Analysis of saline activator solution effects on sperm quality indices of *Barbus sharpeyi* by Image J software

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Abstract

Regarding low fertilization rate of *Barbus sharpeyi*, one of the main Barbus species in south of Iran, saline activator solutions (SAS 1 and 2) were used for increasing of sperm activation and its sperm quality indices were assessed by using Image J software as a fish sperm adapted tools. SAS_1 (45mM NaCl, 5mM KCl, 30mM Tris, 188±2.3 mOsmol/kg) and SAS_2 (50mM NaCl, 30mM KCl, 30mM Tris, 189±1.7 mOsmol/kg) were added to a mixture of 12 male semen comparing with distilled and tap water and their effects on VCL, VSL, ALH, Linearity, %motility, spermatozoa path, % A, B and C spermatozoa type, fertilization, hatching and deformity rate were analyzed in triplicate. Results showed that SAS_2 had the highest VCL (21.37±0.36 µm/s), VSL (11.6±0.3 µm/s) and motility percentage (85.75±1.56 %) among treatments. The highest A type spermatozoa percentage was recorded for SAS_1 (47.16±3.15 %) and SAS_2 (43.89±4.54%). The lowest C type spermatozoa percentage was recorded using SAS_2 (14.24±1.56 %). Also, the highest fertilization rate was achieved from SAS_2 (79.5±0.37%) and SAS_1 (77.74±4%) respectively. Furthermore the lowest larval deformity rate (13.77±1.03%) was shown due to the use of SAS_2. Final conclusion confirmed that SAS_1 and SAS_2 have positive and significant effects ($P < 0.05$) on extending the time length of sperms movement, VCL and VSL of *B. sharpeyi* which consequently resulted in better efficiency of propagation of this species in south of Iran.

Keywords: Sperm, Saline activator solution, Fertilization, *Barbus sharpeyi*, Iran

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Introduction

Barbus sharpeyi is considered as one of the 300 species of barbus-fishes family (Al Mukhtar et al., 2006) and an important fish species in Khuzestan Province of Iran (Mohamadian et al., 2009). In recent years, several reasons such as market value, illegal fishing, pollution of wetlands, lack of efficient methods for larval production and reconstructing natural stocks (Pyka et al., 2001; Al Mukhtar, 2009) resulted in depletion of population and categorized it as an endangered species (Al Mukhtar et al., 2009). Geographical distributions of *B. sharpeyi* are limited to the Iran, Iraq, Turkey, Nile, Victoria and Naser River (Coad, 1995; Kahkesh et al., 2010), which could be a reason for its limited available studies on the reproduction.

Although natural habitat of this species in Iran is Shadegan and Hoor-el-Azim wetlands where *B. sharpeyi* forms the highest percentage of carp fish (AL Mukhtar et al., 2006), Maremzi et al. (2000) reported that *B. sharpeyi* distribution in most water bodies of Khuzestan Province.

Despite of propagation of *B. sharpeyi* in Iran, the fertilization rate and efficiency of reproduction have been reported unsatisfactory (Pyka et al., 2001). Among different species of barbels in Khuzestan, *B. sharpeyi* shows the lowest fertility rate and so study of sex gametes and identifying the causes of their lower fertilization rate until hatching and applying ways to increase the quality of sex gametes of *B. sharpeyi*, can be a valuable step to increase the fertilization rate of this species.

In most hatchery centers the quality of eggs and larva are considered important, while the quality of sperm and eggs affect fertilization success and survival of larvae (Rurangwa et al., 2004). In some species, low sperm quality can be considered as a limiting factor of their breeding (Bozkurt, 2006).

Since the availability of sperm with high quality is essential for achieving the high fertilization rate and larval quality (Verma et al., 2009), implementation of this study can be worthwhile for completing basic information of gamete biology and increasing reproduction efficiency in *B. sharpeyi*.

The sperm quality is determined in accordance with general mobility parameters; sperm concentration and egg fertilization success rate (Alavi et al., 2006; Krol et al., 2006; Cejko et al., 2008). The most important qualitative characteristic of spermatozoa is motility (Lorestani et al., 2006; Kalbassi and Lorestani, 2007) and studies on fish sperm motility have been limited to about 20 species, while many species of fish exist, that lack comprehensive studies on their sperms (Cosson, 2004; Utarabhand and Watankul, 1998). It seems that the increasing duration of sperm motility has a strong positive correlation with ability of fertilization (Utarabhand and Watankul, 1998; Rurangwa et al., 2004).

