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Cold Storage of Milt from Four Species of Flatfish

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The proper conditions for cold storage of milt in four species of flatfish were investigated. Various diluents were tested for the preservation of marbled sole, brown sole, starry flounder and olive flounder milt at 1±0.5℃ for 7 days. The most effective diluent was 1% NaCl for marbled sole and Stein's extender for brown sole, starry flounder and olive flounder. After 7 days of storage in different dilution ratios (milt:diluent), marbled sole milt diluted with 1:3~10 and brown sole, starry flounder and olive flounder milt diluted 1:1~10 maintained good sperm activity index (SAI). SAI was affected by storage volume and presence of antibiotic. SAI was lower after 7 days storage in the volume of 1.5 mL than in 0.5 or 1.0 mL. SAI after 29 days storage was markedly enhanced by addition of antibiotic in marbled sole (400 ppm gentamicin, 400 ppm neomycin, 200~400 ppm streptomycin), brown sole (600 ppm gentamicin), starry flounder (1,000 ppm gentamicin) and olive flounder (200~1,000 ppm gentamicin, 200~400 ppm neomycin, 200~1,000 ppm streptomycin).

Key words: Flatfish, *Limanda yokohamae, Limanda herzensteini, Platichthys stellatus, Paralichthys olivaceus,* Cold storage, Diluent, Dilution ratio, Storage volume, Antibiotic

Introduction

The brown sole, marbled sole, starry flounder and olive flounder are commercially important species in Korea. These flatfish populations have been declining as a result of pollution and overharvest. Therefore, it is necessary to increase production by aquaculture using artificial seedling. However, there is a possibility that male and female gametes for the artificial seedling production are collected at different times or locations, or the collection site and incubation facilities are some distance apart.

Thus, fertilization is often delayed, in that case, milt preservation is demanded. The methods of milt preservation were divided into cryopreservation and cold storage. Cryopreservation technique is to freeze milt at −79℃ or −196℃ using liquid nitrogen, and cold storage is to reduce the metabolic activity of the cells in order to extend their life span. Cold storage of milt have disadvantage to be impossible to store during the long term. However, cold storage is able to prolong life of sperm during the short period without use of special techniques and instruments. So, it can be useful in the practice of artificial propagation and can be complementary to cryopreservation.

Since Barrett (1951) reviewed about cold storage of milt, the studies concerned with it have been accomplished extensively in salmonids (Scott and Baynes, 1980): (1) the storage effect according to diluent and dilution method (Chao et al., 1975; Hara et al., 1982; McNiven et al., 1993; Stoss, 1983), (2) elevation in fertility of sperm by air supply (Stoss and Holtz, 1983), (3) extension of storage period by antibiotic addition (Chao et al., 1992; Saad et al., 1988; Stoss et al., 1978), (4) Changes of

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fertilization by diluent concentration (Erdahl and Graham, 1987).

Two methods are used for cold storage of milt; diluted and undiluted. The use of diluents for milt storage provides better control of the physicochemical conditions during storage. Storage of milt with a diluent of optimal constituents often prolongs their life compared to undiluted storage. The constitution of the diluent considerably influences the success of storage. As is generally known, sperm of marine fishes are immotile in the seminal plasma, and motility is initiated by osmotic shock when they are discharged into seawater (Morisawa, 1985). Thus, a suitable diluent for cold storage of marine fishes milt must meet condition to prevent sperm from activation by osmotic shock and offer appropriate environment for survival of sperm.

Hence, the present work was designed to determine the suitable diluent and various conditions for cold storage of milt in four species of flatfish (marbled sole, brown sole, starry flounder and olive flounder).

Materials and Methods

Fish specimens and milt collection

Marbled sole (*Limanda yokohamae*), brown sole (*Limanda herzensteini*), starry flounder (*Platichthys stellatus*) and olive flounder (*Paralichthys olivaceus*) males were used in this experiment (Table 1). Mature marbled soles, brown soles and olive flounders were captured near the Chumunjin waters, Kangnung, Korea. After transport to Kangnung Marine Hatchery, National Fisheries Research and Development Institute, fishes were held in indoor 2 m^3 tank supplied with seawater. Starry flounders were used, which had been reared for about 1 year in a circular tank (20 m^3) at Uljin Marine Hatchery, National Fisheries Research and Development Institute. Luteinizing hormone releasing hormone analogue [LHRH-a, (pGlu-His-Trp-Ser-Tyr-D-Ala-Leu-Arg-Pro-NHEt, Sigma)] pellets were implanted in dorsal muscle of starry flounder to increase milt amount.

Prior to handling each fish collected, it was first anesthetized with tricainemethane sulfonate (MS-222). The urinary bladder of fish was gently emptied and the genital area was wiped with paper towel before the milt was stripped by hand. Caution was taken to prevent contamination of the milt with urine or fecal material. The milt was pooled and placed on crushed ice immediately after collection and utilized within one hour. Only sperm showing vigorous movement (>90% progressive motility) upon microscopic observation were used in the experiments.

Table 1. Measurements of marbled sole, brown sole, starry flounder and olive flounder male for milt collection (mean ± SD)

Species	Number of specimen	Total length (cm)	Body weight (g)
Marbled sole	26	26.7 ± 3.1	270.8 ± 93.3
Brown sole	36	21.0 ± 1.8	99.2 ± 29.3
Starry flounder	23	34.2 ± 1.5	574.0 ± 96.0
Olive flounder	24	33.1 ± 1.9	382.1 ± 71.9

Sperm motility

To evaluate sperm motility according to conditions of cold storage, sperm activity index (SAI) was investigated. Milt was diluted at the ratio of 1:20 with artificial seawater to activate sperm. Sperm motility was estimated on a glass slide without cover under a light microscope (×200 magnification) at laboratory temperature ranging from 15℃ to 20℃. The degree of motility was estimated using a scale of arbitrary index from I to Ⅳ, and the percentage of sperm corresponding to each index was recorded. The scores of 3, 2, 1 and 0 were allowed for the index of I, II, Ⅲ and Ⅵ, respectively. SAI was calculated according to following formula using the scores and the percentage of sperm corresponding to each index (Table 2).

