Thesis for the Master Degree of Fisheries Science

Sperm Cryopreservation of Red Seabream *Pagrus major*

by

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KOICA-PKNU International Graduate Program of Fisheries Science,

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Pukyong National University

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참돔 *Pagrus major* 정자의 냉동보존

Advisor : Prof. Young Jin Chang

by

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Contents

List of Figures

List of Tables

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Abstract

The aim of this study was to determinate the optimal conditions in sperm cryopreservation in red seabrem *Pagrus major*. Two extenders and five cryoprotectants (CPAs) in four different concentrations (5, 10, 15 and 20%) were evaluated on fresh sperm motility and post-thaw sperm motility. The sperm viability on fresh sperm was estimated immediately after dilution with the CPAs. Glycerol with glucose as extender showed the highest viability comparing with the other CPAs with a viability of 16.8 ±0.6 min showing no different between concentrations, and in post-thaw using glycerol as a CPA and sucrose as an extender, showed the highest values in spermatozoa

activity index, duration of progressive motility, total duration of motility and survival rate. In 5, 10 and 15% gave a spermatozoa activity index of 4.0±0.0 the duration of progresive motilities were 9.8±0.7 min in 5%, 7.9±0.5 min in 10% and 5.7±3.3 min in 15%, respectively. With a total duration motility of 21.7±0.4 min in 5%, 16.1±2.3 min in 10% and 12.2±1.1 min in 15%,. and a survival rate of 82.0±1.7 % in 5%, 74.3±3.5 % in 10% and 60.0±3.6%. in 15% There was no significant difference in sperm viability between all the CPAs except sperm cryopreserved with methanol in all four concentrations. Methanol in all four concentrations turn up to be toxic for red seabream sperm. In conclucion, these methods of cryo-preservation of red seambream sperm are suitable for routine aquaculture application and preservation of genetic resource.

I. INTRODUCTION

The development of routine use of artificial fertilization in aquaculture has raised the requirement for storage of reproductive material. The short period of time for which fish gametes remain in good condition after collection became a significant obstacle to hybridization between species of fish inhabiting different geographical locations, or having different spawning times. The asynchronous maturation of breeders invariably causes problems in aquaculture and has stimulated research to develop a method suitable for the prolonged storage of fish sperm. Cryopreservation is considered as one component in effective strategies to save endangered species by facilitating the storage of their gametes in a bank (Gausen, 1993).

Research on cryopreservation of fish sperm, with focus on cryopreservation protocols, has achieved great advances since the first successful cryopreserving of sperm in herring *Clupea harengus* 55 years ago (Blaxter, 1953). It provides many benefits such as ease of global germplasm shipping and supply, selective breeding and hybridization with desirable characteristics, and conservation of genetic diversity (Van der Walte et al., 1993; Gwo et al., 1999; Tiersch et al., 2000; Ohta et al., 2001)

furthermore, a frozen sperm bank could maintain a continuous and stable supply of gametes for hatchery seedling production or laboratory experiment (Lubzens et al., 1997). Because of the advantages of this technique, fish sperm of over 200 freshwater and 40 marine species have been cryopreserved successfully (Gwo, 2000).

Most of fish sperm cryopreservation researches have focused on freshwater species such as cyprinids, salmonids and catfishes. In recent years, with the rapid development of marine fish aquaculture, some experiments on cryopreservation have also been conducted on marine fish species, especially on the fishes of great commercial value such as turbot *Psetta maxima*, olive flounder *Paralichthys olivaceus,* and halibut *Hippoglossus stenolepis*. Although successful sperm cryopreservation of sperm in straws is advantageous for laboratory use such as gene banking, or small-scale commercial use, but it is impractical for large scale insemination at hatcheries. Therefore, much work remains to be done before cryopreservation of sperm can be successfully employed in large scale application to fish species (Liu et al., 2006).

From the viewpoint of aquaculture the availability of frozen gametes, allows the reduction of production costs by reducing maintenance costs for

breeding animals and their transport from one crop to another center, it is more economical to transport a container of gametes in liquid nitrogen that a group of breeding animals, while avoiding related stress.

Red seabream *Pagrus major* is a marine fish species of considerable commercial importance in the aquaculture industry in South Korea, China and Japan. In recent years, the decline in wild red seabream population has occurred because of overfishing and marine pollution. Therefore, reliable methods for sperm cryopreservation could be beneficial both aquaculture application and conservation of biodiversity. The aim of this study was to establish the optimal condition in sperm cryopreservation of the red seabream.

II. MATERIALS AND METHODS

1. Experimental fish

The breeder animals were obtained from the National Fisheries Research and Development Institute (NFRDI) during the first week of May 2011, which coincided with spawning season of the red seabream. Eight male fish were used for this experiment, those were indoor cultivated in the NFRDI, in a seawater recirculation system, under the control conditions with a 18°C of water temperature and 35 psu of salinity. Each individual was measured from the total length and body weight (**Fig. 1**). Fish were fed twice a day with the commercial feed.

2. Sperm collection

Prior to handling, the breeding animals were first anesthetized in a bath of 200 ppm phenoxy ethanol. Semen was collected from three different stages with a period of 8 days between each other, sperm volume was measured in mL. Semen were collected by gentle abdominal massage (**Fig. 2**) from the anterior portion of the testis towards the genital papilla

Fig. 1. Equipment for measuring specimen size and weight. 1: length data table, 2: analytical balance.

Fig. 2. Collection of red seabream semen *Pagrus major* by gentle abdominal massage.

using a 14 mL tube, to prevent contamination with seawater, urine, mucus, fecal material or blood. The samples were placed immediately on crushed ice and transport to the laboratory in the university to initial the analysis.

3. Biochemical property of seminal plasma

To analyze biochemical properties of seminal plasma, the sperm were separated from the seminal plasma by centrifugation (15,000 rpm for 10 min) (**Fig. 3**). Seminal plasma was centrifuged twice to avoid possible contamination with spermatozoa. The supernatants were frozen and stored in the refrigerator until analysis. The biochemical components of seminal plasma were determined by Fuji Dri-Chem 3500 (Fujifilm Co. Ltd., Japan). The pH and osmolality of seminal plasma were measured by pH meter (Istek, Korea) and the osmometer (Wescor Inc., USA), respectively.

