Sperm characteristics in Grass carp, *Ctenopharyngodon idella*; effect of ions on spermatozoa motility and fertilization capacity

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Abstract

In this study, sperm characteristics (motility parameters and ionic composition of seminal plasma) and effect of ions on motility (duration of motility of sperm and percentage of motile spermatozoa) and fertilization capacity (fertilization rate, hatching rate, survival rate and larvae length) were investigated in *Ctenopharyngdon idella.* The longest duration of motility obtained in solution containing 104 mM NaCl. The highest fertilization capacity was observed in solution containing 112 mML NaCl. Solutions containing different concentrations of KCl caused a decrease on the percentage of motile spermatozoa; fertilization rate and hatching rate, but duration of sperm motility and survival rate were significantly higher in solution containing 440 and 320 mM KCl, respectively. There was a decreasing trend on motility when spermatozoa incubated in solutions containing $MgCl_2$ compared to the control. Similarly, higher motility parameters in terms of percentage of motile spermatozoa and duration of sperm motility were observed in distilled water. The sperm motility just after activation was suppressed by concentrations of $CaCl_2$ of 32 mg/dL or more. The maximum hatching rate, survival rate and larvae length were observed in 32 mg/dL $CaCl_2$. Understanding the effects of these ions is helpful to the aquaculture industry as it allows for the development of optimal artificial reproduction methods and contributes towards the knowledge base of better short-term fish semen preservation conditions.

Keywords: Spermatozoa motility, Seminal plasma, Cations, Fertilization, Grass carp

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Introduction

Several parameters have been dedicated to evaluate motility. Motility is the most commonly used parameter to evaluate sperm quality in fish (Bilard et al., 1995). Most studies refer to duration of motility and percentage of motile spermatozoa (Billard et al., 1987; Cosson et al., 1999). Sperm motility is a prerequisite factor determining semen quality and fertilizing capacity (Alavi et al., 2004). The quality of sperm usually refers to the motility, which is a prerequisite factor determining the semen fertilizing ability (Lahnsteiner et al., 1997). Sperm motility is also influenced by several factors, such as pH (Alavi and Cosson, 2005 a, b), cations (Cosson, 2004; Alavi et al., 2007), osmolality (Cosson, 2004; Alavi and Cosson, 2006; Alavi et al., 2007) and dilution ratio (Alavi et al., 2004) in either aqueous environment or diluent. The spermatozoa of most fish species are immotile in the testis and seminal plasma. Therefore, motility is induced after the spermatozoa are released into the aqueous environment during natural reproduction or into the diluents during artificial reproduction (Alavi and Cosson, 2006). Carp spermatozoa like that of other teleost fishes are immotile in the seminal plasma (Alavi et al., 2005a). In carp, key factor for controlling of sperm motility is osmolality (Morisawa et al 1983; Perchec et al., 1995). Carp spermatozoa immediately become motile when exposed to hypoosmotic media (Morisawa et al., 1983; Morisawa, 1994; Billard et al., 1995). Determination of sperm quality parameters such as seminal plasma composition and sperm motility traits could help us to develop and improve artificial reproduction in fish farms (Alavi et al., 2008). Use of high quality gamete in broodfish is of great importance for ensuring production viable larvae (Bozkurt, 2006). Also, fertilization success is depended on sperm motility, sperm/egg ratio and egg quality (Billard et al., 1995). In addition, sperm quality is a measure of the ability of sperm to successfully fertilise an egg. Any quantifiable parameter that directly correlates with fertilization capacity could be potentially used as a measure of sperm quality (Rurangwa et al., 2004). Although basic reproductive and life-history information on this species is available, data on the effect of cations on spermatozoa motility and fertilization capacity is rare. Therefore, this study aimed to investigate the effects of cations (Na^{+}, K^{+}, Ca^{+2}, Mg^{+2}) on spermatozoa motility and fertilizing capacity of sperm in Grass carp.