Cold storage in various diluents

To investigate cold storage effect of milt in various diluents in four species, diluents tested are as

Table 2. Numerical index for the evaluation of sperm activity index (SAI)

Index	Score	Motility characteristics
I	3	Sperm display forward movement rapidly
II	2	Sperm display forward movement slowly
Ⅲ	1	Sperm display vibrating movement moderately
Ⅳ	0	Immobile sperm

SAI=[(3×sperm percentage of I)+(2×sperm percentage of II)+(1×sperm percentage of Ⅲ)+(0×sperm percentage of Ⅳ)]/100 follows: Alsever's solution, Cortland medium, fructose (0.5 M), glucose (0.5 M), marine fish Ringer's solution (MFRS), Mounib's solution, NaCl (1%), sodium chloride medium, sodium citrate (3.6%), Stein's extender, sucrose (2.8%) (Table 3). Milt was diluted at the ratio of 1:5 (one part of milt:five parts of diluent) with 11 diluents. These diluents were pre-cooled on ice in 1.5 mL Eppendorf tube before adding milt, and after mixing with milt, tubes were capped during storage. Storage treatments were replicated 3 times and were stored in an incubator set at 1 ± 0.5℃. The SAI in each tube were tested daily for 7 days.

Cold storage at various dilution ratios

In order to determine optimal dilution ratio for cold storage of milt in four species, the milt was diluted at the ratio from 1:1 (one part of milt:one part of diluent), 1:3, 1:5, 1:10, 1:20 and 1:40 with a diluent which was proper in each fish species: 1% NaCl in marbled sole, Stein's extender in brown sole, starry flounder and olive flounder. Each mixture was placed in 1.5 mL Eppendorf tube. Storage treatments were replicated 3 times and were stored in a incubator set at 1 ± 0.5℃. The SAI in each tube was tested daily for 7 days.

Cold storage in different volume of diluted milt

In order to evaluate the effect of the storage volume of diluted milt on sperm of marbled sole, brown sole, starry flounder and olive flounder, at first, the milt was diluted at the ratio 1:5 (one part of milt:five parts of diluent). At this time, the diluent to dilute milt of each species is as follows: 1% NaCl in marbled sole and Stein's extender in brown sole, starry flounder and olive flounder. Then, each mixture was placed in 1.5 mL Eppendorf tube as the volume of 0.5 mL, 1.0 mL and 1.5 mL (full). Storage treatments were replicated 3 times and were stored in a incubator set at 1 ± 0.5℃. The SAI in each tube were tested daily for 7 days.

Cold storage with antibiotic

In order to evaluate the effect of antibiotic on

Table 3. Constituents of diluents used in this experiment

Diluents	Constituents	pH	Osmolality (mOsm/kg)
Alsever's solution	20.5 g glucose, 4 g NaCl, 8 g sodium citrate/DW 1,000 mL	6.13	345 ± 3
Cortland medium	0.23 g $CaCl_2$ H_2O, 1.0 g glucose, 0.38 g KCl, 0.23 g $MgSO_4$ $7H_2O$, 7.25 g NaCl, 1.0 g $NaHCO_3$, 0.41 g NaH_2PO_4 H_2O/DW 1,000 mL	6.92	287 ± 7
Fructose (0.5 M)	60.1 g/DW 1,000 mL	6.38	557 ± 4
Glucose (0.5 M)	60.1 g/DW 1,000 mL	6.31	544 ± 5
MFRS	0.346 g $CaCl_2$, 0.597 g KCl, 0.017 g $MgCl_2$, 13.5 g NaCl, 0.025 g $NaHCO_3$/DW 1,000 mL	6.41	458 ± 4
Mounib's solution	6.5 mM glutathione, 100 mM $KHCO_3$, 125 mM sucrose	7.54	317 ± 3
NaCl (1%)	10 g NaCl/DW 1,000 mL	7.12	321 ± 3
Sodium chloride medium	0.4 M NaCl−0.1 M glycine 40 parts, 1.3% $NaHCO_3$ 8 parts	6.75	751 ± 3
Sodium citrate (3.6%)	36 g sodium citrate/DW 1,000 mL	8.16	415 ± 4
Stein's extender	2 g NaHCO3, 1 g glucose, 0.4 g KCl, 7.5 g NaCl/ DW 1,000 mL	7.85	299 ± 3
Sucrose (2.8%)	28 g sucrose/DW 1,000 mL	6.32	83 ± 3

DW: distilled water, MFRS: marine fish Ringer's solution.

extension of storage period of sperm at 1±0.5℃ in four species, at first the milt was diluted at the ratio 1:5 (one part of milt:five parts of diluent). Then, antibiotic (gentamicin, neomycin or streptomycin) was added to each mixture at concentrations of 200, 400, 600, 800 or 1,000 ppm. Storage treatments were replicated 3 times and were stored in an incubator set at 1±0.5℃. The SAI in each tube was tested daily for 7 days and from 7 days forth, SAI was tested at intervals of 2 days.

Statistical analysis

The results were analysed using a one-factor ANOVA. Means were separated by Duncan's multiple range test and were considered significantly different at $P<0.05$.

Results

Cold storage in various diluents

To determine proper diluent for cold storage of milt in four species, milt of each species was diluted with 11 diluents and stored at 1±0.5℃ for 7 days. The results of these experiments were shown in Figs. 1, 2, 3 and 4.

In marbled sole (Fig. 1), immediately after mixing milt with 11 diluents (day 0), SAI in sodium chloride medium and sucrose showed a rapid decrease. Also, SAI declined rapidly in Cortland medium, fructose and glucose at day 1. Sperm stored in NaCl (1%) had significantly high SAI (1.9) at day 7 ($P<0.05$).

In brown sole (Fig. 2), SAI showed high values of 2.1~2.8 just after mixing with 11 diluents. But, at day 1, SAI of sperm stored in Cortland medium, sodium chloride medium and sucrose showed a rapid decrease and reached 0. Also, SAI in fructose, glucose, Mounib's solution and sodium chloride showed low values. SAI remained at 1.8~2.4 in MFRS, NaCl and Stein's extender up to 7 days. Among the three diluents, the best diluent for cold storage of brown sole was determined to be Stein's extender.

