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Effects of Osmolality and Ca^{2+} on Sperm Motility in Marbled Sole, *Limanda yokohamae*

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A series of experiments were conducted to study effects of osmolality and Ca^{2+} on sperm motility in marbled sole, *Limanda yokohamae*.

Spermatozoa were immotile when the milt was mixed with solutions (electrolyte or non-electrolyte) of lower osmolality than the average seminal plasma osmolality (351 mOsm/kg), but became motile after mixing milt with an hyperosmotic solution. However, with the osmolality above 1,639 mOsm/kg, spermatozoa ceased their movement.

When the milt was suspended in the Ca^{2+} free artificial seawater (ASW) containing ethylenglycoltriamine (EGTA), the percentage of motile spermatozoa decreased in proportion to the increase of EGTA. Most spermatozoa were quiescent in a medium containing 3 mM EGTA. The motility of spermatozoa prevented by 3 mM EGTA was recovered by the subsequent addition of Ca^{2+} over the concentration of 0.25 mM. Effects of calmodulin antagonist, trifluoperazin (TFP) on spermatozoa were examined to elucidate the possible functions of calmodulin in sperm motility. TFP immobilized spermatozoa in ASW at the concentration of 0.5 M. These findings suggest that calcium is an effective external factor in the initiation process of sperm motility.

Key words : marbled sole, *Limanda yokohamae*, sperm motility, osmolality, Ca^{2+}

Introduction

Recently, artificial methods of controlling reproduction have been developed to enable the collection of fish gamete at any time. However, aquaculturists have had difficulties in obtaining sperm and eggs simultaneously in adequate ratios for insemination and management of broodstock. Hence, many researchers have felt keenly the necessity of the development of effective techniques for sperm preservation, which is suitable for preservation compared to eggs.

Preserving viable spermatozoa of cultured fish is desirable for making good any deficiencies of supply, enabling breeding to occur whether or not the maturing period of males and females coincide, and establishing to reserve genetic material of known quality for the initiation of selective breeding programs.

Since the first successful trials of cryopreservation of spermatozoa from marine fish were reported by Blaxter (1953) on *Cluper harengus*, the techniques of preservation of fish spermatozoa have been steadily developed to obtain a fertility rate of over 80% in salmonid fish from the latter half of 1970 (Kurokura, 1983). But there is little agreement to results depending upon the kinds and concentrations of diluents and cryoprotectants used, and considerable discrepancy of results between many researchers.

It is well-known that teleost spermatozoa in the testis are immotile, but that spermatozoa have high motility when they are exposed to the environmental water by spawning (Morisawa and Suzuki, 1980; Morisawa, 1994). And one of the main characteristics of fish gametes is their short life span (several minutes) in the medium in which they are emitted (Billard, 1988).

The preservation of spermatozoa activation is the most critical in spermatozoa storage, as fertility declines soon after activation (Jamieson, 1991), and diluents and cryoprotectants for preservation should not be harmful to spermatozoa and yet effectively suppress their motility.

Morisawa and Morisawa (1990) reported that sperm motility in salmonid fish is suppressed by seminal K^+ in the sperm duct and that Ca^{2+} influx by decreasing of K^+ around spermatozoa spawned in freshwater induces cAMP synthesis in the cells, followed by conversion of the axoneme from an immotile state to a motile one by cAMP, resulting in initiation of sperm motility. However, little information is available on the mechanism of sperm motility in marine fish.

Therefore, to obtain the basic information for sperm preservation of marine fish for aquaculture, we investig

ated the effects of Ca^{2+} and osmolality on the initiation of sperm motility in marbled sole, *Limanda yokohamae*.

Materials and Methods

1. Broodstock handling and milt collection

Mature male marbled sole were caught in the sea near Wando Island and reared at 7~12℃ in a rearing tank at Yocheon Hatchery, National Fisheries Research and Development Institute, Korea. The body length, body weight and number of fish used for the experiment were 28.1 ± 5.7 cm, 416.8 ± 82.3 g and 15, respectively.