Materials and methods

The experiment was carried out at the Kasmahi Company, Rasht, north of Iran. Broodstocks (30 mature males and 30 mature females) were captured from reared hatchery, during the spawning season. Fish were transferred to the place of experiment, and acclimated for 2 weeks in 4000 L tanks. Fish used in the experiment ranged from1.5 to 2.5 and 1.5 to 2.0 kg total weight and 83 to 91 and 91 to 97 cm total length for females and males, respectively. Males were intramuscularly injected with Pituitary Gland Hormone (PG) at dose of 0.5 mg kg^{-1}. Also, females fish were intramuscularly injected with a double injection of 2 mg kg^{-1} PG. In first injection,

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10% (0.2 mg/kg) PG was given 12 h before the second injection (1.8 mg/kg). Semen was collected after spermiation, approximately 12 h after induction. Semen of each male was collected and transported to the laboratory under cold conditions (4 ºC) until used for analysis and fertilization. Care was taken to avoid contamination of the semen with water, mucus, blood cells, faeces or urine. Eggs were collected from females that were stripped by gently massaging the abdomen10-12 h after second injection.

To analyze the chemical components of seminal plasma, the seminal plasma was separated from the semen by centrifugation (Eppendorf AG, Germany) and then seminal plasma was collected. Plasma was centrifuged twice to avoid possible contamination with spermatozoa and then samples were frozen at -20$^{\circ C}$ until analysis. Two minerals (Ca^{+2} and Mg^{+2}) of the seminal plasma were measured using a spectrophotometric method (Caretium-XI-921, England). Potassium and sodium were measured using flamephotometer (Jenway PFP, England, Standard kits from Parsazmoon, Tehran, Iran). To examine the effects of ions on sperm motility, Na^{+} (NaCl) (96, 104 and 112 Mm), K^{+} (KCl) (320, 380 and 440 Mm), Mg^{2+} ($MgCl_2$) (11, 19 and 27 mEq/L) and Ca^{2+} ($CaCl_2$) (30, 32 and 35 mg/dL) were used. All solutions were buffered with 30-40 mM Tris–HCl, adjusted to pH 8.5 ± 0.2. Distilled water was served as control. Sperm motility was visually evaluated for the percentage of motile spermatozoa after activation and total duration of motility (in seconds). To induce the initiation of motility, sperm triggered directly in activation solutions at ratio of 1: 2000 and immediately recorded with a 3 CCD video camera (Panasonic 240 Japan) mounted on a dark-field microscope (Leica USA). The duration of sperm motility was measured immediately after initiation of sperm activation until 100 % spermatozoa were immotile. Percentage of motility was defined as the percentage of progressively motile spermatozoa within each activated sample. Progressively motile spermatozoa were defined as actively swimming in a forward motion. Only forward moving sperm was judged motile and sperm cells that vibrated in place were not considered motile (Aas et al., 1991). Analysis of sperm motility was carried out in triplicate for each sample at room temperature (20–22 ºC), using light microscopy under 400 × magnifications.

Freshly ovulated eggs were obtained from females and pooled just prior to assay. To control variation among the qualities of egg pools, the eggs were obtained from same-age females cultivated under the same conditions. Fertilization took place in dry plastic dishes and 50 g of egg (approximately 50000 eggs) was placed into each dish. The fertilization solution (3 g of urea, 4 g of NaCl in 1 L distilled water) was used according to the dry fertilization technique. Batches of eggs were inseminated with solutions containing 96, 104, 112 Mm NaCl, 320, 380 440 Mm KCl, 30, 32, 35 mg/dL $CaCl_2$ and 11, 19, 27 mEq/L $MgCl_2$ respectively. Distilled water was used as control. Following fertilization, the eggs were stirred for 1 h and then eggs rinsed with hatchery water and placed into the incubator.

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Fertilization rate was determined as the percent of the eyed eggs about 6 h after the fertilization. Hatching occurred between 1 – 2 days after fertilization. Following equations was used to calculate fertilization capacity.