In starry flounder (Fig. 3), at day 0, SAI was lower in sucrose (0.8) than in any other diluents (2.1~2.7). Storage for 1 day in diluents except Alsever's solution, MFRS, sodium citrate and Stein's extender produced abrupt decline in SAI. SAI in Stein's extender decreased gradually, but this diluent maintained SAI of 2.1 by 7 days.

In olive flounder (Fig. 4), most diluents except sucrose had high SAI at day 0. However, SAI in sodium chloride medium reached 0 at day 1, and SAI in Cortland medium, fructose, glucose, MFRS, Mounib's solution and sodium citrate dropped to 0 or nearly 0 at day 4. Alsever's solution, NaCl and Stein's extender maintained high SAI above 2.1 by

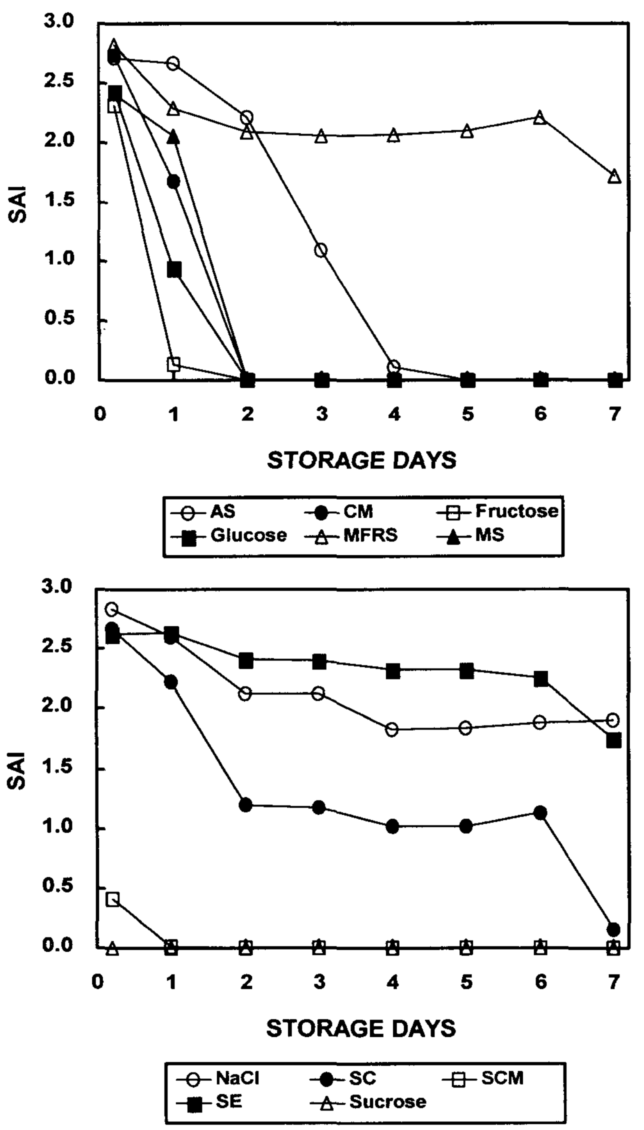

Fig. 1. Variation in SAI of marbled sole sperm stored at 1±0.5℃ for 7 days with 11 diluents. AS: Alsever's solution, CM: Cortland medium, MFRS: marine fish Ringer's solution, MS: Mounib's solution, SC: sodium citrate, SCM: sodium chloride medium, SE: Stein's extender.

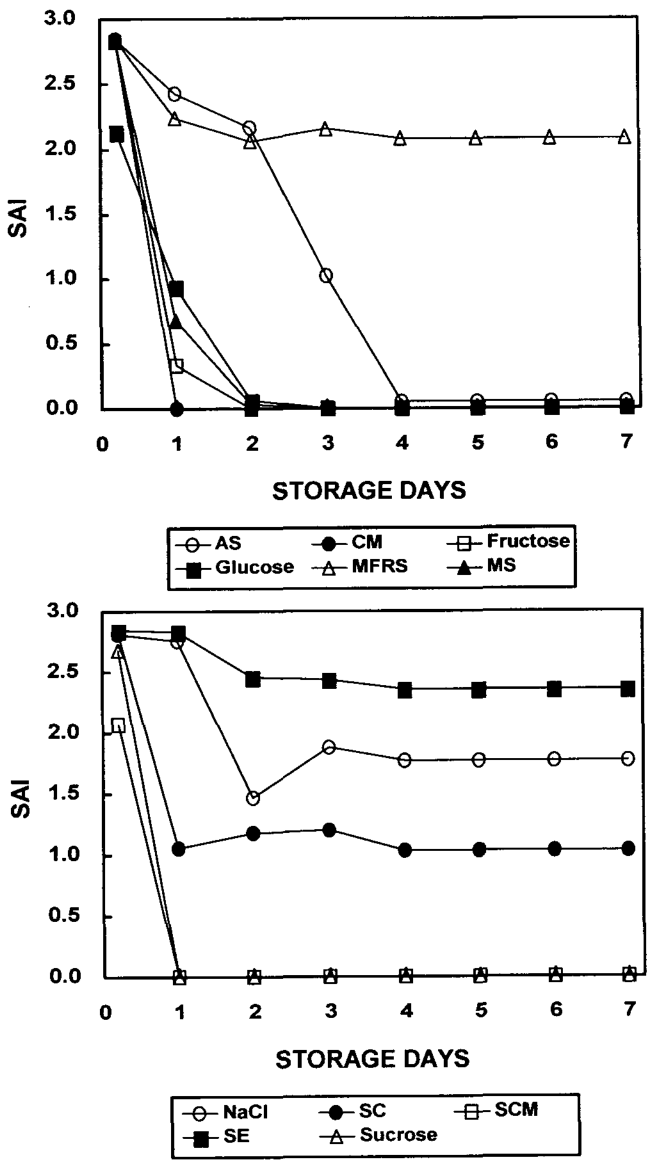

Fig. 2. Variation in SAI of brown sole sperm stored at 1 ±0.5℃ for 7 days with 11 diluents. AS: Alsever's solution, CM: Cortland medium, MFRS: marine fish Ringer's solution, MS: Mounib's solution, SC: sodium citrate, SCM: sodium chloride medium, SE: Stein's extender.

