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Effect of different activation solutions on motility and fertilizing ability of spermatozoa in common carp *Cyprinus carpio* Linnaeus, 1758

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ABSTRACT

The aim of this study was to test the effect of activation solutions containing sodium, (Na^{+}) potassium (K^+), calcium (Ca^{2+}) and magnesium (Mg^{2+}) ions on motility traits and fertilizing ability of spermatoza in common carp, *Cyprinus carpio*. The duration of sperm motility was influenced by activation solution containing NaCl ($p<0.05$) while other ions did not have any significant effect on motitlity. Percentage of motile spermatozoa was not influenced by any of the activation solution used. The results showed that fertilization and hatching rates were influenced ($p<0.05$) by solutions containing all ions except Mg^{2+}. Other parameters such as length of larvae at hatching, larval length during active feeding and survival rate did not show any significant change with use of activation solutions. The results indicated that motility and fertilizing ability of common carp spermatozoa can be suitably manipulated using appropriate activation solutions.

Keywords: *Cyprinus carpio*, Fertilizing ability, Ions, Sperm motility

Introduction

Sperm of freshwater teleosts are quiescent at the osmolality of seminal plasma, referred to as isotonic condition and are immotile immediately after collection (Morisawa *et al.,* 1988). They begin to move when suspended in hypo-osmotic media, and show high motility in freshwater. Sperm quality is a key factor that increases the efficiency of artificial fertilization. The quality of sperm usually refers to its motility which is a prerequisite factor determining the semen fertilizing ability (Lahnsteiner *et al.,* 1997). The percentage of motile sperm and duration of motility are considered to play an important role in sperm-fertilizing ability in fish (Alavi and Cosson, 2005a; Cosson *et al.,* 2008). Several studies haveconfirmed that sperm motility parameters are commonly used to evaluate fertilization success (Lahnsteiner *et al.,* 1998; Mansour *et al.,* 2005). Sperm motility is influenced by several factors, such as pH (Alavi and Cosson, 2005a,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. Artificial media that induce good activation of sperm without exposing them to extreme osmotic conditions will prolong sperm motility (Cosson, 2004). Determination of factors that influence sperm motility traits could help develop and improve artificial reproduction in fish farms (Alavi *et al.,* 2008). The role of ions on sperm motility and fertilizing ability of spermatozoa has been reviewed over the years (Stoss, 1983; Cosson *et al.,* 1991; Billard *et al.,* 1995; Cosson 2004; Linhart *et al.,* 2008). The current study was designed to investigate the effect of activation solutions containing different ions on motility and fertilizing ability of common carp spermatozoa.

Materials and methods

The experiments were carried out in the Kasmahi Cooperative Farm, Rasht, Iran. During the spawning season, broodstock fishes (*Cyprinus carpio*) were caught using a commercial set net, randomly selected and transported to the place of experiment where they were acclimated for 1 week to the following conditions: water temperature 25ºC, pH = 7.5-8, oxygen saturation 80%. A total of 60 males (average weight 4350 ± 43.5 g, average length 61.3±1.41 cm) and females (average weight

5000 ± 81.7 g body weight, average length 63.5 ± 1.29, were maintained. Male broodstock were injected intramuscularly (i.m.) with carp pituitary extract (CPE) homogenised in sterile saline water (7% NaCl) at dose of 0.5 mg kg^{-1}. Females were given a first dose of (0.2 mg kg^{-1} CPE (i.m.) and after 12 h, asecond injection (i.m.) of 1.8 mg kg^{-1} CPE was given.

For preparation of activating solutions, chemicals containing the ions were dissolved in distilled water at the following concentrations: Na^+ (NaCl - 63, 72 and 81 mM), K^+ (KCl - 80, 88 and 96 mM), Mg^{2+} ($MgCl_2$ - 1.2, 1.3 and 1.5 mM and Ca^{2+} ($CaCl_2$ 1.75, 2.5 and 3.25 mM). A magnetic stirrer was used to dissolve the chemicals completely. All solutions were buffered with 30-40 mM Tris–HCl, adjusted to pH 8.5 ± 0.2.

