FISHERIES SCIENCE 2005; 71: 293–298

Cryopreservation diluents for spermatozoa of Sakhalin taimen *Hucho perryi*

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ABSTRACT: To develop a suitable cryopreservation diluent for spermatozoa of the endangered Sakhalin taimen *Hucho perryi*, all possible combinations of cryoprotectants (glycerol, dimethyl sulfoxide [DMSO], methanol) and extenders (fetal bovine serum [FBS], 300 mM glucose solution [GS], artificial seminal plasma for masu salmon) were examined by observing sperm motility 10 s after thawing. Spermatozoa cryopreserved with diluents such as mixtures of 10% glycerol plus 90% FBS, 10% DMSO plus 90% FBS, and 10% methanol plus 90% GS showed the highest motility. The maximal post-thaw motility was observed at 10% among all concentrations (0, 5, 10, 15 and 20%) of these three cryoprotectants. No significant difference among three diluents was observed in motility at 10 s. Mixtures of 10% glycerol plus 90% FBS, 10% DMSO plus 90% FBS, and 10% methanol plus 90% GS are suitable cryopreservation diluents for Sakhalin taimen spermatozoa.

KEY WORDS: **cryopreservation, cryoprotectant, extender, *Hucho perryi*, Sakhalin taimen, spermatozoa.**

INTRODUCTION

The Sakhalin taimen *Hucho perryi* is only distributed in Sakhalin Island, the southern Kurile Islands, the Primorye region of Siberia, and the northernmost island of Japan, Hokkaido.[1] The species is iteroparous and reproducible up to about 16 years of age,[2] and is the largest salmonid in the western Pacific Ocean, attaining weights up to 25–60 kg.[3,4] They are partly anadromous.[5] However, even the anadromous form is often land-locked by artificial dams and obliged to spend the non-breeding period in freshwater lakes. Especially in Hokkaido, they are now very rare, seriously endangered and close to extinction.[6] Therefore, adequate management and conservation of this species is very important.

In teleosts, since cryopreservation is an effective method for gene-banking endangered species, the development of appropriate techniques for sperm cryopreservation is essential. Sperm has been successfully cryopreserved in a relatively large number of fish species,[7] including salmonid species.[8] Recent advances in chromosome manipulation to induce androgenetic diploid genotypes have made it possible to generate individuals from cryopreserved sperm. Bercsényi *et al.*[9] have successfully achieved interspecific androgenesis using genetically-inactivated eggs of one species and normal sperm of another in cyprinids. Gamma ray-irradiated eggs of common carp *Cyprinus carpio* were fertilized with cryopreserved sperm of goldfish *Carassius auratus* and the androgenetic haploid embryos were then heat-shocked to restore diploidy and to produce viable offspring. Babiak *et al.*[10] have also produced viable diploids in androgenetic rainbow trout *Oncorhynchus mykiss* by using cryopreserved sperm. Therefore, sperm cryopreservation is the first step for regeneration of endangered species. Successful sperm cryopreservation has been reported in Danube salmon *Hucho hucho*,[11,12] which is distributed in Eurasia. In the Sakhalin taimen, the optimum diluent for sperm cryopreservation has not been systematically investigated in terms of the combination of cryoprotectant and extender, nor has cryopreservation yet been attempted. The present study was conducted to develop a suitable cryopreservation diluent for Sakhalin taimen spermatozoa. We examined the effects of the combination of cryoprotectant and extender, and those of a concentra-

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Received 20 May 2004. Accepted 6 October 2004.

tion of cryoprotectant on the post-thaw motility of Sakhalin taimen spermatozoa.