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Since 1990, researchers used computer assessment sperm quality analysis method (CASA) for assessing different spermatozoa movement parameters and its methodology in ichthyology and aquaculture has been taken from human clinical fertility (Elofsson et al., 2003; Alavi and Cosson, 2005; Pavlov, 2006). Based on CASA method, the optimum conditions are performed for artificial fertilization, selecting high-quality males, long-term sperm storage experiments, study of water pollution and environmental effects on male population (Kime et al., 2001; Ebrahimi, 2005). However, due to the complexity, high cost and unavailability of CASA system, its usage is not possible in most laboratories, but sperm analysis with Image J software has not considerable finance and is easy to work (Pavlov, 2006; Fauvel et al., 2010). Image J software is an open source software for calculating the most spermatozoa motility parameters (Fig. 1) (Wilson Leedy and Ingermann, 2007; Cabrita et al., 2008).

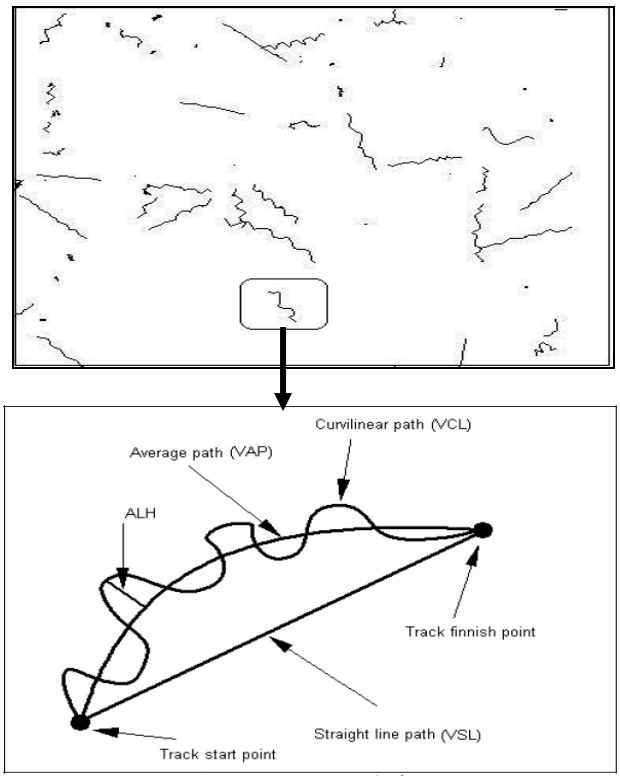


Figure 1: Spermatozoa movement scheme

The aim of this study was to compare the effects of saline activator solutions, distilled water and tap water on various parameters of spermatozoa motility in *B. sharpeyi*, such as VCL (Curvilinear Velocity Length), VSL (Straight Line Velocity), ALH (Lateral Head Displacement),Linearity (VSL/VCL×100),

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% motility, A, B and C spermatozoa types, duration of sperm motility and Spermatozoa path. Also, effects of aforementioned parameters were evaluated on fertilization, hatching and deformity rate.

Materials and methods

This experiment was carried out at the center of endemic fish propagation, Khuzestan province, south of Iran, from beginning of March until June 2010. Twelve male *B. sharpeyi* (4^+ years old) with total weight 916±234.15 g and total length 43.40±4.33 cm were used as semen donors. Also four female *B. sharpeyi* (5^+ years old) with total weight 1577±208.06 g and total length 49.75±2.62 cm were used as oocyte donors. The broodstock was held in 1.5×1×8 m raceways at 23 ^oC.

Hormone injection

The fish were anaesthetized by 100 mg/L MS_{222} and the males and females were injected intraperitonealy at volume of 1 ml/kg. The spermiation of males was stimulated with injection of 10 µg $LHRHa_{II}$ and 2.5 mg metoclopramide per kg^{-1} of body weight. Also females received 4 mg per kg^{-1} b.w carp pituitary extract and 12 hr after the second injection; ovulation was checked by gently massaging the abdomen.

Sperm dilution

The sperm samples were pooled and pre diluted by 100 micro liter fresh sperm: 30 ml immobilizing solution (IMS: 150 mM NaCL, 20 mM Tris–HCl, pH 8.5; 366±7.3 mosM) and were kept at refrigerator temperature until Image J analysis. Pre diluted sperm samples were activated by second dilution step in 50 volume activating solution: 50 volume immobilized spermatozoa (Jing et al., 2009).

Sperm activators

Activator solutions were used for studying with the Image J software and performing *in vitro* fertilization which consist of the Saline Activator Solution 1 (SAS_1: 45 mM NaCL, 5 mM KCl, 30 mM Tris–HCl, pH 8.2; 188 mOsmol/kg), Saline Activator Solution 2 (SAS_2: 50 mM NaCL, 30 mM KCl, 30 mM Tris–HCl, pH 8.5; 189 mOsmol/kg), distilled water and hatchery water. Osmotic pressure of activation and dilution solutions was measured by using a vapor pressure osmometer (OSMOMAT 030 Japan).

