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Cryopreservation of Semen in Dead Yellow Croaker, *Larimichthys polyactis*

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This study demonstrated that cryopreserved semen from dead fish can be used for seedling production. Yellow croakers, *Larimichthys polyactis*, were killed and stored at temperatures of 20°C or 0°C for 6 hours. At 2 hour intervals, semen from these fish was collected using abdominal pressure and evaluated for spermatozoa motility and semen cryopreservation. Semen collected after 6 hours from dead fish stored at 0°C could be cryopreserved and attained fertilization and hatching rates of 15.0±1.2% and 14.8±1.6%, respectively. This study suggests that germ cells such as the semen of dead fish can be cryopreserved and utilized in the restoration of a species.

Key words: Yellow croaker, *Larimichthys polyactis*, Semen, Cryopreservation, Dead fish

Introduction

According to the World Conservation Union, 25% of mammal and amphibian species, 11% of birds, 20% of reptiles, and 34% of the fish species surveyed to date are threatened with extinction. Furthermore, aquatic ecosystems are becoming increasingly vulnerable to disasters caused by runoff from agriculture and industrial sources, oil spills, or sudden environmental changes, leading to the total elimination of stocks from various ecosystems (www.worldwatch.org). In the event of such emergencies, no methods exist to quickly restore a species to its former habitat. This emphasizes the need for cryopreservation techniques, whereby germ cells such as semen of dead fishes can be utilized to restore species and prevent economic losses. The cryopreservation of fish semen has now been tested successfully in a range of marine and freshwater species (Chao and Liao, 2001; Cabrita et al., 2003; Suquet et al., 2000; Babiak et al., 1997; Horváth et al., 2003; Lahsteiner, 2000; Gwo, 2000). Although it has been reported that the spermatozoa of fish remain viable after death (Routray et al., 2006), their cryopreservation and successful use in producing fish has not been reported elsewhere in any species. The yellow croaker, *Larimichthys polyactis*, is an important traditional and commercial fish species in Korea. It 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 investigate the viability of spermatozoa for the cryopreservation of semen collected from dead yellow croakers.

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Materials and Methods

Fish and semen collection

Experiments were carried out at the Aquaculture Physiology Laboratory, Department of Aquaculture, Colleges of Fisheries Science, Pukyong National University, Korea, during July 2009. Fish were held in spawning tank (1 m^3) with through-flowing seawater (32 psu) at a flow rate of 0.2 $L \cdot s^{-1}$ with an air stone, under a simulated natural photoperiod and water temperature of 19.0-20.0°C. Six male yellow croakers (total length (TL): 23.3±0.2 cm; body weight (BW)=128.8±0.6 g) were killed individually and stored at temperatures of 20°C or 0°C for 6 hours. At 2-hour intervals, semen from these fishes was

collected by gently pressing the genital area of the fish. Collected semen then was transferred into 1.5-mL tubes. From here, the motility of the spermatozoa was evaluated, and the semen was cryopreserved.

Spermatozoa motility assessment

Motility of spermatozoa samples was deter-mined immediately after the semen was collected. The percentage of sperm exhibiting rapid, vigorous forward movement was classified under a microscope (Axioskop 2 plus Zeiss, Germany). To measure the motility of spermatozoa, semen was diluted in artificial seawater (ASW; NaCl 27 g, KCl 0.5 g, $CaCl_2$ 1.2 g, $MgCl_2$ 4.6 g, $NaHCO_3$ 0.5 g in a liter of distilled water) at a ratio of 1:100 (1 μL prediluted sperm to 100 μL ASW). Then, 1 μL of solution was placed on a glass slide (Teflon Printed Glass Slide; 21 wells; diameter of each well, 4 mm; Funakoshi Co., Japan) without a cover glass. The motility of post-thawing spermatozoa was observed immediately at 200x magnification under a microscope, and was quantified using a video camera (Carl Zeiss, Germany) and timer (VTG-55B, Germany) connected to a video recorder and player (Samsung VHS, SV-G1000, Korea). The motility of post-thawing spermatozoa was determined by evaluating the percentage of motile and immotile spermatozoa.