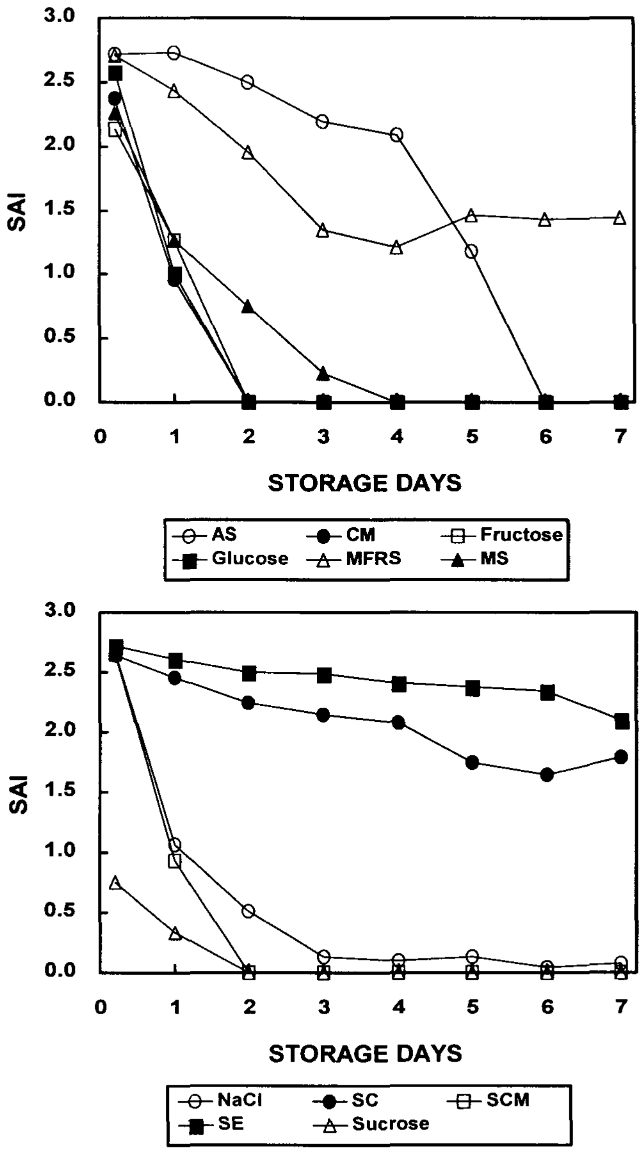

Fig. 3. Variation in SAI of starry flounder sperm stored at 1±0.5℃ for 7 days with 11 diluents. AS: Alsever's solution, CM: Cortland medium, MFRS: marine fish Ringer's solution, MS: Mounib's solution, SC: sodium citrate, SCM: sodium chloride medium, SE: Stein's extender.

7 days. Among the three diluents, the best diluent was determined to be Stein's extender.

Cold storage at various dilution ratios

When the milt diluted at various ratios using a diluent was stored at 1±0.5℃ for 7 days, the results were shown in Fig. 5. In marbled sole (Fig. 5A), the dilution ratio of above 1:10 and below 1:3 rapidly decreased SAI up to day 3. At day 7, SAIs of milt diluted at the ratio from 1:3 to 1:10 were significantly higher than those of other dilution ratios, and significant differences among these dilution ratios were not detected ($P>0.05$).

In brown sole (Fig. 5B), starry flounder (Fig. 5C) and olive flounder (Fig. 5D), SAI remained highly at the dilution ratios of below 1:10 for 7 days, and significant differences among these dilution ratios were not detected ($P>0.05$). However, further in-

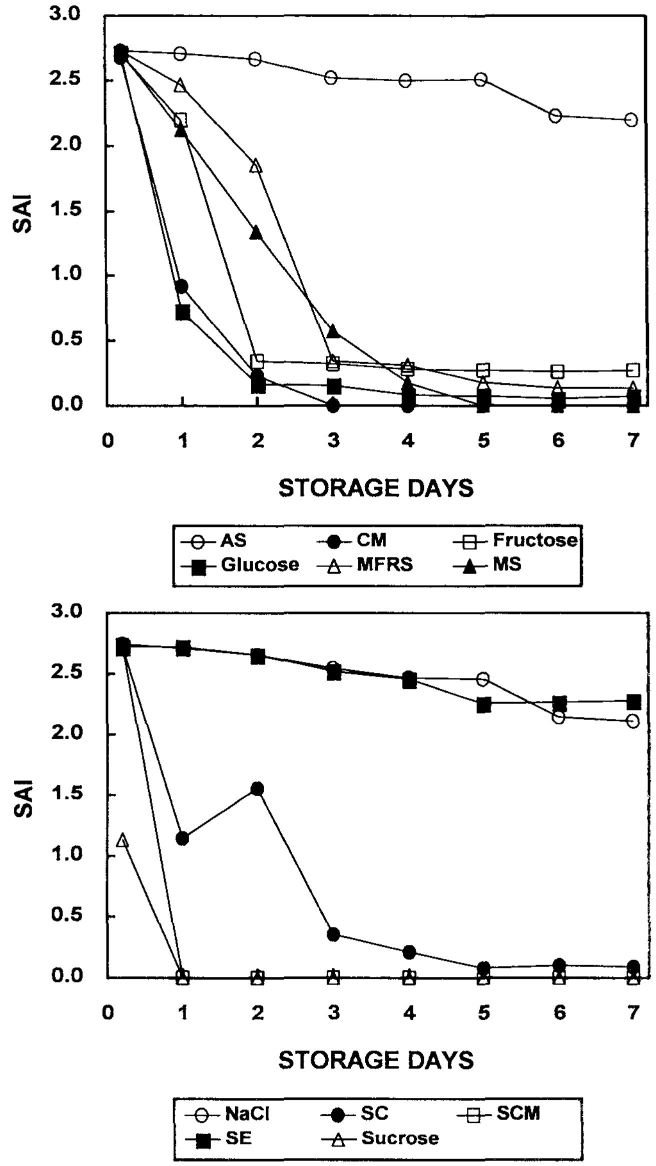

Fig. 4. Variation in SAI of olive flounder sperm stored at 1 ± 0.5℃ for 7 days with 11 diluents. AS: Alsever's solution, CM: Cortland medium, MFRS: marine fish Ringer's solution, MS: Mounib's solution, SC: sodium citrate, SCM: sodium chloride medium, SE: Stein's extender.