4. Spermatozoa motile assessment

The motility of spermatozoa samples was determined immediately after semen collection, the percentage of the motile spermatozoa were check, using a microscope (**Fig. 4**) by diluting the sperm into artificial sea water

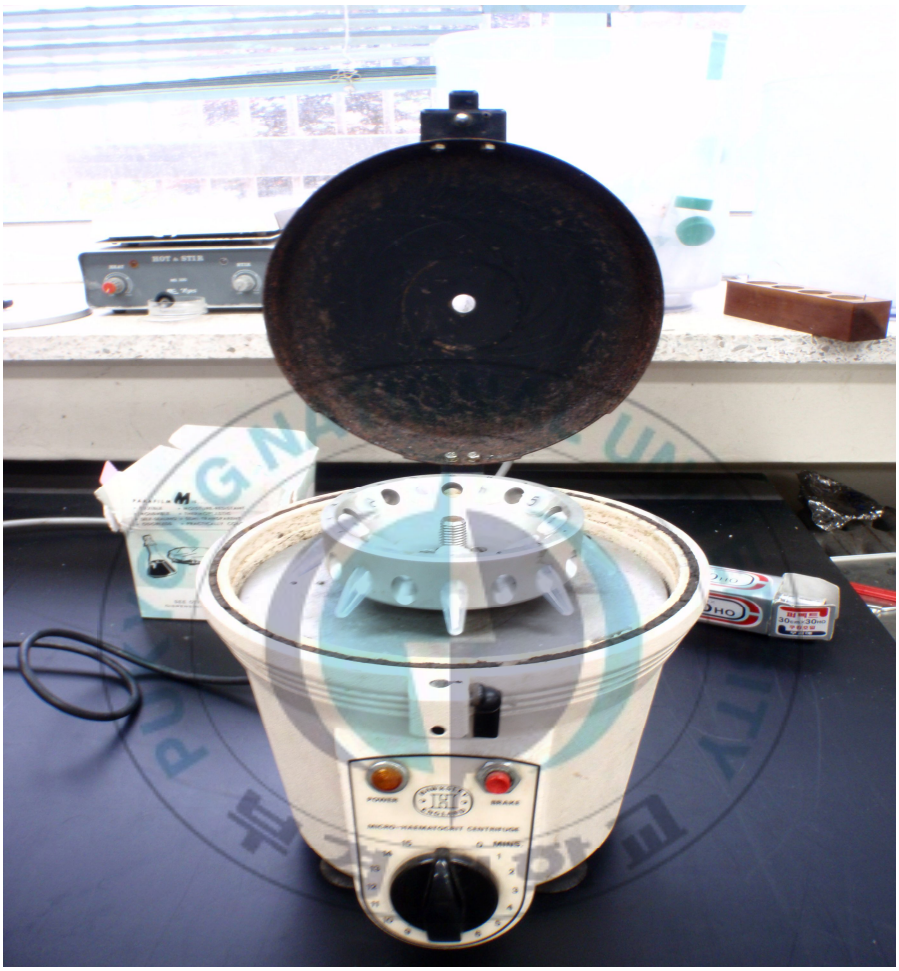

Fig. 3. Centrifugation of the semen of the red seabream *Pagrus major* to get the seminal plasma for use in the experiment.

Fig. 4. A microscope used for the evaluation of sperm motility

(ASW; NaCl 27 g, KCl 0.5 g, $CaCl_2$ g, $MgCl_2$ 4.6 g, $NaHCO_3$ 0.5 g in a liter of distilled water) at the ratio of 1 to 100. The spermatozoa with a total motility above that 90% were use in this study.

To evaluate the post-thawed spermatozoa viability of each cryopreservation condition, spermatozoa activity index (SAI), duration of progressive motility (DPM), total duration of motility (TDM) and survival rate of post-thawed spermatozoa were estimated, by mixing the prediluted semen in ASW at one ratio of 1 to 100 (1 μL presiluted sperm to 99 μL ASW). From here 1μL was put into slide glass (Teflon Printed Glass Slide; 21 wells; diameter of each well, 4 mm; Funakoshi Co., Japan) without a cover slide. The activity of post-thawed spermatozoa was observed immediately at x200 magnification under the microscope, using different electronic equipment such as digital camera to be able to record the spermatozoa activity. The sample was observed three times under the microscope and the time for each one was observed until spermatozoa stopped moving. The SAI of post-thawed sperm was evaluated by the percentage of motile and immotile sperm. 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 were recorded. On this the score of 4, 3, 2, and

1, were allowed into the index from I to IV. SAI was calculated following the formula, using the score and the percentage of sperm corresponding to each index (**Table 1**). The SAI of post-thawed spermatozoa was determined by the distance of moving sperm on one second. TDM was calculated after dilution of post-thawed semen until 100% of spermatozoa stopped moving.

5. Effects of cryoprotective agents on the viability of fresh sperm

The aim of this experiment was to evaluate the effect of four cryoprotectant on fresh sperm viability in red sea bream, four cryoprotectants namely, dimethyl sulfoxide (DMSO), glycerol, ethylene glycol (EG) and methanol (**Fig. 5**) were respectively mixed with two different extenders, namely glucose and sucrose, to obtain concentrations of 5, 10 15 and 20%. The sperm was diluted at the ratio of 1:5 with each CPA solution. Immediately after the dilution the survival rate was observe under the microscope to evaluate the duration of viability, until almost a 90% of the spermatozoa stopped moving.

Table 1. Numerical index for the evaluation of spermatozoa activity index (SAI)

Index	Score	Motility characteristics
I	4	Sperm display forward movement rapidly
II	3	Sperm display forward movement slowly
III	2	Sperm display vibrating movement moderately
IV	1	Immotile sperm

SAI = score x % motile sperm/100.

Fig. 5. Four cryoprotective agents used in this experiment. DMSO: dimethyl sulfoxide, EG: ethlylene glycol.

