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Freezing Protocol Optimization for Gilthead Seabream Sperm, *Sparus Aurata L.*

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Abstract

In this study, it was aimed to determine certain spermatological properties of seabream in order to optimize the freezing protocol and to provide more economical and efficient production in seabream culture from the obtained results. In this study, 10 male and 4 female seabreams were selected. After certain spermatological characteristics were determined, the sperm samples were drawn into 0.25 mL straws following the addition of an appropriate cryoprotectant to the best diluent. Four different diluents (Mounib, saline Mounib, Ringer's, and 1% NaCl) were used to freeze the sperm (Experiment 1). In this experiment, Ringer's solution afforded the highest motility. Then, 10% dimethyl sulfoxide (DMSO), 10% ethylene glycol (EG), and 10% dimethyl acetamide (DMA) were added to the Ringer's diluent to determine the most suitable cryoprotectant (Experiment 2). DMSO afforded the highest motility. Following the freezing of sperm, 2 methods were attempted for thawing: 20 s at 26°C and 15 s at 35°C (Experiment 3). Post-thaw motility values of 69.37% and 67.55%, respectively, were obtained. At the end of this study, the hatching rate was 43.48% for frozen sperm. Finally, the best diluent, best cryoprotectant, and optimum thawing temperature and fertility rates for the freezing of seabream sperm were found.

Keywords: Fertility, freezing protocol, seabream, sperm

Introduction

Controlled fish production has reached a considerable size in order to meet animal protein requirements. Fish can be rehabilitated by genetic potential, crossing between species and strains, and manipulation of gametes. Some of these practices require the preservation of sperm (Akçay et al., 1995).

Frozen semen in fish have not yet found any wide applications. It is believed that the sperm of marine fish are more resilient to freezing, because the ratio of cholesterol/phospholipid in the spermatozoon membrane is high and the ATP content is high at the beginning. Again, it has been emphasized that phosphatidylcholine located in the spermatozoon membrane of marine fish with resistance to cold may be promising (Drokin, 1993; Suquet et al., 2000).

Sperm is not suitable for freezing without dilution. Diluents used for freezing marine fish sperm are mostly salty (concentration: 1–10%) or sugary (concentration: 1–10%) solutes. Diluents should not activate the motility of sperm (Suquet et al., 2000).

Cryoprotectants are divided into two groups: internal and external. Dimethyl sulfoxide (DMSO) is commonly used in intracellular cryoprotectants such as ethylene glycol (EG), propylene glycol (PG), glycerol, dimethyl sulfoxide (DMSO), dimethyl acetamide (DMA), and methanol. It has been suggested that DMSO is more effective than others because it facilitates the rapid penetration of sperm as well as sperm interaction with the phospholipid layer (Suquet et al., 2000).

Because of the small size of fish spermatozoa, penetration can quickly occur after dilution; therefore, there is no need for an

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equilibration stage. The toxic effects of cryoprotectants minimize the effects of the absence of this step. In the study conducted in seabream, it was reported that the fertilization rates were lower when DMSO-added sperm was allowed to acclimatize for more than 2 min (Billard, 1978; Jamieson, 1991). On the other hand, Ogier de Baulny (1997) reported that in rainbow trout, DMSO needed 10 min of penetration to reach the spermatozoa, but it was reported that its use did not affect the fertility.

Glycerol has been successfully used as a cryoprotectant: it has been reported that it is necessary for the equilibration step. Doi et al. (1982) proposed 30-min equilibration because of slow glycerol penetration in the study involving bluefin tuna fish.

There are several factors that affect the success of the freezing strategy, such as dilution, cryoprotectant, equilibration, dilution rate, cooling rate, thawing temperature and duration, and time elapsed after thawing. Another important factor is when the sperm is collected (beginning, middle, and end of the breeding season) (Rideout et al., 2004; Suquet et al., 2000). Evidently, the motility period of the frozen sperm is shorter and its metabolism is different. In addition, fertilized eggs used in artificial insemination are found to have lower hatching rates (Fauvel et al., 1998; Zilli et al., 2005). After 10 s of sperm activation, it has been stated that the motilities of fresh and frozen sperm are almost equivalent, although the motility duration is 50 and 40 s for fresh and frozen sperm, respectively. Again, it has been reported that the same concentrations of fresh and frozen sperm have different fertilization rates and that fresh sperm can fertilize more eggs (Fauvel et al., 1998).

