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Cryopreservation of seabream (*Sparus aurata*) semen and fertility

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Abstract: In this study, the aim was to reveal spermatological parameters of seabream (*Sparus aurata*), freezing ability of semen in different extenders, and fertilization capacity of frozen seabream semen. The study was conducted with 10 male and 6 female brooder seabreams. Female fish were synchronized by photoperiod and water temperature in the breeding season. Four different extenders [Mounib, Salty Mounib, Ringer, and sea water (1% NaCl)] including 10% dimethyl sulfoxide were used to assess the freezing ability of seabream semen. After postthaw spermatological examinations, three successful extenders (Mounib, Ringer, 1% NaCl) were chosen for the fertilization stage and 2 control and 3 experimental groups were formed (control I: fresh semen, control II: naturally fertilized eggs). When postthaw motility values were considered, the best results belonged to Mounib and Ringer extenders at 54.45 ± 1.18% and 58.25 ± 1.97%, respectively. At the fertilization stage, the highest hatching rates were from Mounib and Ringer at 29.20 ± 1.05% and 28.63 ± 1.12%, respectively. The statistical difference between extenders was significant ($P < 0.05$). Mounib and Ringer extenders gave the highest motility and fertility rates. As a result, Mounib and Ringer extenders can be used for freezing seabream semen, and semen frozen with these extenders supplies sufficient fertility results.

Key words: Seabream semen extender, *Sparus aurata*, semen cryopreservation, Turkey, fertility

1. Introduction

Reproduction in fish is under the influence of various external factors. The most important factors are photoperiod, water temperature, water quality (dissolved oxygen, pH, hardness, salinity, alkalinity), floods, water currents, tides and cycles of the moon, weather conditions (atmospheric pressure, rainfall), spawning substrates (aquatic plants, pieces of wood, wards), nutrition, diseases, parasites, and the presence of other fish (1). In natural conditions, these fish are mostly female after 2 years of age (2). Seabream (*Sparus aurata*) has reproductive properties such as protandrous hermaphroditism, asynchronous ovarian development, and multiple daily spawnings (3,4).

Newly spawned seabream eggs are approximately 0.9–1 mm in diameter and are transparent. Normally they contain one single oil drop and have a pelagic structure. Since the eggs have a pelagic structure, only the eggs that can float in water are viable and can form embryos (5,6). The egg membrane is transparent and thin, and the micropile hole is about 14 µm (2,7). After the seabream eggs complete their maturation and they are left in water and hydrolyzed, the egg diameter can be up to 1100 µm (8).

Since fish spermatozoa are not motile when they leave the testes, they should be activated in species-specific ways (fresh water or sea fish species) (9). For spermatozoa activation, a number of extracellular agents were reported. Flagellar activation is provided by hyperosmotic-osmotic (500–1100 mOsm) medium in sea fish and by hypoosmotic (0–100 mOsm) medium in freshwater fish. In salmonids and sturgeon the most important motility activation factor is potassium (K^+) concentration (10,11). It was stated that in seabream K^+ and calcium (Ca^{2+}) had no effect on spermatozoa activation and that the optimal osmolarity for this activation was 1100 mOsm/kg (11).

Penetration happens quickly after extending because fish spermatozoa are very small in structure; thus, an equilibration step is not required. The toxic effects of cryoprotectants can be minimized by not applying the equilibration stage. In a study conducted on seabream, when dimethyl sulfoxide (DMSO)-added semen was left for equilibration for more than 2 min, fertilization rates were lower (12,13).

Fish semen is not appropriate for freezing without extending. The extenders used for freezing sea fish semen are mostly salty (1%–10% concentration) or sugary

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(1%–10%) solutions. Extenders should not activate spermatozoa motility (9). Sea fish semen is considered to be more resistant to freezing and this is because of the higher cholesterol/phospholipid ratio in the spermatozoa membrane and the adenosine triphosphate (ATP) content at the beginning. The phosphatidylcholine that is present in the sea fish spermatozoa membrane may be effective for cold resistance (9,14).

Cryoprotectants are divided into 2 groups: internal and external. Among the internal cryoprotectants ethylene glycol (EG), propylene glycol (PG), glycerol (G), dimethylacetamide (DMA), DMSO, and methanol (M), DMSO is most commonly used. DMSO is more effective when compared with the others because it can penetrate quickly and it interacts with the spermatozoa's phospholipid layer (9).