6. Sperm cryopreservation and thawing

After sperm collection, pooled sperm was mixed with extender supplement cryoprotectants at a ratio of 1:3 (semen: extender), and 0.25 ml of diluted sperm was put in to a 0.25 mL plastic straw, in one end of the straw was sealed with straw powder. Then the straw were placed on a tray, and suspended for 5 min at 3.5 cm above the liquid nitrogen surface with a temperature of -76°C, finally sink into the liquid nitrogen. For thawing, the straws with the sperm were thawed at 37°C water bath for 30 seconds. Immediately after thawing, the evaluation activity began (SAI, DPM, TDM and survival rate). The procedure of sperm cryopreservation and thawing is given in **Fig. 6**.

7. Effect of extenders and cryoprotectants on sperm motility

In this study two extenders were evaluated, including glucose and sucrose (**Table 2**). After cryopreservation storing for two weeks in liquid nitrogen**,** plastic straws were thawed. SAI, DPM, TDM and survival rate were determined as described above.

Five different cryoprotectants will be evaluated for their effects on motility on red sea bream sperm including DMSO, glycerol, DMSO+gly-

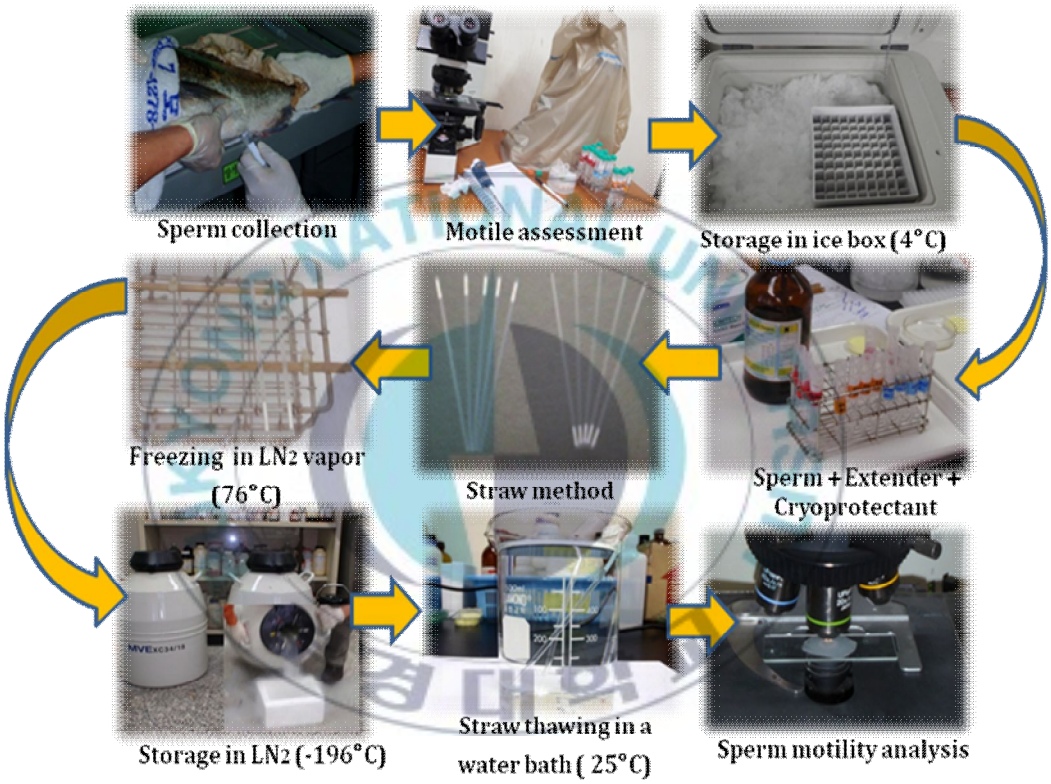

Fig. 6. Procedure of cryopreservation, thawing and motility analysis of red seabream *Pagrus major* sperm. LN_2: liquid nitrogen.

Table 2. Composition of extenders used in the experiments

Composition (g)	Sucrose	Glucose
NaCl	13.5	13.5
KCl	0.35	0.35
$Cacl_2$	0.6	0.6
$MgCl_2$	230	230
$NaHCO_3$	0.25	0.25
Sucrose	10.296	--
Glucose	--	59.452

cerol, EG, and methanol. All this cryoprotectants were at four different concentration of 5, 10, 15 and 20% in mixture of extender and sperm.

8. Statistical analysis

All data will be analyzed by one-way ANOVA. Means were separated by Ducan´s multiple range test and were considered significantly different at P=0.05.

III. RESULTS

1. Size of experimental fish

Total length and body weight of eight breeder male fish used for the experiment were measured. **Table 3** shows the different size and weight of each fish. Being the smaller fish number 7 with total length of 37.3 cm and body weight of 881 g and the biggest fish number 5 with total length of 55.6 cm and 3,435 g of body weight.

2. Sperm volume

The sperm volume that was collect during the spawning season of eight red seabreams is show in **Fig. 7**. For the first week the total is of 51.9 mL knowing that on this week fish number 3 and 4 gave no data. For the second the volume was of 95.3 mL, from all eight fish and for the third week the total was of 80.0 mL from only 6 fish, for the fourth week the total was 70.0 mL from 6 fish, been this the most productive week of the season, for the fifth week the volume was of 50 mL from only 4 fish and the sixth week the last week of the spawning season the total volume was of 28 mL from 4 fish too.