creased dilution ratio slowly led to a decline in SAI during cold storage and at 1:40 dilution ratio, sperm of brown sole and olive flounder lost their motility at day 6 and at day 7, respectively.

Cold storage of different volume of diluted milt

When diluted milt was stored in the volume of 0.5 mL, 1.0 mL and 1.5 mL at 1 ± 0.5℃ for 7 days in four species, the results were shown in Fig. 6.

The SAIs of the diluted milt stored in 1.5 mL were significantly lower than those of other experimental groups after 7 days in four species ($P<0.05$).

Cold storage with antibiotic

As milt of four species was diluted with diluent containing antibiotic, the effect of antibiotic on the sperm of each species was shown in Figs. 7, 8, 9 and 10.

In marbled sole (Fig. 7), SAI remained highly under the presence of antibiotic for 29 days. However, SAI of control group declined more rapidly during storage and reached 0 at day 23. Sperm stored in diluent containing 400 ppm gentamicin, 400 ppm neomycin and 200~400 ppm streptomycin showed significantly high SAI at day 29 ($P<0.05$).

In brown sole (Fig. 8), SAI in milt treated with antibiotic was gradually decreased during storage, although SAIs at some concentrations of neomycin or streptomycin were inferior to control group during some period. However, sperm stored in diluent containing 600 ppm gentamicin had significantly high SAI (1.0) at day 29 ($P<0.05$).

In starry flounder (Fig. 9) and olive flounder (Fig. 10) good SAIs were observed under the presence of antibiotic for 29 days. The rapid decrease of SAI in absence of antibiotic (control) was ovserved during storage. The milt containing 1,000 ppm gentamicin in starry flounder and 200~1,000 ppm gentamicin, 200~400 ppm neomycin, 200~1,000 ppm streptomycin in olive flounder showed the best SAI for 29 days of storage.

Discussion

The main factors, which influence the cold storage of fish milt, are diluent, dilution ratio, volume of preserved milt and antibiotic. Diluents for cold storage of milt from several species have been proposed (Chao et al., 1975; Hara et al., 1982; Scott and Baynes, 1980; Truscott et al., 1968). As an important criterion for these diluents, the sperm should remain immotile in the diluent in order to save the energy of motility demanded for fertilization (Ohta and Izawa, 1996). 1% NaCl in marbled sole and Stein's extender in brown sole, starry flounder and olive flounder were found to be the best among the

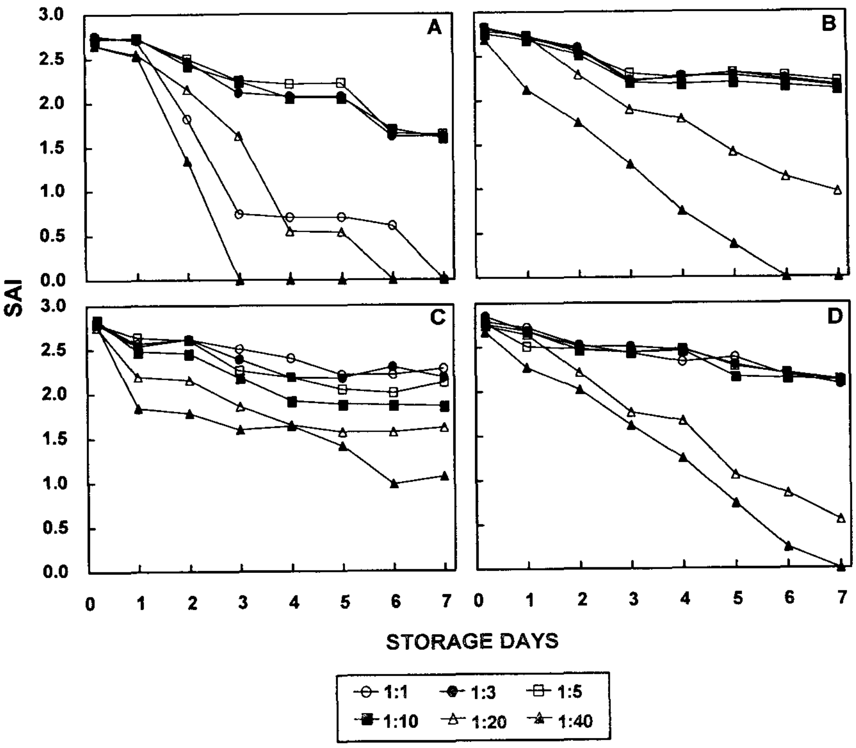

Fig. 5. Variation in SAI of four flatfish sperm stored at 1±0.5℃ for 7 days at various dilution ratios. A: marbled sole, B: brown sole, C: starry flounder, D: olive flounder.