Fish were anesthetized with 200 ppm lidocain. Accumulated urine and feces were removed by gently pressuring areas around the genital orifice and the urinary bladder. Milt was then obtained by abdominal massage and stored in sealed test tubes with crushed ice until needed. Only milt which showed high sperm motility of index 5 by the method of Strussmann et al. (1994) in artificial seawater (ASW)[1] was used in the experiment.

2. Characteristics of milt

Sperm density was evaluated by using two different methods; firstly by measuring spermatocrit value using a microhematocrit centrifuge (Bouck and Jacobson, 1976) and secondly by counting spermatozoa using a hemocytometer chamber after dilution of the milt with eosin solution[2].

Milt was centrifuged at 6,000 g for 10 min. and the resulting supernatant was designated as the seminal plasma. Cation concentrations and osmolality of seminal plasma were analyzed by atomic absorption spectrophotometer (Smith-Mieftje 1000) and osmometer (Fiske "OS™" Osmometer), respectively, and Cl^- concentration was analyzed by ion analyzer (Ciba-Corning Diagnostics M664).

3. Sperm motility

1) Sperm motility accordance by osmolality

Motility was examined after dilution with electrolyte and non-electrolyte solutions of different osmolalities. Estimation of sperm motility was started immediately after dilution and continued at 1-min. intervals for 10 min.

2) Sperm motility in the specific cation free ASW

To examine the effects of cations on the initiation of sperm motility, motility was measured at 1-min. intervals for 10 min. after dilution with the specific cation free ASW.

To determine whether sperm in Na^+ free ASW was suppressed by Na^+ itself or osmolality, sperm motility was measured at 1 min. intervals for 10 min. by gradually adding 1 M NaCl, 1M KCl, respectively. In addition, motility of spermatozoa suspended in solution containing 0.64 M choline chloride in Na^+ free ASW was measured immediately after dilution.

3) The effects of Ca^{2+} on the initiation process of sperm motility

Milt was suspended in Ca^{2+} free ASW containing a variety of concentrations of ethylenglycoltriamine (EGTA) and ASW containing trifluoperazin (TFP) which is an inhibitor of calmodulin, respectively. Sperm motility was immediately measured after dilution.

4) The effects of pH on the initiation process of sperm motility

To study the effects of pH on the initiation process of sperm motility, the pH levels of ASW were adjusted in the range of 4~9 by adding 0.1 M HCl and 0.1 M NaOH. Sperm motility was immediately measured after dilution with ASW of various pH levels.

5) Evaluation of the sperm motility

Sperm motility was examined at a milt-diluent ratio of 1 : 200 on a slide glass without cover glass under a light microscope (×200 magnification). All of the sperm motility were examined with the spermatozoa of five different males.

A numerical index from Strussmann et al. (1994) was used to assess sperm motility. Sperm motility is expressed on a 6-point scale based on the degree of motile spermatozoa.

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[1] NaCl 27.0 g+KCl 0.7 g+$MgCl_2 \cdot 6H_2O$ 9.82 g+$CaCl_2$ $2H_2O$ 1.6 g+$NaHCO_3$ 0.5 g+distilled water 1,000 mℓ

[2] 0.125 M Na_2HPO_4 80.4 mℓ+0.125 M KH_2PO_4 19.6 mℓ+eosin 1 g+aniline blue 1 g

Results

1. Characteristics of milt

Average spermatozoa concentration and spermatocrit were $41.5 \pm 11.4 \times 10^9$ cells/mℓ and 96.0 ± 0.9. Average pH value of milt was 8.0 ± 0.1.

The results of ion concentrations and osmolality of seminal plasma were given in Table 1. Osmolality of seminal plasma (351.1 mOsm/kg) was slightly higher than that of blood plasma (319.7 mOsm/kg).