MATERIALS AND METHODS

Collection of gametes

Twenty-two 7-year-old males of Sakhalin taimen were used in this study. They were raised in outdoor ponds under natural light conditions until the time of final maturation at Ibeshibetsu hatchery of the Akan Fisheries Cooperative Association (Akan, Hokkaido). At the time of collection the fish were anesthetized with 2-phenoxyethanol in order to allow more complete control during the collection process. Ripe males were wiped with a damp towel to avoid contamination of the milt with water and mucus. Semen was collected in a Petri dish by gently pressing the abdomen of the fishes. When the amount of expressible semen from one male was very little, those from up to three males were pooled and used as a single sample. In order to evaluate motility of fresh sperm, semen from each sample was diluted 1:1000 with a 120-mM $NaHCO_3$ solution (thawing solution),[13] and then sperm motility was assessed 10 s after dilution by modified grades that were decided by Ohta *et al.*[14] Samples with percentages of cells with progressive motility under 50% were discarded. The percentage of motile spermatozoa ranged from approximately half to three-quarters of spermatozoa in six samples, whereas more than three-quarters of spermatozoa in another five samples. Sperm concentration was determined by mixing 1 μL of semen with 1 mL of artificial seminal plasma (ASP) for masu salmon.[15] Cell counts were performed using a hemocytometer chamber (Thoma). The sperm concentration of the semen for experiments ranged from 2.9×10^9 cells/mL to 11.6×10^9 cells/mL in 11 samples and the average was $8.1 \pm 0.6 \times 10^9$ cells/mL. Semen was kept in a Petri dish placed on crushed ice and used for experiments within 4 h.

Freezing and thawing

Cooling of semen was carried out at Ibeshibetsu hatchery by the pellet method. Fresh semen was diluted six times with diluent containing cryoprotectant and extender. A total of 100 μL of the diluted semen was dropped within 1 min after dilution on a small depression, about 5 mm in diameter and 3 mm depth, of dry ice (–79°C). After 5 min, frozen pellets were immersed in liquid nitrogen (–196°C), transferred to the laboratory of Hokkaido Fish Hatchery (Eniwa, Hokkaido), and cryopreserved for more than 19 days.

One pellet was taken from the container of liquid nitrogen with pre-cooled tweezers, immersed in 5 mL of a thawing solution[13] at 25°C, mixed for 1 or 2 s on a vortex mixer and then thawed. The pellet melted away within about 7 s.

Using this procedure, the post-thaw motility was examined under the following various parameters: (i) cryoprotectant (glycerol, dimethyl sulfoxide [DMSO], methanol); (ii) concentration of these cryoprotectants (0, 5, 10, 15 and 20%); and (iii) extender (fetal bovine serum [FBS], ASP, 300 mM glucose solution [GS]).

Evaluation of sperm motility

To estimate the post-thaw motility of cryopreserved sperm, an aliquot of the activated sperm was transferred immediately onto a glass slide, and motility was observed without a cover slip. The motility was recorded with a VHS video-recorder (model A-J1; Toshiba Co., Tokyo, Japan) and video camera (model KP-C251, Hitachi Electronics Engineering Co. Ltd., Tokyo, Japan) connected to a microscope (Microphot-FX; Nikon Co, Tokyo, Japan) system (an object lens of ×20). The video recording was started simultaneously with the loss of the pellet's shape in thawing solution. Motility was observed 10 s after thawing.

Spermatozoa were considered motile when the sperm head showed forward movement under the consecutive video frames from 10 s after thawing. Percent motility was determined by assessing the motility of at least 50 randomly selected spermatozoa for each measurement. Measurements at each dilution were repeated twice using two pellets, and the average result was used in the data analysis.

Statistical analyses

All data are represented as mean ± standard error of the mean (SE). Percent motilities were transformed by angular transformation and analyzed statistically using ANOVA and the Tukey test (Honestly significant difference test).[16] A value of $P < 0.05$ was considered to represent statistical significance.

RESULTS

Cryoprotectant and extender

The effects of all possible combinations of various 10% cryoprotectants and 90% extenders in the

