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Inhibitory effect of K^+ and Ca^{2+} concentrations, pH, and osmolality of activation solution on motility of shabut (*Barbus grypus* Heckel 1843) spermatozoa

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Abstract: Evaluation of sperm quality is important in order to understand the main biochemical processes occurring during motility of sperm, to determine the reproductive ability of fish species, and to optimize the environment for spermatozoa. Generally, once differentiated in the gonad the sperm remain there completely quiescent until they are released into an external medium. After activation, motility duration of fish spermatozoa is greatly affected by environmental factors such as pH, ion concentrations, and osmotic pressure. Investigating these factors in relation to fish sperm can help establish good activation or immobilizing media for improving artificial fertilization and preservation. This study aimed to determine the effect of activation solution pH, ions (K^+ and Ca^{2+}), and osmolality on the motility of cultured shabut (*Barbus grypus* Heckel 1843) sperm. The best pH level for activation of spermatozoa was pH 9.0. Ionic factors can stimulate the initiation of sperm activation and duration. Maximum percentage and duration of motile sperm were observed in solutions containing 5 mM K^+ and no Ca^{2+} with an osmolality of 56 mOsmol kg^{-1}. Concentrations of more than 20 mM K^+ and more than 10 mM Ca^{2+} had negative effects on sperm motility.

Key words: *Barbus grypus*, activation solution, sperm motility, K^+, Ca^{2+}, pH, osmolality

1. Introduction

In most fish species with external fertilization modes spermatozoa that are immotile in the testis become instantly motile upon contact with the activating external medium in which fertilization takes place. The quality of sperm usually refers to the motility, which is a prerequisite factor determining the fertilizing ability of semen (1). The chemical characteristics of the motility-inducing medium essentially determine the duration of sperm motility even though several other factors play a role (2). Consequently, osmotic pressure, pH, or ionic content of the activation medium may influence the motility of fish spermatozoa in both freshwater and marine fish species. In particular, parameters of activation mediums such as pH, ion concentration (K^+, Na^+, Ca^{2+}, Mg^{2+}), and osmotic level affect the motility duration of fish spermatozoa (3,4). The pH of an activating solution greatly affects sperm motility. Extracellular and intracellular pH, as well as the ionic composition of the activating solution, influences the initiation and duration of sperm motility (5). The external pH probably influences the intracellular proton concentration, which subsequently affects membrane potential as well as motility behavior (6). In contrast, pH has relatively little impact on carp spermatozoa motility (7), except at an extreme pH via a Na^+/H^+ exchanger (5). Moreover, carp spermatozoa motility can be initiated in a medium with a pH between 6.0 and 9.0 (8). On the other hand, the internal pH of the sperm is approximately 1 unit below the external pH (9). Generally, seminal plasma osmolality inhibits sperm motility in freshwater and marine fishes (10); however, K^+ provides the main inhibiting effect on sperm activation in salmonids (3) and sturgeons (11). The inhibitory K^+ concentration for sperm activation has been reported between 0.1 mM and 2 mM in salmonids (12,13) and between 10 and 40 mM in different carp species (3). Potassium ions also inhibit motility at very low concentrations (in the range of 0.01 mM) in paddle fish and shovelnose sturgeon spermatozoa (14). In most freshwater fish the hypo-osmotic signal induces K^+ and Ca^{2+} channel activities and triggers sperm activation through intracellular Ca^{2+} concentrations (15). Na^+ and Ca^{2+} counteract the inhibitory action of K^+ (3). In salmonids, Ca^{2+} at 10, 1, and 0.001 mM prevented the K^+ inhibitory action at 40, 20, and 2 mM, respectively (16). The Ca^{2+} effect on K^+ inhibitory action has also been studied in paddlefish (17). The response of sperm cells of different

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fish species to activation solutions may be different because seminal plasma Ca^{2+} and K^+ concentrations changed among species. Generally, cyprinid sperm are immotile in the seminal plasma at 300 mOsmol kg^{-1}. Activation is induced when osmolality is lowered for sperm of freshwater species and increased for marine milt (18).