The aim of this research was to detect some of the spermatological parameters of seabream that have major economic importance, freeze seabream semen, and present the success rate of frozen semen in artificial insemination. In the semen freezing stage, the effects of 4 different extenders (Mounib, Salty Mounib, Ringer, and 1% NaCl) that are commonly used in sea fish, 10% DMSO, and a thawing process at 26 °C for 20 s on motility were examined and the frozen semen was evaluated based on fertility and artificial insemination success.

2. Materials and methods

The study was carried out in the breeding facilities of the Bafa Production Center of Kılıç Marine Products Exports and Imports Inc. Ten male and 6 female seabreams, randomly selected, were used.

Brooder seabreams were grown in PVC tanks of 23 t in capacity, with a male/female ratio of around 2:3, and were fed with mollusks such as squids. Along with these, commercial condensed feed was also given. The water temperature of the tank was set at 18 ± 0.5 °C. Water temperature was checked twice a day to keep the temperature changes minimal. The salinity, pH, and dissolved oxygen of the water in the tank were recorded as 36%, 7.5–8.0, and 7–10 mg/L, respectively. The study was carried out with brooders that were synchronized by water temperature and photoperiod in the breeding season in March. The brooders were subjected to 18 h of light and 6 h of dark, which is close to natural conditions. During the study, the ambient temperature was recorded as 15 ± 3 °C.

2.1. Collection of semen and eggs

Water volume of the tank was reduced before collection of the semen for easy capture and rapid anesthesia of the brooders. Phenoxyethanol (final titer of 100–120 mg/L) was used as anesthetic in a small tank of 100 L. Genital areas of the seabreams were cleaned and semen was collected into sterile cups by abdominal (craniocaudal) pressure. The same protocol was administered to the females to collect eggs. Collected semen samples were numbered and transferred to the laboratory in a dark cold Thermos (0–4 °C).

Collected semen was put into a scaled plastic tube and the amount of semen was measured in terms of milliliters. Semen color was evaluated for hematospermia and pyospermia by visual inspection. Semen pH was detected with indicator strips. In microscopic examination, fresh semen motility, postthaw motility, and concentration were determined. For motility examinations, a small drop of semen was put onto a slide and activation solution (Table 1) was added at a ratio of 1:10, quickly mixed, covered, and examined at room temperature (15 ± 5 °C) without a heating plate. Motility evaluation was performed subjectively by 2 different researchers examining 5 different microscopic fields and was noted as a percentage. Spermatozoa concentration was determined by hemocytometer method and recorded as spermatozoa/mL (15).

2.2. Extenders and freezing

Collected semen samples were transferred to the laboratory within 20 min in a cold Thermos. After spermatological examinations, 10 different ejaculates were pooled into 50-mL plastic tubes (concentration examinations of the semen were done after freezing). Pooled semen samples were diluted with 4 different 10% DMSO extenders at a ratio of 1:2 (Table 1) and were cryopreserved in 0.5-mL

Table 1. Composition of the extenders and activation solution per 100 mL.

Mounib	10 mg/mL $KHCO_3$ + 2 mg/mL reduced glutathione + 42.7 mg/mL sucrose + 10 mg/mL BSA
Salty Mounib	10 mg/mL $KHOC_3$ + 19.4 mg/mL NaCl + 6.2 mg/mL $NaHCO_3.2H_2O$ + 2.2 mg/mL glycine
Ringer	6.5 mg/mL NaCl + 0.40 mg/mL KCl + 0.12 mg/mL $NaCHO_3.2H_2O$ + 2 mg/L glucose
1% NaCl	1% NaCl (10 mg/mL)
Activation solution	30 g/L NaCl + 0.8 g/L KCl + 1.3 g/L $CaCl_2$ + 6.6 g/L $MgSO_4$ + 0.18 g/L $NaHCO_3$ (pH 8.2, osmolarity 987 mOsm/kg)

straws. Temperatures of both extenders and pooled semen samples were the same during the dilution stage. A freezing stage was applied in 3–5 min without equilibration. The open ends of straws were closed with polyvinyl alcohol and transferred directly into water (12–13 °C). The straws were frozen 4–6 cm above the liquid nitrogen level for 15 min and were stored in liquid nitrogen (–196 °C). All experiments were carried out in a cold room. The thawing process was applied at 26 °C for 20 s in a water bath.

2.3. Insemination and fertilization

After postthaw examinations, the Salty Mounib extender was not found appropriate, so it was not used in the fertilization stage. The fertilization process was initiated just after the collection of eggs from female seabream. Eggs from 4 different females were set to be 5 g (approximately 5000–6000 eggs) in each group. During the collection of eggs, nontransparent and defective eggs were eliminated. Three experimental and 2 control groups were set for the fertilization stage. Fresh semen of 3 different males was pooled and used for the control groups of the fertilization stage.