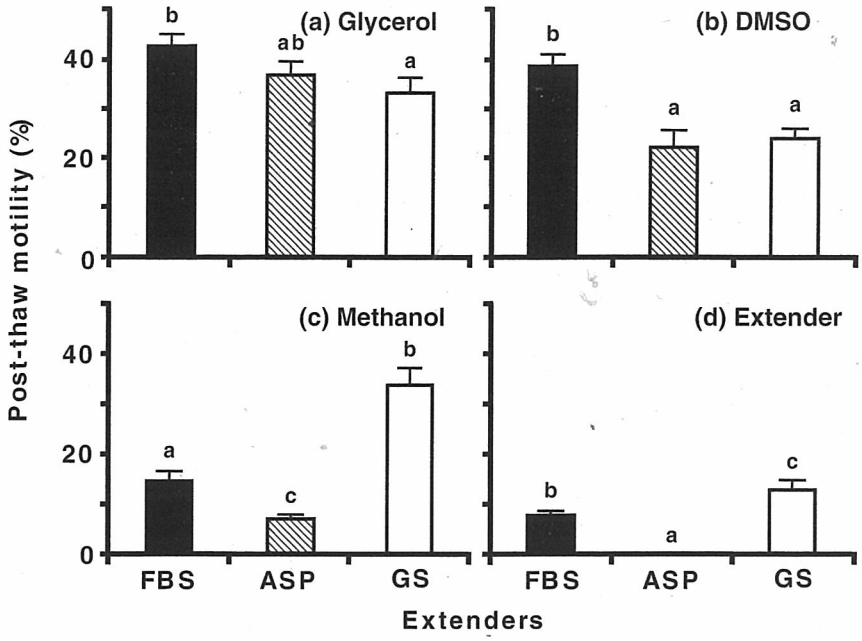

Fig. 1 Post-thaw motility of spermatozoa cooled with 10% cryoprotectants and 90% extenders, or cooled with 100% extenders. (a) glycerol, (b) DMSO, (c) methanol, (d) extender alone. Values represent the mean ± SE for milt from six samples. Within each graph, means with different letters are significantly different ($P < 0.05$).

diluent on motility 10 s after thawing were examined (Fig. 1). In the case of glycerol, cryopreserved sperm showed the high post-thaw percent motility in each extender, especially highest in FBS (Fig. 1a). When DMSO was diluted with FBS, cryopreserved sperm in this diluent showed a significantly higher percent motility than ones with other extenders (Fig. 1b); while sperm cryopreserved with the diluent consisting of methanol and GS showed the highest percent motility among those with other extenders (Fig. 1c). When the diluent lacked cryoprotectant, cryopreserved sperm in GS showed the highest post-thaw percent motility of all the extenders examined (Fig. 1d). A higher post-thaw percent motility was also obtained in FBS than in ASP. The optimum combination between cryoprotectants and extenders was determined in this experiment, that is, glycerol plus FBS, DMSO plus FBS, and methanol plus GS.

The effects of the concentration of three cryoprotectants (glycerol, DMSO and methanol) in these diluents were examined by observing the motility 10 s after thawing (Fig. 2). In the case of glycerol (Fig. 2a), although no significant difference between different concentrations in the range of 5–20% glycerol was observed in terms of the percent motility, the percentages of motile spermatozoa increased with the increasing glycerol concentration, were the highest at 10%, and decreased with more than 15%. In the case of DMSO and methanol (Fig. 2b,c), the percent motility showed a similar tendency and was also the maximal at 10% of all concentrations of them examined.

Comparison of motility between present diluents and other

When sperm was cooled in the above-mentioned three diluents (10% glycerol plus 90% FBS, 10% DMSO plus 90% FBS, and 10% methanol plus 90% GS) and diluent containing 10% DMSO and 90% GS[13] that has been widely used in sperm cryopreservation of salmonid fish, no significant difference among these three diluents was observed in motility 10 s after thawing; however, they showed significantly higher percent motility than that of the diluent consisting of 10% DMSO and 90% GS (Fig. 3).

DISCUSSION

The goal of this study was to develop a suitable cryopreservation diluent for Sakhalin taimen spermatozoa. Since the pellet method has been widely used in a relatively large number of laboratories and salmon hatcheries because of its favorable post-thaw fertility and its simplicity,[17] it was carried out in this study. Fertilization tests were difficult in the Sakhalin taimen because a large number of eggs were required for these experiments. Post-thaw motility was chosen as the criterion to determine a suitable cryopreservation diluent.