For the purpose of averting a shortage of shabut (*Barbus grypus* Heckel 1843) male broodstock, quality of sperm should be determined, and this sperm should be used effectively. One objective of spermatozoa activation medium is to increase spermatozoa motility during fertilization assay. Spermatozoa motility is the most commonly used criterion to evaluate semen quality. However, in numerous fish species with external fertilization the duration of sperm motility is very short. Additionally, studies in most fish species show that the duration and motility of semen may vary seasonally (3).

B. grypus is a cyprinid species in the Mesopotamia Basin and is highly valuable as food in the region. It is an omnivorous species that feeds mainly on detritus (19). Its adaptation to earthen ponds was carried out at the General Directorate of State Hydraulic Works, Fish Production Station, Atatürk Dam Lake, Urfa, Turkey. The aim of *B. grypus* cultivation is the production of fingerlings for restocking dam lakes and the production of cultured fish broodstocks. Although there is a cryopreservation study (20), there is little information about *B. grypus* sperm, especially sperm activation, and it has not been previously studied. Although information on the basic reproductive biology of this species is available, the effect of pH and cations on spermatozoa motility is unknown. This study aims to reveal the minimal Ca^{2+} and K^+ ratio that suppresses sperm activation in *B. grypus*. With the beginning of *B. grypus* culture and the current lack of detailed information on sperm biology, main purpose of this study was to determine the effect of pH, ions, and osmolality on motility of cultured shabut sperm.

2. Materials and methods

2.1. Broodstock management

This study was conducted during spawning season in June 2012 at the aquaculture department of the General Directorate of State Hydraulic Works, Fish Production Station, Atatürk Dam Lake, Urfa, Turkey. Shabut males used in this study were produced in 2006 to enhance the fish stocks of dam lakes in the Euphrates Basin. The broodstocks were held in sand/gravel ponds under a natural photoperiod regime. Twelve mature shabut (mean weight ± SD: 750.3 ± 137 g; mean total length ± SD: 47.3 ± 2.81 cm) were selected randomly from a pond, transferred to the hatchery, and maintained in a 7000-L tank. Six-year-old males produced by the first generation at this hatchery were fasted 48 h prior to sperm collection. Water conditions were as follows: 24.0 ± 0.3 °C, 8.9 ± 0.2 mg L^{-1} oxygen, pH 8.3 ± 0.1, and 1% salinity.

2.2. Sperm collection and motility analysis

Twelve mature males were anesthetized with 2-phenoxyethanol (500 μL L^{-1}) and injected with 1 mg kg^{-1} of carp pituitary extract (CPE). After 24 h males were anesthetized again, and sperm were collected by manual abdominal stripping. The sperm were stored in glass tubes, and only samples uncontaminated with water, blood, urine, or feces were used. Sperm samples were stored in ice until analysis. Motility of sperm was tested microscopically after initiation of sperm activation by subjective estimates of the percentage of progressively motile spermatozoa and the duration of motility (length of time before most spermatozoa became nonprogressive) by a sensitive chronometer. Percentage of sperm motility was assessed using an arbitrary scale in which nonmotile represents 0%, 10%–20%, 20%–30%, 30%–40%, 40%–50%, 50%–60%, 60%–70%, 70%–80%, 80%–90%, >95% of the motile spermatozoa as modified from Borges et al. (21). Motility was evaluated using a light microscope with phase-contrast attachment (Olympus CKX-41, Tokyo, Japan) at 400× magnification.

For the evaluation of motility about 5 μL of semen was placed on a glass microscope slide and 500 μL of activation solution was added. Sperm motility analysis was conducted in pentaplicates for each treatment. All pH and osmolarity measurements were conducted using WTW pH meter 3110 set 2 and Gonotec Osmomat 030 cryoscopic osmometer, respectively. Each semen sample obtained was immediately activated with hatchery water to examine motility percentage and duration. Then the 3 samples with the best motility parameters compared to the others were selected for experiments.