2. Sperm motility

1) Sperm motility accordance by osmolality

The results of sperm motility with the different osmolality solutions were given in Fig. 1. In NaCl solutions, spermatozoa were immotile at the levels below 375 mOsm/kg, but spermatozoa showed vigorous motility at the osmolalities from 574 mOsm/kg to 1,071 mOsm/kg. In KCl solutions, spermatozoa were immotile at the levels below 342 mOsm/kg, but spermatozoa showed vigorous motility at the osmolalities from 523 mOsm/kg to 1,023 mOsm/kg. In $CaCl_2$ solutions, spermatozoa were immotile at the levels below 328 mOsm/kg, but spermatozoa showed vigorous motility at the osmolalities from 513 mOsm/kg to 1,006 mOsm/kg. In $MgCl_2$ solutions, spermatozoa were immotile at the levels below 317 mOsm/kg, but spermatozoa showed vigorous motility with the osmolality above 466 mOsm/kg. Spermatozoa moved vigorously in 100% ASW (1,007 mOsm/kg) and 75% ASW (739 mOsm/kg), and moved weakly in the solution of 50 % ASW (489 mOsm/kg). They ceased their movement in the solutions of 25% ASW (231 mOsm/kg) and distilled water (5 mOsm/kg). In the manitol solutions of non-electrolyte, results of sperm motility showed a similar pattern to those in the electrolyte solutions.

Table 1. Inorganic electrolytes and osmolality of seminal plasma of *Limanda yokohamae*

Property	Seminal plasma
Na^+ (mM)	120.06 ± 0.30
K^+ (mM)	4.75 ± 0.03
Ca^{2+} (mM)	2.90 ± 0.03
Mg^{2+} (mM)	7.12 ± 0.01
Cl^- (mM)	129.37 ± 9.05
Osmolality (mOsm/kg)	351.14 ± 23.42

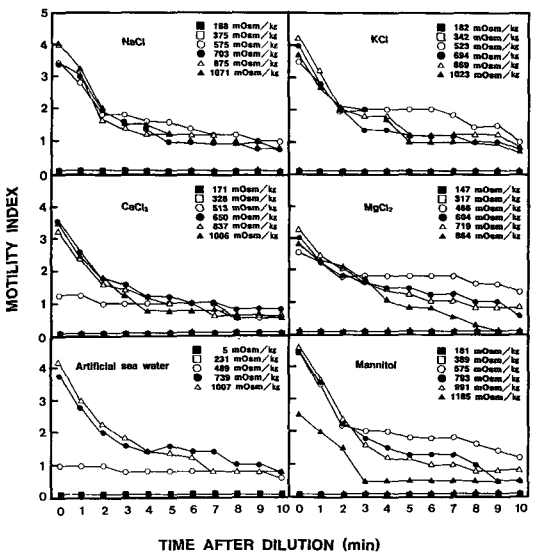

Fig. 1. Motility of *Limanda yokohamae* spermatozoa with the various osmolalities of electrolyte and non-electrolyte solution.

2) Sperm motility in the specific cation free ASW

The results of sperm motility after dilution with Na^+, K^+, Ca^{2+} or Mg^{2+} free ASW, respectively were given in Fig. 2. Spermatozoa in Na^+ free ASW only showed no motility, but spermatozoa in other specific cation free ASW showed vigorous motility immediately after dilution.

The effects of osmolality on sperm motility were given in Fig. 3. When osmolality was raised by gradually adding 1 M NaCl in Na^+ free ASW, spermatozoa in 764~1,101 mOsm/kg moved vigorously, but motility markedly decreased at osmolalities of 498 mOsm/kg and 1,450 mOsm/kg. And it ceased at the osmolality of 1,771 mOsm/kg.

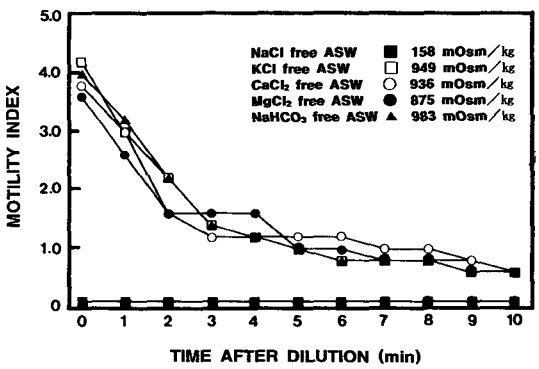

Fig. 2. Effects of five different osmolalities of artificial seawater (ASW) on motility of *Limanda yokohamae* spermatozoa.

