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Cryopreservation of Medaka Spermatozoa

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ABSTRACT—In order to establish a reliable and reproducible method for cryopreservation of medaka spermatozoa, we tested several procedures. The vapor phase of liquid nitrogen (LN), the liquid phase of LN, and dry ice were used for freezing, and dimethyl sulfoxide and N, N-dimethylformamide (DMF) were used as cryoprotectants. The best results were obtained using the following method. Medaka spermatozoa were collected in a plastic tube containing 50 μl of fetal bovine serum supplemented with 10% DMF by squeezing an isolated testis. The sperm suspension was frozen by holding the tube for 10 or 20 min in the vapor phase of LN at a depth of 9 or 10 cm from the top of a container. The frozen sample was immersed and stored in LN. After more than one week of storage, the sample in the tube was rapidly thawed by being incubated in a waterbath for 0.5-1 min at 30°C, and then immediately diluted with 2 volumes of Iwamatsu's solution. Fertilization tests using fresh unfertilized eggs showed that the stored spermatozoa could fertilize 96-100% of the eggs. The hatchability of the fertilized eggs was 84-100%. Thus, this study provides, for the first time, a practical method for preserving medaka spermatozoa.

INTRODUCTION

Small teleost fish, such as medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*), are increasingly being used as vertebrate model systems in studies in various zoological fields, mainly because the fish are readily amenable to genetic and developmental studies (Yamamoto, 1975; Egami *et al.*, 1990; Anders *et al.*, 1991; Kimmel *et al.*, 1991; Westerfield, 1993; Iwamatsu, 1993). For example, Mullins *et al.* (1994) and Solnica-Krezel *et al.* (1994) have chemically induced numerous developmental mutants of the zebrafish. As to the medaka, many spontaneous mutant strains have become available for experimental work (Tomita, 1990, 1992; Ozato and Wakamatsu, 1994). Recently, generation of developmental mutants of the medaka by exposure to radiation and chemicals has also been reported (Shimada and Shima, 1990; Ishikawa and Hyodo-Taguchi, 1995; Ishikawa, 1996; Ishikawa and Hyodo-Taguchi, 1997).

In order to keep a stock of various fish strains, much time and effort need to be invested in the breeding of fish. In some cases, accidental loss of strains has in fact occurred during long-term stocking (Tomita, 1990). Moreover, a large space is required to stock many strains. The approach taken in this study to alleviate these difficulties is to cryopreserve spermatozoa and to thaw them at a later time for *in vitro* fertilization.

It has been well known that the optimum conditions for cryopreservation of fish spermatozoa are highly variable for each fish species (Scott and Baynes, 1980; Leung and Jamieson, 1991). In the case of the zebrafish, a method using skim milk and methanol as cryoprotectants and dry ice for freezing has been used successfully for sperm cryopreservation (Harvey *et al.*, 1982; Westerfield, 1993). However, no reliable procedure for the cryopreservation of spermatozoa has been reported for the medaka, although only one preliminary method has been reported by Kobayashi (1966). In the present study, we describe a reproducible and reliable procedure, by using which viable medaka spermatozoa can be cryopreserved in liquid nitrogen (LN).

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MATERIALS AND METHODS

Chemicals

Two batches of fetal bovine serum (FBS), Wako Hyclone Lot 115607 and IANSA Lot 54220, were obtained from Wako Pure Chemical Industries (Osaka) and from IANSA (Industrializadora Agropecuaria Nacional Sa de cv, Mexico), respectively. Cell Banker (BCL-1) was purchased from Nihon Zenyaku Industries (Fukushima). The composition of Cell Banker has not been disclosed because of the patent application. According to the personal communication of Nihon Zenyaku Industries, it is composed of mainly calf serum supplemented with 10% dimethyl sulfoxide (DMSO). DMSO and N, N-dimethylformamide (DMF) were obtained from Wako Pure Chemical Industries (Osaka).

Fish

The orange-red variety of medaka (*bb*) from a closed colony or of an inbred strain, HO4C (Hyodo-Tguchi, 1980; Hyodo-Taguchi and Egami, 1985), was used. The fish were kept in a room in which the water temperature (26-29°C) and photoperiod (14-hr light/10-hr dark cycle) were controlled. The fish were given a powdered fish food (Tetra-

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min, Tetra Werke Co., Mells, Germany) once a day.

Spermatozoa

The overall experimental scheme is summarized in Fig. 1. An adult male fish, which had been kept without females for about 1 week so that many spermatozoa could be obtained, was killed by cutting the medulla and the testis was isolated. A sperm suspension (about 1×10^3 spermatozoa/μl) was prepared within 30 sec by squeezing, using a pair of forceps, spermatozoa out of the testis into 50 μl of a pre-cooled solution in a plastic tube (Seramu tube, MS-4501W, 1.2 ml, Sumitomo Co., Osaka; or Assist tube, 1.5 ml, Assist Co., Tokyo). The following three solutions were tested for comparison as freezing media: FBS supplemented with 10% DMF, FBS supplemented with 10% DMSO, and Cell Banker.