2.3. Experimental design for effect of pH, K^+, and Ca^{2+} on sperm activation

Sperm obtained from 3 of 12 males were used individually to determine the effect of varying pH on shabut spermatozoa motility. Total duration of sperm motility and the percentage of motile spermatozoa observed after dilution depend on the pH of the dilution medium, distilled water buffered with 20 mM Tris–HCl at pH ranging from 5.5 to 12.0 with 0.5 intervals. The effect of pH on motility of shabut sperm was analyzed using the buffer Tris–HCl (20 mM) adjusted to different pH values with HCl (1 M) and NaOH (1 M) at a dilution rate of 1:100 (5 μL semen:500 μL diluent). Maximum and minimum percentages and total durations of motility were recorded.

K^+, Ca^{2+}, and their combinations were prepared at different concentrations. For K^+, an activation solution was adjusted with 20 mM Tris–HCl buffer, pH 9.0, containing 0, 5, 10, 20, 40, 60, 80, and 100 mM of K^+ made up from KCl with no added Ca^{2+}. To study the effect of Ca^{2+}, shabut

sperm were activated with pH 9.0 containing 0, 5, 10, 20, 40, 60, 80, and 100 mM of Ca^{2+} made up from $CaCl_2$ with no added K^+. To determine interactions between these cations, combinations of K^+ and Ca^{2+} were added to the activation solution. To study K^+ and Ca^{2+} interactions or antagonist activity, 49 different media with different K^+:Ca^{2+} ratios were prepared. In media without added K^+ and Ca^{2+} the sum of 64 activation media were used in the experiments.

2.4. Statistical analysis

Nonparametric Mann–Whitney U-tests following Kruskal–Wallis tests at 0.05 significance levels were used to determine significant variation between sperm motility duration activated by media with no added K^+ or Ca^{2+}. The data obtained from sperm motility percentage and duration using media with no added K^+ or Ca^{2+} were summarized in box plots. Spearman correlation coefficients were used for defining the relationship between osmolality and sperm motility characteristics. Statistics and graphics were performed using SPSS software version 20.0.

3. Results

3.1. Effect of pH on sperm activation

Seminal plasma pH of the samples used in this study measured 8.33 ± 0.29. The effect of different pH levels on the percentage of motile spermatozoa (Figure 1A) and total duration of sperm motility (Figure 1B) were observed after activation. Sperm motility percentage and duration were significantly influenced by pH of activation media. Maximum percentages and total durations of motility were observed at pH 9.0. Although there were no significant differences between percentage of motile spermatozoa at pH 8.5 and 9.0 ($P > 0.05$), motility durations at these pH levels were significantly different ($P < 0.05$). Sperm motility of shabut was inhibited at both pH 5.5 and 12.0. Motility activation started at pH 6.0 (1%) and was completely inhibited at 12.0. Sperm motility increased significantly between pH 8.0 and 10.0. Spermatozoa were also activated at pH 11.5. Motility was greatly affected especially at extreme pH levels; moreover, shabut spermatozoa motility can be activated in medium with a pH between 6.5 and 11. The results of this study show that pH 9.0 is the optimum for inducing sperm motility of shabut.

