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Cold Storage of Sperm in Marbled Sole, *Limanda yokohamae*

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문치가자미, *Limanda yokohamae* 정자의 냉장보존

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A series of experiments were conducted to compare the effects of various diluents in cold storage on the marbled sole, *Limanda yokohamae* sperm.

Various diluents of glucose, *L. yokohamae* serum, marine fish Ringer's solution, sodium citrate and Ca^{2+} free artificial seawater (ASW) (tris-HCl, pH 7.4) containing 3 mM ethylene glycol-bis(2-aminoethyl ether)tetraacetic acid (EGTA) were used to store the sperm at 4℃. The storage effect was evaluated using motility index and survival rate of sperm. Glucose and sodium citrate were found to be better diluents which maintained high motility and survival rate of sperm for a storage period of 10 days.

Some morphological changes of spermatozoa were observed during the cold storage with diluents. In particular, a detachment of the nuclear envelope and of the plasma membrane from the nucleus in spermatozoa was observed. Morphological normality of the stored spermatozoa diluted with 0.3 M glucose was better than that of the stored spermatozoa undiluted or diluted with Ca^{2+} free ASW containing 3 mM EGTA.

Key words : Marbled sole, *Limanda yokohamae*, Sperm, Cold storage, Diluent

Introduction

As fish farming expands and the harvesting of wild stocks becomes more intense, there is a growing need for techniques of sperm storage to facilitate artificial reproduction procedures and to preserve desirable gene pools. The cold storage of unfrozen fish gamete is short-term preservation, while the cryopreservation of frozen fish gamete is long-term preservation. The cold storage of fish sperm, for time periods ranging from a few days to several weeks, is applied mostly in hatcheries to overcome problems such as asynchrony in maturation, difficulties in gamete transportation.

Although considerable research has been carried out on sperm storage in salmonids, comparatively little attention has been given to the various important marine species for cultivation.

In cold storage of fish sperm, two methods are used : undiluted and diluted (Scott and Baynes, 1880 ; Stoss, 1983). The use of diluents provides a better control of the physicochemical conditions during the storage (Stoss, 1983), so that the storage of sperm with a diluent of optimal constituents often prolongs its viability in comparison with undiluted storage (Hogendoorn and Vismans, 1980 ; Hara et al., 1982 ; Withler and Lim., 1982 ; Harvey and Kelley, 1984 ; Takahashi et al., 1987). As the constitution of the diluent considerably influences suc-

cessful storage, the development of an appropriate diluent is indispensable for the efficient cold storage of fish sperm.

In the present study, attempts were made to preserve *Limanda yokohamae* sperm, both undiluted and diluted, in various media at 4℃ in a refrigerator. The storage effect was evaluated using a motility index and survival rate and the morphological change of the spermatozoa.

Materials and Methods

1. Broodstock handling and sperm collection

Mature male *L. yokohamae* was caught in the sea near Yosu and reared at 7~12℃ in a rearing tank at the South Sea Regional Fisheries Institute, National Fisheries Research and Development Institute, Korea.

To collect the sperm, the fish were anesthetized in lidocain (200 ppm). Accumulated urine and feces were removed by gently pressuring the areas around the genital orifice and the urinary bladder. Sperm was then obtained by abdominal pressure and stored in sealed test tubes with crushed ice until use. Only sperm which showed high motility in artificial seawater (ASW)[1] was used in the experiment.

2. Cold storage of sperm

1) Cold storage of sperm in various diluents

Various diluents of glucose, *L. yokohamae* serum, marine fish Ringer's solution (MFRS)[2], sodium citrate and Ca^{2+} free ASW[3] (tris-HCl, pH 7.4) containing 3 mM ethylene glycol-bis (2-aminoethyl ether)tetraacetic acid (EGTA) were used to store the sperm. Milt samples of 1 ml were diluted with each of the diluents at a rate of 1:2 in 6 ml vials. After gentle mixture, the vials were capped with sealing tape to minimize evaporation and contamination. Undiluted milt was also used as a control. Triplicate samples of pooled milt from different males were kept at temperature of 4℃ in a refrigerator. Motility and survival rate of sperm were observed daily for 10 days.

2) Evaluation in motility and survival rate of sperm

To evaluate the storage effects of different diluents, the motility of *L. yokohamae* sperm was examined microscopically (×200) on a glass slide without a cover, after that the sperm have been diluted with ASW at a rate of 1:4.

A numerical index from Strussmann et al. (1994) was used to assess the sperm motility.