Freezing procedures

The sperm suspension in the plastic tube was immediately got frozen using the following three different freezing procedures (Fig. 1).

In the first method, the sperm suspension was frozen in the vapor phase of LN absorbed by a Biological Shipper (Taylor-Wharton Cryogenics, CP35, Theodore, AL, USA). The sperm suspension in the plastic tube was placed in the center of a holder, which had been made from a 50-ml plastic conical tube (Falcon, 2070, Becton Dickinson Labware, Lincoln Park, NJ, USA; Fig. 1). The suspension in the tube was frozen by being held the holder for 10 min (for the Assist tube) or 20 min (for the Seramu tube) in the vapor phase of LN at a depth of 9 cm (for the Assist tube) or 10 cm (for the Seramu tube) from the top (mouth) of the Biological Shipper. During the freezing process, the mouth of the Biological Shipper was covered with a piece of paper so that the temperature in the Biological Shipper did not increase.

In the second method, the sperm suspension was frozen by immersing in the liquid phase of LN. The sperm suspension in the plastic tube was placed in a holder of another kind, which had been made from a 50-ml plastic syringe (Terumo Co., Ltd., Tokyo) and a piece of Styrofoam (Fig. 1). The tube was frozen by being immersed the holder for 10 min (for the Assist tube) or 20 min (for the Seramu tube) in the liquid phase of LN at a position 1 cm from the surface of the LN in a vacuum bottle (LG-2000, Zojirushi Vacuum Bottle Co., Tokyo, 12 cm in inner diameter and 19 cm in inner height). During the freezing, the bottle was covered with a lid.

In the third method, 25 μl of the sperm suspension was dropped directly onto the surface of dry ice, which had been hollowed (7 mm in diameter and 7 mm in depth) in advance using a heated iron stick, and kept frozen for 20 min. The frozen drops were transferred to a plastic tube (the Assist tube) which had previously been kept in LN.

Storage and thawing

All frozen samples were stored in a container (MVE sc 11/7, Minnesota Valley Engineering Inc., New Prague, USA) filled with LN. After storage for 1 hr, 3 days, 1 week, 2 weeks, 6 months, and 10 months, the frozen tubes were taken out from the container and the caps were removed. The frozen samples were rapidly thawed by immersion with agitation in a waterbath for 0.5-1 min at 30°C. The sperm suspensions were immediately diluted with 2 volumes of Iwamatsu' s solution (110 mM NaCl, 5 mM KCl, 0.9 mM $CaCl_2$, 0.8 mM $MgSO_4$, and 1.2 mM $NaHCO_3$; Iwamatsu, 1983).

Semi-quantitative test for sperm motility

About 5 μl of the diluted suspension was dropped onto a slide glass, and the spermatozoa were immediately observed under a microscope at a total magnification of × 200. The sperm motilities, namely, what percentages of the total spermatozoa could swim and whether the spermatozoa swam rapidly or slowly, were recorded. The motilities were graded semi-quantitatively as follows: +, less than about 10% survival; ++, about 10-40% survival; +++, more than about 40% survival.

Fertilization and hatchability tests

Matured and ovulated female fish were killed by cutting the medulla. Unfertilized eggs (5-12) were removed from the ovarian lumen using a small amount (300 μl) of Iwamatsu's solution. The unfertilized eggs were inseminated by an immediate adding of the diluted sperm suspension to the solution containing the eggs. Fertility efficiency was expressed as the percentage of fertilized eggs for all eggs examined. The fertilized eggs were observed under a stereomicroscope once a day, until normal fry hatched about 7-10 days after fertilization. Hatchability was estimated by the percentage of normally hatched fry for all fertilized embryos.

RESULTS

The sperm suspensions in three different media were frozen in the vapor phase of LN (Fig. 1). The frozen spermatozoa were stored for 1-2 weeks in LN and thawed. The viability of the frozen-thawed spermatozoa was examined in the motility, fertility, and hatchability of the fertilized eggs (Table 1).