In particular, the protogynous hermaphrodite, which is seen in many types of the Serranidae family, reaches puberty as a female and remains female until the age of 3–5 years, thereby making the freezing of the sperm to become critical (De Graaf et al., 2004).

In this study, the aim was to determine certain spermatological characteristics of the seabream fish, which has significant economic importance, as well as to determine the success of frozen spermatozoa in artificial insemination. In the freezing of sperm, 4 different diluents (Mounib, saline Mounib, Ringer's, and 1% NaCl), 3 different cryoprotectants (DMSO, EG, and DMA), and 2 different thawing methods (at 26°C for 20 s and at 35°C for 15 s) that are commonly used in marine fish were used to demonstrate the effects on motility and fertilizing ability of the frozen sperm, and their success in artificial insemination were evaluated.

Materials and Methods

The study was approved by the ethical committee of Ankara University with the number of 2008.30.140.

This research was carried out at the breeding facilities of Kılıç Seafood Co. in Bafa Fish Production Center in Akyeniköy, Dalyan, Turkey. The materials used in this study were obtained from the breeding stocks used for larval production in the center. A total of 4 female and 10 male breeding seabreams were randomly selected from 150 male and 100 female seabreams. Randomly selected males weighed between 800 and 1280 g and females weighed between 1100 and 2300 g. The breeding male and female seabreams were grown in round PVC tanks (capacity: 23 tons) at a ratio of 2:3 (female/male). During the breeding season, the water temperature of the tank was maintained at 18±0.5°C. The temperature was checked twice a day to keep the changes at a minimum level. The salinity of the water in the tank was recorded as 36%, pH 7.5–8.0, and dissolved oxygen at 7–10 mg/L. The study was carried out in January, which corresponds to the mid-breeding season for geographical breeding. The brooders were subject to 18 h of light and 6 h of dark. The outdoor temperature during the study was recorded to be 15±3°C. During the breeding season, the fish were mainly fed with wet feed (squid, sepia, etc.) and commercial concentrates.

Sperm and egg collections

Before the sperm samples were collected, the water volume of the tank was reduced to allow the fish to swiftly enter anesthesia and be easily caught. Phenoxyethanol was used as the anesthetic agent. The fish were slowly anaesthetized, and their responses were observed. Fish were placed in small 100-liter tanks in a semi-anesthetic state and brought to complete anesthesia with a final titer of 100–120 mg/L of 2-phenoxyethanol. The males, taken under anesthesia, were held with the help of a towel and the genital area was dried with a paper towel. The genital area was cleaned, the head was held upright, and sperm was collected under a slight craniocaudal pressure on the upper abdominal region. Graduated plastic tubes (15 mL) were used for sperm collection. When a change in color or viscosity of the sperm was noticed, urea contamination was suspected; such samples were not used for this study. The collected sperm was numbered and transferred to the laboratory in a dark, cold thermos (0–4°C).

In order to obtain eggs from female fish, the conditions specified for sperm collection were observed. The eggs collected from the female fish were put in 50 ml plastic containers.

Spermatological examination

Certain spermatological characteristics were determined after sperm collection. The sperm samples were collected in graduated plastic tubes and the amount in milliliters (mL) was measured from the scale on the tube. In the microscopic examination of the sperm, parameters such as fresh spermatozoa motility, post-freezing spermatozoa motility, and spermatozoa density were determined.

Spermatozoa motility was detected in two stages: fresh and after freezing. The motility test was carried out at the ambient temperature of 15±3°C without using a heating table. For the motility assay, a small drop of sperm was placed on the slide;

then, a 1:10 ratio of activation solution (30 g/L NaCl, 0.8 g/L KCl, 1.3 g/L $CaCl_2$, 6.6 g/L $MgSO_4$, and 0.18 g/L $NaHCO_3$; pH 8.2; 987 mOsm/kg) was added and rapidly mixed. After contact with the activation solution, the coverslip was used for 4–8 s and the motility was determined under 40× magnification. Motility evaluation was subjectively performed in five different microscopic areas and recorded as a percentage value.

