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Research Article

The effect of cryopreservative extender developed for Mesopotamian spiny eel (*Mastacembelus mastacembelus* Banks & Solander, 1794) on sperm motility and fertilization rate after thawing

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Abstract

This study was aimed to determine the effect of a freezing extender developed for Mesopotamian spiny eel *(Mastacembelus mastacembelus* Banks & Solander, 1794). Mesopotamian spiny eel was caught from May to July 2017 in the Atatürk Dam Lake and Euphrates Basin (meridians) in Şanliurfa, Turkey. During the study, the animals (n=10; 8 males, 2 females) were caught with gill nets (22 mm×22 mm, mesh size) and kept under natural lake water [temperature (22.00–23.60ºC), oxygen (8.45–8.98 mg/l), pH (8.14–8.50) and salinity (0.22 ppt)]. Semen was collected from eight males (406.46±166.68 g) weekly by abdominal massage after the hormonal injections of human chorionic gonadotropin treatment. Sexual maturation and spermiation of MSE males (n=8) were stimulated by weekly injections of human Chorionic Gonadotropin (hCG; Pregnyl, Merck Sharp Dohme, Turkey) [(1 International Unit (IU) hCG/body weight (g)/week]. At the 7th week of hormonal treatment, milt from eight mature males was carefully collected by abdominal massage, avoiding contamination with urine and feces into the petri dish, 24 h after the hormone applications. The motility of the collected spermatozoa and the spermatozoa density were evaluated on day of collection. Sixteen milliliter pooled ejaculate (2 milliliter per male) was split into two equal aliquots and diluted with the ArŞaDo extender (NaCl (163mM), $NaHCO_3$ (9.4mM), $MgCl_2$ (0.84mM), $CaCl_2$ (8.26mM) and KCl_2 (18.16 mM)) and the P1 (NaCl (125 mM), $NaHCO_3$ (20 mM), $MgCl_2$ (2.5 mM), $CaCl_2$ (1 mM) and KCl2 (30 mM)) extenders. After two weeks, straws were thawed in a water bath at 40°C for 13 s and post-thaw sperm motility was immediately estimated subjectively. For the activation, saline solution (0.9% NaCl) was used. Sperm motility of thawing samples was significantly higher in ArŞaDo extender than in P1 extender (42.66±1.17% *vs*. 32.96±1.27%, respectively). The fertilization rate in ArŞaDo extender was higher than sperms preserved in the P1 extender (82.03±1.94% vs. 73.43±1.45%, respectively). In conclusion, the ArŞaDo extender improved the post-thawing spermatozoa motility percentage and fertilization rate in Mesopotamian spiny eel males.

Keywords: Cryopreservation, Diluent, Freezing extender, Mastacembelidae

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Introduction

Mesopotamian spiny eel (*Mastacembelus mastacembelus*-MSE) is a freshwater fish, belongs to the Mastacembelidae family and is commonly found in Tigris and Euphrates River Systems (Geldiay and Balık, 1988). Out of the spawning period (between end of the May to July), they live in deep-water environment. During the spawning period, they are found on the shore, near the surface of the water to mate and lay eggs. These fish are caught from their natural aquatic environment for the commercial fishing.

In recent years, the culture and propagation of MSE has gained increasing attention in Turkey. However, MSE semen freezing typically remains a challenge as a suitable extender is not available. Fresh sperm collection in all seasons is difficult since this species has seasonal spawning and lives in deep waters after spawning season. In addition, captured male fish does not always have high quality milt during spawning season (Aral *et al.,* 2007). The maturation of the MSE in the captive condition is only achieved by expensive and long hormonal treatments and the final maturation in both sexes can still be unsynchronized (Asturiano *et al.,* 2006; Asturiano *et al.,* 2016). As a result, it is essential to do sperm cryopreservation in this species. Additionally, sperm cryopreservation will facilitate its transportation distant locations.

Extenders, which typically species-specific, perform a substantial role as a fluid nutrient medium developed for spermatozoa to preserve the integrity of the sperm against external factors (e.g., freeze and osmotic shock, oxidative stress, and cell injury by ice crystals). In addition, they determine the fertilization capacity, increase the milt volume to the desired level and provide the milt preservation requirements regarding osmotic pressure, pH, ion component (Chang, 1997; Zidni *et al.*, 2020; Şahinöz and Doğu, 2020). Therefore, extenders have critical role in increasing semen quality during the milt cryopreservation. Milt with high quality can be achieved with better diluents under optimized conditions.

