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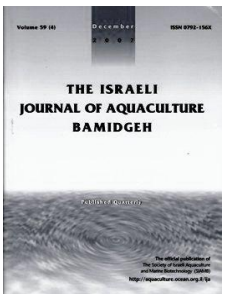

Storage of Yellow Croaker *Larimichthys polyactis* Semen

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Abstract

Storage of semen is useful for genetic studies and artificial breeding of fish. The aims of the present study were to find the best extender, dilution ratio, temperature, and antibiotic for cold storage of yellow croaker *Larimichthys polyactis* semen. The semen of yellow croaker was stored with artificial seminal plasma or marine fish Ringer's solution as extenders, diluted at 1:1 (semen:extender), 1:3, or 1:5, maintained at 0°C, 2°C, or 4°C, and preserved with gentamycin or neomycin at concentrations of 200, 400, 600, 800, or 1000 ppm. The most effective conditions for storage were artificial seminal plasma extender in a dilution ratio of 1:3 at 0°C, in which the preserved sperm maintained motility for 14 days. The best concentration of antibiotic was 600 ppm of gentamycin or 200 ppm of neomycin, and the preserved sperm retained motility for 26 days. These results demonstrate that spermatozoa of yellow croaker can be preserved.

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Introduction

Storage reduces the metabolic activity of sperm cells and extends their life span (Lim et al., 2005, 2006). Storage of fish semen is a simple technique that allows sperm to be available at various times for fertilizing eggs produced by hormone-induced females, facilitating reproductive productivity (Marques and Godinho, 2004). Sperm storage can eliminate the need to keep males available for artificial fertilization and allows faster easier handling of semen when eggs are available (Christensen and Tiersch, 1996).

Studies on the storage of semen of aquatic species are important for four reasons. First, semen storage can be used to maintain genetic lines, aid recovery of developed populations, and maximize aquaculture production (Segovia et al., 2000). Second, it can be useful in artificial propagation and complement cryopreservation (Lim et al., 2005; 2006). Third, it can prolong the life of spermatozoa for short periods without the use of special techniques and instruments (Chang et al., 2002). Fourth, it is beneficial when male and female gametes are collected at different times or locations, or when the collection site and incubation facility are some distance apart and fertilization is delayed (Lim et al., 2005, 2006).

The storage of semen is affected by extenders, dilution ratio, temperature, and antibiotics (Lim et al., 2005; 2006). Storage temperature is a major factor that affects the viability of fish gametes *in vitro*. Fish semen can be stored at temperatures above freezing without the addition of cryoprotectants (McNiven et al., 1993; Marques and Godinho, 2004) but viability of gametes and embryos can be prolonged by maintaining them close to 0°C to reduce their metabolic rates. However, the ability to tolerate low temperatures varies among temperate and tropical fish species (Leung and Jamieson, 1991) and microorganisms in gamete samples can cause transfer of disease agents from wild and hatchery sources. In addition, the presence of microorganisms in stored samples can endanger valuable germ-plasm by decreasing fertilizing capacity and lowering cell quality and viability (Segovia et al., 2000). To limit these potential problems, antibiotics are commonly added to the cold storage of semen.

Yellow croaker, *Larimichthys polyactis*, is an important traditional commercial fish species in Korea that migrates to the East China Sea in winter and returns to the Yellow Sea to spawn in spring (Kim et al., 1997). The objectives of the present study were to (a) evaluate the effects of extender, dilution ratio, temperature, and type of antibiotic in yellow croaker and (b) compare them with other fish species.

Materials and Methods

Fish and milt collection. Experiments were carried out at the National Fisheries Research and Development Institute, Korea, during June 2008 and June 2009, coinciding with the peak spawning activity for yellow croaker (Lim et al., 2010). Yellow croaker males (23.3±0.2 cm, 128.8±0.6 g) were maintained separately from females in 2-m^3 tanks supplied with 17-21°C sea water of 32-33 ppt salinity, 5-6 mg O_2/l, and a flow rate of 0.2 l/s. Fresh semen samples were obtained by serial waves of abdominal pressure and stored in 1.5-ml Eppendorf tubes on ice until use.

Analysis of spermatozoa. The motility of fresh spermatozoa from each male was determined immediately after semen was collected. The percent of spermatozoa exhibiting rapid, vigorous, forward movement was determined under a microscope by diluting the semen in artificial sea water (NaCl 27 g, KCl 0.5 g, $CaCl_2$ 1.2 g, $MgCl_2$ 4.6 g, $NaHCO_3$ 0.5 g per liter distilled water) at a ratio of 1:100. Samples with a movable spermatozoa ratio of more than 80% were pooled and used in this study.