The concentration of spermatozoa was determined by means of a hemocytometric method and expressed as spermatozoon/mL (Tekin, 1994).

The pH indicator papers (pH 5.5–9) were used to determine the pH of the sperm.

Sperm freezing

The sperm diluted with an appropriate diluent and cryoprotectant was filled into 0.25 mL straws; the open ends of the straws were sealed with polyvinyl alcohol and immediately transferred into water (12–13°C). The straws were left in the water for 1–2 min for hardening of polyvinyl alcohol and were immediately transferred into a Styrofoam box for freezing without equilibration. The straws placed on the shelves were frozen at 4–6 cm above the liquid nitrogen level for 10 min at -110 to -120°C. As soon as the freezing process was completed, the straws were immersed in liquid nitrogen (-196°C) and stored in a container.

Experiment 1 (Diluent selection)

After 10 different ejaculates were separately examined, they were pooled in a 50 mL plastic tube. The pooled sperm was diluted in the ratio of 1:3 with 4 different diluents to be frozen in split samples. DMSO (10%) was added to all the diluents, and freezing was started within 3–5 min.

Diluents Used:

Diluent I:	Diluent II:
Mounib Medium;	Saline Mounib Medium;
10 mg/mL $KHCO_3$	10 mg/mL $KHCO_3$
2 mg/mL reduced glutathione	19.4 mg/mL NaCl
42.7 mg/mL sucrose	6.2 mg/mL $NaHCO_3$.$2H_2O$
10 mg/mL bovine serum albumine (BSA)	2.2 mg/mL glycine

Diluent III: Ringer Solution;	Diluent IV; Seawater
6.5 mg/mL NaCl	1% NaCl (10 mg/mL)
0.40 mg/mL KCl	
0.12 mg/mL $CaCl_2$	
0.2 mg/mL $NaHCO_3$. $2H_2O$	
2 mg/mL glucos	

During dilution, it was ensured that the sperm and diluents were maintained at similar temperatures (8–14°C). The diluent that yielded the best motility among the four different diluents in the study was chosen as the main diluent, and the study was continued with the selected diluent.

Experiment 2 (Cryoprotectant selection)

After the selection of the best diluent, three different cryoprotectants were added to this diluent; the cryoprotectant that yielded better post-thaw motility results was determined.

Used Cryoprotectants:
Cryoprotectant I: 10% DMSO
Cryoprotectant II: 10% EG
Cryoprotectant III: 10% DMA
The study was continued with the cryoprotectant that afforded the highest motility.

Experiment 3 (Thawing temperature and duration)

Thawing was performed in a water bath in 2 different ways: 20 s at 26°C and 15 s at 35°C. The sperm samples were subjected to a combination of the best diluent and best cryoprotectant and they were thawed at two different temperatures. The post-thaw motility values were determined. The aim was to perform the fertilization experiments with the thawing procedure that yielded the best motility. For this purpose, the frozen sperm samples were thawed in a water bath at the desired temperature and time, and the group with the highest post-thaw motility was used in fertilization.

Artificial insemination and fertilization

After the best diluent, cryoprotectant, thawing temperature, and time were determined, fertilization trials were performed. The fertilization process was immediately started after the eggs were collected from the females. Eggs from 4 different females were adjusted to 5 g (about 5000–6000 eggs) in each group. During egg collection, nontransparent broken eggs were separated by inspection and not used in this study. During fertilization, four different groups were formed: one experimental and three controls.