Table 3. Measurements of adult male red seabreams *Pagrus major*

Fish number	Total length (cm)	Body weight (g)
1	52.5	2,867
2	42.0	1,377
3	40.1	1,134
4	35.8	760
5	55.6	3,435
6	38.7	1,072
7	37.3	881
8	51.8	2,484

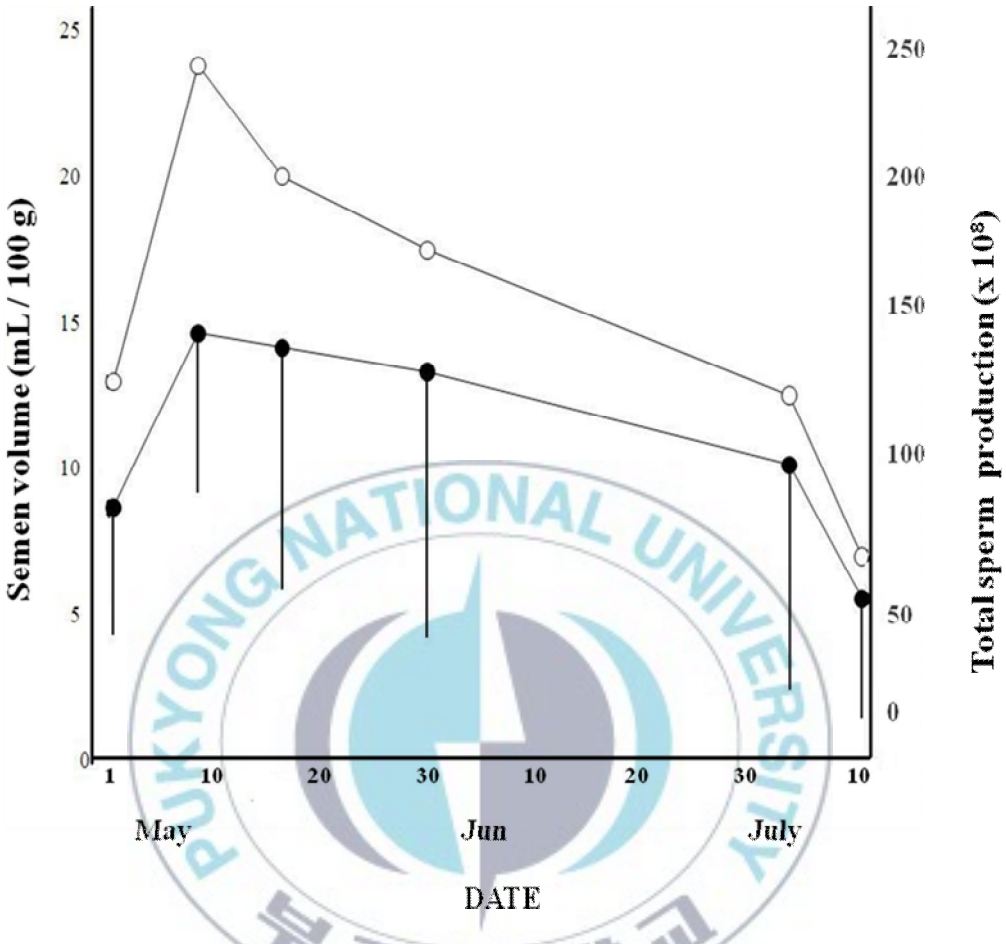

Fig. 7. The results of semen volume (●) and total sperm production (○) during the spawning season.

Giving a total of 375.2 mL during the spawning season. The relationship between weight and sperm was shoen in **Fig. 8.** The high volume of sperm were from fish number 1 with a total volume. of 60.7 mL, with a total body weight of 1997.5 g, and fish number 5 with a total body volume of 51.2 mL, with a total body weight of 3373.70 g. and the lowest were fish number 4 with a total volume of 11.42 mL, with a total body weight of 747.5 g. and fish number 6 with a total volume of 12.o mL, with a total body weight of 1044.9 g.

3. Biochemical property of seminal plasma

The biochemical properties of the seminal plasma were given in **Table 4** Na^+ (171.8±3.6 mmol/L) and Cl^- (137.4±1.6 mmol/L) were predominant ions in the seminal plasma, and the lowest concentration was show in glucose (3.0±0.6 mmol/L).

4. Effects of cryoprotective agents on the viability of fresh sperm

To evaluate the effect of CPAs on fresh sperm viability, sperm viability was estimated immediately after dilution with the CPAs on two different

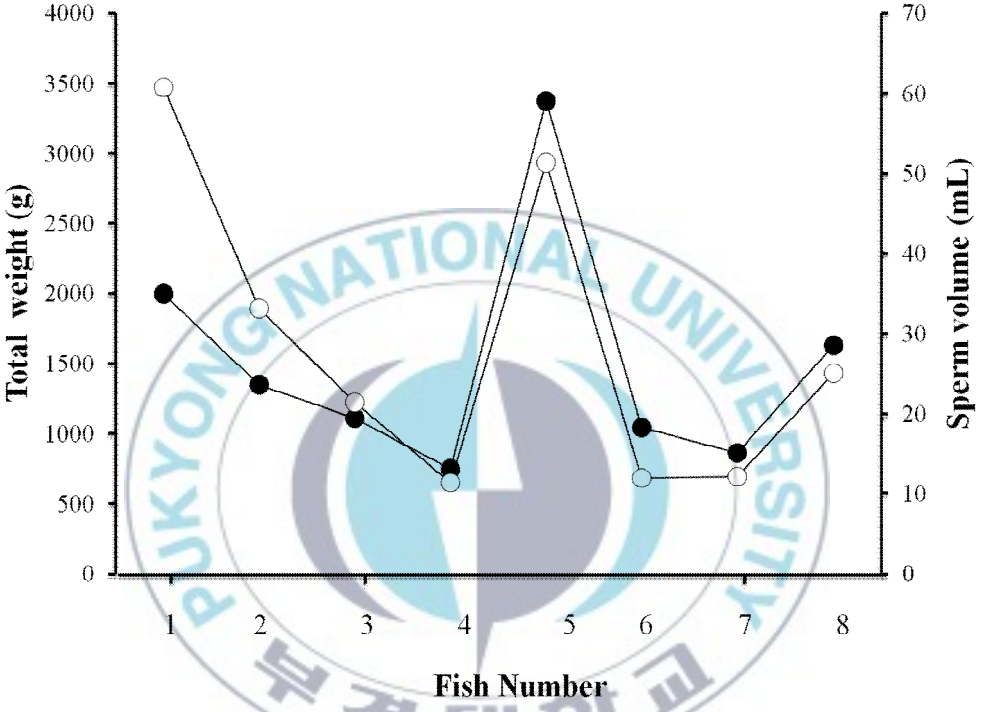

Fig. 8. Relationship between fish weight (g, ●) and sperm volume (mL, ○) in red seabream *Pagrus major* during the spawning season.