In the present study, the results of sperm motility 10 s after thawing suggest that 10% glycerol in FBS, 10% DMSO in FBS, and 10% methanol in GS are the optimum diluents. Lahnsteiner *et al.*[11] demon-

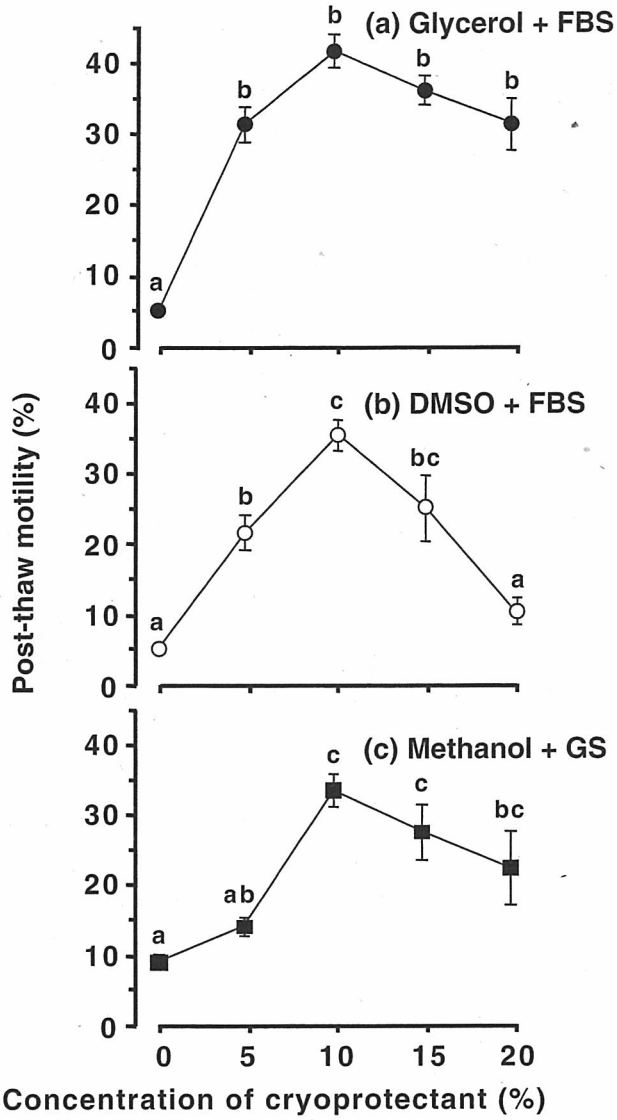

Fig. 2 Post-thaw motility of spermatozoa cooled with three diluents containing 0–20% each cryoprotectant. (a) glycerol + FBS, (b) DMSO + FBS, (c) methanol + GS. Values represent the mean ± SE for milt from five samples. Within each graph, means with different letters are significantly different ($P < 0.05$).

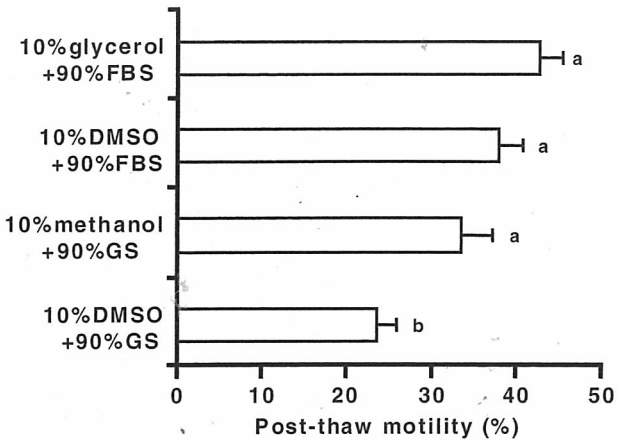

Fig. 3 Post-thaw motility of spermatozoa cooled with four diluents 10 s after thawing. Values represent the mean ± SE for milt from six samples. Means with different letters are significantly different ($P < 0.05$).