Cryopreservation of semen and thawing

Cryopreservation of semen was carried out following the standard protocols of Lim et al. (2007; 2008) and Le et al. (2008). The collected semen was mixed with yellow croaker artificial seminal plasma extender (ASP; NaCl 9.92 g, KCl 0.77 g, $CaCl_2$ 0.13 g, $MgCl_2$ 0.05 g, glucose 0.01 g in a liter of distilled water), which was made following the basic biochemical properties of yellow croaker seminal plasma (Le et al., 2009) with supplementary cryoprotectant (10% ethylene glycol) at a ratio of 1:3 (semen: extender). Then, 0.25 mL of diluted semen was sucked into 0.25 mL plastic straws (FHK, Fujihira Industry Co., Tokyo, Japan), and the straws were placed on a tray suspended 3.5 cm above the surface of liquid nitrogen for 5 min before being plunged into liquid nitrogen (-196°C). Straws containing diluted semen were thawed in a 37°C water bath for 30 seconds. Immediately after thawing, spermatozoa motility was determined as described above.

Fertilization of eggs

Cryopreserved semen was subjected to fertilization tests using eggs taken from three mature female yellow croakers. To collect unfertilized eggs, mature females (27.5±1.9 cm TL and 269.1±57.1 g BW) were collected, and eggs were extracted by the application of gentle abdominal pressure. Immediately after the semen was thawed, it was used to fertilize the eggs. Five hundred eggs were placed into a 15 cm-diameter dish, and 80 μL of thawed spermatozoa (0.6×10^9 cells/mL) was immediately dropped onto the eggs. The fertilized eggs were incubated in natural seawater (32 psu) at 19°C, and dead eggs, which became opaque, were removed from each dish after fertilization. When the fertilized eggs had developed into gastrula-stage embryos, the fertilization rate (number of gastrula-stage embryos/ numbers of eggs) was calculated. The hatching rate is expressed at the percentage of hatched fry from the fertilized eggs. The fertilization and hatching rates (%) of post-thawing spermatozoa with different storage temperature and times after death were examined. The process of *in vitro* fertilization followed the above description, with a spermatozoa-to-egg ratio of ca. 960,000 to 1.

Statistical analyses

All data are shown as mean ± standard error of mean (SEM). Spermatozoa motility, fertilization rate, and hatching rate were analyzed using one-way analysis of variance (ANOVA). Tukey's multiple-range tests were applied to evaluate significant differences. Values of $P<0.01$ were considered statistically significant. Statistical analyses were computed using SPSS 16.0 software.

Results and Discussion

Motility of pre-freezing and post-thawing spermatozoa from dead yellow croakers at different time intervals and storage temperatures is shown in Table 1. Spermatozoa motility decreased from 0 hour to 6 hours after fish death. After the fish had been dead for 6 hours, the spermatozoa motility was 0% in pre-freezing and post-thawing semen when the dead fish had been stored at 20°C. When the dead fish had been stored at 0°C, spermatozoa motility was 20.0± 2.9% before freezing and 5.0±1.2% after thawing.

The results obtained from fertility tests of post-thawing spermatozoa of dead yellow croakers at different time intervals and storage temperatures are presented in Table 2. Fertilization was affected by cryopreservation of semen collected from fish stored at temperatures of 0°C and 20°C after death. However, higher fertilization rates were observed for sperm extracted 0, 2, 4, and 6 hours after death from fish stored at 0°C compared with those stored at 20°C.