Artificial seminal plasma (ASP) diluents are the most widely used formulations for European (*Anguilla anguilla*) and Japanese eel (*Anguilla japonica*) sperm (Morisawa *et al*., 1983; Suquet *et al*., 1993; Lin *et al*., 1996; Astuarino *et al*., 2006; Peñaranda *et al*., 2009; Koh *et al*., 2017; Müller *et al.*, 2018; Herranz-Jusdado *et al*., 2019, Zhang *et al.*, 2020). To improve the freezing protocol of Zig-zag eel (*Mastacembelus armatus*) semen, another subspecies of the Mastacembelidae, 0.9% NaCl solution was tried (Hossain *et al*, 2011; Rahman *et al.*, 2016). However, there are no studies on artificial seminal plasma (ASP) diluents containing egg yolk to increase spermatozoa motility or fertility of the milt in MSE.

The spermatozoa motility in an extender is necessary for its freezing and main criteria for the evaluation of an extender. Seminal fluids normally have high levels of K^+, Na^+ and Cl^- ions, but

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they contain low concentrations of $Ca^{2+}$and Mg^{2+} ions (Morisawa *et al.*, 1983; Suquet *et al.*, 1993; Lin *et al.*,1996). Ions, pH and osmolality have a powerful impact on sperm motility. Spermatozoa in fish are immobile in the male sperm duct, and they gain motility after ejecting into the aquatic environment (Alavi and Cosson, 2006). It was reported that sperm of freshwater species are triggered in hypotonic solutions and those of marine species in hypertonic solutions (Morisawa *et al*., 1983; Rahman *et al*., 2016).

The European eel sperm diluent developed by Tanaka *et al.* (2004) contained NaCl, $NaHCO_3$, and soy lecithin. P1 extender, used in previous studies, has been improved for freezing ability of European eel sperm (Astuarino *et al.*, 2006; Peñaranda *et al.*, 2009). Researchers aimed to preserve the sperm motionless in extenders like the male sperm duct (Ohta *et al.*, 2000; Herranz-Jusdado *et al.*, 2019). Ohta *et al.* (2000), studied on ASP extender for Japanese eel sperm. Another extender examined in the Japanese eel to sperm freezing was K30 ASP (Koh *et al.*, 2017). Japanese eel sperm extenders were based on freezing the sperm of European eel. However, the P1 extender was designed to freeze the European eel sperm (Perez *et al*., 2003). All extenders in these studies contained 10 % Dimethyl Sulfoxide (DMSO) as cryoprotectant. In addition, other cryoprotectant like methanol, N-N, dimethyl-formamide (DMF), N-N, dimethyl-acetamide (DMA) could be added to extenders in eel sperm freezing protocols.

In many fish species, different sperm extenders have been developed to freeze sperm. There is no sperm extender for MSE that grow in Turkish freshwater. Therefore, in this study, we concentrated on the new extender for freezing of MSE sperm that was named as ArŞaDo extender. Additionally, the present research aimed to formulate a useful freezing extender specific for MSE sperm and to examine the motility and fertilizing capacity of the frozen sperm.

Materials and methods

Animals

The protocol was approved by the Experimental Animal Ethics Committee from the DOLLVET-HADYEK, Şanlıurfa, Turkey (Protocol Number: 2017/l1). The MSE was caught from May to July 2017 in the Atatürk Dam Lake and Euphrates Basin (meridians) in Şanliurfa, Turkey. During the study, the animals (n=10, 8 males, 2 females) were caught with gill nets (22 mm×22 mm, mesh size) and kept under natural lake water [temperature (22.00–23.60ºC), oxygen (8.45–8.98 mg/L), pH (8.14–8.50) and salinity (0.22 ppt)]. Physico-chemical parameters of the sampling areas were measured with YSI environmental test tool (YSI 85, Yellow Springs, Ohio, USA). Then, these fish were placed in 50 L plastic containers and transported to the laboratory of Fisheries Department of Bozova Vocational High School (VHS).