Table 4. Biochemical properties of sperm and seminal plasma in red seabream *Pagrus major*

Property	Minimun	Maximun	Mean	SE
Sperm vol (mL)	0.9	1.5	1.1	0.1
K^+(mmol/L)	2.5	5.2	3.8	0.6
Na^+(mmol/L)	167.0	183.7	171.8	3.6
Cl^- (mmol/L)	132.8	139.7	137.4	1.5
Ca^{++} (mg/100mL)	11.7	15.1	12.2	0.8
Mg^{++} (mg/100mL)	3.8	4.8	4.2	0.3
Glucose (mg/100mL)	1.2	4.2	3.0	0.6
pH	7.9	8.3	.8.0	0.1
Spermatocrit(%)	42.2	65.6	55.0	4.8

extenders (sucrose and glucose) containing five CPAs at various concentrations (**Fig. 9**). After mixing the sperm with the extender sucrose containing DMSO, DMSO+glycerol, ethylene glycol and methanol at various con-centrations, immediately the sperm movement stared, for the CPA DMSO+glycerol the motility was decreased after a few minutes, comparing to the others CPAs, DMSO+glycerol at the concentration of 20% on sucrose extender, the motility was for of 5.4 min and the higher for DMSO +glycerol was with a concentration of 10% with a motility of 10.02 min showing a low relative different between each other, however DMSO along showed a high motility in all concentrations being the highest concentration 15% with a 16.6 min of motility. Comparing with the others CPAs the relative different was really high, knowing that for glycerol it was no different between concentrations with a duration of spermatozoon movement of 16.8 ±0.6 min.

When sperm was mixed with glucose as extender on all five CPAs, immediately the sperm movement stared, for this extender the viability was higher comparing with sucrose, even though the DMSO+glycerol was the lowers between all the CPAs showing no differences between concentration, but comparing to glycerol the different of motility was high. Showing for

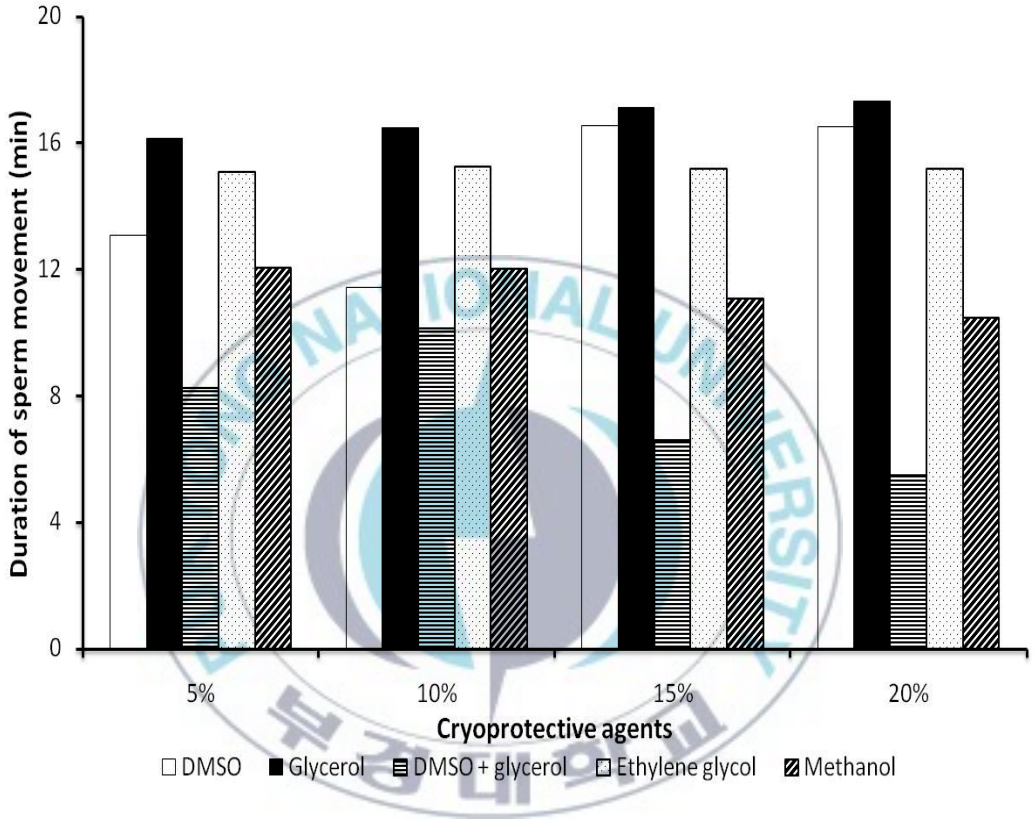

Fig. 9. Duration of sperm movement by the immersion in several cryoprotective agents with sucrose as extender.

DMSO+glycerol 9.9±0.6 min and glycerol had a 18.0±0.3 min of duration of sperm movement (**Fig. 10**).

When we compared the two extenders (**Fig. 9 and 10**) glucose and sucrose we can see that glycerol gave the best result in all four concentrations with no relative different between each other.

5. Sperm cryopreservation and thawing

The cryopreserved effect of DMSO, glycerl, DMSO+glycerl, EG and methanol at different concentrations in sucrose extender where show in **Table 5**. The highest sperm viability was observed in glycerol on 5, 10 and 15% in red seabream, SAI of 4.0±0.0 duration of progresive motility of 9.9±0.6 for 5%, 7.9±0.5 for 10% and for 15% 5.7±3.3 min. the total duration motlity of 21.7±0.4 for 5%, 16.1±2.3 for 10% and for 15 % 12.2±1.1 min. and a survival rate of 82.0±1.7 for 5%, 74.3±3.5 for 10% and for 15% a rate of 60.0±3.6%. there was no significant difference in sperm viability between all the CPAs except sperm cryopreserved with methanol in all four concentrations ($P<0.05$). Cryopreserved with EG at all four concentration 5, 10, 15 and 20% the sperm had no motility.