In order to estimate the survival rate, an eosin-nigrosin staining technique (Blom, 1950 ; Fribourgh, 1966) was used to determine the percentage of live sperm in each sample.

One-way analysis of variance and Tukey test (Zar, 1984) were used to test for difference at $P<0.05$.

3) Fine structure of spermatozoa in pre- and post-storage

To examine the external shape of fresh spermatozoa after stripping, samples of the spermatozoa were fixed for 2 hr in 5% glutaraldehyde and 10% sucrose, buffered to pH 7.2 with phosphate. Later, dehydrated samples were gold coated and examined under a scanning electron microscope (JSM-T330A).

The morphological changes of the spermatozoa between pre- and post-storage were also examined using a transmission electron microscope (TEM). The sperm was stored for 7 days at 4℃ with undiluted, 0.3 M glucose and Ca^{2+} free ASW (tris-HCl, pH 7.4) containing 3 mM EGTA, respectively. Specimens of fresh spermatozoa were used as a control. Spermatozoa from each treatment were fixed for 2 hr at 4℃ in 2.5% glutaraldehyde, buffered to pH 7.2 with phosphate. Samples were then washed with the same buffer. They were post-fixed for 2 hr in 1% osmium tetroxide. After dehydration through an ascending ethanol series, the samples were embedded in Spurr resin for TEM obser-

[1]NaCl 2.7 g+KCl 0.07 g+$CaCl_2$ 0.12 g+$MgCl_2$ 0.46 g+$NaHCO_3$ 0.05 g+distilled water 100 ml

[2]NaCl 1.35 g+KCl 0.06 g+$CaCl_2$ 0.025 g+$MgCl_2$ 0.035 g+$NaHCO_3$ 0.002 g+distilled water 100 ml

[3]NaCl 2.7 g+KCl 0.07 g+$MgCl_2$ 0.46 g+$NaHCO_3$ 0.05 g+distilled water 100 ml

vation. Ultrathin sections were contrasted with alcoholic uranyl acetate and lead citrate (Reynolds, 1963). They were then examined with TEM (JEM 1200 EX-II, 60~80 Kv, JEOL). Changes in the nucleus, nucleus membrane and plasma membrane of stored spermatozoa were compared with the fresh spermatozoa.

Results

1. Cold storage of sperm in various diluents

The changes in motility and the survival rate of sperm after 10 days of storage at 4℃ are shown in Figs. 1~3. Sperm motility was extended following 10 days of cold storage in all diluents. A high motility index of 3.0~4.0 was observed for the first 3 days in 0.3 M glucose and a motility index of 2.0~3.0 for the followings 4 days. A relatively high motility index of 2.3~3.0 was observed for 2 days in undiluted sperm and for 1 day in 0.1 M glucose, 0.5 M glucose, 0.05 M sodium citrate and 0.15 M sodium citrate. But, the sperm motility index for the sperm diluted with *L. yokohamae* serum, MFRS, and Ca^{2+} free ASW with and without 3 mM EGTA was unsatisfactory. In the mean time, the changes in the survival rate were similarly to those in motility. After 10 days of storage, the sperm preserved in 0.05 M and 0.15 M sodium citrate showed the highest survival rate of 66.9% and 63.2%, respectively ; whereas the sperm, which was preserved in *L.*

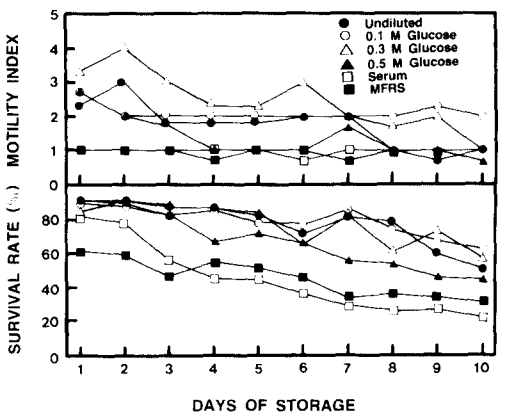

Fig. 1. Changes in the motility and survival rate of *Limanda yokohamae* sperm stored with five different kinds of diluents at 4℃. MFRS : marine fish Ringer's solution.