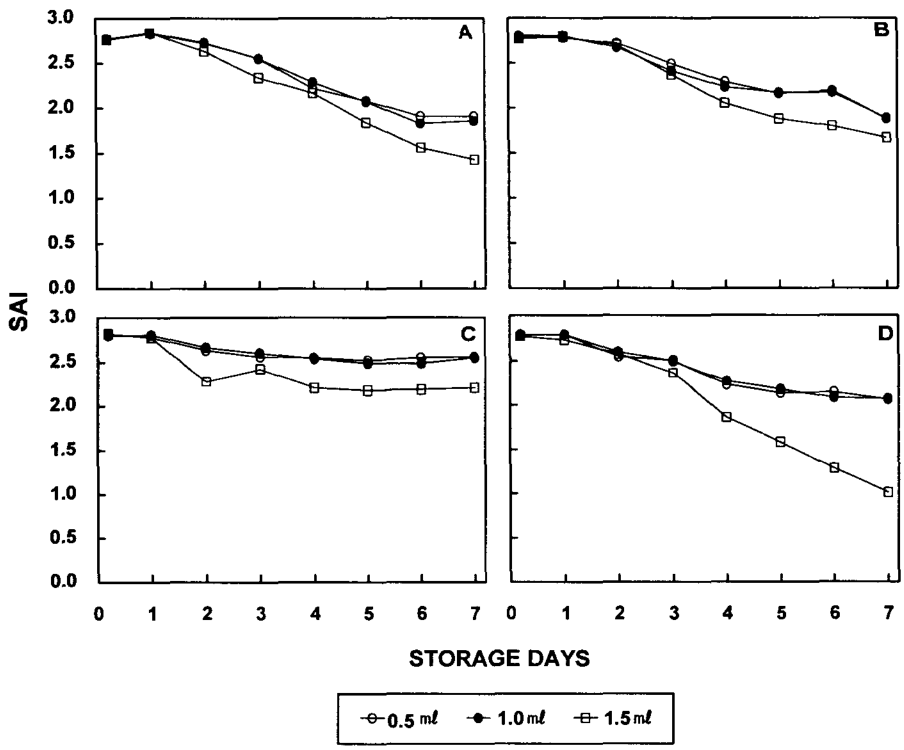

Fig. 6. Variation in SAI of four flatfish sperm stored at 1±0.5℃ for 7 days at different volume of diluted milt. A: marbled sole, B: brown sole, C: starry flounder, D: olive flounder.

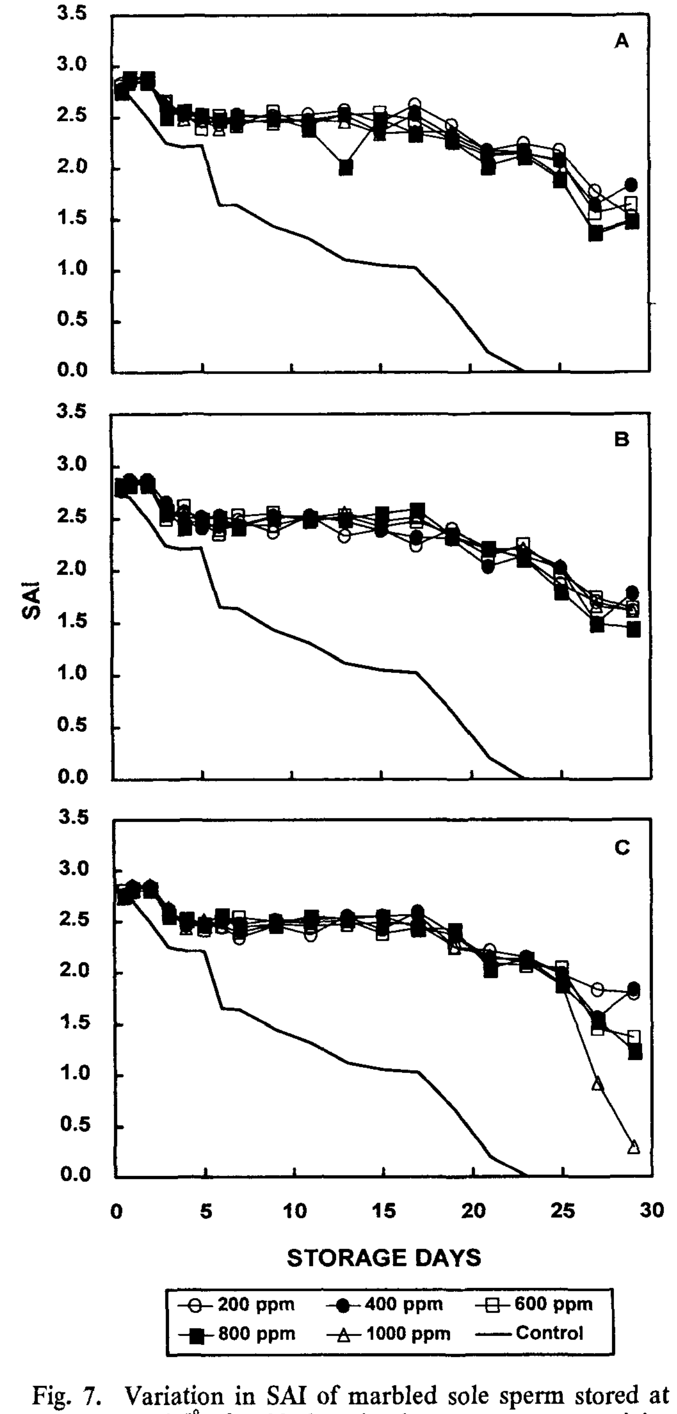

Fig. 7. Variation in SAI of marbled sole sperm stored at 1 ± 0.5℃ for 29 days in the presence gentamicin, neomycin or streptomycin, or absence of antibiotic (control). A: gentamicin, B: neomycin, C: streptomycin.