Before collecting gametes, fish were wiped dry with towel to prevent contamination by water, faeces, urine and mucus. Semen of each male was collected from anaesthetised males and sperm batches transported to the laboratory under cold conditions (4ºC) for analysis and fertilization. Sperm motility was immediately recorded after activation, using a 3 CCD video camera (Panasonic 240 Japan) mounted on a dark-field microscope (Leica USA). For sperm motility estimation Na^+ (NaCl), K^+ (KCl), Mg^{2+} ($MgCl_2$) and Ca^{2+} ($CaCl_2$) were used. Distilled water was used as control. The duration of sperm motility was measured immediately after initiation of sperm activation until 100% spermatozoa were non-motile. Percentage of motility was estimated as the percentage of progressively motile spermatozoa in each activated sample. Only actively forward moving spermatozoa were judged progressively motile and sperm cells that vibrated in the same place were not considered motile (Aas *et al.,* 1991). Analysis of sperm motility was carried out in triplicate for each sample at room temperature (20-22ºC), using light microscopy under 400X magnification. Fresh eggs were obtained from females and pooled just prior to assay. With gentle squeezing of the abdomen, eggs were extruded into clean dry dishes. The eggs of individual females were pooled in order to minimise variation in gamete quality. Fertilization was effected in dry plastic dishes. The diluted semen with solutions containing Na^+ (NaCl, 63, 72 and 81 mM), K^+ (KCl, 80, 88 and 96 mM), Mg^{2+} ($MgCl_2$, 1.2, 1.3 and 1.5 mM) and Ca^{2+} ($CaCl_2$, 1.75, 2.5 and 3.25 mM) were added to dishes containing pooled eggs and then mixed. The fertilization solution (3 g of urea and 4 g of NaCl in 1 l distilled water) was used according to the dry fertilization technique. Following fertilization, the eggs were stirred for 1 h, rinsed with hatchery water and placed in an incubator. Fertilization rate was determined as the percentage of eyed eggs about 6 h after fertilization. Hatching occurred between 1–2 days at water temperature 20-24.5°C. Following equations were used to calculate fertilization capacity:

Fertilization rate = Number of fertilized eggs/Total number of eggs× 100 (Bromage and Cumaranatunga, 1988)

Hatching rate = Number of hatched eggs/Total number of eggs × 100 (Hanjavanit *et al.,* 2008)

Statistical analysis

After controlling normality of data byShapiro-Wilk's test, data were analysed using one-way ANOVA, followed by Duncan's multiple range test to assess the significant difference if any between treatments.

Results and discussion

The longest duration of sperm motility was observed in solution containing 72 mM NaCl (Fig. 1A). Duration of sperm motilitywas not influenced by the activation solutions, KCl, $MgCl_2$ and $CaCl_2$ and higher values were observed in distilled water, but the values were not statistically different (Fig.1B, C). The maximum percentage of motile spermatozoa was observed in distilled water compared to solutions containing ions (Fig. 1 A, B, C). A decreasing trend was observed in percentage of motile spermatozoa when dose of solutions containing $CaCl_2$ gradually increased (Fig.1 D). Fertilization rate and hatching rate showed significant differences ($p<0.05$) when sperm was subjected to solutions containing NaCl, KCl and $CaCl_2$ (Tables 1). But the same were not influenced by solution containing $MgCl_2$ (Tables 1, $p>0.05$). The larval length at hatching, larval length during active feeding and survival rate did not change with use of activation solutions.