Group I	*Group II (control)*
Frozen sperm 30 µL	Diluted sperm 30 µL with inactivation solution at 1/3 ratio; 3.5 g/L NaCl, 0.11 g/L KCl, 0.3 g/L $MgCl_2$, 1.68 g/L $NaHCO_3$, 10 g/L BSA, pH 7.5
Group III (control)	*Group IV (control)*
Fresh sperm 10 µL	Natural Fertilized Egg

For fertilization, 5 g (about 5000–6000 pieces) eggs were placed in 50 mL plastic containers and the sperm was added into them. After about 15–20 s of gentle stirring, the activation solution (30 g/L NaCl, 0.8 g/L KCl, 1.3 g/L $CaCl_2$, 6.6 g/L $MgSO_4$, and 0.18 g/L $NaHCO_3$; pH 8.2) was added to activate the sperm, and the eggs were allowed to gain water and swell. The amount of activation solution was approximately 3 times that of the eggs; therefore, 15 mL was added to all the groups. The activation solution was slowly poured from the edge of the plastic

containers. The activation solution was kept in water at 18°C so that it would be at the same temperature as those of the tank and eggs. During fertilization, the ambient temperature was measured to be 16.5°C. Three minutes after the initiation of fertilization, they were transferred to incubators with a 500-micron eye opening. This transfer to incubators was gradually undertaken to minimize the possibilities of temperature and osmotic shocks.

In order to minimize the external effects on eggs, fertilization had to be completed; the placement of all the groups into incubators required a total of 20–30 min. The sperm used in the control groups were obtained from a mixture of sperm collected from 3 different males with 90% motility. In fertilization, the average spermatozoon/egg ratio was determined; this value for the frozen sperm (30 µL) and fresh sperm (10 µL) was calculated to be approximately 1.5×10^4. The fertilization experiments were repeated three times.

An air tube was immersed in each incubator to provide oxygen to each group and prevent the eggs from sticking together. The eggs were constantly displaced in the incubator due to the weight of the air tube attached to the bottom of the incubator. During the incubation period, the water temperature was measured twice a day (mean: 17.9±0.4°C), and a dark environment was provided.

Twenty-four hours after the eggs were hatched, ventilation was stopped and the hatched eggs were left there for 3–5 min so that the defective and unfertilized eggs collapsed. They were then removed from the incubators through a glass pipe system with the help of a flashlight. The thin-tipped glass pipe system was disinfected and washed with sterile seawater. The eggs collected under vacuum were separated at the other end of the system in a 500-micron mesh (Figure 1). The collected eggs were weighed with an electronic balance (precision: 0.01 g). Before weighing, the water in the eggs was drawn. On the 24th and 48th hours, the eggs were noted according to the groups and reached the larvae state after 70 h.



Figure 1. Incubators with 500-µm mesh openings

All the experiments were performed in triplicate.

Statistical analysis

The mean value and standard errors of the data obtained in this study were calculated. One-way analysis of variance (ANOVA) test was used to demonstrate the differences between the diluents, cryoprotectants, and fertility. Tukey's test was used to determine the groups for which the difference was significant. Student's t-test was used to determine the difference between the thawing temperatures.

Results

In this study, the sperm volume, motility, concentration, and pH were determined from the main spermatological features in the ejaculates collected from 10 male seabreams (Table 1).

It was observed that the motility values of the 10 different ejaculates were very close to each other. The average motility of the native sperm was recorded to be 90.5%. The examinations were made by evaluating at least 5 different areas in a microscope with a 40× lens. In the preliminary studies, the motility of the sperm activated with 37% (seawater and NaCl) solution was significantly lower. For this reason, 30% activation solution was used. The sperm was assessed within 10 s of activation. From the time of observation, the rate of motility rapidly increased until 20 s, reached maximum motility at 20–30 s, and retained the maximum motility for a short period of time (~1–2 min); then, it started to decrease.

It was observed that there was a considerable difference in the concentration values between the ejaculates. According to this, the lowest, highest, and average spermatozoa concentrations were 11.6×10^9, 32.2×10^9, and 21.55×10^9 spermatozoon/mL.

