イトウ凍結精子における適切な耐凍剤の検討

誌名	水産増殖
ISSN	03714217
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発行元	水産増殖談話会
巻/号	52巻2号
掲載ページ	p. 171-175
発行年月	2004年6月

Suisanzoshoku **52**(2), 171－175 (2004)

Cryopreservation of Sakhalin Taimen *Hucho perryi* Spermatozoa: Effect of Cryoprotectants on Post-thaw Fertility

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Abstract: To develop the cryopreservation diluent for spermatozoa of the endangered Sakhalin taimen, we examined the effect of the nature of cryoprotectants on post-thaw fertility. Fresh semen was diluted 4 times with each diluent, comprising 90% 300 mM glucose and 10% each cryoprotectant (dimethyl sulfoxide (DMSO), methanol, or glycerol). The 100 μl of diluted semen was dropped on dry ice to freeze and immersed in liquid nitrogen for over 2 h. Four pellets were thawed in 16 *ml* of a 120 mM $NaHCO_3$ solution at 30℃. Egg batches of 20 g were immediately inseminated with thawed semen (Sperm to egg ratio was $5.0 \times 10^6 : 1$). Semen cryopreserved with methanol showed the highest fertilization rate (82.0 ± 0.9%) among all the cryoprotectants examined. The fertilization rate of semen diluted with DMSO (45.1 ± 2.2%) was significantly higher than that with glycerol (12.7 ± 2.0%). Semen cryopreserved with methanol yielded almost similar post-thaw fertility to that of 50 μl of fresh semen (78.4 ± 2.4%) at the sperm to egg ratio of $2.5 \times 10^6 : 1$. These results indicate that methanol is the suitable cryoprotectant for Sakhalin taimen spermatozoa.

Key words: *Hucho perryi*; Cryopreservation; Spermatozoa; Cryoprotectant

In Japan, wild populations of the Sakhalin taimen *Hucho perryi* are only distributed in some rivers and lakes in Hokkaido (Kimura 1966). The species is anadromous (Kawamula et al. 1983), iteroparous (Gritsenko et al. 1974), and reproducible up to about 16 years of age with large body size occasionally reaching *c.* 60 kg (Berg 1962; Khatkevich 1973). They are currently very rare and are classed as endangered in Japanese river systems primarily because of severe habitat degradation including stream channelization (Japanese Environment Agency 1999). Therefore, adequate management and conservation of this species are strongly required. Sperm cryopreservation is an effective method for gene-banking endangered teleost species.

In Danube salmon *Hucho hucho* distributed in Eurasia, sperm cryopreservation has already been succeeded using the straw (Lahnsteiner et al. 1996) and pellet method (Glogowski et al. 1997). These studies indicated that methanol (Lahnsteiner et al. 1996), DMSO (Glogowski et al. 1997) and glycerol (Glogowski et al. 1997) were effective cryoprotectants of cryopreserved spermatozoa. In endangered Sakhalin taimen, however, sperm cryopreservation has not been systematically optimized. In this study, we examined the effect of the nature of cryoprotectants on post-thaw fertility in order to determine the suitable cryoprotectants for sperm cryopreservation of Sakhalin taimen.

Received October 8, 2003: Accepted May 21, 2004.

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Materials and Methods

Collection of gametes

Six-year-old males ($n = 9$) and females ($n = 19$) of the 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 Fishermen's Cooperative Association. Semen was collected by gently pressing the abdomen of the fishes. Motility of spermatozoa from each individual male was analyzed by modified criteria that were decided by Ohta et al. (1995b). Samples in which more than 50% of spermatozoa exhibited progressive motility were used for the experiments. The percentage of motile spermatozoa ranged between about 50% and 75% in six males, while over 75% of spermatozoa was motile in three males. Sperm concentration was determined by mixing 1 μl of semen in 1 *ml* of artificial seminal plasma (ASP) for masu salmon *Oncorhynchus masou masou* (Ohta et al. 2000). Cell counts were performed using a hemocytometer chamber (Thoma). Sperm concentration ranged between 7.5×10^9 and 13.2×10^9 cells/*ml* in nine males and the average was $10.5 \pm 0.7 \times 10^9$ cells/*ml*. Semen from nine males were pooled for the following experiments, because the amount of expressible semen from one male was very little. Pooled semen was kept in a petri dish placed on crushed ice and used for experiments within 4 h. Eggs were collected into a sieve by pressing the abdomen of the fishes gently and the ovarian fluid was discarded. They were pooled, well mixed, separated into egg batches of 20 g and stored for a maximum of 1 h at a temperature of 10 − 12℃ before the onset of the experiments.