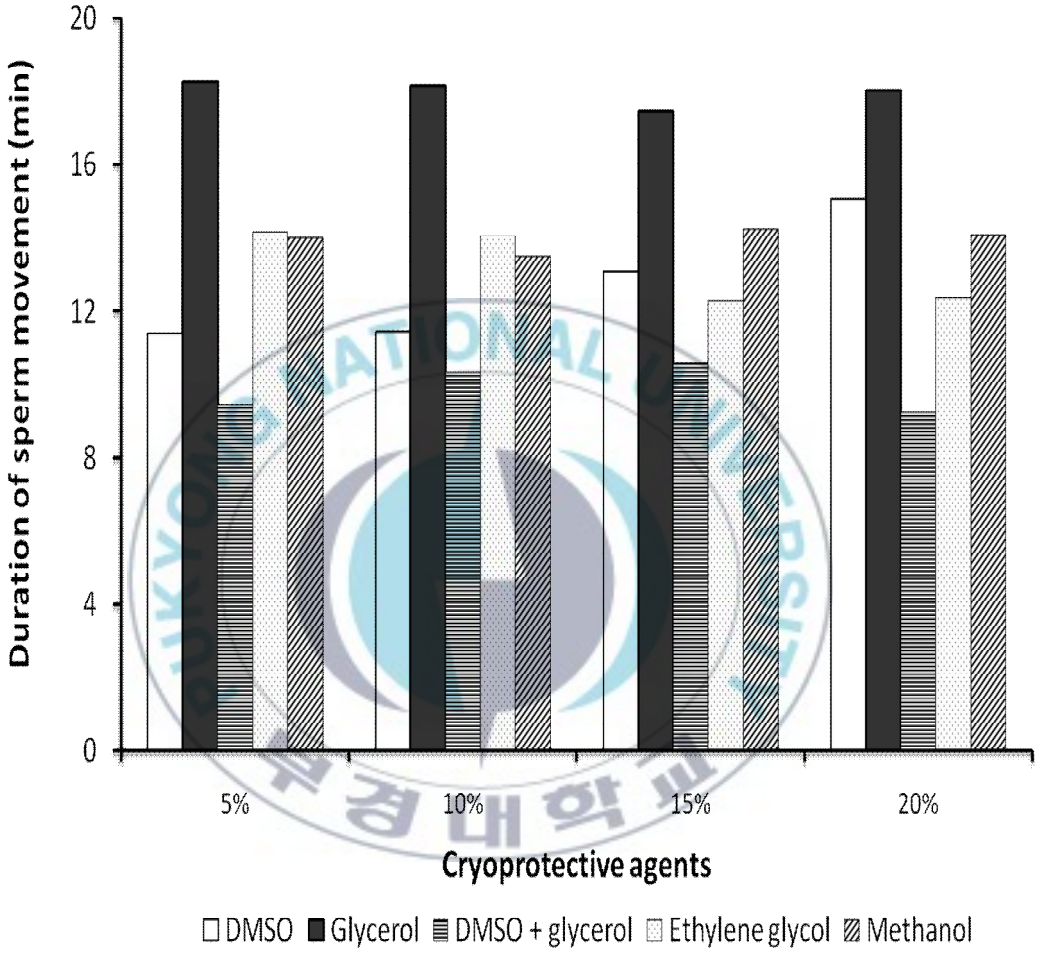

Fig. 10. Duration of sperm movement by the immersion in several cryoprotective agents with glucose as extender.

Table 5. Effect of extenders on post-thawed spermatozoa motility in red seabream *Pagrus major* with sucrose as extender containing various cryoprotective agents (CPAs) dissolved at different concentrations

CPAs	Conc. (%)	SAI	DPM (min)	TDM (min)	Survival rate (%)
DMSO	5	3.0 ± 0.0 [a]	6.2±0.1 [a]	16.4±0.7 [a]	48.0±1.0 [a]
	10	2.7 ±0.1 [a]	5.2±0.0 [a]	11.3±0.9 [a]	51.7±1.5 [a]
	15	2.7 ±0.6 [a]	7.9±0.6 [a]	13.3±2.6 [a]	56.0±7.9 [a]
	20	2.7 ±0.2 [a]	6.3±0.2 [a]	10.3±0.9 [a]	56.0±7.5 [a]
Glycerol	5	4.0 ±0.0 [a]	9.9±0.7 [a]	21.7±0.4 [a]	82.0±1.7 [a]
	10	4.0 ±0.0 [a]	7.9±0.5 [a]	16.1±2.3 [a]	74.3±3.5 [a]
	15	4.0 ±0.0 [a]	5.7±3.3 [a]	12.2±1.1 [a]	60.0±3.6 [a]
	20	3.0 ±1.0 [a]	5.4±1.0 [a]	11.5±4.8 [a]	53.3±7.5 [a]
DMSO + glycerol	5	2.0 ±0.0 [a]	3.7±0.5 [a]	6.0±0.6 [a]	36.0±10.4 [a]
	10	3.0 ±0.0 [a]	6.1±0.6 [a]	18.3±5.2 [a]	58.6±6.7 [a]
	15	2.8 ±0.1 [a]	6.7±0.5 [a]	17.4±1.5 [a]	46.3±9.2 [a]
	20	2.6 ±0.1 [a]	7.6±0.4 [a]	19.1±1.0 [a]	45.7±4.7 [a]
Methanol	5	1.5 ±0.2 [b]	2.2±0.6 [b]	4.6±0.6 [b]	38.6±2.1 [b]
	10	1.2 ±0.3 [b]	2.2±0.2 [b]	4.7±0.3 [b]	35.0±3.5 [b]
	15	1.3 ±0.3 [b]	1.9±0.5 [b]	4.9±0.4 [b]	37.0±6.6 [b]
Ethlylene glycol	5	0.0 ±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	10	0.0 ±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	15	0.0 ±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	20	0.0 ±1.0	0.0±0.0	0.0±0.0	0.0±0.0

Data were expressed by mean±S.E. Value within followed by the superscript letter “a” are not significantly different (P>0.05).DMSO: dimethyl sulfoxide, DPM: duration of progressive motility, SAI: sperm activity index, TDM: total duration of motility.