3.2. Effect of K^+ and Ca^{2+} on sperm activation

Both motility percentage (Figure 2A) and duration of progressive sperm (Figure 2B) were significantly affected by increasing K^+ concentrations. Sperm activation was totally inhibited at pH 9.0 and 100 mM K^+ concentration. Sperm motility activation started at no added K^+ (about 80%, 42.3 ± 2.5 s) and reached the maximum at 5 mM K^+ (>95%, 57.7 ± 2.5 s). Sperm motility rapidly decreased after 20 mM K^+ and was inhibited at 100 mM K^+. The sperm motility just after activation was almost completely suppressed by 100 mM K^+ or more. K^+ ions increase sperm motility up to 5 mM K^+ in shabut. The positive effects of K^+ ions were seen in flagellar movement, which was found to be directly controlled by ion concentration. There were no significant differences in sperm motility duration among activation solutions between 10 and 20 mM K^+ and 20 and 40 mM K^+ ($P > 0.05$). The results of this experiment show that 5 mM K^+ is the optimum concentration for inducing sperm motility.

Effects of different concentrations of Ca^{2+} solution at pH 9.0 on sperm motility percentage and duration are presented in Figures 3A and 3B, respectively. The maximum percentage of motile spermatozoa was observed in 5 mM Ca^{2+}, while the highest motility durations of sperm were at no added Ca^{2+} (42.3 ± 2.5 s), 5 (40.0 ± 2.0 s), and 10 (40.0 ± 1.0 s) mM Ca^{2+}. The percentage of motile spermatozoa dramatically decreased at more than 10 mM

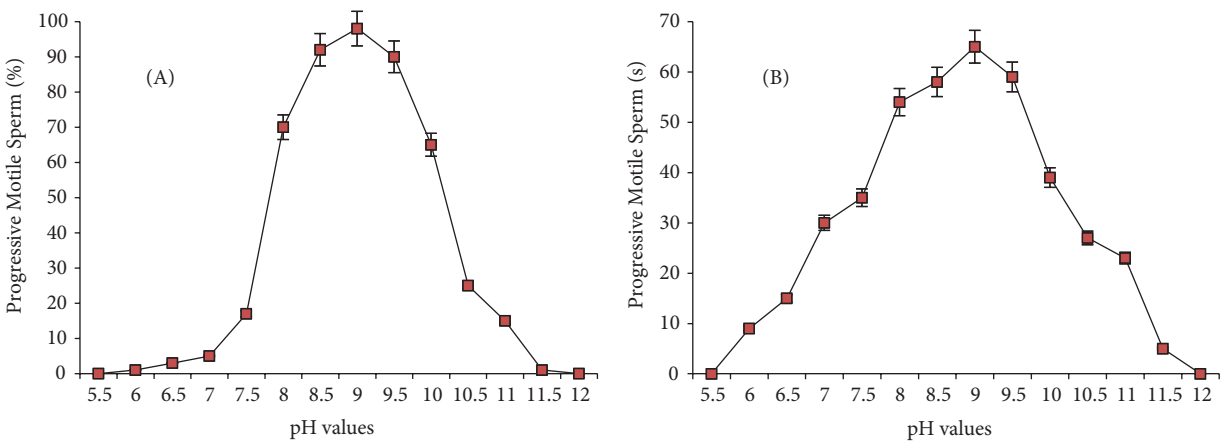

Figure 1. The percentage (A) and duration (B) of progressive motile sperm as a function of activation solution pH (mean ± error bars with percentage).