Study of sperm motility with Image J software

Effects of the activating solutions on some sperm motility factors such as VCL, VSL, Linearity, % motility, ALH, path of spermatozoa movement and percentage of A, B and C spermatozoa types were analyzed by Image J software in three replicates. Image J equipment included a microscope (SAIRAN Company) with video camera (Samsung, SCC-B2007P, China) connected to a computer system (512 GB of RAM, processor 3.8 full Co. AMD) and the objective lens 10X Olympus (USA). Also a sperm chamber (code: MS090056, India) were used for

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microscopic observation. This chamber produced a thin microscopic space (10µm depth) and spermatozoa cells were arranged to one layer for better analysis.

Amount of 100 micro liters diluted sperm were placed in the chamber of sperm meter and then 100 micro liters of activator solutions (SAS_1, SAS_2, Distilled Water or Hatchery Water) were added. For Each activator, at least three video clips were recorded. The first minute of all video clips were cut to 12 pieces of 5 seconds part, by VCD Cutter software and were converted to row frames by Image J software. Each second of clips consisted of 30 frames. Image J software gives specific codes to each spermatozoid cell and follow position of all spermatozoid cells from one frame to the next one. The motility parameters of *B. sharpeyi* spermatozoa were analyzed based on the method of Pavlov (2006); Wilson Leedy and Ingermann (2007) and Fauvel et al. (2010).

Also, on the basis of Lahnsteiner et al. (1996) method, spermatozoa were categorized to 3 types: A (with a velocity more than 20 µm/s), B (velocity between 5 to 20 µm/s) and C (with a velocity less than 5 µm/s). During the present study, 21600 frames, 720 pictures of movement path and motility characteristics of 20860 spermatozoa cells were analyzed with Image J software.

Fertilization protocols

In each treatment 10 ml of pooled fish oocytes (from 4 females) mixed with 10 micro liters of pooled fresh sperm (from 12 males) and 1 cc activating solutions (SAS_1, SAS_2, Distilled Water or Hatchery Water) were added and fertilized in triplicate.

Adhesiveness of eggs was removed with continuous stirring for 0.5 h in the hatchery tap water and then was rinsed twice in a 5% solution of tannic acid for 20 s for the final elimination of stickiness (Horva´th et al., 2007). For incubation of fertilized eggs, a special zuger-type incubator with a capacity of 2 L and 9 cm in diameter were designed and eggs were transferred to it until hatching were recorded. The average water flow in incubators was 543.33± 38.44 ml/min.

After 12 h following fertilization, the embryonic development stages of eggs were calculated. For this purpose about 500 eggs in each replication were taken out of the incubator with siphoning. Then, eggs with a nervous belt were assumed as a fertilized egg. After hatching, the numbers of obtained larvae were counted for calculating hatching and deformity rate (Pyka et al., 2001).

Statistical Analysis

Normality of variance was tested by the Kolmogorov–Smirnov and all data were shown as mean± SE (SEM). One-way ANOVA was used to determine the effects of different activators on motility parameters, incubation survival, hatching and deformity percentage of larvae. Means were separated by Duncan's New Multiple Range Test, and were considered

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significantly at $P<0.05$. Statistical analysis was conducted using SPSS software version 16.0.

Results

The peculiarities location defined by Image J software with plotted based upon the calculated x, y coordinates from the videotape was achieved with the use of hatchery water (A), distilled water (B), SAS_1 (C) and SAS_2 (D), were shown in Figure 2.