Fertilization rate: $\frac{\text{Fertilized eggs number}}{\text{Total eggs}} \times 100$ (Brommage and Cumalantunga, 1998)

Hatching rate: $\frac{\text{Larvae number}}{\text{Fertilized eggs number}} \times 100$ (Hanjavanit et al., 2008)

The normal distribution of the data was tested using the Shapiro-Wilk's test. Data were analysed using one-way ANOVA (SPSS 16.0). The Ducan test was used for post hoc comparisons. Results are presented as mean ± SE. Differences with a $p<.05$ were considered significant.

Results

Sperm characteristics and ionic compositions of the seminal plasma of Grass carp are shown in Table 1. Maximum (44 ± 1.5 s) and minimum (20 ± 3.1 s) duration of sperm motility was observed in solutions containing 104 and 96 mMNaCl, respectively (Fig. 1 a). Also, higher percentage of motile spermatozoa was obtained after triggering the motility in distilled water (85 ± 4 %) compared to other concentrations of NaCl (Fig. 1 b). In case of KCl, duration of motility of sperm was significantly higher (29 ± 4 s) in solution containing 440 mM KCl, but it values was not statistically different (Fig. 2 a). The best motility parameter in terms of the percentage of motile spermatozoa was observed after activation in distilled water (84 ± 4 %) compared to solutions containing KCl (Fig. 2 b). The maximum percentage of motile spermatozoa (85 ± 5 and 85 ± 4 %) and duration of motility of sperm (26 ± 9 and 25 ± 2.5 s) were observed in distilled water compared to solutions containing $MgCl_2$ and $CaCl_2$, respectively (Fig. 3 a, b and Fig. 4 a, b). The sperm motility just after activation was totally suppressed by concentrations of $CaCl_2$ of 32 mg/dL or more (Fig. 4 a, b).

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Table 1: Sperm characteristics of Grass carp

Variables	Minimum	Maximum	Mean ± SE
Sperm of duration of motility (sec)	35	17	26 ± 9.0
Percentage of motile spermatozoa (%)	90	80	85 ± 5.0
Sodium ($mmol^{-1}$)	43	34	38 ± 3.9
Potassium ($mmol^{-1}$)	55	28	45 ± 10.17
Magnesium ($mmol^{-1}$)	11.6	3.1	5.7 ± 3.44
Calcium ($mmol^{-1}$)	6.8	1.1	3.3 ± 2.47

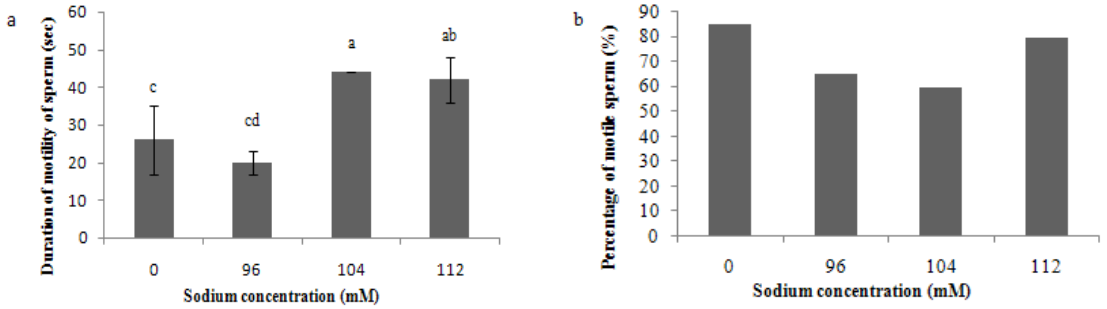

Figure 1: Effect of different concentrations of NaCl on duration of sperm of motility (a) and percentage of motile spermatozoa (b) in Grass carp. Values with the different alphabetic letters are significantly different