Sperm viability in glucose show in was significantly higher ($P<0.05$) when the sperm was cryopreserved with DMSO, glycerol, DMSO+glycerol, and EG at 5, 10 15 and 20% (**Tables 6, 7, 8 and 9**). No significant difference was detected between the CPAs. But drastic decrease un sperm viability was observed in methanol after freezing and thawing.

The highest sperm viability was observed on glycerol 5, 10 and 15% using sucrose as extender and on EG with glucose as extender showing in **Table 10** having a motility for 5% of 4.0±0.0 and for 10% 4.0±0.0 min, duration of progressive motility of 19.2±0.1 for 5% and for 10% 18.7±0.4 min, total duration motility of 34.0±0.1 for 5% and for 10% 35.1±1.0 min. and a survival rate of 85.0±3.5 for 5% and for 10% 80.0±3.6%. Comparing glycerol with sucrose as extender and EG with glucose as extener thers no significant differences between each one ($P > 0.05$).

Table 6. Statistical values calculated from SAI using one way ANOVA among the cryoprotectives agents with sucrose as extender in this study.

Cryoprotectant	Glycerol	DMSO+glycerol	Methanol
DMSO	0.234[a]	0.194[a]	0.008[b]
Glycerol			0.034[b]
DMSO+glycerol	0.428[a]		0.003[b]

Values followed by the letter "a" are not significantly different (P>0.05).

DMSO: dimethyl sulfoxide.

Table 7. Statistical values calculated from survival rate using one way ANOVA among the cryoprotectives agents with sucrose as extender in this study.

Cryoprotectant	Glycerol	DMSO+glycerol	Methanol
DMSO	0.276[a]	0.386[a]	0.0008[b]
Glycerol			0.011[b]
DMSO+glycerol	0.175[a]		0.025[b]

Values followed by the letter "a" are not significantly different (P>0.05).

DMSO: dimethyl sulfoxide.

Table 8. Statistical values calculated from SAI using one way ANOVA among the cryoprotectives agents with glucose as extender in this study.

Cryoprotectant	Glycerol	DMSO+glycerol	EG	Methanol
DMSO	0.264[a]	0.734[a]	0.662[a]	0.007[b]
Glycerol			0.633[a]	0.009[b]
DMSO+glycerol			0.921[a]	0.009[b]
EG				0.032[b]

Values followed by the letter "a" are not significantly different (P>0.05).

DMSO: dimethyl sulfoxide, EG: ethylene glycol.

Table 9. Statistical values calculated from survival rate using one way ANOVA among the cryoprotectives agents with glucose as extender in this study.

Cryoprotectant	Glycerol	DMSO+glycerol	EG	Methanol
DMSO	0.124[a]	0.758[a]	0.244[a]	0.003[b]
Glycerl			0.311[a]	0.006[b]
DMSO+glycerol			0.170[a]	0.0005[b]
EG				0.001[b]

Values followed by the letter "a" are not significantly different (P>0.05).

DMSO: dimethyl sulfoxide, EG: ethylene glycol.

Table 10. Effect of extenders on post-thawed spermatozoa motility in red seabream *Pagrus major* with glucose as extender containing various cryoprotective angentes (CPAs) at different concentrations

CPA	Conc. (%)	SAI	DPM (min)	TDM (min)	Survival rate (%)
DMSO	5	2.4±0.3 [a]	5.9±0.7 [a]	11.1±1.0 [a]	45.0±7.8 [a]
	10	2.9±0.2 [a]	9.0±0.4 [a]	19.0±0.5 [a]	70.3±1.2 [a]
	15	4.0±0.0 [a]	17.9±0.7 [a]	32.5±3.1 [a]	90.7±2.5 [a]
	20	3.9±0.1 [a]	12.8±1.5 [a]	26.0±2.7 [a]	88.7±2.1 [a]
DMSO + glycerol	5	4.0±0.0 [a]	14.6±2.2 [a]	27.5±3.6 [a]	83.3±4.5 [a]
	10	3.8±0.1 [a]	11.5±2.4 [a]	16.7±4.7 [a]	80.0±2.6 [a]
	15	3.9±0.0 [a]	15.1±2.6 [a]	28.3±1.0 [a]	86.3±2.3 [a]
	20	2.8±0.2 [a]	8.5±1.2 [a]	16.6±2.2 [a]	76.3±3.1 [a]
Glycerol	5	3.2±0.3 [a]	8.4±0.9 [a]	16.1±6.0 [a]	69.7±8.5 [a]
	10	2.9±0.1 [a]	9.4±0.1 [a]	20.0±1.9 [a]	50.0±3.0 [a]
	15	1.8±1.5 [a]	3.8±3.3 [a]	7.2±6.3 [a]	57.2±12.7 [a]
	20	3.7±0.1 [a]	8.3±0.8 [a]	20.3±0.9 [a]	79.0±5.2 [a]
Methanol	5	2.0±0.0 [b]	5.6±0. [b]	9.7±0.3 [b]	49.3±1.5 [b]
	10	2.0±0.0 [b]	5.3±0.2 [b]	8.7±0.3 [b]	42.3±2.9 [b]
	15	1.9±0.1 [b]	4.3±0.1 [b]	8.2±0.1 [b]	40.3±1.2 [b]
	20	1.6±0.1 [b]	3.6±0.6 [b]	7.5±0.4 [b]	32.0±5.6 [b]
Ethylene glycol	5	4.0±0.0 [a]	19.2±0.1 [a]	34.0±0.9 [a]	85.0±3.5 [a]
	10	4.0±0.0 [a]	18.7±0.4 [a]	35.1±1.0 [a]	80.0±3.6 [a]
	15	3.4±0.4 [a]	10.0±0.6 [a]	25.9±0.7 [a]	71.7±5.0 [a]
	20	2.7±0.3 [a]	5.6±0.5 [a]	10.7±0.4 [a]	67.7±2.5 [a]

Data were expressed by mean±S.E. Value within followed by the superscript letter “a” are not significantly different (P>0.05).DMSO: dimethyl sulfoxide, DPM: duration of progressive motility, SAI: sperm activity index, TDM: total duration of motility.