For each sample, the total length (TL) was measured using a measuring board, and total weight (W) was measured in grams by digital analytical scales.

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According to our previous studies, this measure (a length of≥20 cm) was chosen because approximately 95% of the MSE with adult gonad have a length of 20 cm or more (Aral *et al*., 2007).

At that time, the identified samples were carefully moved to an aquarium. They were placed in a 120-liter tank at the laboratory. The tank contained the dam water. The used fish were kept under natural photoperiod with 20.0 to 24.0ºC. The used tanks (separately for males and females) were merely maintained, well-aerated by running lake water. Fish were fed daily with a commercially available dry diet (48% protein, 8% ash, 0.57% Crude fiber and 22% fat) (URL1). This feed contains a similar formulation to the recommended feed for freshwater eel (https://www.aller-aqua.com/).

Male maturation

The protocol by Ohta *et al*. (1997) was applied for sexual maturation induction and ovulation of MSE. After 10 days of acclimation period, male fish anesthetized with benzocaine (60 ppm). The maturation and sperm collection were carried out in the fresh water laboratory, the Harran University, Bozova VHS. Sexual maturation and spermiation of MSE males (n=12) were stimulated by weekly injections of human Chorionic Gonadotropin (hCG; Pregnyl, Merck Sharp Dohme, Turkey) [(1 International Unit (IU) hCG/body weight (g)/week]. Mean fish weight is 406.46±166.68 g. At the 7th week of hormonal treatment, milt from eight mature males was carefully collected by abdominal massage, avoiding contamination with urine and feces into the petri dish, 24 h after the hormone applications.

Evaluation of sperm motility

In maximum10 minutes after the sperm collection, the sperm of all collected ejaculates were analyzed for motility and sperm density in triplicates. Sperm motility was assessed by observing fewer than 100X magnification using 10X optical systems. Motility was determined by carefully observing motion under 100X magnification using 40X optical systems at room temperature (22 to 24°C). Sperm motility was expressed in percentage. Briefly, 1µl samples of milt from each male were activated with 9.5 µL of sperm activating medium (saline solution; 0.9 %), ArŞaDo and P1 extenders, respectively. Hemocytometric method was used to determine spermatozoa concentration which was expressed as number of spermatozoa$\times 10^9$/mL. The spermatozoa with motility values over 80% were selected for cryopreservation. For further use, spermatozoa were stored in a refrigerator (2-4°C).

Sperm extender

The ArŞaDo extender contained NaCl (163mM), $NaHCO_3$ (9.4mM), $MgCl_2$ (0.84mM), $CaCl_2$ (8.26mM) and KCl_2 (18.16 mM). In addition, the ratios of egg yolk and DMSO added to the extender are 20% and 10%, respectively. The pH and osmolality of the freezing medium were arranged 8.0, and 340

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mOsm/kg, respectively (Single-Sample Osmometer, Advanced, Model 3250).

The P1 extender was used (Peñaranda *et al*., 2009) as the control of the freezing extender. P1 extender contained NaCl (125 mM), $NaHCO_3$ (20 mM), $MgCl_2$ (2.5 mM), $CaCl_2$ (1 mM) and KCl_2 (30 mM) and its pH was adjusted to 8.5. The P1 extender also contained 20% egg yolk instead of 20% FBS and 10% DMSO.

Freezing-thawing of the milt samples

Every sample was cryopreserved and thawed through following similar protocols. Overall, sperm samples (n=8) were mixed and properly used for cryopreservation and fertilization tests. Ejaculates pooled were diluted with a saline solution (0.9%-control), ArŞaDo and P1 extender.

