature under appropriate conditions, as same as sperm from internal fertilizers. Here LA was shown to be effective on storage of sperm, in addition to FBS and BSA which were used for the spermatogonia culture system [7, 8]. LA has been reported to be a potential antioxidant [17]. Oxidative stress can lead to sperm damage, deformity and eventually male infertility in human [18]. Furthermore, near-anoxia induces immobilization and sustains viability of sperm stored in ant queens [19]. These evidences provide the possibility that avoidance of oxidative stress is one of the key factors for sperm survival.

Among the phylogenetic distance between zebrafish and medaka, most species in teleost are present [16]. Therefore, this storage method may be widely applicable to other teleost fishes and can be used to store the sperm. There are many internal fertilizing teleosts in different orders, such as guppy, Shiner perch (Cymatogaster aggregata), Helicolenus dactylopterus and Sebastes schlegeli, which store sperm in the female reproductive tract for a long time [20–22]. This system may be acquired in parallel evolution. It is interesting to see if there are any commonalities in the fluid conditions to store sperm, which may be similar to the present medium conditions. Comparative analysis of its fluid composition will lead to improvements in storage medium that allow longer-term sperm storage.

Since sperm is a transcriptionally silent cell, motility depends on the activation and/or inhibition of key signalling pathways [23]. However, the complete sperm motility process is far from fully known. The molecular basis for maintaining fertility of sperm is also not well understood. Since a vast number of sperm are easily prepared, a number of chemicals can be screened simultaneously in vitro by this sperm
storage method. Therefore, this sperm storage method would become a new tool for identification of factors and chemicals that affect sperm motility and fertility, which facilitate understanding key mechanisms that secure sperm motility and fertility. In addition, many mutants and transgenic lines have been established in zebrafish and medaka. The storage of their sperm for more than 7 days at ambient temperature makes it possible to ship these cells without dry-ice. This sperm storage method will be benefit to deposit important lines in the stock center and accelerate their exchange in these animals.

Conclusions

The present study shows that external fertilizers zebrafish sperm can be stored in the storage medium at room temperature for 28 days and phylogenetic distant medaka sperm for 7 days. Thus, it is revealed that sperm even from external fertilizer teleost can survive for a long period of time at physiological temperature under appropriate conditions.

Declarations

Ethics approval and consent to participate

The use of zebrafish and medaka for experimental purposes was approved by the Committee on Laboratory Animal Care and Use at the National Institute of Genetics (approval identification numbers 28-13, 29-13 and 30-6) and by the committee at Nagoya University (approval number 11). The experiments were conducted in accordance with the guidelines of the National Institute of Genetics and Nagoya University.

Consent for publication

Not applicable.

Availability of data and material

All data generated or analyzed during this study are included in this published article [and its supplementary information files].

Competing interests

The authors declare that they have no competing interests.

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Author's contributions
N. S. conceived and designed the research, and K. T., N. S. wrote the manuscript. K. T., T. N., T. K., Y. I. performed the experiments. T. M. reviewed the manuscript. The authors read and approved the final manuscript.

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References


Figures
Effect of FBS, BSA and glucose on the fertility of stored sperm. Pooled sperm from several males were stored in L-15 containing penicillin/streptomycin (PS) with/without FBS, BSA and glucose (Glu) at 4°C for 4 days, and used to fertilize 100-200 oocytes. A Effect of FBS. B Effect of BSA. C Effect of glucose (Glu). The colour of circles in each panel indicates the same batch of the experiment using the same sperm. The same batch of oocytes from a single female was used for each pair of medium conditions. Error bars indicate the standard deviation. **p<0.01; *P<0.05 (paired t-test).
Effect of LA, GSH and the stored temperature on the fertility of stored sperm. Pooled sperm from several males were stored in L-15 containing penicillin/streptomycin, FBS, BSA, glucose with/without LA or GSH, and used to fertilize 100-200 oocytes. A Comparison of stored sperm at 4°C and 23°C in the LA containing medium for 4 days. B Comparison of stored sperm at 4°C without LA and 23°C with LA for 4 and 7 days. C, D Effect of LA and GSH on the stored sperm for 18 days (C) and 28 days (D) at 23°C. The colour of
circles in each panel indicates the same batch of the experiment using the same sperm. The same batch of oocytes from a single female was used for each series of medium conditions. Error bars indicate the standard deviation. **p<0.01; *P<0.05 (paired t-test).

**Fig. 3**

Development of fertilized embryos from stored sperm for 28 days. A, B Adult female (A) and male (B) fish (F1) that were obtained by in vitro insemination of stored vas::EGFP sperm to wildtype oocytes. C, D Transmission of the EGFP gene to the next generation. EGFP expression in gonads was detected in almost half of the next generation (F2) at 12 dpf from F1 female (C) and F1 male (D) mating with the wildtype. Upper panels indicate EGFP-positive gonads (arrow heads), and lower panels negative. E, F Genomic PCR analysis of the EGFP gene in the next generation. The gene was transmitted in almost half
of 30 progenies (F2) from F1 female (E) and F1 male (F) mating with the wildtype. Upper panels indicate amplification of \textit{EGFP} and lower panels amplification of genomic \textit{spo11} locus. Genomic DNAs collected from sperm of the \textit{vas::EGFP} line (Tg) and wildtype (In: \textit{India}) were used as controls. NC: negative control with water. L: ladder.

**Fig. 4**

Storage of medaka sperm in L-15 containing FBS, BSA, glucose and LA. Sperm from individual males were divided and stored in the zebrafish medium (ZM) (L-15 containing FBS, BSA, glucose, and LA), medaka medium-1 (the same components of ZM, except twice of LA), and medaka medium-2 (diluted L-15 with the same amount of inorganic balanced salt solution as that of L-15 and added same supplements of ZM) at 4°C or 23°C. Ten to twenty oocytes were used for fertilization. The colour of circles indicates the same batch of the experiment using the same sperm. Pooled same batch of oocytes were used for each series of medium conditions. Error bars indicate the standard deviation. **p<0.01 (paired t-test).**

**Figure 4**

Storage of medaka sperm in L-15 containing FBS, BSA, glucose and LA. Sperm from individual males were divided and stored in the zebrafish medium (ZM) (L-15 containing FBS, BSA, glucose, and LA), medaka medium-1 (the same components of ZM, except twice of LA), and medaka medium-2 (diluted L-15 with the same amount of inorganic balanced salt solution as that of L-15 and added same supplements of ZM) at 4°C or 23°C. Ten to twenty oocytes were used for fertilization. The colour of circles indicates the same batch of the experiment using the same sperm. Pooled same batch of oocytes were used for each series of medium conditions. Error bars indicate the standard deviation. **p<0.01 (paired t-test).**
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