Table 1. Spermatological characteristics of fresh seabream sperm (n=10)

Fish no	Volume (mL)	Motility (%)	Concentration ($x10^9$/mL)	pH
1	2	95	16.6	7.25
2	10	95	14.1	7.50
3	9.2	85	17.8	7.25
4	13	90	29.5	7.50
5	12	90	30.5	7.25
6	4.5	90	11.6	7.25
7	3.5	85	23.6	7.25
8	4	95	32.2	7.25
9	6	90	22.3	7.00
10	3.5	90	17.3	7.50
Mean±SD	6.77±1.3	90.5±1.2	21.55±2.3	7.3±0.05

SD: standard deviation

The highest post-thaw motility was obtained from the frozen sperm with Ringer's solution (Table 2). Therefore, Ringer's solution was selected as the main diluent, and the study was continued with this diluent.

After the selection of Ringer's solution, the best cryoprotectant was determined by separately adding 10% DMSO, 10% EG, and 10% DMA to this diluent. The success of the cryoprotectants is listed in Table 3. The best motility in this study was obtained from 10% DMSO. The difference between the cryoprotectants was found to be significant ($p<0.05$).

Table 2. Post-thaw motility rates obtained from different diluents

Diluent	Post-thaw motility (%)
Mounib	66.75±1.18[a]
Saline mounib	10.75±0.75[c]
Ringer	68.25±1.97[a]
Seawater 1%	50.75±0.75[b]

[a, b, c]Difference between group averages with different letters is significant at $p<0.05$.

Table 3. Post-thaw motility rates obtained from different cryoprotectants

Cryoprotectant	Post-thaw motility (%)
DMSO	61.66±1.66[a]
EG	42.66±1.45[b]
DMA	23.33±1.66[c]

[a, b, c] Difference between group averages with different letters is significant at $p<0.05$. DMSO: dimethyl sulfoxide; EG: ethylene glycol; DMA: dimethyl acetamide

Table 4. Post-thaw motility rates obtained from different thawing temperatures and times

Thawing temperature and time	Post-thaw motility (%)	Significance grade
26°C 20 s	69.37±1.22	-
35°C 15 s	67.55±0.57	-

- Difference between the group averages is insignificant at $p>0.05$

Table 5. Obtained fertility rates from experimental or control groups

Fertilization groups	Amount of sperm used	Fertility (%)	Significance grade
Frozen sperm (experimental)	30 µL	43.48±1.87	-
Diluted sperm (control)	10 µL	44.57±1.40	-
Fresh Sperm (control)	10 µL	45.42±1.30	-
Natural fertilized (control)	-	43.49±2.73	-

Difference between the group averages is insignificant at $p>0.05$

The post-thaw motility (%) data for thawing temperatures and times of 26°C for 20 s and 35°C for 15 s are listed in Table 4. There are marginal differences in the motilities at these two thawing temperatures. The thawing procedure applied at 26°C for 20 s afforded a better result with a slight difference. The difference between both these water temperatures was not significant ($p>0.05$).

Artificial insemination was carried out after separating 4 groups with 5 g eggs collected from 4 females. The best post-thaw motility was obtained from Ringer's solution containing 10% DMSO. Since there was no statistical difference between the 2 thawing temperatures and times, 26°C for 20 s was used to thaw the frozen sperm because it had a temperature close to that of the eggs.

For fertilization trials, four different groups were formed: one of them was experimental and three of them were controlled: frozen sperm (Group I), diluted (with inactivation solution) sperm (Group II), fresh sperm (Group III), and naturally fertilized eggs (Group IV). At the 24th and 48th hours, dead eggs were collected from the bottom and weighed. Nearly 100% of the eggs left in the control at the 70th hour had hatched. The fertility rates obtained after artificial insemination are listed in Table 5. The difference between the frozen sperm and control groups was not significant ($p>0.05$).

Discussion

After anesthesia, the sperm were collected by the abdominal massage method: the average volume was 6.77 mL. Barbato et al. (1998) reported that the volume of sperm that can be collected from seabream was 0–3.1 mL, whereas Cabrita et al. (2005a) reported the collection of 1 ml sperm via aspiration. The volume of the sperm collected in this study (6.77 mL) was higher than that in Barbato et al. (1998), which was attributed to the difference in the quality of breeds between both these studies. Despite the use of 2–3-year-old breeding male fish (weight: 800–1280 g) in this study, Barbato et al. (1998) used fish weighing 118–444 g (1 year old) in their study. Further, the fact that sperm collection conditions and season affect the volume of sperm should also be considered.