The milt samples were frozen according to previous studies (Peñaranda *et al*., 2009; Müller *et al*., 2004). Briefly, 1 mL of the milt was mixed with 3 mL of the cryoprotective medium (1:3; v:v; milt:extender). The cryoprotective medium was added to the milt dropwise and the mixture was equilibrated at 20°C for 15 min. After that, the diluted sperm was cooled enough at about 15 °C for 30 min. Each sample was then loaded into 250 µL straws (IMV Technologies, L'aigle, and France) and ends sealed with sealing gel. The straws were first exposed to liquid nitrogen vapor (-60 to 85°C) for 5 min on the freezing ramp at 5 cm above the elevated surface of the liquid nitrogen. Straws were direct plunged into liquid nitrogen (-196 ºC) to freeze and stored for subsequent analyses. For thawing, the straws were taken out of liquid nitrogen and allowed to stand into a water bath at 40 ºC for 13 s for thawing (Herranz-Jusdado *et al*., 2019). Each thawed milt was then divided into two aliquots for evaluating the post-thaw motility percentage and fertilization rate. The post-thaw motility percentage and fertilization rate were analyzed. Sperm samples were frozen in duplicates.

Motility percentage and fertilization rate of fresh and frozen spermatozoa with two independent extenders and saline solution (0.9 %-control) were analyzed under completely randomized design (CRD).

Egg fertilization

The protocol by Ohta *et al*. (1997) was applied for induction of sexual maturation and ovulation of MSE. Briefly, four female fish received weekly intramuscular injections of carp pituitary extract (CPE), (Argent, USA) for 13 weeks. Prior to administration, CPE was homogenized in a mortar and dissolved in 0.9 % NaCl solution. At 24-h after the last CPE injection, 17, 20 β-dihydroxy-4-pregnen-3-one (DHP) was injected (2 mg/kg body weight) to stimulate final maturation and induce ovulation within 14–23 h. After 12-h post-DHP injection, all females were carefully checked for ovulatory response every 4 h by exerting gentle pressure on the abdomen of fish. The eggs of two females were collected into dry plastic bowls via abdominal massage and used for fertilization test. The fertilization test with fresh and cryopreserved sperm was

[DOR: 20.1001.1.15622916.2022.21.2.21.9]

carried out at Harran University, Bozova VHS, by using dry fertilization method. Eggs were distributed into 5 cm petri dishes in batches (1g) of approximately 1100-1300 eggs so that they would form a monolayer of eggs. Each batch of eggs was fertilized with four straws (250µL) of thawed spermatozoa and 1000 µL of fresh spermatozoa to obtain ~500x10^3 spermatozoa per egg as previously suggested (Fauvel *et al*., 1992). Saline solution (salinity 0.9%) was used to activate the gametes. Eggs were rinsed with the saline solution. Following the insemination, eggs were incubated in three replicates in petridish at 22°C for 3 to 6 h (using 235x235 Silicone Heater Plate (220V 500W), Hobimekatronik, Turkey). The fertilization success was examined via a dissecting microscope (x10) on 100 randomly chosen eggs. It was assumed that eggs were fertilized while they were at the four-cell stage or greater embryos (Schiavone *et al*., 2006).

Statistical analysis

Results obtained from all variables of laboratory analyses were tested for normality of residues and homogeneity of variance. If data did not present the normal distribution and homogeneity of variances, the transformation arcsine square was applied. ANOVA was preferentially used to assess marked differences in extender efficiency on spermatozoa motility and fertilization rate in each fresh and frozen spermatozoa. As follows, ANOVA was efficiently performed considering a completely randomized design by the effect of the sperm type (fresh and frozen sperm) and the extender effect (saline, ArŞaDo and P1). When some significant difference was observed in a main effect, comparisons between the treatments were made using the Tukey's multiple comparisons test. Analysis results of *p*-values<0.05 were considered significant. The results were shown as the mean±standard error. Differences were considered significant at $p<0.05$.

Results

Sexual maturation and spermiation

The results showed there was significant difference ($p<0.05$) in sperm motility among control (saline), ArŞaDo and P1 extender. The highest motility percentage was gained in the ArŞaDo extender when compared to P1 [($p<0.001$(Fig. 1)].

The effect of freezing extender on post-thaw sperm motility

The fresh sperm was slowly activated when diluted with ArŞaDo extender without DMSO and egg yolk. However; prepared samples did not show the sperm motility before instantly freezing when diluted in the ArŞaDo extender, containing DMSO and egg yolk.

The frozen-thawed sperm motility was significantly decreased in ArŞaDo (47.5%) and P1 (60.0%) (Fig. 2) as compared to control (saline).