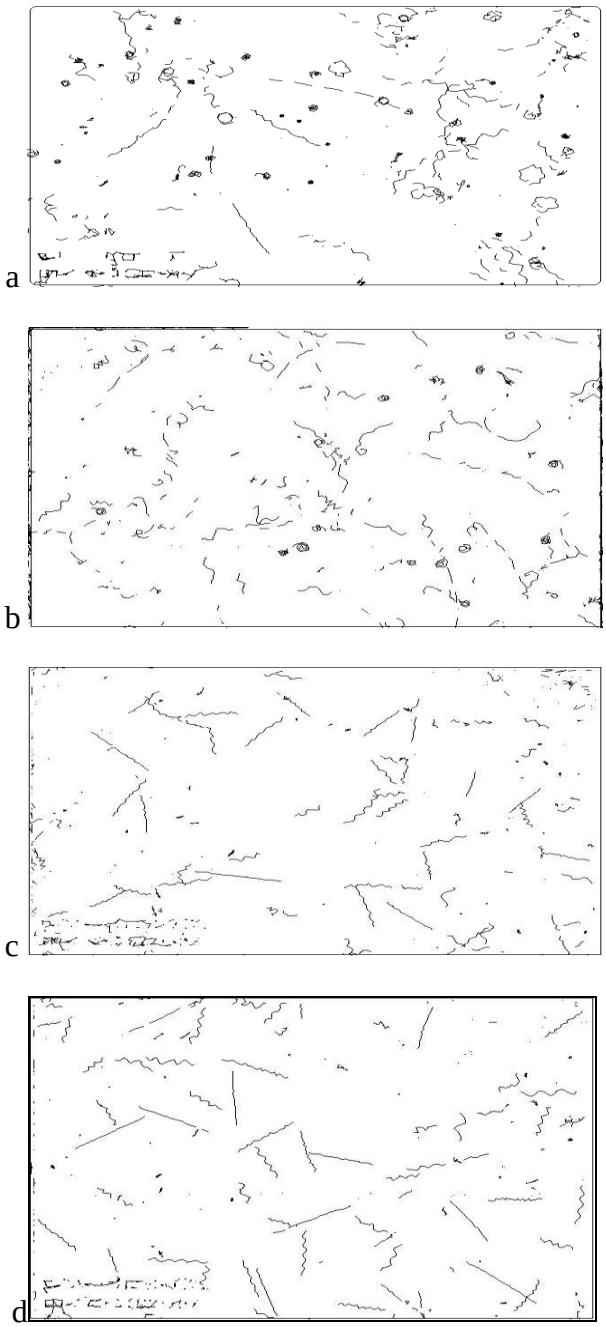


Figure 2: Image of all video frames in 1 second of video taken at 30 frame per second in hatchery water (a), Distilled water (b), SAS_1 (c) and SAS_2 (d).

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As seen in the Figure 2 the higher percentage of sperm linearity movement in SAS 1 and 2 revealed their better potential for fertilization than the sperm movement in tap and distilled water.

The results of spermatozoa analysis in *B. sharpeyi* with the Image J software were shown in Table 1. The VCL amount was from 5.44±0.29 to 21.37±0.36 µm/s. There were significant differences among groups in VCL and the highest VCL was related to SAS_2 ($P < 0.05$, Table 1). There were no significant differences between VCL of distilled and tap water ($P > 0.05$, Table 1). They created the lowest VCL rate, but had significant differences with SAS_1 and SAS_2 ($P < 0.05$, Table 1). The Lowest and highest VSL amounts were detected in tap water and SAS_2 ranging from 6.32±0.19 to 11.6±0.3 µm/s, respectively. Also we observed significant differences ($P < 0.05$, Table 1) between VSL amounts of SAS_1 (10.630.33± µm/s) and SAS_2 (11.6±0.3µm/s). The lowest levels of VSL were related to tap water (6.32±0.19 µm/s) and showed significant differences with other groups ($P < 0.05$, Table 1). The highest incubation survival (Fig. 3) and hatching rate (Figure 4) were achieved by using SAS1 and 2, with no significant differences ($P > 0.05$), while tap water showed the lowest survival and hatching rate which had significant differences with other treatments ($P < 0.05$).

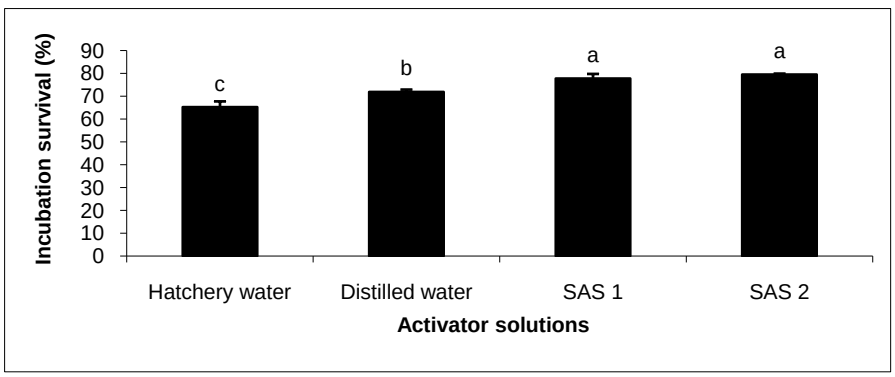


Figure 3: Effect of different activator solutions on (%) incubation survival of *B. sharpeyi*