IV. DISCUSSION

Sperm properties of red seabream were compared with another teleost fish species as shown in **Table 11**. Like in most teleost fish, the sodium and the chloride ions predominate in seminal plasma of red seabream. However the potassium in red seabream it was really low compered to **Table 11** muskellunge *Esox masquinongy* (27.9mmol/L) (Lin et al., 1996), marbled sole *Pleuronectes yokohamae* (4.04±0.98 mmol/L), brown sole *Pleuronectes herzenseini* (10.96±4.07 mmol/L), starry flunder *Platichthys stellatus* (7.47±3.19 mmol/L) and olive flounder *Paralichtyus olivaceus* (20.75±8.99 mmol/L) (Chang, 2001), gold fish *Carassius auratus auratus* (7.02 mmol/L) and common carp *Cyprinus carpio* (82.4 mmol/L) (Morisawa, 1983). In cyprinid fishes the seminal plasma contains almost equally high sodium ans potassium levels (about 70 mmol/L) (Morisawa, 1983), but in the seminal plasma of red seabream, the sodium concentration is more than 10 times higher, that the potassium concentration. Such tendencies were exhibited in marbled sole, brown sole, starry flounder and olive flounder (Chang, 2001).

Table. 11. Biochemical properties of seminal plasma in teleosts

Species	Na^+	K^+	Mg^{++}	Ca^{++}	Cl^-	Author
Oncorhynchus mykiss	104	25.3	1.1	1.4	135	Holtz et al. (1979)
	107	25.8	0.8	2.6	-	Holtz et al. (1977)
	133	20	-	-	130	Schlenk and Hahmann (1938)
Oncorhynchus keta	141	66	3.6	1.0	134	Morisawa et al. (1979)
Salmo salar	103	22	0.9	1.3	-	Hwang and Idler (1969)
Salmo clarki	107	38.6	1.5	0.3	156	Cruea (1969)
Cyprinus carpio	94	67.8	0.02	12.5	-	Clemens and Grant (1965)
Vimba vimba	107	38.7	1.2	0.3	-	Kusherova (1972)
Ctenopharyngodon idella	811	35.1	1.6	1.0	-	Gosh (1985)
Stizostedion vitreum	167	4.8	2.0	0.4	132	Gregory (1970)

Glucose concentration of red seabream was 3.0±0.6 mg/100mL. This value was optimal compared to *Alburnus alburnus* (2.18 mg/100 mL), zarte, *Vimba vimba* (6.05 mg/100 mL) (Lahnsteiner et al., 1994). Fish spermatozoa are capable of utilizing extracellular carbohydrates through oxidation (Gregory, 1968). Additionally, the pH value in seminal plasma may have some conditions which maintain highly spermatozoa viability during spawning period.

Publisher method for cryopreservation of fish sperm is similar, although, not standardized. The sperm is diluted with a diluents, and cryoprotectant is added to protect against freezing and thawing damage. The diluted sperm was frozen and stored in liquid nitrogen. The factor that affect cryopreservation of sperm in fish bare as sallow: time and method of sperm collection, composition ratio, freezing method rate, thawing method and rate.

Selecting a suitable extender is the key factor in a successful cryopreservation of fish sperm. In this study two extenders (sucrose, glucose) were tested for cryopreservation of red seabream sperm.

In previous researches on cryopreservation of fish sperm Hank's buffered salt solution (HBSS) was the perfect extender for red seabrem sperm (Liu et al., 2006), followed by Ringer solution and modified Mounib medium

(MNM) when 15% DMSO as used as cryoprotectant. In this study sucrose gave the best result with glycerol and glucose with EG. Cryoprotectants are also playing an important role in cryopreservation particularly for long-term preservation. In this study, five cryoprotectants (DMSO, glycerol, DMSO+glycerol, EG and methanol) were used in cryopreservation of fish sperm. Cryoprotectants are needed to protect the sperm cell from cold and hot shock treatments and prevent cell dehydration. They can also prevent ice formation during pre-freezing, but the same levels of cryoprotectants can be lethal to unfrozen cell (Jamieson, 1991; Chao and Liao, 2001; Suquet et al., 2000). Cryoprotectants, on the other hand, have some disadvantages in that it can induce protein denaturation at higher temperature and cause cryoprotectant toxicity in cellular systems (Le, 2010). Chen and Tian (2005) reported that the toxicity of methanol was lowest among four cryoprotectants. Because of small volume of fish sperm (Suquet et al., 1993), sperm can be cryopreserved successfully using cryoprotectants ant low concentration. Therefore, the toxicity of the cryoprotectants in not considered as an important factor in selecting a suitable cryoprotectant for the cryoprotectants may be a more important factor than toxicity in selecting a suitable cryoprotectant for the cryopreservation of fish sperm. Since

spermatozoa are very sensitive cells and are easily affected by diluents such as extenders and cryoprotectants, studies on the cryoprotectants and extenders, in terms of spermatozoa preservation are important to determine the most suitable extenders, cryoprotectants and their concentration for certain fish species. DMSO at various concentrations is generally used as a cryoprotectant for animal cells, for example 10% DMSO resulted in a high percentage of motile spermatozoa of starry flounder *Platichthys stellatus* (Lim et al., 2007) and filefish (Le et al., 2008). However, 10% methanol was more effective that 10% DMSO for cryopreservation of rainbow trout *Oncorhynchus mykiss* spermatozoa and Danube salmon sperm (Kusuda, 1994). Also 10% EG was found to be a more effective cryoprotectant that DMSO, methanol and glycerol for oliver flounder sperm (Chang, 2001) and yellow croaker *Larimichthys polyactis* (Le, 2010). However on this study EG with sucrose as extender give 0 motility and methanol in all concentration with both extenders give the lowest result in the viability of red seabrem sperm. For this specie glycerol in all concentration with both extender sucrose and glucose, were the best suitable, showing a higher SAI, and survival rate. Glycerol had demonstrated good results in some species such as olive flounder (Zhang et al., 2003), summer whiting *Sillago ciliate*

(Young et al., 1992), and European catfish *Silurus glani* (Ogier de Baulny et al., 1999).

In conclusion satisfactory motility in cryopreserving red seabream sperm could be achieved with gycerol with sucrose and glucose as extenders in all four (5, 10, 15 and 20%) concentrations.

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