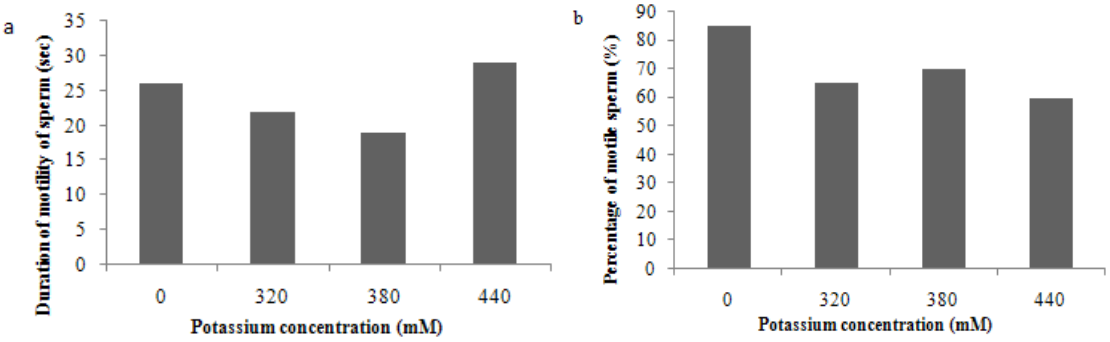

Figure 2: Effect of different concentrations of KCl on (a) duration of sperm of motility and percentage of motile spermatozoa (b) in Grass carp.

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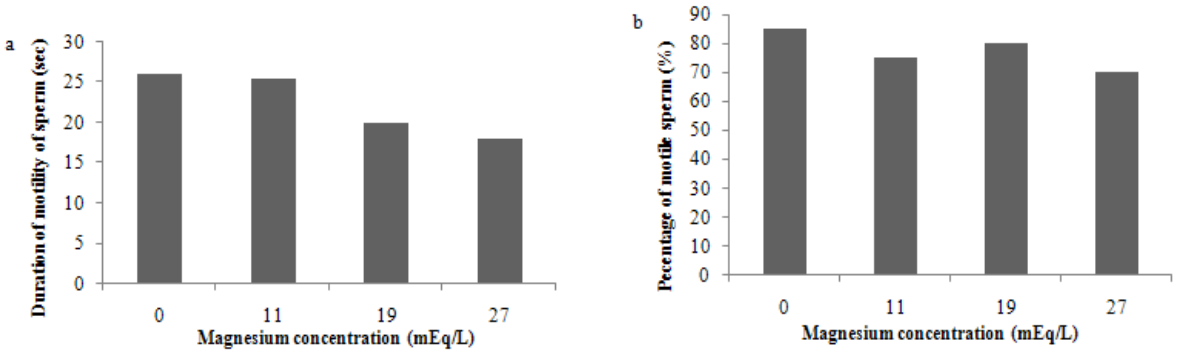

Figure 3: Effect of different concentrations of $MgCl_2$ on duration of sperm of motility (a) and percentage of motile spermatozoa (b) in Grass carp.

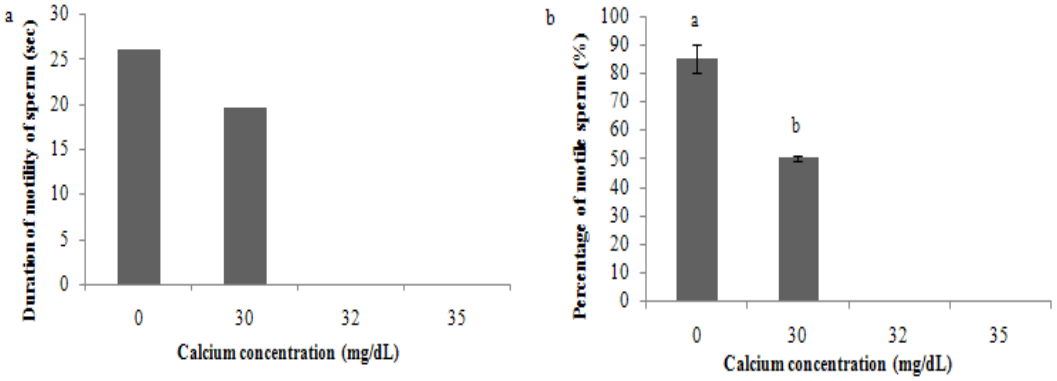

Figure 4: Effect of different concentrations of $CaCl_2$ on duration of sperm of motility (a) and percentage of motile spermatozoa (b) in Grass carp. Values with the different alphabetic letters are significantly different.