[DOR: 20.1001.1.15622916.2022.21.2.21.9]

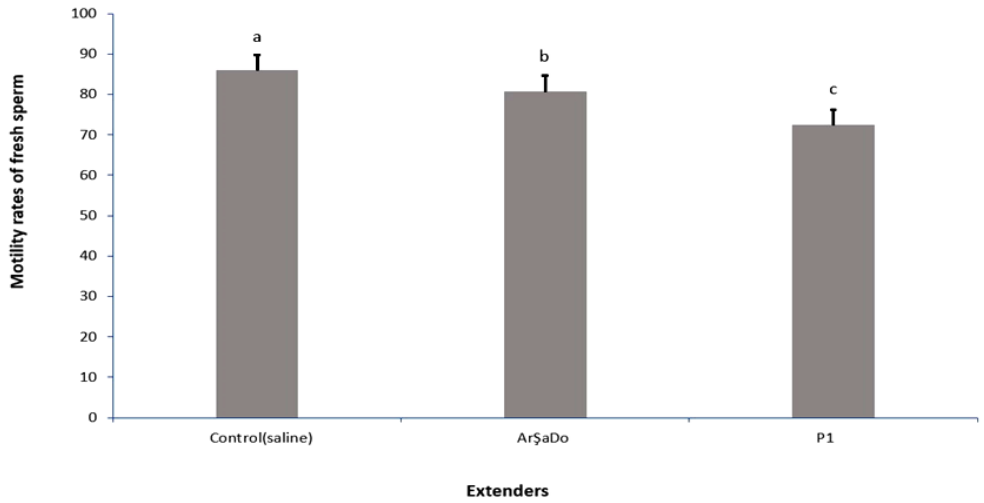

Figure 1: Motility rates of fresh MSE (*M. mastacembelus*) sperm. Values are the means±SE of three replicates. Bars marked with different lower case letter are significantly different (p<0.001)

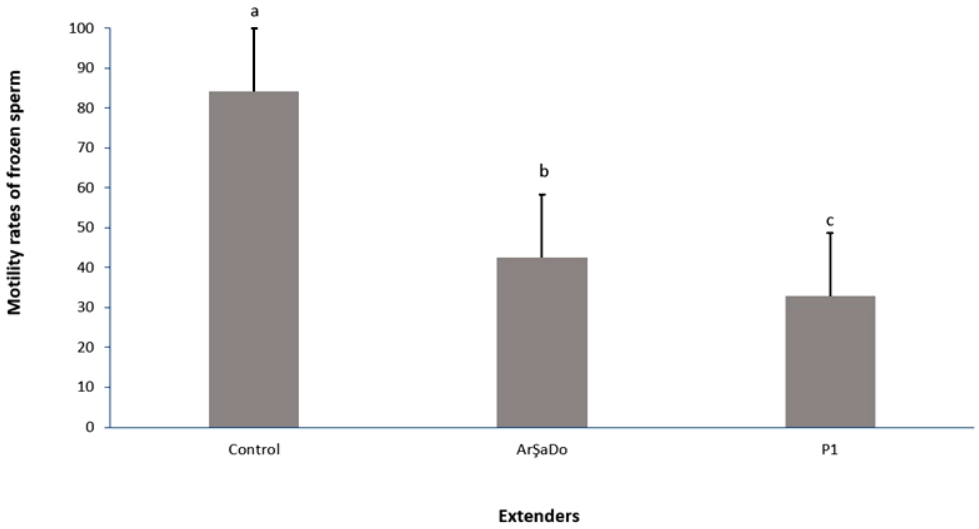

Figure 2: Frozen-thawed sperm motility percentages of MSE (*M. mastacembelus*.) with two different extenders. Values are the means±SE of three replicates. Bars marked with different lower case letter are significantly different (p<0.001).

the highest Sperm motility was in the control group in fresh sperm among all the groups tested (Figs. 1 and 2).

ArŞaDo extender yielded the highest fertilization and that was followed by P1 extender (p<0.001) (Fig. 3).

The results of the study have shown that extenders have a significant effect on the fertilization. ArŞaDo gave the highest fertilization rate when compared to P1 extender (p<0.001) (Fig. 4).