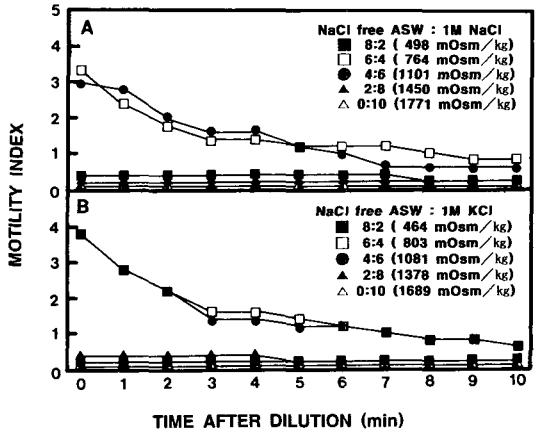

Fig. 3. Effects of five different osmolalities made by adding Na^+ (A) and K^+ (B) to NaCl free ASW on motility of *Limanda yokohamae* spermatozoa.

Changes of motility while raising osmolality by gradually adding 1 M KCl in Na^+ free ASW showed similar patterns. When spermatozoa were suspended in the solution containing 0.64 M choline chloride in Na^+ free ASW, they showed high motility, and the osmolality of the solution was 1,184 mOsm/kg (Fig. 4).

3) The effects of Ca^{2+} on the initiation process of sperm motility

When the milt was suspended in Ca^{2+} free ASW containing EGTA, the percentage of motile spermatozoa decreased with the concentration of EGTA. Most spermatozoa were quiescent in medium containing 3 mM EGTA. The motility of spermatozoa, which had been ceased by adding 3 mM EGTA, was recovered by the subsequent addition of Ca^{2+} over the concentration of 0.25 mM (Fig. 5).

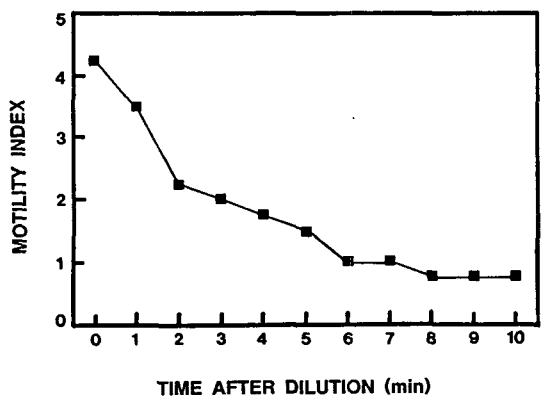

Fig. 4. Effects of osmolality of 1,184 mOsm/kg made by adding 0.64 M choline chloride to NaCl free ASW on motility of *Limanda yokohamae* spermatozoa.

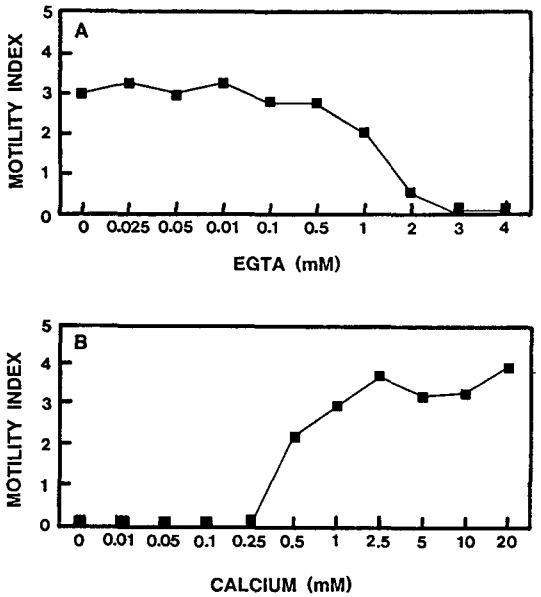

Fig. 5. Effects of EGTA and Ca^{2+} on motility of *Limanda yokohamae* spermatozoa. A : 10 $\mu\ell$ of milt was diluted with 2,000 $\mu\ell$ Ca^{2+} free ASW containing various concentrations of EGTA. B : 10 $\mu\ell$ of milt was diluted with 2,000 $\mu\ell$ Ca^2 free ASW containing 3 mM EGTA and then appropriate concentrations of $CaCl_2$ were added.