In cyprinids, because of high osmolality, spermatozoa are immotile in the seminal plasma, but some ions such as Na^+, K^+ and Mg^{2+} can interfere (Morisawa and Morisawa, 1988). Osmotic shock induces initiation of motility in sperm of teleosts leading to fertilization in external spawning grounds (Morisawa and Suzuki, 1980). In fact, the motility of spermatozoa is related to their sensitivity to osmolality and to ions (Cosson *et al.,* 1999). However, the environment controlling the regulatory mechanism seems to be different. Hypotonic shock triggers initiation of sperm motility in freshwater teleosts such as cyprinids (Morisawa *et al*., 1983). In the present study, the longest duration of sperm motility was observed when semen was incubated in solutions containing 88 mM KCl and 72 mM NaCl. The results of our study indicated that Na^+ ion plays a key role in the activation of sperm as was reported in *Cyprinus carpio* (Krasznai *et al.,* 1995), Java carp, *Puntius javanicus* (Morita *et al.,* 2002), common barbel, *Barbus barbus* (Alavi *et al.,* 2009) and bunnei, *Barbus sharpeyi* (Alavi *et al.,* 2010). Sperm motility in fish is

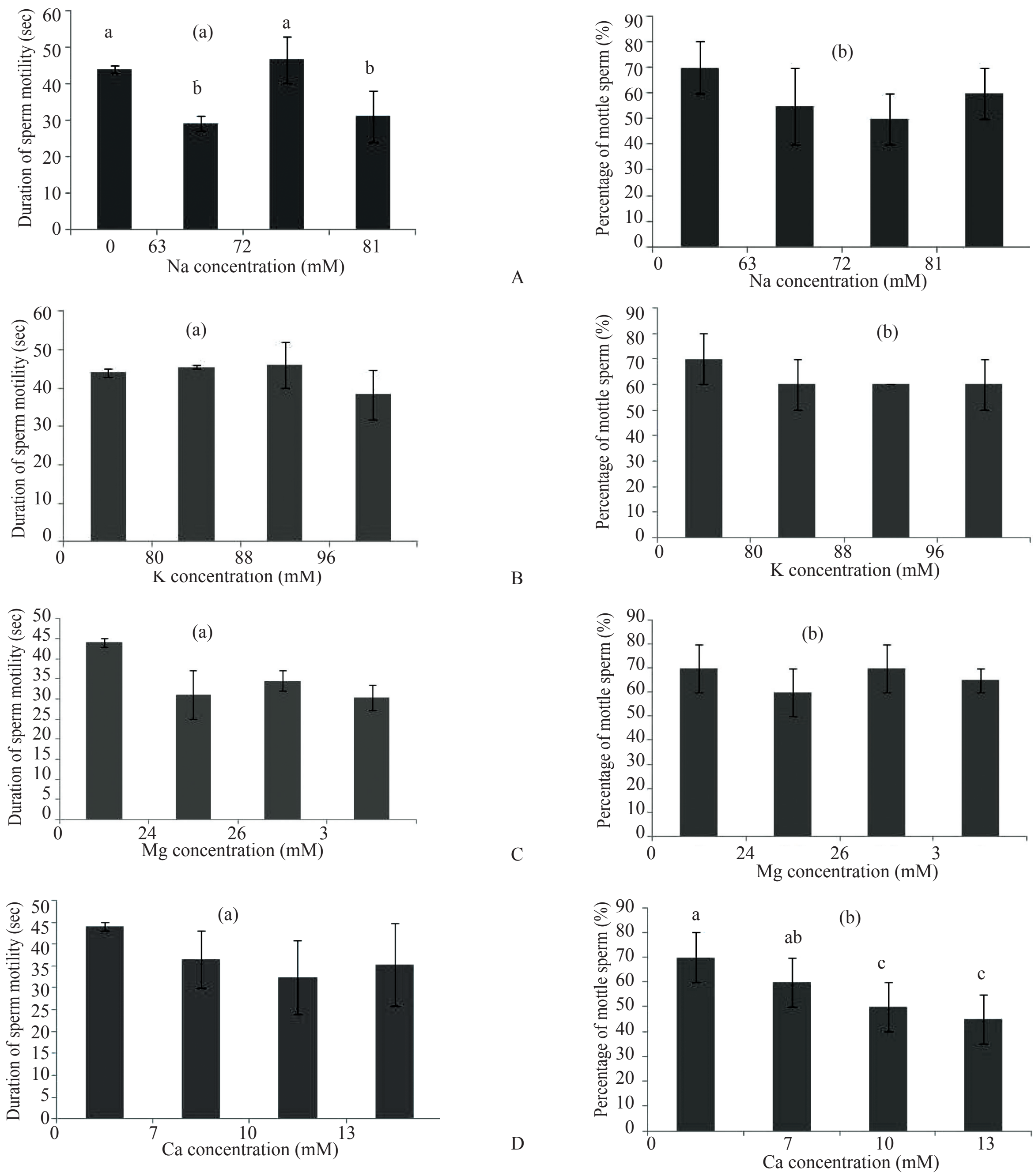

Fig. 1. Duration of sperm motility (a) and percentage of motile spermatozoa (b) of common carp in activation solution containing different ions. A :Na^+ (NaCl); B : K^+ (KCl); C: Mg^{2+} ($MgCl_2$); D: Ca^{2+} ($CaCl_2$). Values with the different alphabets are significantly different ($p<0.05$).

controlled by their sensitivity to cation concentration and this phenomenon is related to cationic channel activities in the membrane and governs the motility mechanisms of axonemes (Alavi and Cosson, 2006). Sperm motility is also influenced by several factors like pH, temperature and osmolality (Cosson, 2004; Alavi and Cosson, 2006).