Fertilization rate was the highest in the control group in fresh sperm among all the groups tested (Figs. 3 and 4).

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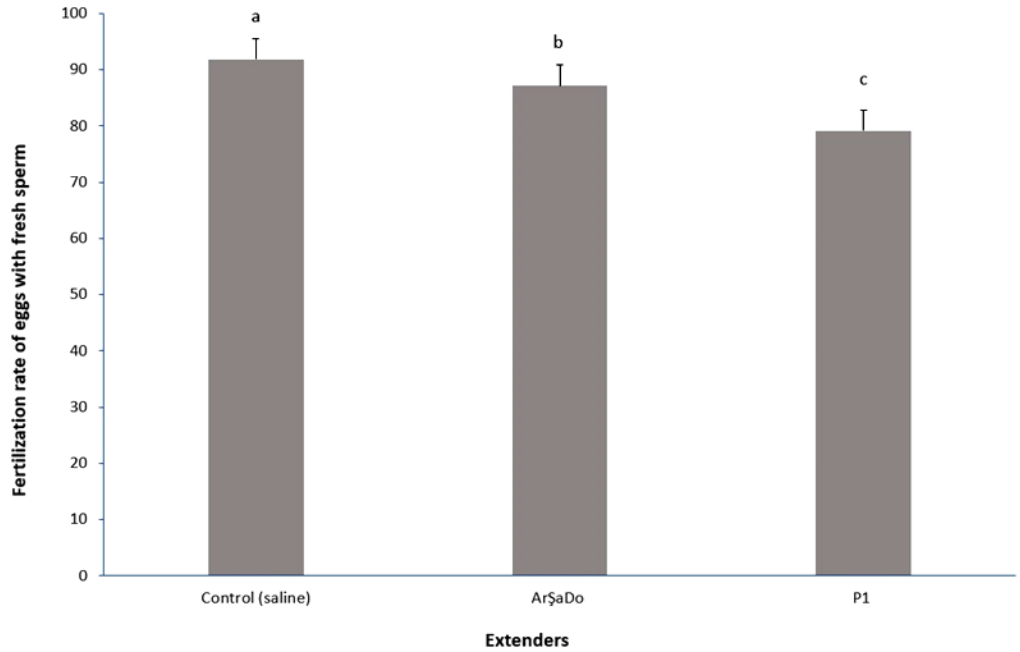

Figure 3: Fresh MSE (*M. mastacembelus*) sperm fertilization rates with two different extenders and saline solution. Values are the means±SE of three replicates. Bars marked with different lower case letter are significantly different (p<0.001).