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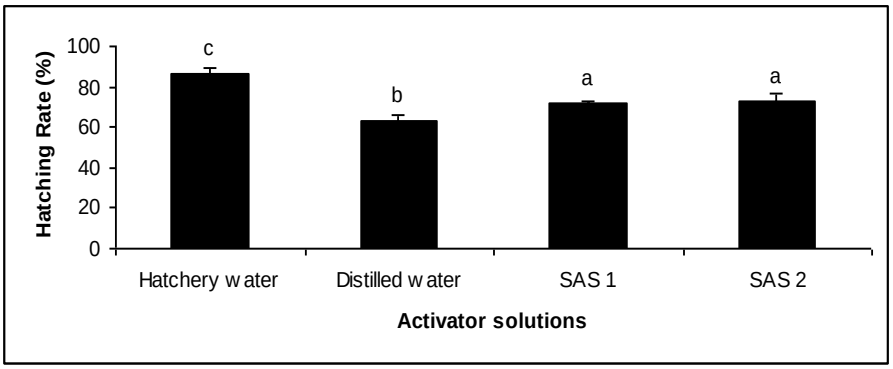


Figure 4: Effect of different activator solutions on (%) hatching rate of *B. sharpeyi*

However, significant differences were detected among treatments regarding larval deformity ($P < 0.05$, Fig. 5). Application of SAS_2 caused the lowest larval deformity (13.77±1.03 %), whereas usage of distilled water led to high levels of larval deformity (21.071±1.09 %). spermatozoa linearity movement results showed that there were no significant differences among SAS_1, SAS_2 and distilled water ($P > 0.05$, Table 1), but tap water had the lowest linearity movement (38.88±2.1 %) showing significant differences with other groups ($P < 0.05$, Table 1). Significant differences were distinguished relating to ALH values among treatments ($P < 0.05$, Table 1). The lowest (1.22±0.033µm/s) and highest (2.77± 0.073µm/s) amounts of ALH were belonged to SAS_2 and tap water, respectively.

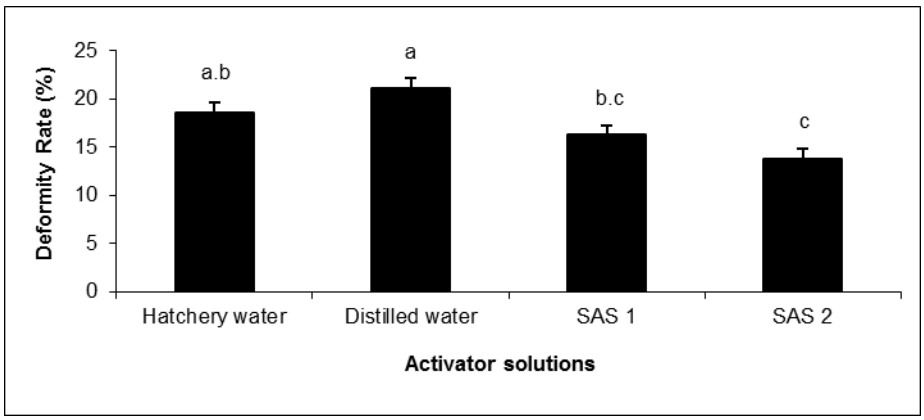


Figure 5: Effect of different activator solutions on (%) deformity rate of *B. sharpeyi*

We also detected significant differences concerning motility percentages among activators ($P < 0.05$, Table 1). The results showed that the highest (85.75±1.56) and lowest (74.58±2.65) percentages of spermatozoa motility were found in SAS_2 and tap water, respectively.

The highest total duration of sperm motility and forward motility were

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achieved by using SAS_1 144.66±3.17 second) and SAS_2 (144.33±10.2 second) ($P > 0.05$, Table 1). Also, the lowest total duration of sperm motility and forward motility were assessed using distilled and tap water (Table 1).

Table 1: Effect of different activator solutions on motility parameters of *B. sharpeyi* (µm/s)

Activator / Motility parameters	Hatchery water	Distilled water	SAS 1	SAS 2
VCL (µm/s)	[c]16.14±0.31	[c]15.44±0.29	[b]20.16±0.44	0.36 [a]±21.37
VSL(µm/s)	[d]6.32±0.19	[c]8.38±0.23	[b]10.63±0.33	[a]11.6±0.30
Linearity (%)	[b]38.88±2.1	[a]53.43±2.37	[a]52.84±2.39	[a]53.32±2.08
ALH (µm/s)	[a]2.77±0.073	[b]2±0.06	[c]1.59±0.036	[d]1.22±0.033
Motility (%)	[c]74.58±2.65	[b.c]79.57±1.76	[a.b]81.78±2.09	[a]85.75±1.56
Duration of motility (s)	[b]68.66±2.88	[b]62±2.30	[a]144.66±3.17	[a]144.33±10.20
Forward motility (s)	[b]25±2.64	[b]20±1.52	[a]34±1	[a]34±1.52

A frame by frame analysis of the videotapes showed that most of cells in tap water possessed agitated motion (with a VCL less than 3 µm/s, Table 2, Fig. 2a) compared with other treatments. The highest C type spermatozoa were achieved with application of tap water (Table 2).