Effects of cations (Na^+, K^+, Ca^{2+} and Mg^{2+}) on fertilizing capacity of Grass carp sperm are given in Tables 1 – 4, respectively. The highest hatching rate, fertilization rate, survival rate and larvae length were achieved with solution containing 112 mM NaCl (Table 2). Higher fertilization rate and hatching rate were observed after the activation of sperm in distilled water compare to different concentrations of KCl. Also, a similar trend in survival rate and larvae length was observed (Table 3). The highest hatching rate and survival rate were obtained in solution containing 11 mg/dL $MgCl_2$. Fertilization rate and larvae length did not affect by different concentrations of $MgCl_2$ (Table 4). Fertilization rate was not influenced by solutions containing various concentrations of $CaCl_2$, and highest value was observed in distilled water. Maximum hatching rate was detected in solution containing 30 mg/dL $CaCl_2$. A similar pattern was observed in

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survival rate and larvae length, but their values were not statistically significant (Table 5).

Table 2: Effect of different concentrations of NaCl on fertilization capacity of Grass carp sperm.

Sodium concentration (Mm)	Fertilization rate	Hatching rate	Larvae length	Survival rate
Distilled water	60 ± 0 [ab]	65 ± 18.03 [abc]	4.3 ± 0.4	90.03 ± 0.95
96	70 ± 5 [c]	70 ± 5 [ab]	3.47 ± 0.24	92.2 ± 1
104	70 ± 5 [c]	19.67 ± 2.89 [d]	4.15 ± 0.19	90.4 ± 1.23
112	94.3 ± 3.06 [a]	75 ± 5 [a]	4.6 ± 0.22	93.2 ± 0.81

Values with the different alphabetic letters are significantly different ($p<.05$)

Table 3: Effect of different concentrations of KCl on fertilization capacity of Grass carp sperm.

Potassium concentration (Mm)	Fertilization rate	Hatching rate	Larvae length	Survival rate
Distilled water	90 ± 0	65 ± 18.03 [a]	4.3 ± 0.39	90.03 ± 0.95
320	75 ± 5	55 ± 5 [b]	4.3 ± 0.13	90.3 ± 1.19
380	80 ± 5	3.23 ± 5.77 [c]	0	0
440	70 ± 5	1.67 ± 2.89 [cd]	0	0

Values with the different alphabetic letters are significantly different ($p<.05$)

Table 4: Effect of different concentrations of MgCl2 on fertilization capacity of Grass carp sperm

Magnesium concentration (mEq/L)	Fertilization rate	Hatching rate	Larvae length	Survival rate
Distilled water	90 ± 0	65 ± 18.03	4.3 ± 0.40	90.03 ± 0.93
11	80 ± 5	70 ± 5.00	3.62 ± 0.15	93.6 ± 1.53
19	90 ± 5	60 ± 5.00	3.83 ± 0.08	91.4 ± 0.47
27	80 ± 5	65 ± 5 .00	3.17 ± 0.16	92.07 ± 0.9

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Table 5: Effect of different concentrations of CaCl2 on fertilization capacity of Grass carp sperm.

Calcium concentration (mg/dL)	Fertilization rate	Hatching rate	Larvae length	Survival rate
Distilled water	90 ± 0 [a]	65 ± 18.03 [b]	4.3 ± 0.4	4.33 ± 0.29
30	60 ± 5 [b]	80 ± 5.00 [a]	4.61 ± 0.3	4.66 ± 0.23
32	50 ± 5 [bc]	15 ± 5.00 [c]	4.09 ± 0.18	3.93 ± 0.08
35	5 ± 5 [d]	0.67 ± 1.16 [d]	0	0