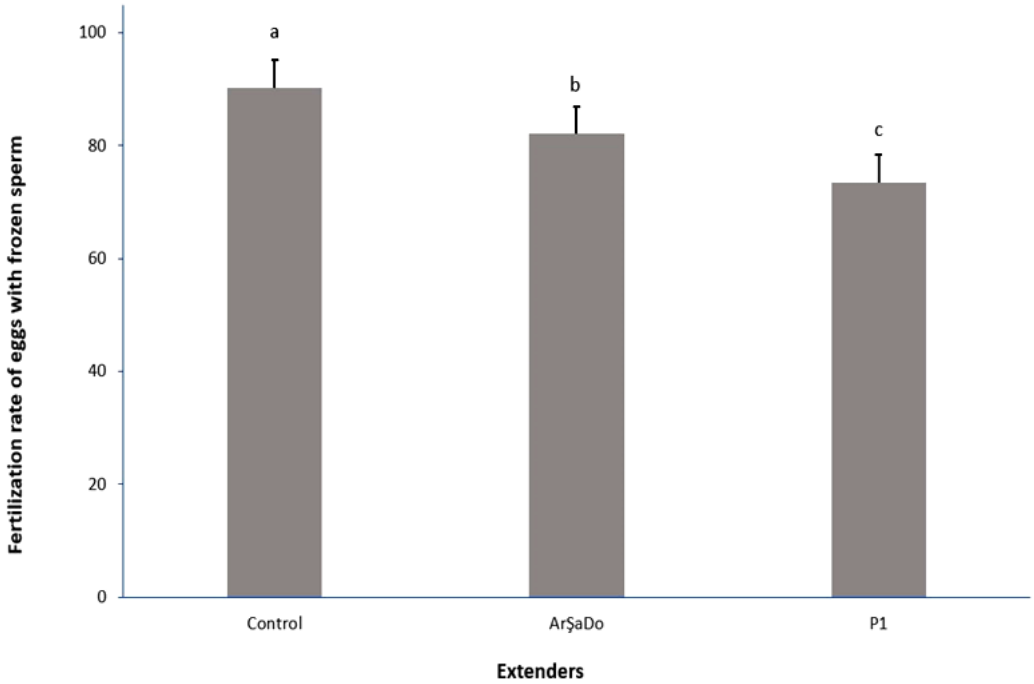

Figure 4: The frozen-thawed fertilization rates of MSE (*M. mastacembelus*) with two different extenders and saline solution. Values are the means±SE of three replicates. Bars marked with different lower case letter are significantly different (p<0.001).

Discussion

Sperm motility is commonly accepted as one of the critical markers to evaluate sperm quality and fertilization potential (Gage *et al*., 2004; Rurangwa *et al*., 2004; Gallego and Asturiano, 2019). The higher motility of the spermatozoa usually has the superiority in accessing

[DOR: 20.1001.1.15622916.2022.21.2.21.9]

the egg within a shorter time for fertilization activity (Butts *et al*., 2020).

The motility in fresh spermatozoa after collection ranged between 80 and 95% in hormone-induced MSE. Mean motility percentage of fresh sperm was reported 67% in MSE (Aral *et al*., 2007), 73±10% in the European eel (Müller *et al*., 2004), up to 80 % in European silver eels after 15-29 h HCG's booster injection (Palstra *et al*., 2005). In addition, the sperm motility of hormone-induced *M. armatus* and European eel males were between 70 and 95% (Rahman *et al.,* 2016; Herranz-Jusdado *et al*., 2019; Müller *et al*., 2004; Herranz-Jusdado *et al*., 2018). Our results were similar to those obtained on sperm motility in previous studies.

After the freezing-thawing process, spermatozoa had lower motility (from 47 to 60%) than fresh samples. Fresh samples (65% and up) had significantly higher motilities than those the frozen with P1, M1, M4 and M5 extenders (22 to 38%) (Peñaranda *et al*., 2009; Herranz-Jusdado *et al*., 2019; Müller *et al*., 2004; Herranz-Jusdado *et al*., 2018). Similarly, Müller *et al*. (2004) declared that the evaluated mean motility of fresh sperm samples for cryopreservation in European eel was 73 %, while the post-thaw motility of frozen sperm was 36%. Similar effects were observed in different studies. Hossain *et al*. (2011) reported that 0.9% NaCl solution containing either 10% Me_2SO or methanol yielded about 50 % post-thaw motility of frozen *M. armatus* sperm. In another study, Alsever's solution with Me_2SO gave the best post-thaw (65%) motility and showed that it was acceptable for sperm preservation of the species (Rahman *et al*., 2016). Motility of fresh and frozen sperm was determined 70% and 38% in the European eel diluted with P1 extender, respectively (Herranz-Jusdado *et al*., 2019). A dramatic decline in post-thaw sperm motility had typically observed in these published studies. This decrease in motility could naturally depend on many key components like Na^+, $Ca2^+$, K^+ ions, pH levels in fish milt and osmolality of the preferred medium (Billard *et al*., 1995; Alavi and Cosson, 2006; Rahman *et al*., 2016).

In aqueous media $NaHCO_3$ is dissociated in several ways: CO_2^+ H_2CO_3 (free-CO_2), HCO_3^- and $CO_3^{-2}$and their balance has interfered with pH. There are possible factors for these dissociations. For instance, if the medium is acidic, most of the HCO_3^- will be transformed into free-CO_2, and this free-CO_2 will act as an inhibitor determinant of sperm motility. Because of these effects of the necessary components, in the current study the concentration of $NaHCO_3$ in the ArŞaDo extender was implemented about the half of $NaHCO_3$ in P1. In addition, the ArŞaDo extender is alkaline. Very few of the HCO_3- may be efficiently converted into free-CO_2 in alkaline condition. This low free-CO_2 might be typically acted as a weak inhibitor factor of the motility (Tanaka *et al*., 2004). In the present study, a lower concentration of $NaHCO_3$ for sperm freezing extender could be attributed to the maintenance of function during freezing and thawing.