strated the successful cryopreservation of the Danube salmon spermatozoa using diluent containing 10% methanol as permeating cryoprotectant and 90% extender (103 mM NaCl, 40 mM KCl, 1 mM $CaCl_2$, 0.8 mM $MgSO_4$, 20 mM HEPES [pH 7.8], 1.5% bovine serum albumin, 7% egg yolk, 0.5% sucrose) by the straw method. Glogowski *et al.*[12] also showed that the diluents containing 10% DMSO, 300 mM glucose, 25 mM KCl, and 10% yolk or that containing 20% glycerol, 300 mM glucose, and 10% yolk, were the most suitable for the cryopreservation of the Danube salmon spermatozoa by the pellet method. They obtained 70–90% eyed stage embryos after fertilization with cryopreserved spermatozoa as compared to the control fertilization rate. Thus, methanol, DMSO and glycerol are likely to be effective cryoprotectants for spermatozoa in Danube salmon. This is consistent with the results of the present study. On the other hand, although glycerol has been known to be inadequate as cryoprotectant of other salmonids[18–20] and striped bass *Morone saxatilis* sperm[21] because of the resultant low fertility, the diluent with glycerol in this study gave high post-thaw motility. The discrepancy between the present result and the previous studies[18–21] is not clear at present. Glycerol may cause lower fertility after insemination. Further studies, especially fertilization experiments, will be required.

In this study, the optimum concentration of cryoprotectants was 10%. It was the same as that in previous studies of Danube salmon,[11,12] and was covered within limits of the partial concentrations of cryoprotectants (5–20%),[7] at which cryoprotectants displayed the beneficial effect of cryoprotection on cryopreserved sperm. The concentration (5–20%) of glycerol did not affect post-thaw motility; whereas the limits of effective concentration of DMSO and methanol, allowing better post-thaw motility to be achieved, were smaller than that of glycerol. These results might be caused by the nature (for example, molecular weight) of cryoprotectants examined. Harvey[22] reported that cryoprotection was maximal when 5% methanol plus 15% powdered milk as intracellular and extracellular cryoprotectants were used

for cryopreservation of Tilapia *Sarotherodon mossambicus* spermatozoa, and either cryoprotectant alone provided lower protection ability than their combination. Therefore, the limits of the protecting effect of methanol could be improved by adding extracellular cryoprotectants in the present diluents.

These three diluents recommended in this study gave a significantly higher motility than that of a mixture of 10% DMSO and 90% GS. Therefore, they were considered to be suitable cryopreservation diluents for the Sakhalin taimen spermatozoa and would be available as the cryopreservation diluents for sperm in other salmonids. Since fertilization tests could not be conducted in this study, the relationship between motility and fertility remains unclear. It is known that the post-thaw fertility and percent motility of cryopreserved spermatozoa were positively correlated in rainbow trout,[8] amago salmon *Oncorhynchus masou ishikawae*,[23] northern pike *Esox lucius*[24] and zebrafish *Brachydanio rerio*.[25] When the effects of GS containing 10% methanol or 10% DMSO were examined on the post-thaw fertility in a preliminary experiment, cryopreserved spermatozoa in the Sakhalin taimen showed a significantly higher fertility in the former ($82.0 \pm 0.9\%$) than in the latter ($45.1 \pm 2.2\%$).[26] Thus, the effect of methanol on post-thaw motility is likely linked up with its fertility. Further investigation should be conducted to clarify the relationship between motility and fertility of cryopreserved spermatozoa in the Sakhalin taimen as well as to decide the best diluent.

In conclusion, this study indicated that mixtures of 10% glycerol plus 90% FBS, 10% DMSO plus 90% FBS, and 10% methanol plus 90% GS would be suitable as the cryopreservation diluent for Sakhalin taimen spermatozoa.

ACKNOWLEDGMENTS

We wish to thank Mr Shizuo Kimura, Nanae Fresh Water Laboratory, Field Science Center for Northern Biosphere, Hokkaido University, and the staff of the Akan Fisheries Cooperative Association for their support. We also wish to thank Mrs Yukie Hujimoto and the staff of Hokkaido Fish Hatchery for their support. This study was supported in part by grants from Japanese Fisheries Resource Conservation Association.

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