Eggs (5 g) were put into 50-mL plastic tubes and semen was added for the fertilization stage. After nearly 10–15 s of gentle mixing, activation solution was added to activate the spermatozoa and to swell the eggs. Amount of activation solution was determined to be 3 times the amount of eggs; thus, 15 mL of activation solution was added to each group. The activation solution was slowly poured into the plastic tubes from the edge. The temperature of the activation solution was maintained at the same level as the eggs, water in the tank, and semen (approximately 18 °C). Three minutes after the initiation of fertilization with the activation solution, the mixture was transferred slowly and gradually into incubators with 500-μm mesh in order to minimize temperature changes and osmotic shock.

To minimize external effects on eggs, the completion of fertilization and transfer of all the groups into the incubators was performed within 20–30 min. Semen collected from 3 different males that had 90% or higher motility values was mixed and used for the control groups. In the fertilization, the spermatozoa/egg (sp/egg) ratio was determined as 4–8 × 10^3 sp/egg mean values. The amounts of semen in groups were set as follows: frozen semen, 12 μL, and fresh semen, 7 μL (Table 2). Fertilization experiments were repeated 3 times.

Vent pipes were placed in the incubator in order to provide oxidation for each group and to prevent the eggs from sticking together. A dark environment was provided during the incubation period and water temperature was measured by electronic thermometer twice a day.

After the eggs were incubated for 24 h, vent pipes were taken out, and 3–5 min later defective and unfertilized eggs were removed with a glass tube system. The eggs were weighed with a sensitive electronic scale. Eggs collected at 24 and 48 h were noted with respect to their groups and at 70 h the larvae ratios were obtained. At 70 h, the bottom of the incubator was assessed for presence of defective or unfertilized eggs.

2.4. Statistical analysis

In this study, standard errors and average values of the data were calculated. One-way analysis of variance (ANOVA) was used to demonstrate the differences between extenders and fertility, and the Duncan test was used to determine group differences (SPSS 10.0 for Windows, SPSS Inc., Chicago, IL, USA).

3. Results

Semen samples collected from 10 male seabreams were evaluated in terms of spermatological parameters such as semen amount, spermatozoon motility, spermatozoa concentration, pH, and semen color (Table 3).

In this study, Salty Mounib (19.4% salt) was unsuccessful due to its activating the spermatozoa and low postthaw motility results (10%–15%). Thus, it was not included in the fertilization stage. In the present study, the highest postthaw motility results were obtained from Mounib and Ringer (Table 4). The statistical difference between extenders was significant ($P < 0.05$).

Table 2. Artificial insemination groups.

Group	Extender	Amount of semen (4–8 × 10^3 sp/egg)
Group I	Mounib	Frozen semen, 12 μL
Group II	Ringer	Frozen semen, 12 μL
Group III	1% NaCl	Frozen semen, 12 μL
Group IV (control)	-	Fresh semen, 7 μL
Group V (control)	-	Naturally fertilized egg

Activation solution: 30 g/L NaCl, 0.8 g/L KCl, 1.3 g/L $CaCl_2$, 6.6 g/L $MgSO_4$, 0.18 g/L $NaHCO_3$, pH 8.2.

Table 3. Spermatological parameters of seabream semen (n = 10).

Fish no.	Amount (mL)	Motility (%)	Concentration ($\times 10^9$/mL)	pH	Color
1	4.50	95	16.60	7.25	Milky
2	10.00	95	14.10	7.50	Milky
3	9.50	85	18.20	7.25	Milky
4	13.00	90	29.15	7.50	Milky
5	12.00	90	30.15	7.50	Milky
6	4.50	90	11.90	7.25	Milky
7	3.50	85	23.60	8.00	Milky
8	4.00	95	31.20	7.25	Milky
9	6.00	90	22.55	7.00	Milky
10	3.50	90	17.50	7.50	Milky
Mean ± SE	7.05 ± 1.17	90.5 ± 1.17	21.53 ± 2.20	7.40 ± 0.08	-

Table 4. Postthaw motility of the extenders.

Extenders	Postthaw motility (%)
Mounib	54.45 ± 1.18^a
Salty Mounib	12.14 ± 1.38^c
Ringer	58.25 ± 1.97^a
1% NaCl	44.75 ± 0.75^b

$^{a, b, c}$: The difference between mean values of groups having different superscripts is significant at $P < 0.05$.