The highest a type spermatozoa percentage were evaluated in SAS_1(47.16±3.15) and SAS_2 (43.89±4.54) with no significant difference ($P > 0.05$, Table 2). Also, the lowest C type spermatozoa percentage was recorded in SAS_2 (14.24±1.56) showing significant differences ($P < 0.05$, Table 2).

Table 2: Effect of different activators on A, B and C spermatozoa type of *B. sharpeyi* (%)

Activator / Sperm Type	Hatchery water	Distilled water	SAS 1	SAS 2
A (%)	3.76 [b]±31.73	[b]30.94±5.27	[a]47.16±3.15	[a]43.89±4.54
B (%)	[a]42.84±1.81	[a]48.61±3.65	[a]34.63±4.05	[a]41.05±4.17
C (%)	[a]25.41±2.65	[a.b]20.42±1.76	[b.c]18.18±2.07	[c]14.24±1.56

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The highest VCL and VSL amounts in A and C type spermatozoids were related to SAS_2 (Tables 3 and 4). There were no significant differences ($P > 0.05$, Table 3) regarding VCL amounts of C type spermatozoids between SAS_2 (3.59±0.049 µm/s) and distilled water (3.52±0.043 µm/s). Maximum amounts of VCL, VSL and Linearity movement of A type spermatozoa in different treatments were shown in Table 5.

Table 3: Effect of different activators on VCL of A, B and C type of spermatozoa in *B. sharpeyi* (µm/s)

Activator / Sperm Type	Hatchery water	Distilled water	SAS 1	SAS 2
A (µm/s)	^{b}33.16±0.54	^{b}32.77±0.48	^{b}32.26±0.55	^{a}35.60±0.45
B (µm/s)	^{b}12.19±0.15	^{c}11.09±0.13	^{a}13.60±0.22	^{b}12.08±0.16
C (µm/s)	^{b}3.17±0.04	^{a}3.52±0.043	^{c}3.49±0.093	^{a}3.59±0.049

Table 4: Effect of different activators on VSL of A, B and C type of spermatozoa in *B. sharpeyi* (µm/s)

Activator / Sperm Type	Hatchery water	Distilled water	SAS 1	SAS 2
A (µm/s)	0.83 $^{b.c}$±30.89	$^{a.b}$31.94±0.67	^{c}29.56±0.79	^{a}34.15±0.64
B (µm/s)	^{c}10.22±0.15	^{c}10.53±0.15	^{a}11.62±0.18	^{b}11±0.15
C (µm/s)	^{c}1.37±0.04	^{b}1.66±0.04	^{c}1.23±0.07	^{a}1.99±0.05

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Table 5: Effect of different activators on maximum VCL, VSL and Linearity between A type spermatozoa of *B. sharpeyi* (µm/s)

Motility parameters / Activator	VCL (µm/s)	VSL(µm/s)	Linearity (%)
Hatchery water	[a]64.09±0.67	[b]43.47±0.67	[c]65.14±5.67
Distilled water	[c]55±0.67	[b]43.85±0.67	[a.b]76.11±5.13
SAS $_1$	[a.b]60.14±0.67	[b]45.53±0.67	[a.b]74.99±4.21
SAS $_2$	[a]68.60±0.67	[a]60±0.67	[a]86.71±4.03

VCL and VSL amounts of *B. sharpeyi* spermatozoa at various times after sperm activation by different solutions were shown in Table 6.

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Table 6: Effect of activator solutions on VCL and VSL of *B. sharpeyi* spermatozoa at different times after sperm activation during their movement along (µm/s)