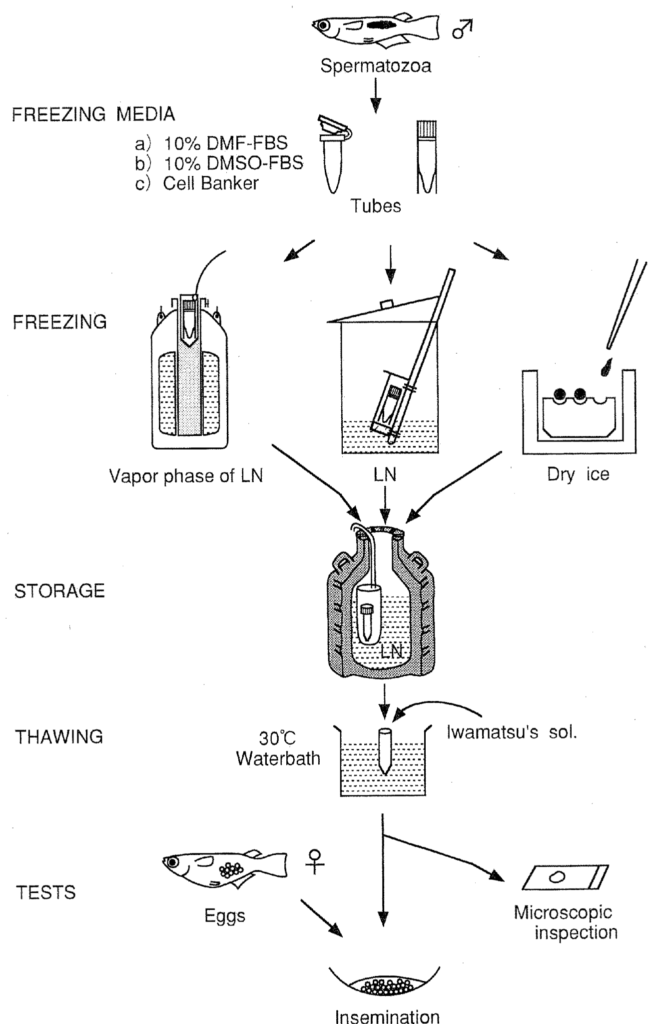

Fig. 1. The overall experimental scheme of the present study. Medaka spermatozoa were suspended in freezing media, frozen in liquid nitrogen (the vapor or liquid phase) or using dry ice, stored in liquid nitogen, and thawed. The viability of the stored spermatozoa was examined on the motility and fertility.

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Table 1. Viability of frozen-thawed and control (unfrozen) spermatozoa

Procedure	Medium	Number of fish	Motility (Speed)	Fertility (% ± SEM)	Hatchability (% ± SEM)
Vapor phase of LN	10% DMF-FBS	6	+++ (Fast)	96 ± 2.4	84 ± 8.2
	10% DMF-FBS	3*	+++ (Fast)	100 ± 0	100 ± 0
	10% DMSO-FBS	21	++ (Fast)	60 ± 7.2	71 ± 8.1
	Cell Banker	4	+ (Slow)	78 ± 13	82 ± 15
Liquid phase of LN	10% DMF-FBS	16	+++ (Fast)	75 ± 8.0	75 ± 8.0
		3*	+++ (Fast)	52 ± 25	66 ± 8.1
Dry ice	10% DMF-FBS	4	++ (Variable)	60 ± 4.7	59 ± 6.1
Unfrozen control	Iwamatsu's Sol.	8	+++ (Fast)	98 ± 1.4	91 ± 3.2
	10% DMF-FBS	8	+++ (Fast)	93 ± 4.6	93 ± 5.3
	10% DMSO-FBS	8	+++ (Fast)	91 ± 3.9	91 ± 2.5
	Cell Banker	4	+++ (Fast)	93 ± 4.8	76 ± 10

Fish from a closed colony were usually used in the experiments.
* An inbred strain HO4C was used.

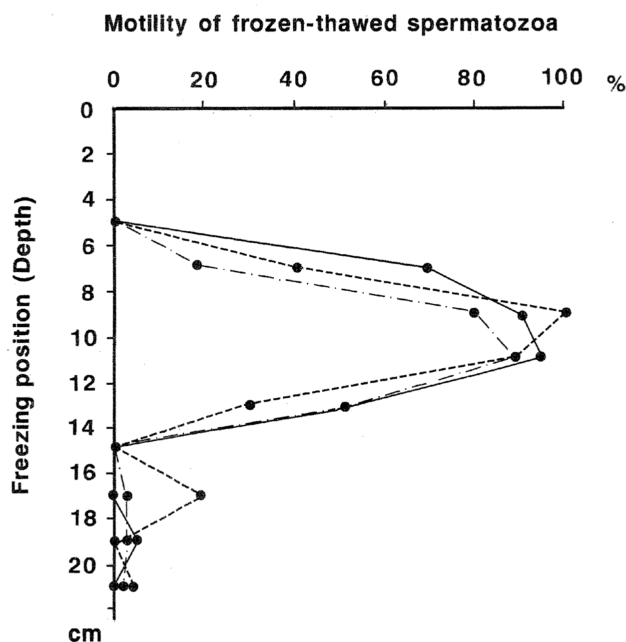

Fig. 2. Motilities of frozen-thawed spermatozoa, which were frozen at various positions (depth from the mouth) in the Biological Shipper. The spermatozoa, which had been suspended in FBS containing 10% DMF, in the Seramu tubes were frozen in the vapor phase of liquid nitogen, stored in liquid nitrogen for 1-2 weeks, and thawed. The motilities of the stored spermatozoa were examined and the percentages of motilities were approximately recorded. A series of experiments was repeated three times (three kinds of lines).