Table 1. Spermatozoa motility of pre-freezing and post-thawed of dead yellow croaker, *Larimichthys polyactis* at different intervals of time and storage temperatures

Time after death (h)	Fish stored at 0°C		Fish stored at 20°C	
	Pre-freezing motility (%)	Post-thawed motility (%)	Pre-freezing motility (%)	Post-thawed motility (%)
0	89.0±2.1[a]	71.3±1.7[a]	87.0±1.5[a]	70.0±2.3[a]
2	66.0±3.8[b]	48.3±4.1[b]	35.3±2.6[b]	18.3±3.3[b]
4	33.3±1.7[c]	18.3±4.4[c]	7.3±1.5[c]	3.3±0.9[c]
6	20.0±2.9[d]	5.0±1.2[d]	0.0±0.0[c]	0.0±0.0[d]

Data were expressed as mean±S.E. with three replicated. Values within column followed by different superscript letters are significantly different ($P<0.01$).

Table 2. Fertilization rate and hatching rate of post-thawed semen of dead yellow croaker, *Larimichthys polyactis* at different intervals of time and storage temperatures

Time after death (h)	Fish stored at 0°C		Fish stored at 20°C	
	Fertilization rate (%)	Hatching rate (%)	Fertilization rate (%)	Hatching rate (%)
0	47.9 ± 1.7[a]	38.6 ± 4.1[a]	48.9 ± 0.7[a]	37.8 ± 5.2[a]
2	33.3 ± 4.4[b]	22.7 ± 1.9[b]	11.7 ± 1.7[b]	13.1 ± 1.7[b]
4	22.0 ± 1.5[bc]	20.7 ± 1.2[b]	2.3 ± 0.3[c]	0.0 ± 0.0[c]
6	15.0 ± 1.2[c]	14.8 ± 1.6[b]	0.0 ± 0.0[c]	0.0 ± 0.0[c]

Data were expressed as mean±S.E. with three replicated. Values within column followed by different superscript letters are significantly different ($P<0.01$).

Semen collected 6 hours after the death of fish and stored at 0°C showed a 15.0±1.2% fertilization rate and 14.8±1.6% hatching rate.

Collection of semen from dead fish has been tested either after fishing (in the case of the red tilefish, *Branchiostegus japonicus* (Fujinami et al., 2003)) or for experimental purposes to mimic the accidental death of males under fish farm conditions (rainbow trout, *Oncorhynchus mykiss*, brown trout, *Salmo trutta fario*, chinook salmon, *O. tschawytscha*, herring, *Clupea harengus*, mackerel, *Rastrelliger neglectus* (Billard et al., 1981)). Fertilization rates dropped during the 5-12 hours after the death of fish, even when males were kept at 4°C (Billard et al., 1981). A lack of oxygen is proposed to explain these dramatic losses compared with the relatively good preservation obtained when sperm was collected immediately after death and stored *in vitro*. In red tilefish, however, semen collected from the testes 5-8 hours after death could be stored *in vitro* for up to 7 days and still display high motility. These studies confirm that motility losses induced by anoxia are reversible, provided that cells are thoroughly oxygenated. This reversibility of motility and fertilizing ability losses after oxygen deprivation was experimentally demonstrated in rainbow trout (Bencic et al., 2000). The author proposed that losses in semen functionality may be connected to decreases in seminal fluid pH induced by high levels of carbon dioxide. These data emphasize that poor initial sperm quality can be overcome by appropriate storage conditions.

In the present study, the motility of spermatozoa was reduced drastically when the storage time of cadavers was increased (0°C or 20°C) (Table 1). Spermatozoa motility is used to predict fertilizing ability, and prolonged post mortem storage leads to reduced fertilization success in rainbow trout (Dietrich et al., 2005). These results clearly demonstrate that when semen from sacrificed fish was used, the time of post mortem storage significantly altered sperm motility characteristics.

Fertility results using cadaveric sperm drawn from *H. fossilis* specimens preserved at -20°C for 240 days have been reported without cryopreservation (Koteesswaran and Pandian 2002). However, fertilization and hatching rates of 15.0±1.2% and 14.8±1.6%, respectively were found in the present study after fish had been dead for 6 hours and stored at 0°C (Table 2). This study suggests that germ cells such as the semen of dead fish can be cryopreserved and utilized for the restoration of a species. These results can be applied to restore yellow croaker populations.

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