	Hatchery Water		Distilled Water		SAS_1		SAS_2	
Motility parameters	VCL	VSL	VCL	VSL	VCL	VSL	VCL	VSL
Time after activation								
5	^{a}1.56±25.75	^{a}12.37±1.03	^{a}28.30±1.72	^{a,b}13.53±1.13	^{a,b,c,d}20.92±1.48	^{b,c,d,e}9.84±0.62	^{c}8.82±0.66	^{f}4.98±0.38
10	^{a,b}23.81±1.64	^{b,c,d}6.98±0.61	^{b}21.53±1.63	^{c}10.35±1.21	^{b,c,d}19.24±1.50	^{d,e}8.06±1.13	^{b}15.89±0.84	^{e,f}7.46±0.64
15	^{b,c}21.48±1.39	^{c,d}6.77±0.62	^{b}21.55±1.42	^{a}14.16±1.35	^{a,b,c}22.44±1.50	^{a,b}13.42±1.25	^{b}18.54±1.01	^{e,f}6.77±0.63
20	^{c,d}18.72±1.26	^{b,c}8.02±0.83	^{b}21.86±1.31	^{a}14.33±1.21	^{a,b,c}21.68±1.06	^{a,b,c}12.14±1.69	^{b}17.70±1.32	^{d,e}9.29±1.19
25	^{d}18.38±1.19	^{b}8.87±0.87	^{c}18.47±1.14	^{b,c}11.39±1.01	^{b,c,d}19.70±1.46	^{a,b,c}12.39±1.30	^{a}25.85±1.55	^{a,b}15.22±1.39
30	^{d}17.69±1.17	^{b,c,d}7.52±0.82	^{d}15.11±0.97	^{c,d}9.34±0.89	^{a}25.06±1.94	^{a}14.68±1.72	^{a}25.12±1.53	^{a,b,c}14.16±1.39
35	^{d}16.16±0.95	^{c,d}6.78±0.71	^{d}14.47±0.77	^{d,e}7.72±0.62	^{a,b}23.91±2.37	^{a}15.69±2.02	^{a}26.55±1.46	^{a}16.05±1.28
40	^{e}13.02±0.67	^{d,e}5.97±0.52	^{d,e}13.72±0.67	^{d,e,f}7.26±0.52	^{c,d}17.14±1.20	^{c,d,e}9.55±1.09	^{a}23.29±1.26	^{a,b,c}14.88±1.09
45	^{e}12.30±0.71	^{e,f}4.28±0.38	^{d,f}11.29±0.59	^{e,f,g}5.69±0.48	^{a,b,c,d}20.02±1.12	^{b,c,d}10.76±0.92	^{a}24.96±1.24	^{a,b,c}13.64±1.07
50	^{e}12.11±0.63	^{e,f}4.4±0.38	^{e,f}11.59±0.58	^{f,g}5.17±0.39	^{b,c,d}18.66±1.20	^{c,d,e}8.91±0.85	^{a}24.66±1.33	^{b,c,d}12.39±1
55	^{e}10.82±0.71	^{e,f}4.22±0.44	^{f}9.93±0.46	^{g}4.65±0.31	^{b,c,d}19.41±1.13	^{d,e}8.38±0.79	^{a}23.22±1.08	^{c,d}11.81±0.79
60	^{e}10.53±0.73	^{e,f}2.77±0.31	^{f}10.22±0.56	^{g}4.35±0.36	^{e}15.99±0.86	^{e}6.71±0.58	^{b}18.64±0.83	^{d,e}9.51±0.59
Total	16.14±0.31	6.32±0.19	15.44±0.29	8.38±0.23	20.16±0.44	10.63±0.33	21.37±0.36	11.60±0.30

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Results showed that in tap water the highest VCL (25.75±1.56 µm/s) and VSL (12.37±1.03 µm/s) were calculated at 5 seconds after sperm activation. Also 5 seconds after sperm activation in distilled water, the highest amount of VCL was recorded (28.30±1.72 µm/s), but the lowest rate of VCL (9.93±0.46 µm/s) was observed 55 seconds after sperm activation. The highest amount of VCL (28.30±1.72 µm/s) was calculated at 5 seconds after sperm activation. We also detected the highest VSL in distilled water between 15 (14.16±1.35 µm/s) to 20 (14.33±1.21 µm/s) seconds after sperm activation, whereas the lowest rate (4.35±0.36 µm/s) was assessed after 60 seconds.

Also, the highest VCL (25.06±1.94 µm/s) and VSL (15.69±2.02 µm/s) values were recorded at 30 and 35 seconds after activation of sperm in SAS_1, respectively. The results showed that SAS_2 had the highest VCL (26.55±1.46 µm/s) and VSL values (16.05±1.28 µm/s) at 35 seconds after sperm activation.

Discussion

Initial investigations of fertilization rate in barbus fish of Iran, indicated that *Barbus sharpeyi* had the lowest fertilization success, which also reported by Pyka et al. (2001). Also so far, there is no comprehensive published information about the effect of saline activator solutions on different sperm parameters of *B. sharpeyi*. Present study provided useful information about completing the *in vitro* fertilization of *B. sharpeyi*. In some species, environmental conditions cause stress in broodstock fish, thus optimum conditions for achieving sexual maturity and reproduction process may not be provided, and consequently, hormone application for inducing sexual maturity is mandatory (Metwally and Fouad, 2008). It seems that this factor could be one of the causes of low fertilization rate in *B. sharpeyi*.