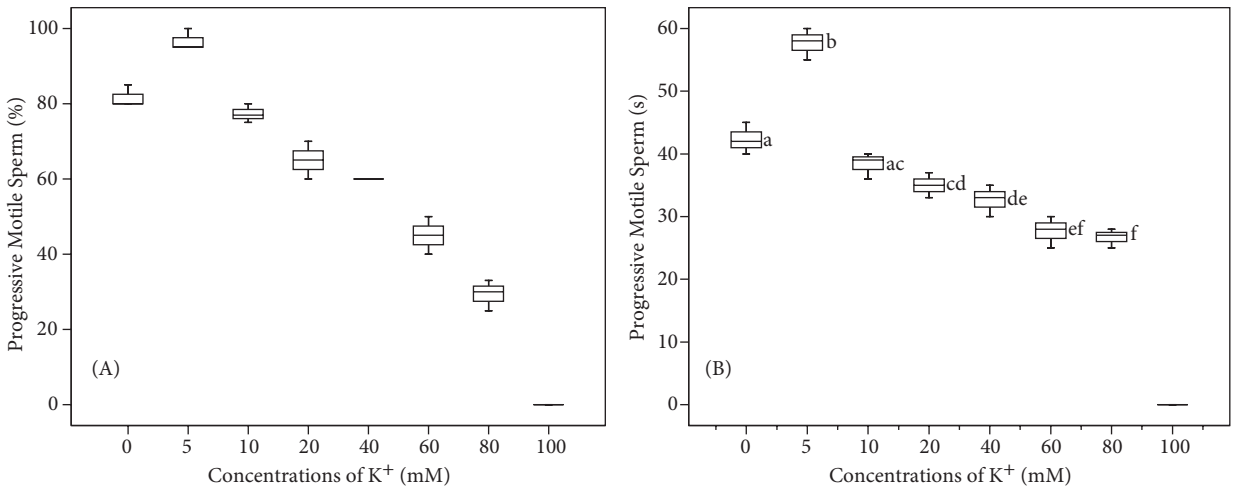

Figure 2. Effects of gradually increasing K^+ ion concentrations on progressive motility percentage (A) and duration (B) in shabut sperm. In the progressive motile sperm box plots common superscripts indicate the differences ($P < 0.05$).

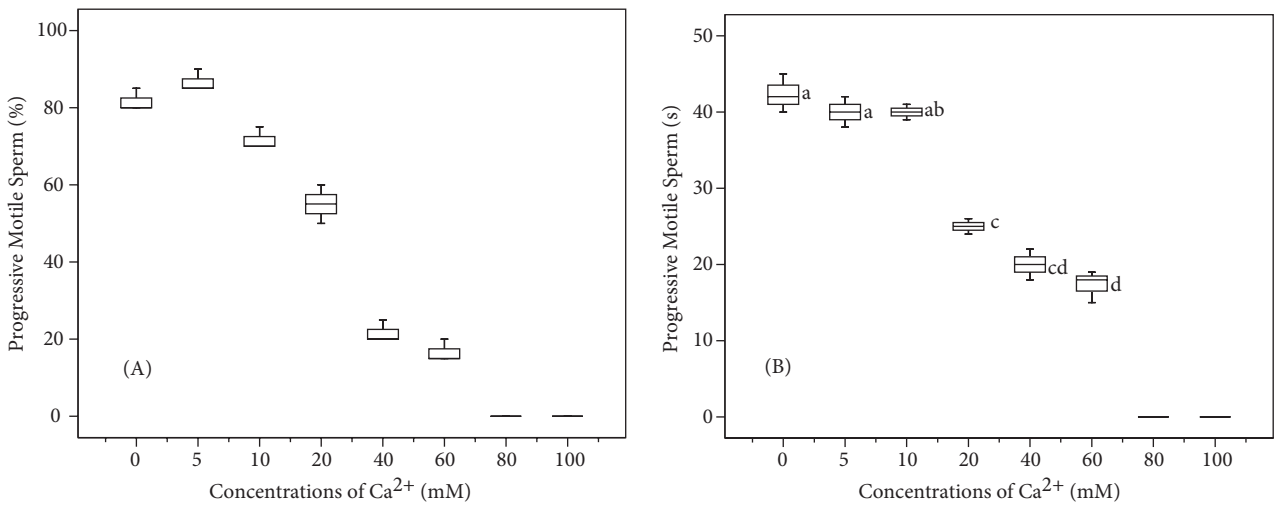

Figure 3. Effects of gradually increasing Ca^{2+} ion concentrations on progressive motility percentage (A) and duration (B) in shabut sperm. In the progressive motile sperm box plots common superscripts indicate the differences ($P < 0.05$).