To evaluate the viability of cold-stored spermatozoa, the movable spermatozoa ratio and spermatozoa velocity were estimated. Prediluted semen was diluted in artificial sea water at the ratio of 1:100 (1 µl prediluted sperm to 99 µl artificial sea water). Then, 1 µl was put onto a Teflon printed glass slide with 21 wells (diameter of each well, 4 mm; Funakoshi Co., Japan) without a cover slide and the activity of the cold-stored spermatozoa was immediately observed at 200× magnification under a microscope (Axioskop 2 plus Zeiss, Germany). Activity was examined using a video camera (Carl Zeiss, Germany) and timer (VTG-55B, Germany) connected to a video recorder and

player (Samsung VHS, spermatozoa velocity-G1000, Korea). Each sample was observed under the microscope three times for 1.5 min. The cold-stored spermatozoa heads were recorded by video tape together with a video timer. The movable spermatozoa ratio was determined as the percent of motile spermatozoa while spermatozoa velocity represents the distance sperm moved in 1 s.

Effect of extender on motility. To determine the optimal extender, semen was diluted at a ratio of 1:3 (semen:extender) with marine fish Ringer's solution or artificial seminal plasma (Table 1). The composition of the artificial seminal plasma extender was based on the biochemical properties of yellow croaker seminal plasma (Le et al., 2011). Mixtures were placed in 1.5-ml Eppendorf tubes and stored in a refrigerator at 0°C. Storage treatments were replicated three times. The movable spermatozoa ratio and spermatozoa velocity in each tube were tested at 2-4-day intervals until the spermatozoa stopped moving.

Table 1. Constituents (g/l distilled water) and properties of extenders: artificial seminal plasma and marine fish Ringer's solution.

	Extender	
	Artificial seminal plasma	*Marine fish Ringer's solution*
KCl	0.77	0.60
NaCl	9.92	13.50
$CaCl_2$	0.13	0.35
$MgCl_2$	0.05	0.02
$NaHCO_3$	-	0.03
Glucose	0.01	-
pH	7.60	7.70
Osmolality (mOsm/kg)	335	444

Effect of dilution ratio on motility. To determine the optimal dilution, semen was diluted in artificial seminal plasma at ratios of 1:1, 1:3, and 1:5 (semen:extender). Mixtures were placed in 1.5-ml Eppendorf tubes and stored in a refrigerator at 0°C. Treatments were replicated three times. The movable spermatozoa ratio and spermatozoa velocity in each tube were tested at 2-4-day intervals until spermatozoa stopped moving.

Effect of temperature on motility. To determine the optimal temperature, semen was diluted at a ratio of 1:3 (semen:artificial seminal plasma), placed in 1.5-ml Eppendorf tubes, and stored in a refrigerator at 0°C, 2°C, or 4°C. Treatments were replicated three times. The movable spermatozoa ratio and spermatozoa velocity in each tube were tested at 2 or 4-day intervals until spermatozoa stopped moving.

Effect of antibiotics on motility. To evaluate the effect of antibiotics, semen was diluted at the ratio of 1:3 (semen:artificial seminal plasma), gentamycin or neomycin was added to mixtures at concentrations of 200, 400, 600, 800, or 1000 ppm. Treatments were replicated three times and stored in a refrigerator at 0°C. The movable spermatozoa ratio and spermatozoa velocity in each tube were tested at 4-6-day intervals until spermatozoa stopped moving.

Statistical analysis. Data were expressed as means±standard deviations. Statistical evaluation was performed by one-way ANOVA using SPSS version 16.0. Means were separated using Tukey's multiple range test and differences were considered significant at $p<0.01$.

Results

Effect of extender on spermatozoa motility. Semen stored in artificial seminal plasma retained its movable spermatozoa ratio and spermatozoa velocity significantly longer than semen stored in marine fish Ringer's solution (Fig. 1). Sperm stored in artificial seminal plasma remained motile for 14 days, while sperm stored in marine fish Ringer's solution remained motile for only 10. Since artificial seminal plasma seemed to be more effective than marine fish Ringer's solution, it was used in subsequent experiments.

Effect of dilution ratio on spermatozoa motility. Semen stored in artificial seminal plasma at the ratio of 1:3 (semen:extender) retained its movable spermatozoa ratio and spermatozoa velocity significantly longer than semen stored at ratios of 1:1 or 1:5 (Fig. 2). Semen stored in the 1:3 dilution remained motile for 14 days, in contrast to semen stored at 1:1 (10 days) or 1:5 (12 days). Thus, the dilution ratio of 1:3 was used in subsequent experiments.