Semen cryopreservation

The pellet method has been world-widely used. Fresh semen was diluted 4 times with the diluent. Each diluent consists of 90% 300 mM glucose and 10% cryoprotectant such as dimethyl sulfoxide (DMSO), methanol, or glycerol. The 100 μl of diluted semen were dropped to freeze on small dimples, about 5 mm in diameter and 3 mm depth, of dry ice (−79℃) within one min after dilution. After 3 min, frozen pellets were immersed in liquid nitrogen (−196℃) for at least 2 h. The cryoprotectant concentration, the dilution ratio, and the pellet volume employed in this study were decided in a preliminary experiment. These conditions were the same as those in previous studies of Danube salmon (Glogowski et al. 1997), amago salmon *O. masou ishikawae* (Ohta et al. 1995a), masu salmon (Ohta et al. 1995a), and Atlantic salmon *Salmo salar* (Stoss and Refstie 1983).

Fertilization assays

In fertilization assays, four pellets were taken from the container of liquid nitrogen with pre-cooled tweezers, immersed in 16 *ml* of a 120 mM $NaHCO_3$ solution (thawing solution, Stoss and Refstie 1983) at 30℃, mixed for 1 or 2 s on a vortex mixer and then thawed. The pellet melted away within about 7 s. Egg batches of 20 g (210 to 217 eggs) were immediately inseminated with the thawed semen.

To determine a minimum volume of fresh semen required for satisfactory fertility in egg batches, eggs were inseminated with different volume of semen. Each volume (1 to 400 μl) of semen was diluted up to 400 μl with the extender consisting of 90% 300 mM glucose and 10% DMSO. As the diluted control without DMSO, semen of 100 μl was also diluted 4 times with the ASP. Egg batches of 20 g (211 to 220 eggs) were inseminated with diluted semen with total 400 μl and then immediately 16 *ml* of thawing solution at 10℃ were added.

The thawed or fresh semen and eggs were gently mixed for 15 s; about 1 min later, well water was added to complete the fertilization process. Eggs were incubated in plastic boxes filled with fresh water at about 8℃ for more than 14 h, and fixed with Bouin solution to investigate the fertilization rates. The fertilization rate was expressed as the percentage of cleaved embryos at the 8 to 32 cell stage in relation to the total number of eggs examined. All fertilization experiments except for both fresh semen of 400 μl and semen diluted with ASP were carried out in

triplicate using pooled semen.

Statistical analyses

All data are represented as mean ± SE. Fertilization rates were transformed by angular transformation and analyzed statistically using an ANOVA and the Tukey test (Honestly significant difference test, Zar 1999). A value of $P < 0.05$ was considered to represent statistical significance.

Results

Fertilization by minimum amount of fresh semen

Fertilization rates were examined, when egg batches were inseminated with various volume of fresh semen (Fig. 1). The percentages of fertilized eggs increased with increased volume of semen. When more than 50 μl of fresh semen was used, fertilization rates reached plateau (78.4 − 86.7%). Therefore, the amount of semen equivalent to 100 μl of fresh semen was used in the following fertilization experiments with cryopreserved semen.