Ca^{2+} and was precisely suppressed in concentrations of 20 mM Ca^{2+} or more. Duration of motility was observed as 25 ± 7.9 s at 20 mM Ca^{2+}. Ca^{2+} in the range of 0 mM–5 mM had a positive effect, but activation solutions that had over 10 mM Ca^{2+} started to inhibit the motility duration of shabut spermatozoa. There was no difference in the sperm motility period among samples in the range of no added Ca^{2+} and 5 and 10 mM Ca^{2+} ($P > 0.05$). Sperm motility was completely inhibited by 80 mM Ca^{2+} or more.

3.3. Effect of concentration combinations of K^+–Ca^{2+} and osmolality on sperm activation

Osmolality values of the samples ranged between 201 and 263. The initiation of sperm movement in shabut obviously depends on osmolality of activation media in addition to pH values. Osmolality values of the solution used in this study ranged between 52 and 481 and are shown in the Table. Regarding the osmolality values of activation media that could activate sperm motility, there was a noticeable decrease in motility parameters over 185 mOsmol kg^{-1}. In addition, sperm cells were quiescent above 250 mOsmol kg^{-1} regardless of the ionic composition of solutions. There were correlations between these activating osmolality values and progressive motility percentage and also duration, respectively, $r = -0.92$ and $r = -0.86$ ($n = 35$, $P < 0.05$). Results of sperm motility percentage and duration obtained from the presence of K^+–Ca^{2+} combinations in the activating solutions are shown in Figures 4A and 4B, respectively. Concentrations higher than the combinations

Table. Osmolality (mOsmol kg^{-1}) average values* in activation medium prepared in 20 mM Tris–HCL buffer, pH 9.0; 3 replicates were prepared for each solution.

	0 mM Ca^{2+}	5 mM Ca^{2+}	10 mM Ca^{2+}	20 mM Ca^{2+}	40 mM Ca^{2+}	60 mM Ca^{2+}	80 mM Ca^{2+}	100 mM Ca^{2+}
0 mM K^+	52	62	80	105	163	203	256	302
5 mM K^+	56	77	88	115	172	217	272	310
10 mM K^+	75	88	100	124	176	227	284	319
20 mM K^+	94	103	116	146	196	248	297	337
40 mM K^+	126	140	149	182	231	279	325	376
60 mM K^+	164	177	190	216	265	316	363	413
80 mM K^+	199	209	223	250	302	344	398	447
100 mM K^+	233	247	260	289	335	382	432	481

*The standard deviations of all average values are <1.