Eggs collected from 6 brooder female seabreams were used for artificial insemination (Figure 1). The inseminations were performed with 2 control and 3 experimental groups, which gave successful results for semen freezing.

Three experimental and 2 control groups were formed. These groups were as follows: for the experimental groups, semen frozen with Mounib extender (group I), Ringer extender (group II), and 1% NaCl (group III); and for the control groups, fresh semen (group IV) and naturally fertilized eggs (group V). Groups I–IV were added with approximately 5 g of eggs. The fertilized and unfertilized eggs were evaluated under light microscope at 48 h (Figure 2). For the calculation of the fertility of the groups, unfertilized eggs were collected and weighed at 24 and 48th h. At 70 h, the hatching rate of the remaining eggs was determined as 100%. According to the results, the fertility rates of semen frozen with Mounib and Ringer extenders were 29.20% and 28.63%, respectively, and the statistical difference of these rates was not significant. The 1% NaCl extender gave the lowest hatching results at 24.9% (Table 5).

4. Discussion

In this study, 4 different extenders were used for the freezing process. However, the Salty Mounib extender was unsuccessful and thus it was not included in the fertilization experiments. Fertilization experiments were done with 3 different experimental groups (frozen semen) and 2 different control groups. The fertilization results were somewhat similar to the motility rates of the groups. The statistical difference between the experimental groups was significant, whereas it was not significant between the control groups. When the control groups were compared with the experimental groups the difference was statistically significant.

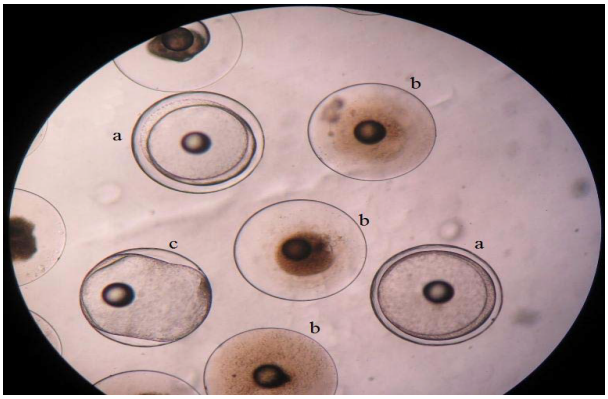

Figure 1. Eggs collected after insemination. a- Fertilized egg (blastomere stage), b- unfertilized or defective egg, c- different blastomere stage.

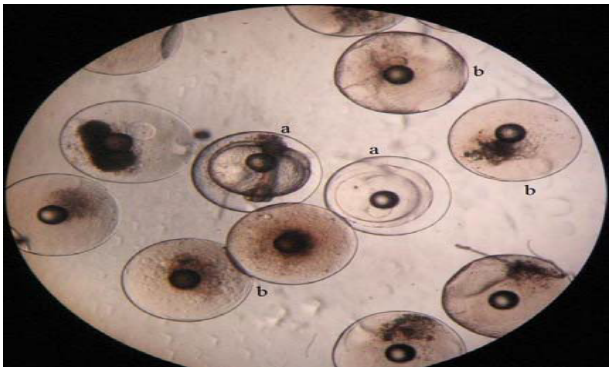

Figure 2. Fertilized and unfertilized eggs at 48 h. a- Fertilized eggs (embryo stage), b- unfertilized eggs.

Table 5. Hatching rates at artificial insemination.

Group no.	Amount	Extender	Hatching rate (%)
I	12 µL	Mounib: 2–0.5 mL	29.20 ± 1.05[a]
II	12 µL	Ringer: 2–0.5 mL	28.63 ± 1.12[a]
III	12 µL	1% NaCl: 2–0.5 mL	24.90 ± 1.16[b]
IV	7 µL	Fresh semen	46.50 ± 1.34[c]
V	-	Naturally fertilized eggs	50.06 ± 0.95[c]

[a, b, c]: The difference between mean values of groups having different superscripts is significant at $P < 0.05$.

In the present study, the amount of collected semen (7.05 ± 1.25 mL) was higher than that of the research conducted by Barbato et al. (16) (0–3.1 mL); the reason for this might be quality of the brooders used. In this study, seabreams of 2–3 years old and weighing 900–1300 g were used, whereas Barbato et al. (16) used seabreams of approximately 1 year old weighing 118–444 g. Therefore, the difference in amounts of collected semen may be explained by the use of seabreams of different ages and weights.