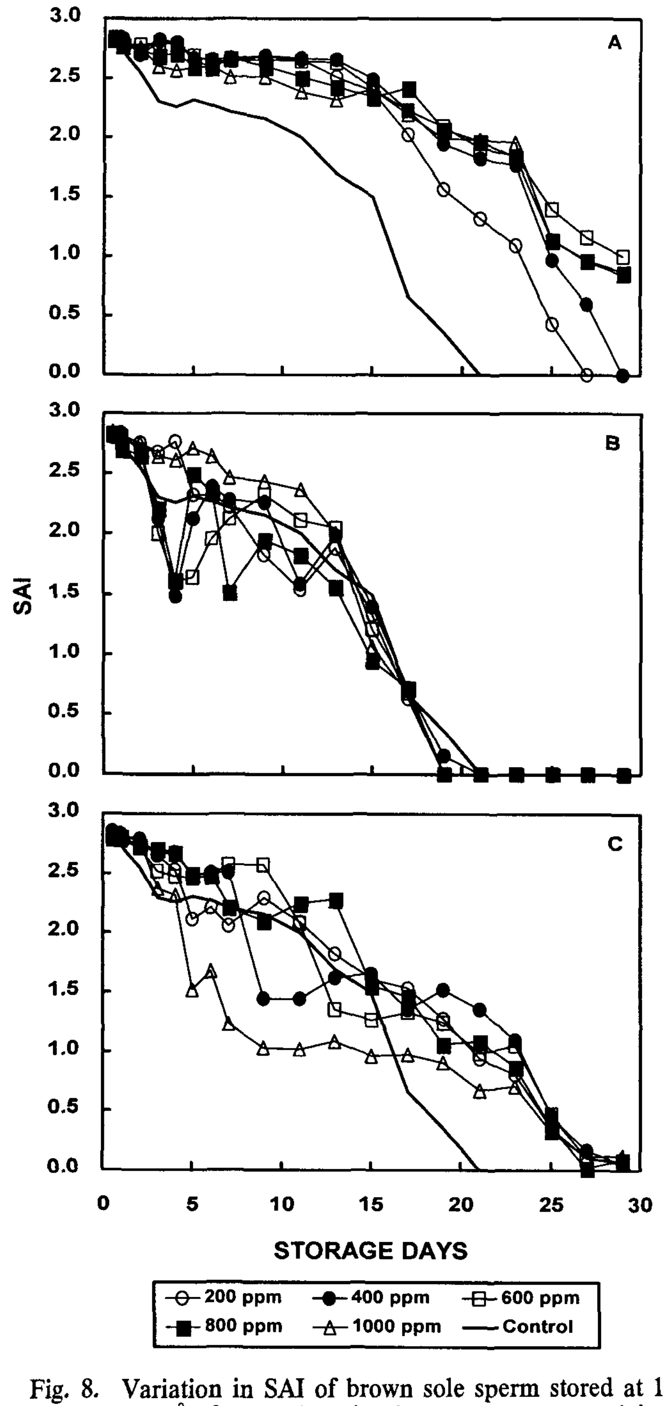

Fig. 8. Variation in SAI of brown sole sperm stored at 1 ± 0.5℃ for 29 days in the presence gentamicin, neomycin or streptomycin, or absence of antibiotic (control). A: gentamicin, B: neomycin, C: streptomycin.

11 diluents tested in maintaining good SAI for 7 days of storage at 1 ± 0.5℃. Hara et al. (1982) found blood serum to be a superior diluent for milkfish, *Chanos chanos* sperm. Chao et al. (1975) reported that 6, 10, and 12% sodium citrate were good diluents in maintaining motility of grey mullet, *Mugil cephalus* sperm at 5℃. The optimal diluent for cold storage seems to be species-specific. Therefore, the

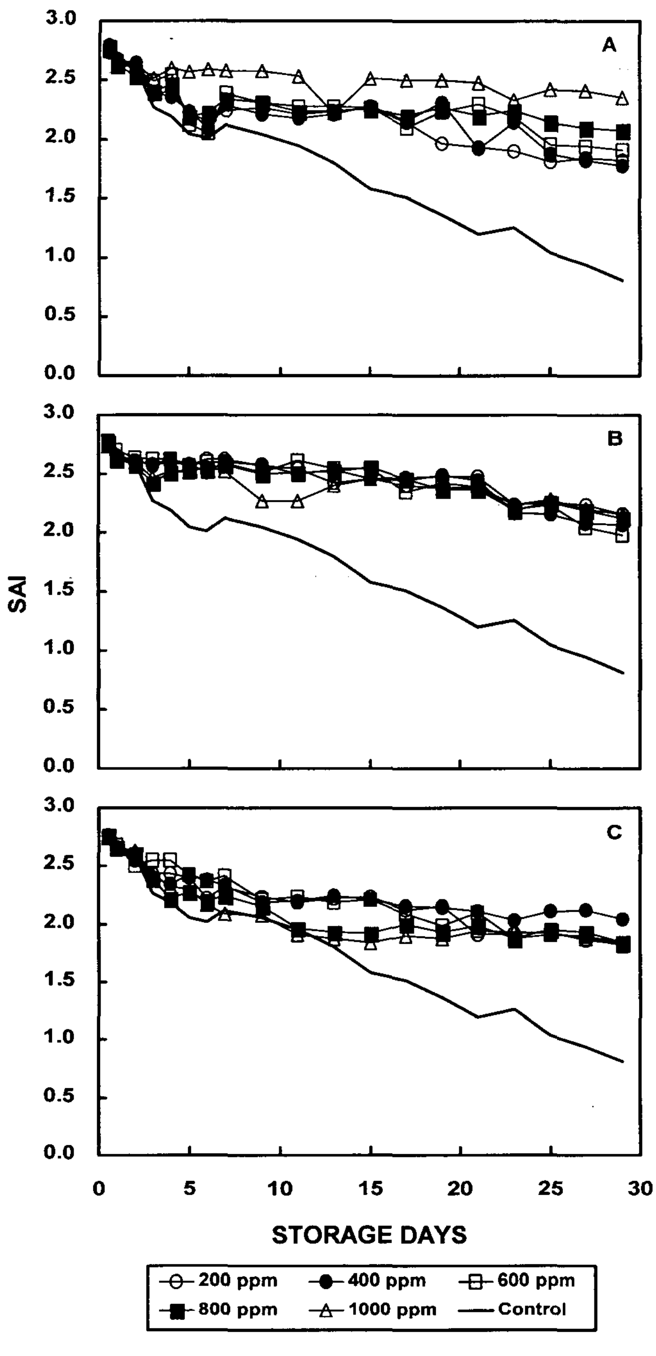

Fig. 9. Variation in SAI of starry flounder sperm stored at 1±0.5℃ for 29 days in the presence gentamicin, neomycin or streptomycin, or absence of antibiotic (control). A: gentamicin, B: neomycin, C: streptomycin.