Discussion

The present study provides the first report about Grass carp sperm response to ionic effects. The initiation of motility of fish spermatozoa is triggered after releasing of sperm into an aqueous environment (in natural reproduction) or into activation medium (in artificial reproduction) (Cosson et al., 1999). In fact, the motility of spermatozoa is related to their sensitivity to osmolality and to ions (Cosson et al., 1999). Therefore, determination of optimum parameters for activation medium is very important to increase the efficiency of artificial reproduction (Alavi et al., 2004). Ciereszko et al. (2002) reported activation inhibition of spermatozoa motility in sea lamprey at the NaCl and KCl concentration of higher than 40 mM just after initiation of movement. In our experiment, the longest duration of sperm of motility was observed when the sperm was incubated in solutions containing 440 Mm KCl and 104 Mm NaCl respectively. In Grass carp, we suggested that K^+ and Na^+ ions plays key role in the activation sperm such as *Cyprinus carpio* (Krasznai et al., 1995) and Java carp, *Puntius javanicus* (Morita et al., 2006), Common barbel, *Barbus barbus* (Alavi et al., 2009) and Bunnei, *Barbus sharpeyi* (Alavi et al., 2010). K^+ ion can increase sperm velocity and motility in carp (Billard and Cosson, 1992). In cyprinids, K^+ increases percentage of motility (Morisawa et al., 1983). Also, K^+ channel inhibitors decrease or suppress the motility of common carp sperm considerably (Krasznai et al., 1995). However, further study is needed to investigate the role of K^+ ions in the activation of sperm motility in cyprinids. Alavi et al. (2010) showed that percentage of motile spermatozoa was significantly higher in an activation medium containing NaCl compared with that in distilled water. Cosson et al. (1991) reported that the potential for motility in common carp spermatozoa recovered after incubation in K^+ rich media, in which the spermatozoa are immotile. This improved potential was observed in solutions containing 50 - 200 mM KCl or NaCl (Redondo-Muller et al., 1991). In this study, in distilled water the Grass crap sperm motility in terms of percentage of motile spermatozoa was more than solutions containing cations. Jing et al., (2009) reported longest motility duration for

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samples activated with de-ionized water in zebra fish (freshwater aquarium fish) sperm. There is limited information about the effects of Mg^{2+} ions on spermatozoa motility in teleosts (Alavi et al., 2004), including the *C. idella*. In this study, different concentration of $MgCl_2$ had no effect on percentage of motile spermatozoa, while studies showed key role of Mg^{2+} in the initiation of activation of sperm motility, especially in demembranated sperm (Cosson et al., 1999). Lim et al. (2011) reported spermatozoa motility parameter of *Larimichthys polyactis* in solution containing 0.2 mol $MgCl_2$ was higher after activation. They also were observed $MgCl_2$ concentration of more than 0.2 mol significantly decreased duration of motility. This is the first report about the negative effects of Mg^{2+} on motility characteristics of Grass carp spermatozoa when different concentrations of Mg^{2+} were tested. The data in the literature indicated that external Ca^{2+} ions are a prerequisite for the initiation of live sperm motility in carp (Krasznai et al., 2000). Spermatozoa motility was observed after 30 s when 10^{-4} mol $CaCl_2$ was added to the activation solution (Krasznai et al., 2000). In the present experiment, the sperm motility (duration of sperm motility and percentage of motile spermatozoa) just after activation was suppressed by concentrations of $CaCl_2$ of 32 mg/dL or more. Although published data confirm a key role for Ca^{2+} in the activation of sperm in fish (Alavi et al., 2004; Alavi and Cosson, 2006). It has already been confirmed that sperm motility plays an important role to determining the fertilizing ability of sperm (Billard, 1992; Billard et al., 1995). The spermatozoa of most freshwater fishes have a limited duration of motility (Saad et al., 1988). The proportion of motile cells decreased faster with time in undiluted sperm samples than diluted ones. It is possible to enhance the fertilizing capacity of the fish by using suitable activating mediums that increase the duration of motility. In this study, the highest sperm motility was obtained in range of 104-112 Mm NaCl, consequently maximum fertilization rate and hatching rate were observed after the activation of sperm in above mentioned doses. Similarly, hatching rate was higher when semen subjected to 30 mg/dL $CaCl_2$. With regard to our results can be conclude that Na^+ and Ca^{+2} ions are influencing factors involved in fertilizing capacity of Grass carp spermatozoa.

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