A hyperosmotic osmotic environment in marine fish and a hypoosmotic environment in freshwater fish initiate flagellar activation. The most important trigger of motility activation in salmonids and sturgeon is K^+ density (Billard, 1983; Zilli et al., 2008). However, such a finding has not been reported for seabream.

In seabream sperm, 50% motility with 500 mM NaCl and 90% motility with 550 mM NaCl could be obtained. Activation can also be achieved with a nonionic dissolved 1100 mM solution

(Zilli et al., 2008). Cabrita et al. (2005b) used artificial seawater for the activation of spermatozoa in seabream. Unlike these researchers, Barbato et al. (1998) and Fabbrocini et al. (2000) used 1:10 seawater (37%) for motility activation. In many studies on marine fish, seawater (36–38%) has been used for motility activation (Chen et al., 2004; Sansone et al., 2002; Zilli et al., 2005). In this study, 30% activation solution (30 g/L NaCl, 0.8 g/L KCl, 1.3 g/L $CaCl_2$, 6.6 g/L $MgSO_4$, and 0.18 g/L $NaHCO_3$) has been used for sperm activation.

In this study, the average motility obtained from fresh sperm was recorded to be 90.5%. Cabrita et al. (2005a) found that the native sperm was 95% motile. For the studies conducted by Fauvel et al. (1999) in seabass, they reported that the native sperm had motility of 90% or more. The findings of motility obtained from the current study are similar to those obtained from the abovementioned studies.

In this research, the hemocytometric method was used to determine the concentration of seabream sperm. This method was used by Barbato et al. (1998), Çevik (2000), and Hatipoğlu (2007). Fauvel et al. (1998, 1999) and Ciereszko and Dabrowski (1993), on the other hand, used the spectrophotometric method to determine the spermatozoa concentrations.

Due to the fact that fish sperm are much more concentrated than mammalian sperm, low dilution rates make counting difficult. This can also increase errors in the count. In this study, the average concentration of seabream sperm was recorded to be 21.55×10^9 spermatozoon/mL. Barbato et al. (1998) reported that the concentration of seabream sperm changed to 11.6–26.7×10^9 spermatozoon/mL with a mean value of 17.95×10^9 spermatozoon/mL. On the other hand, according to Cabrita et al. (2005a), the concentration of seabream sperm was 5.5–17.5×10^9 spermatozoon/mL. The concentrations obtained from this study were higher than those from both the abovementioned studies. The cause of this difference was considered to be the age and nutrition of the breeders used, different periods of breeding seasons, and other environmental factors (water temperature, pH, and dissolved oxygen value).

In fish, it is very important to know the pH of the sperm as that in other species with regard to the applicability of procedures involving the sperm (Akçay et al., 1995; Suquet et al., 1993).

In this study, pH indicator paper was used to determine the pH of the sperm: the mean value was found to be 7.3±0.05. This method was the same as that applied by Çevik (2000) and Hatipoğlu (2007). Cabrita et al. (2005a) used an electronic pH meter.

Diluents used for freezing marine fish sperm are mostly salty (concentration: 1–10%) or sugary (concentration: 1-10%) solutes. Diluents should not activate spermatozoon motility (Suquet et al., 2000). The sperm's dilution rate varies between 1:1 and 1:20 depending on the research and type of fish used. When the dilution rate increases, the post-thaw motility rate and duration of motility decrease. Chambeyron and Zohar (1990) reported that the post-thaw motility was lower for sperm diluted at a ratio of 1:50 as compared to the ones diluted at lower concentrations.

In this study, Mounib, saline Mounib, Ringer's, and 1% NaCl solutions were used as the diluents. These diluents are used for many species of fish. In the freezing of seabream sperm, 1% NaCl is generally used as the diluent (Barbato et al., 1998; Cabrita et al., 2005a, 2005b; Fabbrocini et al., 2000).