The FBS supplemented with 10% DMF gave the best results as a freezing medium: motilities of +++, fertilities of 96-100%, and 84-100% of hatchabilities. The fertilities were unchanged even after a storage for 10 months at least. Unfrozen spermatozoa in control experiments gave similar motilities, fertilities, and hatchabilities (Table 1). Hence, the cryopreservation of viable medaka spermatozoa was satisfactory with this particular procedure using 10% DMF in FBS as a cryoprotectant and the vapor phase of LN for freezing.

In order to test other freezing procedures, the sperm suspensions in 10% DMF in FBS were frozen in the liquid phase of LN or using dry ice (Fig. 1). Table 1 summarizes the results. For freezing in the liquid phase of LN or using dry ice, more than half of the eggs (52-75%) were fertilized by the frozen-thawed spermatozoa, and 59-75% of the fertilized eggs developed normally. However, the viabilities of the frozen-thawed spermatozoa obtained using these two procedures were inferior to those obtained using freezing in the vapor phase of LN.

For freezing in the vapor phase of LN, we found that the motilities of frozen-thawed spermatozoa were variable depending on the freezing positions in a Biological Shipper (Fig. 2). The best position was found to be 9-11 cm (for Seramu tubes) or 8-10 cm (for Assist tubes) from the top (mouth) of the Biological Shipper. For freezing in the liquid phase of LN, we also found that freezing position in the container was highly important. The best position was found to be 1 cm from the surface of LN (data not shown).

DISCUSSION

In the present study, several procedures for cryopreserving medaka spermatozoa were compared. We tested three freezing media containing sera, since sera have been used successfully for cryopreservation of tissue culture cells (Dougherty, 1962) and of milkfish, *Chanos chanos*, sperm (Hara *et al.*, 1982). We have found that freezing spermatozoa in 10% DMF-FBS in the vapor phase of LN is most satisfactory. The spermatozoa cryopreserved using this procedure fertilized virtually 100% of eggs, which is comparable to the fertilization efficiency of untreated spermatozoa (Table 1).

Kobayashi (1966) reported a method for cryopreservation of medaka spermatozoa using glycerol as a cryoprotectant and dry ice for freezing. However, only 40% of spermatozoa were motile at best with this procedure. Moreover, the ability of the stored spermatozoa to fertilize eggs was not described. Our present study, therefore, provides a feasible method for

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cryopreserving medaka spermatozoa for the first time.

Sperm damage caused by freezing and thawing is generally believed to be due to the formation of intracellular ice crystals and the osmotic effects (Horton and Ott, 1976; Graham *et al.*, 1978; Scott and Baynes, 1980; Leibo, 1986). In order to minimize the damage, cryoprotectants are added to freezing solution and suitable freezing and thawing rates are selected. DMSO has been frequently used as a cryoprotectant. In the present study, however, the results obtained using DMF were superior to those obtained using DMSO for an unknown reason (Table 1). Moreover, the chorions of medaka eggs changed from transparent to opaque in the solution containing DMSO, and consequently it was difficult to inspect embryos through the chorions. Hence, we recommend the use of DMF as a cryoprotectant.

We have noticed that the most critical step is the selection of the optimum depth for freezing positions in a container (Fig. 2). This may be because the temperature in the container is varied at different freezing positions. Consequently, the freezing rate may differ depending on the freezing position. It seems highly important to select the right position where the freezing rate is the most optimal for cryopreserving spermatozoa. The freezing rate is also possibly dependent on the size and the material of the freezing tube and holder. Thus, it is highly important for assuring the reproducibility to use a fixed type of freezing tube and of holder in a series of experiments.

The present study provides procedures by which viable medaka spermatozoa can be readily available for various research purposes at any time of the year and any place in principle. The present method is the most suitable for backing up medaka stocks to insure against an accidental loss. Moreover, this method may be useful for keeping stocks of many medaka mutant strains by preserving their spermatozoa without breeding many living animals. In order to examine this possibility, we are now conducting further experiments for long-term storage of frozen spermatozoa.

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