When *L. yokohamae* spermatozoa were exposed to the various concentrations of TFP in ASW, they were immotile at 0.5 mM TFP concentration in ASW (Fig. 6).

4) The effects of pH on the initiation process of sperm motility

Average motility index in *L. yokohamae* spermatozoa following activation in ASW of pH ranging from 4 to 9 were given in Fig. 7. Intensive motility (index 3.75~4.00) was induced at pH 5 to 9, and motility was slightly reduced by 2.5 of motility index at pH 4.

Discussion

Although studies on fish sperm motility have a long history of over 100 years (Barrett, 1951), detailed information for the mechanisms on the most important factors

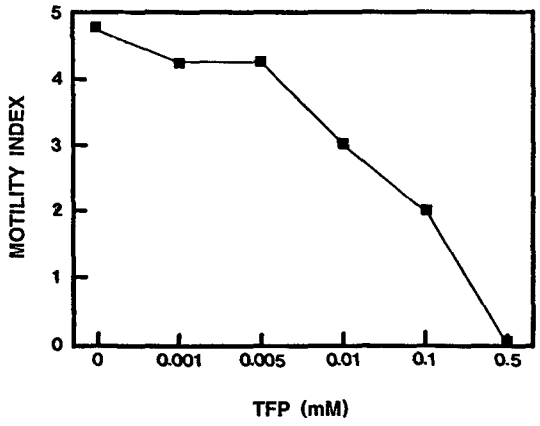

Fig. 6. Effects of trifluoperazine (TFP) on motility of *Limanda yokohamae* spermatozoa.

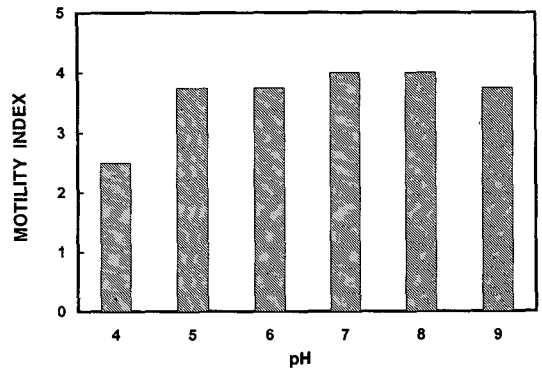

Fig. 7. Effects of pH on motility of *Limanda yokohamae* spermatozoa.

in examining the effects of preservation are still limited and under investigated (Billard and Cosson, 1992).

In our study, spermatozoa of *L. yokohamae* showed high motility in the solutions with a wide range of pH 5~9 levels, but low motility at pH 4. This result is in agreement with that of Strussmann et al. (1994). They showed that the spermatozoa of pejerrey, *Odontesthes bonariensis* moved vigorously in the solutions with pH 5.4~8.3 levels. Thus, pH appears to have little, if any, effects on the initiation process of sperm motility. Spermatozoa in testis of male fish having the external fertilization are immotile until just before spawning to preserve ATP which is their source of energy for motility. Motility is initiated when they are spawned in the aquatic environment such as seawater, brackish water, or freshwater (Morisawa and Morisawa, 1990). In the internal fertilization species of guppy and topminnow, spermatozoa become motile when spermatozoa cells or an assembly of spermatozoa, for example a sperm ball, a spermatophore, are introduced into the female reproductive tract (Morisawa and Morisawa, 1990). As mentioned above, in oviparous teleost, motility occurred only when the milt was diluted with a solution of lower osmolality in the freshwater species including cyprinid or with a solution of higher osmolality in the marine species (Morisawa and Suzuki, 1980).