Mounib diluent (Mounib, 1978) has been used in many studies and fish species with or without BSA. For example, Mounib solution was successfully used by Chen et al. (2004) for turbot, Rideout et al. (2004) for codfish, and Fauvel et al. (1998) and Zilli et al. (2005) for seabass. The Ringer's diluent used in this study was also successfully applied with some changes or simply in many species of fish. It has been successfully used by Dreanno et al. (1997) and Chen et al. (2004) for turbot and Chao et al. (1975) for grey mullet. The saline Mounib solution used in this study was obtained by adding 10 mg/ml $KHCO_3$ to the Mounib solution (Liu et al., 2006). In their study involving red seabream, Liu et al. (2006) obtained higher motility values with saline Mounib solution (63.6%) than that using normal Mounib solution (42%). Considering genetic proximity, it was thought that this diluent should be investigated, and this diluent was also used for seabream sperm. It has been reported that 1% NaCl solution used in this study has been widely and successfully used in studies related to freezing of seabream sperm. According to the findings, there was no significant difference between Mounib and Ringer's solutions with regard to affording the highest post-thaw motility values. It was found that the Ringer's solution selected as the main diluent in this study afforded higher motility values and that the motility duration was longer than that obtained when using the Mounib solution.

The lowest motility in this study was observed in the saline Mounib solution (10.75%) as opposed to the study in red seabream (Liu et al., 2006). The most important cause of the failed results may be attributed to the fact that 19.4% NaCl and 10% DMSO increase osmolarity in this diluent and enable most of the spermatozoa to become active. For this reason, it is important to consider the likelihood that spermatozoa should not be activated until they are placed in liquid nitrogen vapor for freezing within 3–5 min.

In this study, another finding was that 1% NaCl solution was a successful diluent with post-thaw motility of 50.75%, but Ringer's (68.25%) and Mounib (66.75%) diluents were more successful for use with seabream sperm. In the study by Fabbrocini et al. (2000), they selected 5% DMSO as the cryoprotectant after performing toxicity tests before freezing. In the same study, the cooling rate of 10°C/min was found to be better than 15°C/min. With regard to the thawing temperature, they reported

that 30°C was optimal. Cabrita et al. (2005a) also applied the same procedure; the only difference was BSA was added to the diluent.

In this study, the post-thaw motility values were different from those obtained from other studies because of the differences in the dilution ratio, cryoprotectant ratio, and freezing and thawing procedures between the studies. It is also considered that the differences in spermatozoa concentration and the duration of freezing are effective. In the study by Fabbrocini et al. (2000), the best post-thaw motility (67%) obtained with 1% NaCl and 10% DMSO was similar to the results obtained with Ringer's solution and 10% DMSO in this study (68.25%).

In studies involving freezing fish sperm, it has been reported that DMSO generally affords the best results among the intracellular cryoprotectants of EG, PG, glycerol, DMSO, DMA, and methanol (Holtz, 1993; Suquet et al., 2000). The fact that DMSO is more effective than others is due to its rapid penetration and its interaction with the phospholipid layer of sperm. It has been reported that the toxic effects of DMSO change depending on the fish species (Suquet et al., 2000). In this study, 10% DMSO, EG, and DMA were added to Ringer's solution. The difference between these three cryoprotectants was statistically significant. According to this, the post-thaw motility values obtained from frozen sperm without equilibration were determined to be 61.66% with DMSO, 42.66% with EG, and 23.33% with DMA. The reason that these results were different from the results obtained in other studies is that the diluents that determine the activity of the cryoprotectants are different. In this study, the cryoprotective effect of 10% DMA was found to be very low.

It was observed that the cryoprotective role of DMSO was high and that about 80% motility of fresh sperm during cryopreservation could be maintained, similar to that in some other fish species. This finding was supported by Maisse et al. (1998) and Tırpan et al. (2016), giving similar results with 85% cryopreservation success in seabream sperm.

The rapid passage of the thawing step is necessary to prevent crystallization (Suquet et al., 2000). In this study, thawing was applied for 20 s at 26°C and 15 s at 35°C. Barbato et al. (1998) thawed seabream sperm at a temperature of 26°C for 2 min. Fabbrocini et al. (2000) applied the thawing process at a temperature of 30°C at the gradient of 15°C/s.