It has been shown that the ionic composition of the activating solution influences the initiation and duration of sperm motility (Marian *et al.,* 1993). As previously reported, sperm motility is also influenced by the ionic composition of the diluent (Billard and Cosson, 1992). Ciereszko *et al.* (2002) reported activation inhibition of spermatozoa motility in sea lamprey at NaCl and KCl concentrations higher than 40 mM, just after initiation of movement, while concentrations over 20 mM were inhibitory. Morisawa *et al.* (1983) found that duration of motility was slightly decreased at higher KC1 concentrations. In tilapia

Table 1. Effect of different concentrations of ions on fertilization ability of carp sperm

Ion concentration (mM)	Fertilization rate (%)	Hatching rate (%)	Larval length at hatching (mm)	Larval length during active feeding (mm)	Survival rate (%)
Distilled water	78.3 ± 2.8 ab	35 ± 5 c	2.5 ± 0.29	4.3 ± 0.29	87.4 ± 5.1
Na+ (NaCl)					
63	70 ± 5 bc	70 ± 2.1 a	2.5 ± 0.19	4.4 ± 0.22	90 ± 1.2
72	60 ± 5 d	45 ± 5.8 b	2.1 ± 0.16	4.7 ± 0.15	81.6 ± 1.5
81	80 ± 2 a	43.3 ± 2.8 b	2.8 ± 0.14	4.9 ± 0.06	80 ± 0.48
K+ (KCl)					
80	65 ± 5 c	43.3 ± 5 b	2.8 ± 0.10	4.3 ± 0.31	90 ± 1.16
88	70 ± 7 ab	45 ± 2.89 b	2.7 ± 0.14	4.3 ± 0.40	89.6 ± 0.29
96	70 ± 8 ab	65 ± 7.5 a	2.7 ± 0.16	4.9 ± 0.33	82.9 ± 2.5
Mg2+ ($MgCl_2$)					
2.4	75 ± 5	45 ± 5	2.6 ± 0.19	4.9 ± 0.13	91.3 ± 1.6
2.6	85 ± 4.05	41 ± 4.1	2.5 ± 0.08	4.8 ± 0.16	90 ± 1
3	80 ± 5	42 ± 3.08	2.8 ± 0.17	4.5 ± 0.17	82.6 ± 2.08
Ca2+ ($CaCl_2$)					
7	85 ± 5 a	46 ± 2.89 a	2.5 ± 0.17	4.6 ± 0.33	81.6 ± 0.38
10	70 ± 7 b	31.6 ± 3.6 c	2.7 ± 0.17	3.9 ± 0.08	84.6 ± 1.53
13	61.6 ± 5 c	30 ± 4.01 c	2.8 ± 0.15	4.4 ± 0.44	80 ± 0

Values in same columns with different superscripts are significantly different ($p<0.05$).