It was reported that 1% NaCl extender, which was used in this study, was successfully used in other studies related to freezing seabream semen. Thus, 1% NaCl extender was tested and we evaluated whether it was appropriate for seabream semen or not. According to the results, no significant difference between the Mounib and Ringer extenders, which gave the best postthaw motility results, was observed. The lowest motility in this research was obtained from the Salty Mounib extender (10.75%), as 19.4% NaCl and 10% DMSO, which are the contents of the Salty Mounib extender, increase osmolarity and activate the spermatozoa before the freezing stage. NaCl (1%), which was used as an extender for freezing seabream semen in many other studies (17–19), was used at a 1:2 ratio in this study and since it gave 44.75% motility the 1% NaCl extender was unsuccessful. In the present research, the different results obtained may be due to differences in dilution ratios (1:2), cryoprotectant rates (10%), and freezing-thawing procedures. Differences in spermatozoa concentrations and cooling rates of freezing may also alter the results.

Fabbrocini et al. (17) investigated the efficiency of different cryoprotectants such as EG, PG, G, and DMSO and reported that 5% DMSO was least toxic for seabream spermatozoa. Barbato et al. (16) noted that 15% DMSO was useful to freeze seabream semen. Despite the fact that usage of DMSO at low concentrations has negative effects on motility, after postthaw examinations DMSO provides long-term motility and is less toxic than other cryoprotectants. It was also revealed that among the cryoprotectants EG reduces motility least. In this study, 10% DMSO, reported to be the most appropriate concentration for *Centropristis striata* semen by DeGraaf et al. (20), was also appropriate for seabream semen.

The incubation rates of fresh and frozen semen obtained from this research (46.50% and 24.90%–29.20%, respectively) were lower than the fertilization rates of fresh and frozen semen reported by Cabrita et al. (18) (77% and 75%, respectively). The difference that occurred may be due to Cabrita et al. (18) reporting the fertilization rates instead of the incubation rates. Another important factor is that the same researchers inseminated 2000 eggs (2 g) with 350 µL of semen extended at a ratio of 1:6. When semen is subjected to fertilization of fewer eggs with a higher ratio of extender, it will be easier for spermatozoa to reach the eggs. This is thought to have an important positive impact on fertilization ratios. The usage of different ratios of extenders and cryoprotectants (1% NaCl, 5% DMSO) may cause an important effect on fertilization.

Many researchers used 1% NaCl as a seabream semen extender (16–19,21). Cabrita et al. (18,19) reported that to prevent spermatozoa gathering and to protect plasma membrane, 10 mg/mL BSA should be added to the extenders used by Fabbrocini et al. (17) for cryopreservation,. However, according to the data of this research, Ringer and Mounib extenders are more successful than 1% NaCl extender.

In contrast with Salte et al. (22), an important decrease was observed in the incubation rates of eggs fertilized by frozen semen. No comparison could be made about this condition because the same rate of spermatozoa per egg was used in all groups.

Gametes that are collected by abdominal massaging may not be qualified (defective, immature). Therefore, fertilization with gametes collected by abdominal massaging has more disadvantages than natural breeding. In the present study, the incubation results are significantly different from the incubation results of the breeding facility. The reason for this significant difference may be gametes with different qualities. After the seabream eggs complete their maturation and they are hydrolyzed, the diameter of eggs increases up to 1100 µm (8). In this study, the diameters of collected eggs were measured as 940–1000 µm regardless of eggs being fertilized and viable or unfertilized and defective. The reason for low incubation rates might be the eggs being damaged during collection by abdominal massage. Similarly, Atay and Bekcan (23) also noted that eggs might be damaged during abdominal massage.

As a result, in the present study the best results in terms of postthaw motility and fertility were obtained from Ringer and Mounib extenders. This study is one of the pioneering studies in Turkey with respect to determination of spermatological parameters of seabream semen, semen extended with appropriate extenders, and lastly obtaining fertility with frozen semen groups. Worldwide, there are limited numbers of studies about spermatological parameters, freezing, and fertility of seabream semen. This study will be an initial step for further studies and will aid in determining the ideal protocols for seabream semen to be frozen and used in artificial insemination.

In order to determine the ideal procedure for freezing seabream semen, additional studies should be done of various subjects, such as trying different cryoprotectants with different concentrations, different cooling rates of freezing, thawing at different temperatures and times, and detection of postthaw manipulation period of the spermatozoa and spermatozoon viability duration.

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