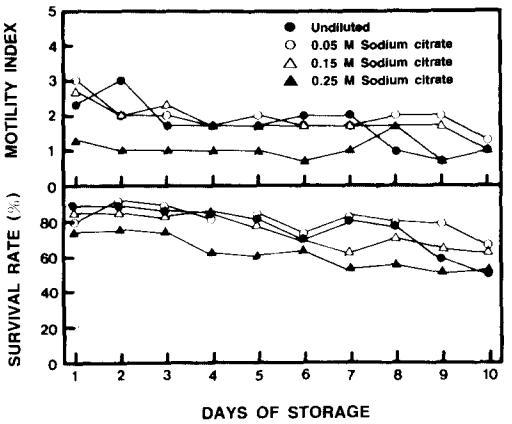

Fig. 2. Changes in the motility and survival rate of *Limanda yokohamae* sperm stored with three different concentration of sodium citrate diluents at 4℃.

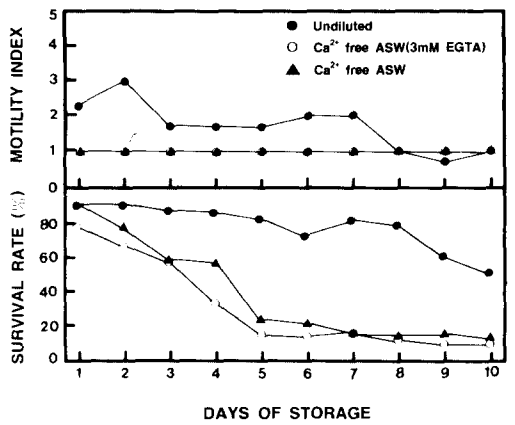

Fig. 3. Changes in the motility and survival rate of *Limanda yokohamae* sperm in diluent of Ca^{2+} free artificial seawater (ASW) containing 3 mM EGTA stored at 4℃.

yokohame serum and Ca^{2+} free ASW with or without 3 mM EGTA, showed the lowest survival rate of 21.9%, 12.7% and 8.5%, respectively (Table 1).

2. Fine structure of spermatozoa in pre- and post-storage

The external shape of spermatozoa of *L.*

Table 1. Effects of various diluents on motility and survival rate of *Limanda yokohamae* sperm

Diluted with	Days of storage	Motility index	Survival rate[1] (%)
0.1 M glucose	10	2.0	62.2 ± 5.1^{bc}
0.3 M glucose	10	1.0	56.8 ± 9.9^{bc}
0.5 M glucose	10	0.7	44.5 ± 7.8^{cd}
0.05 M sodium citrate	10	1.3	66.9 ± 8.9^{b}
0.15 M sodium citrate	10	1.0	63.2 ± 7.3^{bc}
0.25 M sodium citrate	10	1.0	53.5 ± 5.8^{bc}
L. yokohamae Serum	10	1.0	21.9 ± 3.2^{ef}
MFRS[2]	10	1.0	30.9 ± 0.9^{de}
Ca^{2+} free ASW[3]	10	1.0	12.7 ± 1.3^{ef}
Ca^{2+} free ASW (3 mM EGTA)[3]	10	1.0	8.5 ± 3.3^{f}
Undiluted	0	3.3	95.1 ± 2.2^{a}
	10	1.0	50.3 ± 8.2^{bcd}

[1]Values within the same column with different superscripts are significantly different ($P<0.05$).
[2]Marine fish Ringer's solution.
[3]Adjusted to pH 7.4 with tris-HCl.

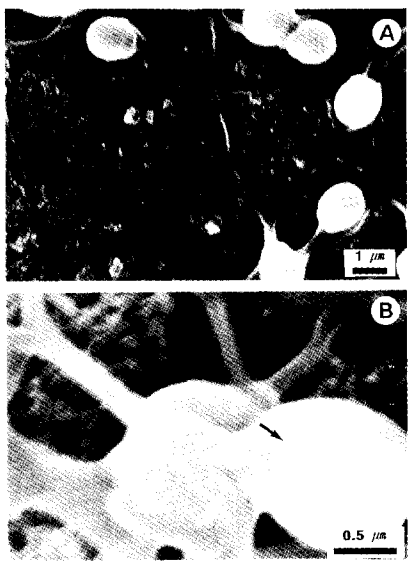

Fig. 4. The external shape of fresh spermatozoa of *Limanda yokohamae*. Ⓐ : Spematozoa. Ⓑ : Spermatozoon showing mitochondrion (an arrow).

yokohamae in pre-storage are shown in Fig. 4-A. The spermatozoon of the *L. yokohamae* had a round-shaped head, a midpiece containing mitochondria and a tail (Fig. 4-B).