In the study on seabass sperm, frozen sperm in 0.25 ml straws were thawed at 35°C for 5 s (Fauvel et al., 1998, 1999; Zilli et al., 2005). De Graaf et al. (2004) thawed frozen black seabass sperm belonging to the same family with seabass in 0.5 mL straws at 20°C for 30 s. In this study, although there was no significant difference between the values obtained for 20 s at 26°C and 15 s at 35°C, it was found that the straws thawed at 26 °C for 20 s gave numerically higher motility values.

In this study, the fertilization rates obtained from the experimental and control groups were very close to each other and no statistical difference was observed. In other words, the fertility rates of the frozen sperm were very successful.

In the study by Barbato et al. (1998), an average value of 300 µL semen from 4 male seabreams were obtained for use in fertilization. The eggs to be used in fertilization were collected by using the catheterization method (4 g). In fertilization, 10 different groups were inseminated with the frozen sperm. Each group was created with an average of 165 eggs. The insemination dose was calculated to be 2.6×10^5 spermatozoa per egg. Consequently, they reported that the hatching rates varied from 13.6% to 58.6%. In this study, the highest rates among the 10 groups (44.4–58.6%) were obtained from the least processed groups with an egg–spermatozoon contact time of 5 min. When the egg–spermatozoon contact duration increased from 5 to 10 min, it was observed that the fertilization rate significantly decreased ($p<0.05$). The same researchers reported that increasing the egg–spermatozoon contact time resulted in significant reductions in the hatching rates. This is because DMSO (15%) has a toxic effect on eggs. In addition, it is thought that low fertility rates were obtained due to the lower number of eggs (165±6) and the quality of eggs used may have created a difference between the groups.

In the study by Cabrita et al. (2005a), the hatching rates (45% and 43%) obtained were lower than the fertilization rates (77% and 75%) with respect to fresh and frozen sperm, respectively. The important cause for this difference is that the fertilization rates were considered instead of the hatching rates. Another important factor that should be considered is that the same researchers investigated the fertilization of eggs with 350 µL frozen sperm diluted at 1:6 ratio. In the dry fertilization technique performed with more sperm and less eggs, more sperm contact with eggs was considered to have a positive impact on the fertilization rate. In addition, the different diluents and cryoprotectant ratios might have caused significant effects on fertilization. Galli et al. (2009) applied GnRH primarily to collect eggs from female seabreams. For fertilization, they used insemination doses of 2.6×10^5 and 1×10^5. As a result, the fertilization rates obtained from fresh and frozen sperm varied between 22.6% and 40%.

In this study, the hatching rates of the frozen sperm (Group I), diluted (with inactivation solution) sperm (Group II), fresh sperm (Group III), and naturally fertilized eggs (Group IV) were determined to be 43.48%, 44.57%, 45.42%, and 43.49%, respectively. It is assumed that the differences between the values obtained from this research and the values obtained by other studies might be due to the age of fish, egg collection, egg quality, solutions used for activation, and amounts of these solutions. In addition, the differences in diluent and cryoprotectant rates used in the studies may have influenced the obtained results.

It was determined that the hatching rates obtained in this study were fairly different from the hatching rates obtained in the fish farm. The reason for the severity of this difference is considered to be primarily caused by the elimination of poor-quality eggs (unfertilized and atresic) and the laying of eggs that have only completed oogenesis. It should be noted that the hatching rate obtained is also significantly affected by environmental factors (water temperature and temperature change, pH of the water, concentration of dissolved oxygen, salinity, and light). Another important factor is the feeding of the breeders and dietary differences.

As a result, Ringer's solution as the best diluent and DMSO as the best cryoprotectant were selected for the freezing of seabream sperm. For thawing the frozen sperm, two different temperatures and durations were applied, and the differences between them were not significant. The fertility rates obtained with the best diluent, cryoprotectant, and thawing temperature and duration were successful and close to the results of the control groups. It should be noted that this study can facilitate the determination of an ideal process for freezing seabream sperm for subsequent use in artificial insemination, and it can provide guidelines for other studies.

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