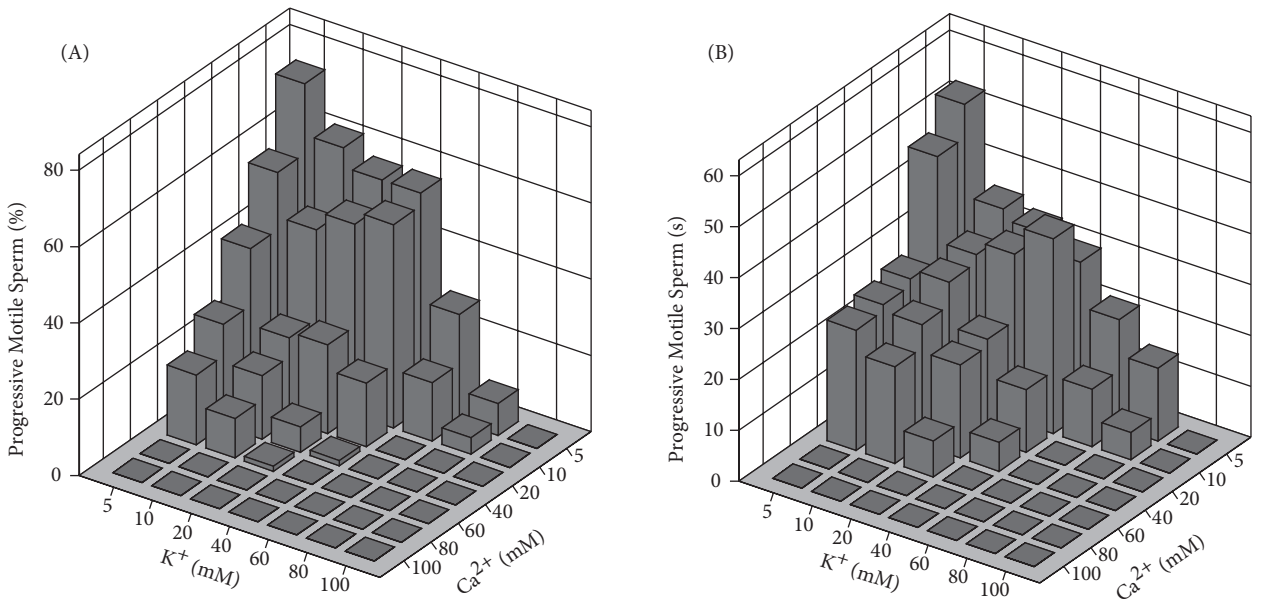

Figure 4. Effects of concentration combinations of K^+ and Ca^{2+} ions on progressive motility percentage (A) and duration (B) in shabut sperm.

5 mM K^+ and 80 mM Ca^{2+} and 60 mM K^+ and 20 mM Ca^{2+} could not induce initiation of sperm motility. Spermatozoa subjected to activation media containing low concentrations of K^+ and Ca^{2+} show high motility compared to those at higher concentrations.

4. Discussion

Shabut reproduction has been ongoing since 2006 at the General Directorate of State Hydraulic Works, Fish Production Station, Atatürk Dam Lake, Urfa, Turkey. According to our experience, testis development and sperm maturity were highest in June. Spawning started in late May and became more intensive from June to early July at Atatürk Dam Lake. The age of sexual maturity in shabut at the Atatürk Dam Lake is about 4+ for females and 3+ for males (22). The present study revealed that the effects of pH and Ca^{2+}:K^+ ratio on shabut sperm motility and the stimulating concentrations can inhibit initiation of sperm motility.

The motility of spermatozoa is related to their sensitivity to pH, osmolality, and ion concentrations, factors that can be used to identify optimum parameters for activating medium, which is very important for increasing the efficiency of insemination (23). To obtain the best fertilizing ability of spermatozoa and optimum hatching ability, fertilization assays should be conducted with good, manipulated sperm. This is necessary to prevent using more spermatozoa for fertilization than required.

Osmolality and pH values of seminal plasma of shabut samples used in this study were 238 ± 32 and 8.33 ± 0.29, respectively. The pH of the activating solution affects sperm fertilizing capacity (5). Optimum sperm motility is at pH 9.0 in shabut, as in *Oncorhynchus mykiss* and *Scophthalmus maximus* (24). Optimum sperm motility was observed at pH 8.5 in *Cyprinus carpio* (25). The duration of sperm motility in shabut decreased instantly at a pH lower than 8.0 and higher than 10.0. The percentage of motile cells did not change over the pH range 6.0–9.0 in *Cyprinus carpio* (4). Maximum and minimum percentages and total durations of motility were reported in *Acipenser persicus* at pH 8.0 and 6.0, respectively (13). With regard to the results, the alkaline conditions of activation solution enhance the motility parameters of shabut sperm. Motility percentage and duration were gradually affected along with an increase in pH. An increase in intracellular pH has been suggested as a conserved step in the activation of sperm motility (6). Extracellular and intracellular pH, as well as the ionic composition of the activating solution, influences the initiation and duration of sperm motility (26). Alteration of internal pH as a possible mechanism interfering with motility was investigated in spermatozoa (9). In this study optimum pH for motility activation of shabut sperm was 8.5, preferably 9.0 (Figures 1A and 1B).