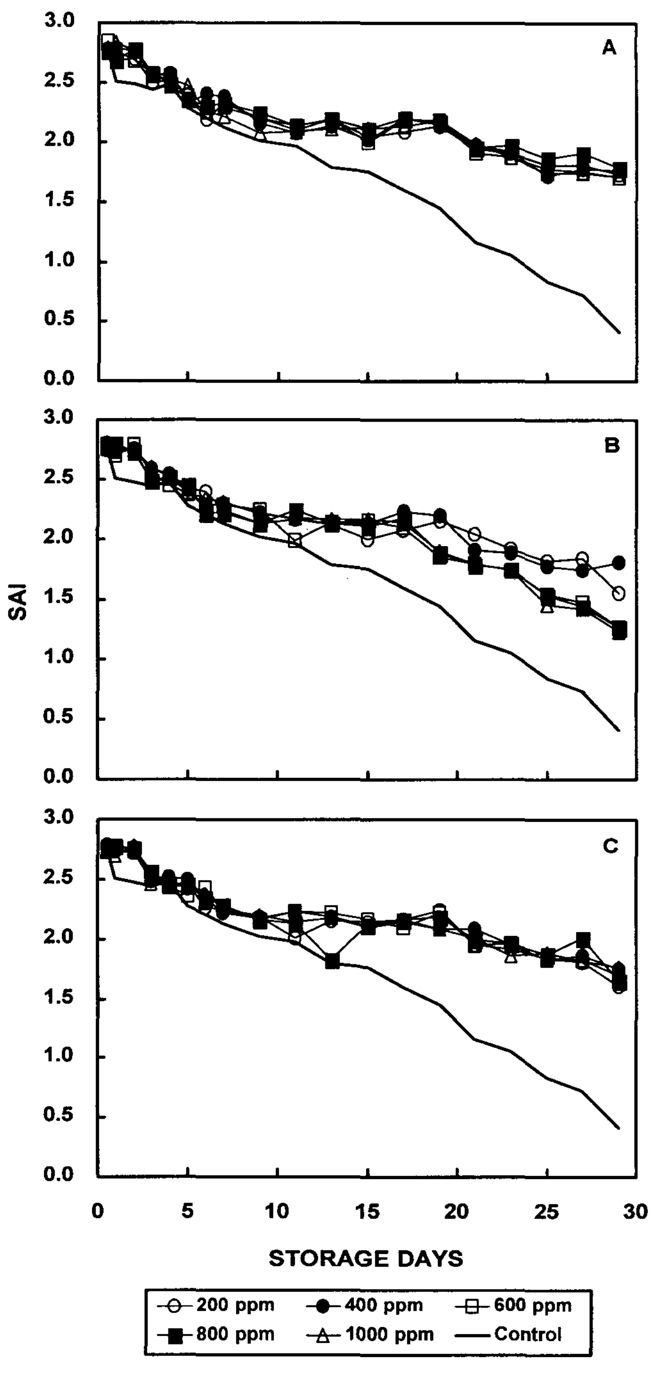

Fig. 10. Variation in SAI of olive flounder sperm stored at 1±0.5℃ for 29 days in the presence gentamicin, neomycin or streptomycin, or absence of antibiotic (control). A: gentamicin, B: neomycin, C: streptomycin.

use of diluent, which is equipped with condition to inhibit initiation of sperm motility or is similar to composition of seminal plasma, will raise the effect of cold storage of milt.

Good retention of SAI was obtained at low dilution ratio (>1:10) in flatfish milt diluted with optimal diluent. Perhaps, this was partially due to a direct reduction of milt viscosity and CO_2 concentration in milt (thus reducing the toxic effect of CO_2 on sperm) (Yao et al., 1999). SAIs at higher dilution ratios (>1:20) were decreased during storage in flatfish milt. It appears that "dilution shock" takes place motility initiation in high dilutions and thereby decreases storage time as proposed by Erdahl and Graham (1987).

Oxygen or air is important to increase of storage period in cold storage of milt. Stoss et al. (1978) reported that pure oxygen was superior to air or other gas for survival of sperm. But, in the carp, oxygen did not produce much better results than storage in the air (Saad et al., 1988). In this study, air supply were not allowed in the storage volume of 1.5 mL (Because the Eppendorf tube was filled with milt). Therefore, SAI decline of 1.5 mL may be due to lack of air or oxygen. In the future, more detail work is necessary to examine the effect of oxygen or air of sperm survival in cold storage of flatfish milt. This observation supports the opinion of Stoss et al. (1987) who suggested that in deep milt samples with small surface area, the perishing cells at the bottom of the container exert a detrimental effect on those located close to the surface, because in these, motility ceased within rather short period. So, the decrease of SAI in the storage volume of 1.5 mL in flatfish seems to be due to a detrimental effect of sperm at the bottom of the container.

The addition of antibiotic for cold storage of milt in four species of flatfish maintained the life of sperm for a long time. The use of antibiotic is essential for the prolongation of storage time, as bacterial contamination of sperm from excreta can be avoided (Stoss and Refstie, 1983) and contributes to raise the motility and survival rate of sperm as well as fertilization rate (Chao et al., 1992; Saad et al., 1988; Stoss et al., 1978). Although antibiotic inhibits bacterial growth, they have detrimental effect on sperm of rainbow trout, *Salmo gairdneri* at levels of 9,000 IU penicillin or 9,000 μg streptomycin per mL (Stoss et al., 1978). In this study, SAIs of brown sole milt containing neomycin or streptomycin were inferior to control group. Thus, neomycin and streptomycin seem to be detrimental to brown sole sperm. Chao et al., (1992) reported that streptomycin at either of 500, 1,000 or 1,500 ppm was worth recommending for cold storage, when compared to other antibiotic and concentrations in grouper, *Epiniphelus malabaricus*. Saad et al. (1988) found that survival rate of carp, *Cyprinus carpio* sperm was markedly enhanced by the addition of antibiotic (50 μg/mL streptomycin+50 IU bipenicillin). The milt of flatfish used in this study also maintained high SAI by addition of antibiotic.

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