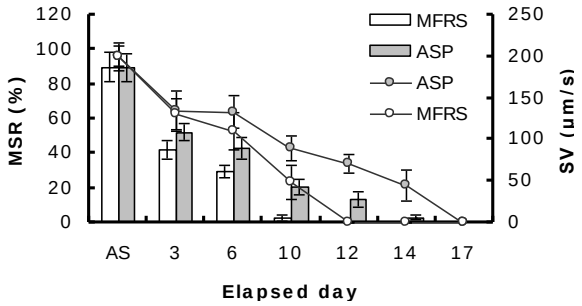

Fig. 1. Movable spermatozoa ratio (MSR; bars) and spermatozoa velocity (SV; lines) of yellow croaker semen stored at 0°C with one of two extenders: artificial seminal plasma (ASP) or marine fish Ringer's solution (MFRS); means±SD, n = 3.

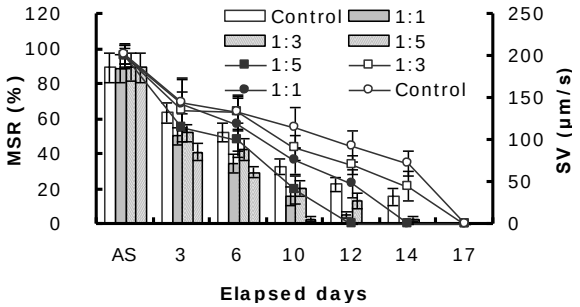

Fig. 2. Movable spermatozoa ratio (MSR; bars) and spermatozoa velocity (SV; lines) of yellow croaker semen stored in dilutions of 1:1, 1:3, or 1:5 (semen:artificial seminal plasma) at 0°C; means±SD, n = 3.

Effect of temperature on spermatozoa motility. Semen at stored at 0°C retained motility 17 days, compared to only 12 days for semen stored at 2°C or 10 days for semen stored at 4°C (Fig. 3). Thus, 0°C was used for the subsequent experiment on antibiotics.

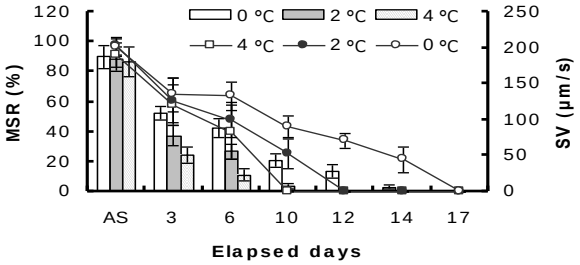

Fig. 3. Movable spermatozoa ratio (MSR; bars) and spermatozoa velocity (SV; lines) of yellow croaker semen stored in a dilution of 1:3 (semen:artificial seminal plasma) at 0°C, 2°C; or 4°C, means±SD, n = 3).

Effect of antibiotics on spermatozoa motility. The highest movable spermatozoa ratios and spermatozoa velocities after cold storage of spermatozoa at 0°C in a 1:3 (semen:extender) dilution in artificial seminal plasma and one of two antibiotics was obtained in the 600 ppm gentamycin and 200 ppm neomycin treatments (Fig. 4). The viability values of spermatozoa in the highest concentration of each antibiotic and the control (no antibiotic) significantly differed.

Results show that yellow croaker semen was best preserved when stored in a 1:3 dilution of semen:artificial seminal plasma as the extender at a temperature of 0°C, and with the addition of 600 ppm gentamycin or 200 ppm neomycin.

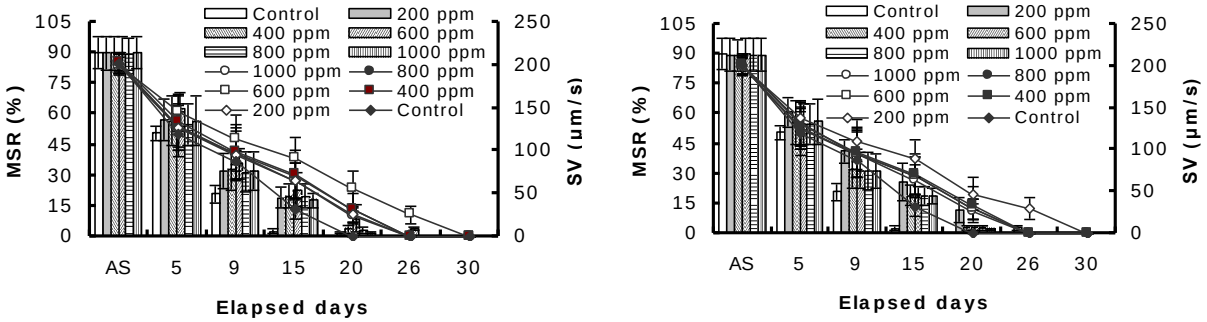

Fig. 4. Changes of movable spermatozoa ratio (MSR; bars) and spermatozoa velocity (SV; lines) of yellow croaker *Larimichthys polyactis* semen after cold storage at 0°C in 1:3 (semen:artificial seminal plasma) and one of two antibiotics: (a) gentamycin or (b) neomycin at different concentrations; control = no antibiotic; means±SD, n = 3.