Effect of cryoprotectants on the post-thaw fertility

Post-thaw fertilities of cryopreserved semen were examined, when semen was diluted with 90% 300 mM glucose and various 10% cryoprotectants and then frozen (Fig. 2). Methanol gave the highest fertilization rate (82.0 ± 0.9%) among all the cryoprotectants examined. DMSO gave the significantly higher fertilization rate (45.1 ± 2.2%) than glycerol (12.7 ± 2.0%). The fertilization rates in methanol and DMSO treatment were almost similar to those of 50 μl (78.4 ± 2.4%) and 10 μl (56.0 ± 2.3%) of fresh semen, respectively. Sperm to egg ratio is 5.0×10^6 : 1 in cryopreserved semen, 2.5×10^6 : 1 in fresh 50 μl semen, and 0.5×10^6 : 1 in fresh 10 μl semen.

Discussion

When the post-thaw fertilities of cryopreserved semen were compared with three diluents containing DMSO, methanol, or glycerol, which are reported as effective cryoprotectants for other salmonid spermatozoa (Stoss 1983; Leung and Jamieson 1991; Piironen 1994; Lahnsteiner

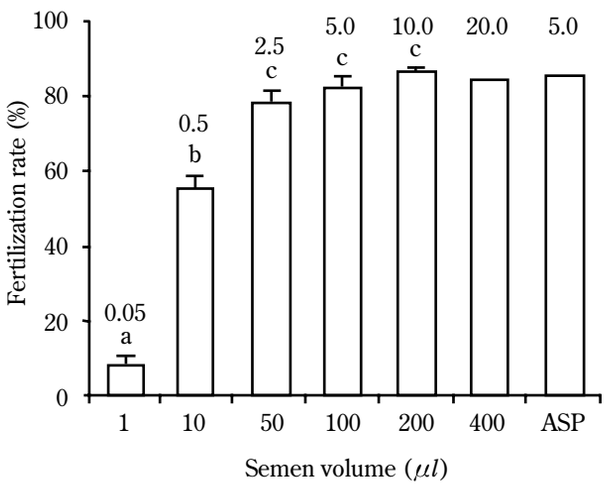

Fig. 1. Comparison of fertilization rates when egg batches of 20 g were inseminated with the different volume of fresh semen. ASP indicates fertilization rate on condition that egg batches were inseminated with 100 μl of fresh semen diluted 4 times with the ASP without DMSO. The numbers above each column indicate the number ($\times 10^6$) of spermatozoa per egg. Columns and bars represent the means and SE, respectively, of three experiments (1 to 200 μl); experiments (400 μl and ASP) were once. Means with different letters are significantly different ($P < 0.05$).

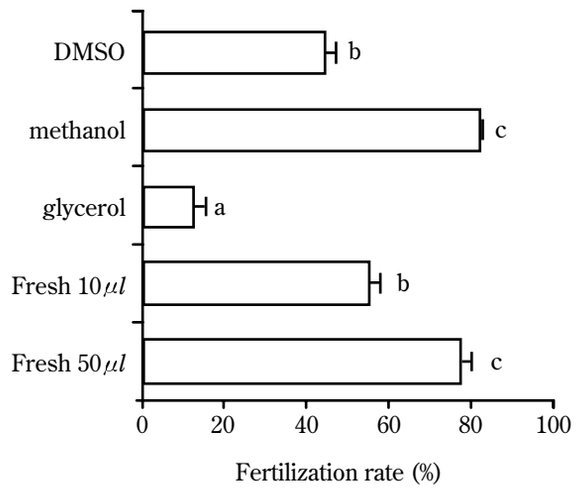

Fig. 2. Effects of cryoprotectants on the post-thaw fertility. Fresh 10 and 50 μl indicate the fertilities of 10 and 50 μl of fresh semen, respectively. These values are obtained by the same procedure in Fig. 1. Sperm to egg ratio is 5.0×10^6 : 1 in cryopreserved semen, 0.5×10^6 : 1 in fresh 10 μl semen, and 2.5×10^6 : 1 in fresh 50 μl semen. Values represent the means ± SE of three experiments. Means with different letters are significantly different ($P < 0.05$).