[DOR: 20.1001.1.15622916.2022.21.2.21.9]

Previous studies accurately aimed to increase the cryopreservation success proportionally by using additives like Fetal Bovine Serum (FBS), Bovine Serum Albumin (BSA), soya lecithin or egg yolk. The mentioned supplements have been routinely used in cryopreservation protocols for various fish species (Cabrita *et al*., 2005; Herranz-Jusdado *et al*., 2019; Hossain *et al*., 2011; Cabrita *et al*., 2011). Egg yolk, FBS and BSA supplementation to the extenders, are used to prevent osmotic shock in the cell membrane during freezing and thawing (Peñaranda *et al.*, 2009). The P1 extender contains 25% (variable v/v) of FBS (Peñaranda *et al*., 2009). To reduce the experimental variation, we properly handled 20% (v/v) egg yolk instead of 25% (v/v) FBS in P1. As a result, motility percentage of fresh sperm in ArŞaDo and P1 extenders were about 80% and 72%, respectively; meanwhile, the thawed samples of these treatments had 42% and 32%, respectively. These results have sufficiently shown the beneficial effect of egg yolk addition on the fresh and the post-thawed sperm motility. The spermatozoa motility percentage changes following the cryopreservation process and this is valid for fish species, as well. A proper preferred protocol for sperm cryopreservation in European eel, provided spermatozoa motility of about 30% (Herranz-Jusdado *et al*., 2019). This is consistent with the motility values derived in this present study with the extenders. Indeed, the necessary addition of egg yolk could cause frozen-thawed sperm motility to be the highest in the European eel. The marked decrease in post-thaw motility percentage could be caused by dilutions rates. An inadequate protection of the intact membrane by the various substances (e.g., protein, glucose and specific ions) represented in the seminal fluid produces the low post-thaw motility. Marco-Jiménez *et al*. (2006) suggested that noticeable differences in the spermatozoa cell membrane during freezing are highly accompanied and inter-related with the seminal plasma concentration and cryoprotectant. In the present study, egg yolk was added to increase spermatozoa's membrane fluidity. Therefore, a beneficial synergetic effect on the spermatozoa motility was detected when egg yolk was added to the ArŞaDo extender, typically inducing a considerable improvement on the determined percentage of motile spermatozoa of around 10%, compared to P1.

Effective fertilization with cryopreserved semen is one of the principal targets of the study. The power of the cryopreserved semen to fertilize the eggs represents the performance of spermatozoa motility and fertilization capacity of both fresh and frozen–thawed spermatozoa (Lahnsteiner *et al.*, 1996; Magyary *et al.*, 1996; Rurangwa *et al*., 1998; Honeyfield and Krise, 2000). The results of the present study agreed with the fertilization success rates reported by Rahman *et al.* (2016).

In previous studies, Rahman *et al.* (2016) reported 74-82% fertilization rate in *M. armatus* frozen-thawed spermatozoa. Dumorné *et al.* (2018)

[DOR: 20.1001.1.15622916.2022.21.2.21.9]

observed 73.9±17% in pink cusk-eel (*Genypterus blacodes* Forster, 1801) fresh sperm. The proportional rate of fertilization in the Japanese eel after artificial insemination with fresh and frozen sperm with K 30 ASP was 84% and 60 to 72%, respectively (Nomura *et al.*, 2018). The fertilization percentages of the European eel artificial inseminated with fresh sperm were between 69 and 94%, while fertilization rates decreased to 0-33% with the eggs inseminated with frozen-thawed milt (Asturiano *et al*., 2016; Herranz-Jusdado *et al.*, 2018; Herranz-Jusdado *et al.*,2019).

This is the first report on the successful fertilization tests carried out by using a new extender for MSE. The ArŞaDo extender developed for MSE sperm cryopreservation has shown to provide sufficient sperm motility and fertility after thawing. As a result, the ArŞaDo is usable for freezing MSE milt together with DMSO as a cryoprotectant.

Declaration of competing interest

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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