In addition to pH, other environmental factors such as osmotic pressure and ions stimulate the motility of spermatozoa through changes in the properties of the plasma membrane including potential and ionic conductibility. Understanding the effects of these factors and the development of optimal artificial insemination methods improves the aquaculture industry. These developments also contribute to improving the knowledge base regarding short- and long-term fish semen preservation conditions (27). These findings suggest that control of shabut sperm motility is due to ionic content of the medium but that shabut sperm show sensitivity to the osmolality and ion concentrations of the medium. The osmolality values of all solutions used in this study are represented in the Table to contribute to the knowledge of osmolality with regards to activation media and fish sperm motility. To date, little information has been available on the sperm biology of shabut. Activation media isosmotic to seminal plasma have been made for artificial insemination, and in these media extreme pH and the presence of high potassium (100 mM) and calcium (80 mM) concentrations inhibit sperm motility. Shabut sperm can be activated in solution with high concentrations of K^+ (80 mM) and a similar situation appears in common carp sperm (3).

In the presence of 0.1 mM K^+ *Acipenser baeri* sperm were inhibited, and the addition of 0.5 mM K^+ caused 50% inhibition of motility in *A. fulvescens* sperm and prevention of the activation of spermatozoa motility in *P. spathula* at concentrations of 0.5–5.0 mM (13,17). Motility of salmonid sperm is sensitive to very low concentrations of K^+ (more than 1 mM) (28), which are lower than levels for sturgeon sperm (more than 2 mM) (29). K^+ can control activation in sturgeon sperm at very low concentrations (0.01–0.3 mM) in *A. baeri*, *A. persicus*, *P. spathula*, and *A. fulvescens* sperm (12,13). Induction of motility in rainbow trout spermatozoa is impossible after dilution in low-concentration K^+ solutions (18). Spermatozoa from fishes other than salmonids, such as carp (30), show much less sensitivity to K^+. Low concentrations in the range of 0–10 mM did not affect sperm activation in shabut. Motility of shabut sperm decreased in more than 10 mM K^+ concentrations. The best motility parameters in terms of percentage of motile cells (>95%) and duration of sperm motility (57.6 ± 2.5 s) were observed after dilution of spermatozoa in 5 mM K^+ (Figures 2A and 2B).

The results obtained for induction of sperm motility suggest the following hypothesis: the inhibition of motility in shabut is mainly due to Ca^{2+}. In cyprinids K^+ ions also increase sperm motility and velocity, and K^+ channel inhibitors clearly inhibited flagellar motion (15,31). The effects of K^+ ions were investigated in demembranated flagella; axonemal motility was found to be directly controlled by ion concentration (32). It is also clear that K^+ concentrations in diluents used for cryopreservation strongly influence the potential motility of carp spermatozoa (33) and treatment of extender with K^+ increased the fertilization rate after cryopreservation in carp (34). The information provided by this study could aid further studies on the cryopreservation of shabut sperm.

Consequently, environmental conditions activate the motility of spermatozoa. To date, the mechanism regulating motility in common carp sperm has been studied; however, shabut sperm has not been previously studied. This study indicated that high K^+ concentrations, especially >80 mM, in activation solution inhibit sperm motility in shabut. Sperm motility can be initiated by raising the concentration of Ca^{2+} ions in many fish species, such as in cyprinids. Extracellular Ca^{2+} (more than 5 mM) commences inhibition of live sperm motility initiation in shabut. Carp sperm motility is fully initiated in media with osmotic pressure below 150–200 mOsmol kg^{-1}, but sperm motility of shabut is inhibited by both K^+ and Ca^{2+} solutions higher than 185 mOsmol kg^{-1}. According to these results and based on the efficiency of fertilization solution for optimum motility of shabut spermatozoa, we suggest using solution containing 5 mM K^+ at pH 9.0 for the artificial reproduction of shabut.

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