2000; Chao and Liao 2001), methanol was considered to be the optimum one. Cryopreserved semen with methanol showed post-thaw fertility approximately two times higher than those with DMSO. Lahnsteiner et al. (1996) reported that 10% methanol was the most effective cryoprotectant of cryopreserved spermatozoa in Danube salmon by the straw method. This is consistent with the present results. Glogowski et al. (1997) also demonstrated that the diluent containing 10% DMSO, 300 mM glucose, 25 mM KCl, and 10% hen egg yolk gave the highest post-thaw fertility ($87.5 \pm 2.1\%$) by the pellet method, whereas it gave the lowest fertility ($1.5 \pm 0.3\%$) under the condition without KCl and egg yolk. It is well known that, in salmonid fishes (Morisawa and Morisawa 1988; Ohta et al. 2000), shishamo smelt *Sipirinchus lanceolatus* (Ohta et al. 1995b) and ayu *Plecoglossus altivelis altivelis* (Tsuji et al. 2000), K^+ ions play a crucial role in the inhibition of sperm motility in incubation media such as ASP. External cryoprotectants such as egg yolk are also known to stabilize the sperm cell membrane against cryodamage (Cabrita et al. 2001). Since KCl and egg yolk were not used in this study, their effects on cryopreserved spermatozoa were unknown. Addition of K^+ ions or extracellular cryoprotectants to the diluent may improve the post-thaw fertility.

In this study, sperm to egg ratios were $5.0 \times 10^6 : 1$ (sperm concentration, 6.4×10^7 cells/ml), $2.5 \times 10^6 : 1$ (3.2×10^7 cells/ml), and $0.5 \times 10^6 : 1$ (0.6×10^7 cells/ml) at insemination using cryopreserved semen, $50 \mu l$ and $10 \mu l$ of fresh semen, respectively. Four pellets of cryopreserved semen with diluent containing methanol showed similar fertilization rate to $50 \mu l$ of fresh semen, while those with DMSO to $10 \mu l$ of fresh semen. These results suggest that about half of spermatozoa diluted with the diluent containing methanol would retain the fertilizability after freezing and thawing. More detailed analysis concerning the relationship between the volume of cryopreserved semen and fertility is necessary to discover the reliable cryopreservation efficiency of Sakhalin taimen spermatozoa. Lahnsteiner (2000) reported that sperm to egg ratios, required for reliable fertility in the range of 90－100% of fresh semen, were species specific and amount to approximately $(2.5 - 5.5) \times 10^6 : 1$, when the fertilizing capacity of post-thaw semen were compared among six salmonid species. Since the sperm to egg ratio at insemination using cryopreserved semen with the diluent containing methanol in this study was within limits of that as described by Lahnsteiner (2000), methanol was considered the most effective cryoprotectant of cryopreserved spermatozoa in Sakhalin taimen by the pellet method in terms of post-thaw fertility.

Acknowledgments

We thank Mr. Shizuo Kimura, Nanae Fresh Water Laboratory, Field Science Center for Northern Biosphere, Hokkaido University, and the staff of the Akan Fishermen's Cooperative Association and Hokkaido Fish Hatchery for their support. We also thank two anonymous reviewers for improving earlier version of the manuscript. This study supported in part by grants from Japanese Fisheries Resource Conservation Association.

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イトウ凍結精子における適切な耐凍剤の検討

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イトウ精子の凍結保存における適切な保存液を開発するため，受精率を指標に耐凍剤の種類を検討した。300 mM グルコースを90%，耐凍剤（DMSO，メタノール，グリセリン）を10%含む保存液で精液を4倍に希釈し，予めドライアイスに開けた小孔に100 μl ずつ滴下し凍結した。滴下3分後に各ペレットを液体窒素に投入し，2時間以上浸漬した。各種ペレット4粒を30℃の120 mM $NaHCO_3$ 16 *ml* 中で解凍し，20 g の卵に媒精した。卵は8℃の水中に14時間以上静置した後，ブアン氏液で固定し，卵割の有無を指標に受精率を算出した。受精率は耐凍剤がメタノールの場合に最も高く，DMSO，グリセリンの順に有意に低下した。メタノールを含む保存液で凍結した精液は，DMSO を含む保存液で凍結した精液より約2倍高い受精率を示した。以上の結果は，メタノールがイトウ精子の耐凍剤として適していることを示唆している。