Oreochromis niloticus, high concentrations of KCl (approximately 100 mM) had no effect on motility activation (Morita *et al.,* 2002). In contrast to the findings of Cosson *et al.* (1999) and Le *et al.* (2011), in the present study the duration of sperm motility in *C. carpio* did not prolong in activation solutions containing $MgCl_2$ and $CaCl_2$. There is limited information about the effects of Mg^{2+} ion on spermatozoa motility in teleosts (Alavi *et al.,* 2004). Studies on the intracellular mechanisms of sperm motility in teleosts confirm a key role for Mg^{2+} in the initiation of sperm motility activation, especially in demembranated sperm (Alavi *et al.,* 2004; Cosson, 2004). It has been shown that Mg^{2+} ions can overcome to a large extent the inhibition by K^+ or H^+ ions (Baynes *et al.,* 1981). Morita *et al.* (2002) reported that Mg^{2+} had no effect on sperm motility of tilapia, *O. niloticus*. Previous studies confirm a key role for Ca^{2+} in the activation of sperm in fish (Alavi *et al.,* 2004; Alavi and Cosson, 2006). Increased Ca^{2+} ion seems to play an important role in the initiation process of sperm motility. Morita *et al.* (2002) demonstrated that Ca^{2+} ion plays a significant role in motility activation of tilapia sperm. In the present study, sperm motility was unchanged in terms of the percentage of motile spermatozoa in solution containing ions compared to distilled water. In case of $CaCl_2$ a decreasing trend was observed in percentage of motile spermatozoa when Ca^{2+} ion concentration increased gradually. Alavi *et al.* (2004) have demonstrated that composition of activation solution can influence the period of sperm motility as well as the percentage of motile sperm. In cyprinids, K^+ increases percentage of sperm motility (Morisawa *et al.,* 1983). Also, K^+ channel inhibitors decrease or suppress the motility of common carp sperm considerably (Krasznai *et al.,* 1995).

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. Motility is an important function of the male gamete, which allows sperm to actively reach and penetrate the female gamete in organisms with internal and external fertilization. Numerous studies suggest that the characteristics of motility of fish spermatozoa are related to their fertilizing capacity (Billard and Cosson, 1992; Billard *et al.,* 1995; Alavi *et al.,* 2005). On the other hand, the fertilizing ability of spermatozoa depends on several parameters of the semen, including concentration of spermatozoa (Ginzburg, 1968), velocity of the spermatozoa (Schlenk and Kahmann, 1938), duration of motility (Billard, 1983), percentage of motile spermatozoa (Billard, 1983), motility pattern of the sperm during activation movement (Tsvetkova *et al.,* 1996) and the biochemical composition of the seminal plasma (Ciereszko *et al.,* 2000). The motility of the spermatozoa of freshwater fish is usually of short duration once initiated. In the present study, the proportion of motile sperms decreased faster with time in undiluted sperm samples than in diluted ones and hatching rates showed significant diffrences ($p<0.05$) in solutions containing NaCl, KCl and $CaCl_2$. This is in agreement with earlier reports of high fertilizing ability of sperm when semen was diluted in saline solutions rather than in freshwater (Alavi *et al.,* 2005; 2009). It is thus possible to enhance the fertilizing capacity of the fish spermatozoa using suitable activating solutions that increase the duration of motility. Cationic factors can stimulate the initiation of activation of sperm, but the biological sensitivity of sperm to cationic concentrations must be considered during determination of diluent composition in fish farms.

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