Discussion

The movable spermatozoa ratio and spermatozoa velocity in yellow croaker semen changed during storage, indicating that semen quality also changes. Semen quality is most accurately measured by fertilization percentage (Dreanno et al., 1999; Lahnsteiner, 2000; Linhart et al., 2000; Lim et al., 2005). This method of analysis, however, could be used in our experiments because yellow croaker females did not produce mature eggs until the experiment was completed. When fertilization trials cannot be performed, movable spermatozoa ratio and spermatozoa velocity are used as an indicator of semen quality (Lim et al., 2005, 2006).

The main factors that influence the cold storage of fish semen are extender, dilution ratio, temperature, and antibiotic. Selecting a suitable extender is a key factor in the successful cold storage of fish semen (Jenkins-Keeran and Woods III, 2002; DeGraaf et al., 2004; Lim et al., 2006). In this work, artificial seminal plasma and marine fish Ringer's solution were tested as extenders for cold storage of yellow croaker semen. While artificial seminal plasma worked effectively, marine fish Ringer's solution extender (which was suitable for tiger puffer *Takifugu rubripes* semen; Chang et al., 1997) resulted in low movable spermatozoa ratio and spermatozoa velocity, suggesting the highly specific nature of extenders.

Dilution of collected semen offers several benefits. Reducing semen density is especially important when dealing with testicular semen as it reduces problems associated with urine contamination and maintains motility for several days if artificial insemination is delayed (Bobe and Labbe, 2009). The semen:extender ratio 1:3 was better than 1:1 and 1:5, similar as in Atlantic cod *Gadus morhua*, haddock *Melanogrammus aeglefinus* (DeGraaf and Berlinsky, 2004a), and rainbow smelt *Osmerus mordax* (DeGraaf and Berlinsky, 2004b). However, in the case of African catfish *Clarias gariepinus*, 1:5 was better than 1:3 (Erdahl et al., 1984).

Air, especially pure oxygen, is necessary for maintaining cellular activity (Chereguini et al., 1997; Basavaraja and Hegde, 2005). Pure oxygen is superior to air or other gases for survival of sperm (Stoss et al., 1978). Viability of spermatozoa stored in plastic bags inflated with oxygen was prolonged in European catfish *Siluris glanis* (Babiak et al., 1996), channel catfish *Ictalurus punctatus* (Christensen and Tiersch, 1996) and Deccan mahseer *Tor khudree* (Basavaraja and Hegde, 2005). In our study, air could not enter the storage volume because the Eppendorf tubes were filled with semen. Therefore, movable spermatozoa ratio and spermatozoa velocity may have dropped because of the lack of air or oxygen. More detailed work is necessary to examine the effect of oxygen and air on sperm survival in cold storage of yellow croaker semen.

Storage temperature affects the viability of fish gametes (Basavaraja and Hegde, 2005). Temperatures of 0-4°C are widely used. Low temperatures drastically reduce bacteria growth, which may explain why below 6°C is better than higher temperatures (Bobe and Labbe, 2009). In this work, cold-stored yellow croaker semen remained motile for 14 days at 0°C, 10 days at 2°C, and 6 days at 4°C. Semen survived one to several days at 1-4°C in salmons (Basavaraja and Hegde, 2005), 30 days at 0°C or 25 days at 2-4°C in flounder, *Paralichthys olivaceus* (Lim et al., 2005), and 30 days at 0°C, 25 days at 2°C, and 16 days at 4°C for starry flounder *Platichthys stellatus* (Lim et al., 2006). The milt of the estuarine *Mystus gulio* was stored up to 7 days at 4°C (Basavaraja and Hegde, 2005). Spermatozoa stored at 4°C and room temperature retained motility up to 84 and 24 h, respectively, in fringe-lipped carp, *Labeo fimbriatus* (Basavaraja and Hegde, 2005), while motility was maintained for 7 days in liquid nitrogen (-40°C) in the pike *Sander lucioperca* L. (Bokor et al., 2008).

Addition of antibiotics to the undiluted semen or the storage extender improves storage duration (Billard et al., 2004; Bobe and Labbe, 2009). Antibiotics are essential for prolonging storage time since bacterial contamination of sperm from excreta is common (Chao et al., 1992; Chang et al., 2002). The movable spermatozoa ratio and spermatozoa velocity of the yellow croaker semen in this study was also prolonged by addition of antibiotics.

In conclusion, semen of yellow croaker can be preserved. The optimal conditions for storage were artificial seminal plasma extender diluted at the ratio of 1:3 (semen:extender), supplemented with 600 ppm gentamycin or 200 ppm neomycin, and stored at 0°C.

Acknowledgements

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