The changes in the fine structure of the spermatozoa after 7 days of cold storage at 4℃ are shown in Fig. 5. The fresh spermatozoon had a dense chromatin and no acrosome (Fig. 5-A). Compared with spermatozoa in pre-storage, spermatozoa stored in undiluted spermatozoa showed no nuclear chromatin injury. But there was a detachment of the nuclear envelope and of the plasma membrane from the nucleus of the spermatozoa (Fig. 5-B). And the spermatozoa in Ca^{2+} free ASW containing 3 mM EGTA showed ruptures in both the nucleus and plasma membrane (Fig. 5-C). In contrast, the spermatozoa in 0.3 M glucose showed no morphological changes in the nucleus and plasma membrane (Fig. 5-D).

Discussion

Since Blaxter (1953) reported on the uses of diluted seawater as a diluent for herring sperm, other media had been tested as diluents for fish sperm preservation (Kubota, 1961 ; Wolf, 1963 ; Chao et al., 1975 ; Hara et al., 1982 ; Palmer, 1994). In the present study, diluents of simple composition such as glucose and sodium citrate was found to be suitable for cold storage of the *L. yokohamae* sperm. In particular, stored spermatozoa diluted with 0.3 M glucose for 7 days showed no morphological changes. These are similiar to the results on cold storage of grey mullet spermatozoa (Chao et al., 1975).

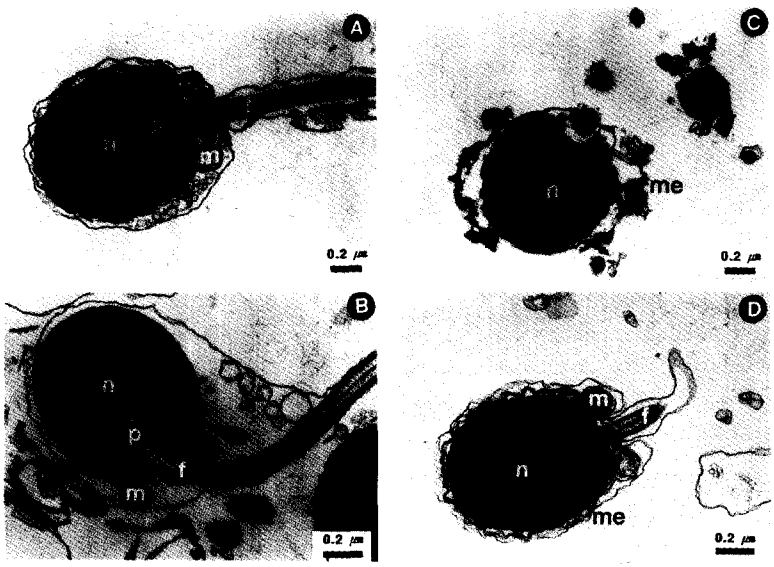

Fig. 5. The ultrastructural changes in *Limanda yokohamae* spermatozoa during cold storage at 4℃. Ⓐ : Fresh spermatozoa in pre-storage. Sagittal section of head showing cell membrane, centriole, compact chromatin, mitochondria and flagellum. Ⓑ : Stored spermatozoa undiluted for 7 days at 4℃. Sagittal section of head showing compact chromatin, centriole and flagellum. Ⓒ : Stored spermatozoa diluted with Ca^{2+} free artificial seawater containing 3 mM EGTA for 7 days at 4℃. Ⓓ : Stored spermatozoa diluted with 0.3 M glucose for 7 days at 4℃. Sagittal section of head showing cell membrane, compact chromatin, mitochondria and flagellum. f : flagellum, m : mitochondrion, me : cell membrane, n : nucleus, p : proximal centriole.

Existing diluents, which are at once isotonic to seminal fluid and contain metabolic substrate, have been used for fish sperm storage. Kho et al. (1997) who investigated on the prevention of the entrance of external Ca^{2+} into the cell by the use of EGTA, a chelator of calcium ion, suggested that Ca^{2+} will be an indispensable factor in the initiation of sperm motility. The sperm, which was preserved in Ca^{2+} free ASW containing 3 mM EGTA in order to minimize the consumption of ATP, revealed low motility and survival rate for the storage periods and showed the injuries to the nucleus and plasma membrane. From the results of these experiments, it was hypothesized that motility is suppressed by chelating of external Ca^{2+}, and the lysis of the plasma membrane was induced by tris (hydroxymethyl) aminomethane. Therefore, further studies on the optimum buffer solution for cold storage of sperm will be necessary in order to find out a more desirable method of cold storage utilizing EGTA.

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