Spermatozoa of *L. yokohamae* showed a high motility with the electrolyte or non-electrolyte solution of 466~1,185 mOsm/kg in the present study. The results suggested that they were initiated by the hyperosmotic solutions above 351 mOsm/kg, which is the average osmolality of the seminal plasma.

The initiation of movement in rainbow trout spermatozoa is probably due to a change in the membrane potential after dilution with freshwater and a decrease of K^+ concentration; this opens Ca^{2+} channels and the initiation process of motility follows the entrance of Ca^{2+} into the cell (Christen et al., 1987; Tanimoto and Morisawa, 1988; Cosson et al., 1989; Morisawa and Morisawa, 1990).

The motility of spermatozoa was suppressed in Na^+ free ASW in our study. Spermatozoa became more motile, as osmolality was raised in the range of 764~1,101 mOsm/kg. In addition, when spermatozoa were suspended in the solution containing 0.64 M choline chloride in Na^+ free ASW, with the osmolality of 1,184 mOsm/kg, spermatozoa showed high motility. These results suggested that the motility of spermatozoa was suppressed not by the absence of Na^+ but by the osmolality of the diluent.

Up to now, there have been few attempts to clarify the relationship between calcium chelating agents and sperm motility in fisheries livestock. Young and Nelson (1974) showed that EGTA suppressed sperm motility and calcium stimulated motility in sea urchin spermatozoa. This suggests that calcium is an important factor for the motility. However, they were unable to demonstrate the role of calcium in the actual initiation of sperm motility. Attempts to demonstrate the role of calcium in the actual initiation of sperm motility failed because attempts to restore motility to the immobilized spermatozoa by addition of calcium ions following treatment with EGTA were

unsuccessful.

In the present study, when spermatozoa were diluted with Ca^{2+} free ASW containing EGTA, sperm motility declined concomitantly with the decrease of calcium as EGTA concentrations were increased, finally halting completely at EGTA concentrations of over 3 mM. The motility of the quiescent spermatozoa was restored by the addition of calcium, suggesting that calcium is an important factor for sperm motility. In addition, when *L. yokohamae* spermatozoa were exposed to TFP in ASW, sperm motility was inhibited at the concentrations above 0.5 M. This result suggests that the motility may be suppressed by the inhibition of spermatozoa calmodulin with TFP.

In summary, the initiation process of motility in *L. yokohamae* spermatozoa diluted in hyperosmotic solutions to seminal plasma is drived from influx of extracellular Ca^{2+} by some changes of the plasma membrane.

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삼투질농도와 Ca^{2+}이 문치가자미 (*Limanda yokohamae*) 정자의 운동성에 미치는 영향

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문치가자미의 성숙한 수컷 15마리를 재료로 하여 삼투질농도와 Ca^{2+}이 정자의 운동성에 미치는 영향을 파악하였다.

문치가자미 정자는 전해질 및 비전해질 용액에서 정장의 삼투질농도인 351 ± 23.4 mOsm/kg 이하의 저장액에서 운동이 억제되었으나, 삼투질농도 466~1,185 mOsm/kg에서는 활발한 운동을 보였으며, 1,698 mOsm/kg 이상에서 다시 운동이 중지되었다.

인공해수를 희석액으로 하여 pH를 달리하였을 때, 정자는 pH 5~9에서 운동지수 3.75~4.00로 운동성이 활발하였으나, pH 4에서는 운동지수 2.50로 미약하였다.

Ca^{2+}의 특이 chelator인 EGTA가 포함된 Ca^{2+} 결여 인공해수 (tris-HCl, pH 7.4)에 정액을 희석하였을 때, EGTA의 농도 증가에 따라 정자 운동성이 낮아졌으며, 3 mM 이상의 농도에서는 완전히 정지되었다. 운동이 억제된 정자에 Ca^{2+}을 서서히 첨가한 결과, Ca^{2+} 농도 0.25 mM 이상부터 운동이 재개되었다. 또한 인공해수속의 정자는 calmodulin 억제제인 TFP의 농도의 증가에 따라 운동성이 감소하여 0.5 mM에서 완전히 정지하였다.