In fishes, period of sperm motility, velocity and duration of forward motility are affected by various parameters such as temperature, pH, ions (including sodium, potassium, calcium and ...), osmolality, dilution rate, season of stripping (Bobe and Labbé, 2009; Mylonas et al., 2010) and structure of spermatozoa (Psenicka et al., 2009). Understanding the effect of above-mentioned parameters would be useful to obtain the best method of artificial insemination (Alavi et al., 2007; Rosengrave et al., 2008).

Our results showed that spermatozoa activation with distilled and tap water caused more whirling movements, lower VSL and VCL values (Figure 2 and Table 1), which can be occurred due to deformity of spermatozoa flagella with the unsuitable activators (Cosson et al., 1999). Also, Le Comber et al. (2004) reported that VSL of spermatozoa can be decreased through activation by distilled water. However, presence of immotile spermatozoa can affect motility ability of normal spermatozoa and fertilization capability (Aas et al., 1991). Also different

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investigations of fish reproductive materials, especially fish sperm, showed that usage of saline activator solutions caused the protection of sperm flagella structure and increased the time of motility, thus researchers used these solutions instead of tap water for activating spermatozoa (Billard, 1992; Cosson et al., 1999; Kalbassi and Lorestani, 2007). Application of the saline activator solutions including SAS_1 and SAS_2 increased Linearity movement, VCL and VSL values, which consequently led to elevation of incubation survival (Fig. 3), hatching (Fig. 4) and deformity rates (Fig. 5).

After activation of fish spermatozoa, their movements will be started immediately in the straight path with very low curvature, but during the final stages of sperm motility or when sperm is in risk of contamination, or also when activation of spermatozoa with unsuitable activators is performed, spermatozoa path is more curved and consequently form concentric circles, in this regard, linearity and percentage of motile cells are assessed as indicators of fertilization ability (Rurangwa et al., 2004). In freshwater fish with external fertilization, spermatozoa are released into a hostile environment where they become generally activated, and survive for a short period of 1 to2 minutes (Holt and Van Look, 2004). In the present study, the duration of spermatozoa motility was recorded from 1 to 3 minutes after activation with the different activators (Table 1). Also, VCL and VSL values in tap and distilled water strongly decreased through the time following activation, which is in accordance with results of Ravinder et al. (1997), while slower decrease was detected by applying SAS_1 and SAS_2 (Table 6).

Alavi et al. (2009) reported that qualitative parameters of *Barbus barbus* spermatozoa declined suddenly after activation; also, the highest period of motility was recorded under osmotic pressure of 215 to 235 mos.kg, but activation of spermatozoa by SAS_1 and SAS_2 under osmotic pressure of 188 to 189 mos.kg created the optimal conditions for extending the duration and forward motility of *B. sharpeyi* spermatozoa (Table 1).

In species with large egg size, any factor which causes a small decrease of spermatozoa motility can lead to fertility rate reduction (Kim et al., 2001). Results of incubation survival and hatching rate with use of tap water showed the lowest rates among treatments (Fig. 3 and 4). That can be related to the high level of C type spermatozoa percentage and low levels of VCL, VSL and A type spermatozoa (Tables 1 and 2). Also, VCL, VSL, duration of spermatozoa motility and percentage of motile cells were the lowest and that may relate to the higher consumption of energy and deformity of spermatozoa tail by tap and distilled water (Perchec et al., 1995, Cosson et al., 1999).

The lowest percentage of motile cells were obtained with use of tap water

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for activation of spermatozoa compared with other treatments (Table 1), that also can lead to decline of fertilization rate in *B. sharpeyi*. Among all treatments, tap water had the highest ALH (Table 1) and sinusoidal path (Fig. 2), while SAS_2 had the lowest sinusoidal motion and the highest VCL, VSL, Linearity and motility percentage. On the other hand, SAS_2 caused the highest and lowest percentage of A and C type spermatozoa, respectively. Also, these factors may cause the better state of SAS_2 including increasing the chances of fertilization and declining larval deformity rate. VCL and VSL amounts in tap water and VSL in distilled water reached their maximum levels at 5 seconds after spermatozoa activation and then declined rapidly (Table 6). Whereas, VCL had the highest rate during 25 to 55 seconds after activation of sperm in SAS_2 and remained constant throughout that time. VCL and VSL in SAS_2 were about 1.5 and 4 times more than those in tap water in 60 seconds after sperm activation, respectively. VCL and VSL in SAS_1 were more than in distilled and tap water at the same time (Table 6). In common carp, VCL and percentage of mobile spermatozoa were equal to 139 µm/s and 98% in distilled water, respectively (Linhart et al., 2005), but present study showed that VCL and motile spermatozoa percentage in *B. sharpeyi* were lower than that of common carp. VCL and VSL were almost the same in most freshwater fish (salmon, carp and cat fish) and average VCL was approximately 110 µm/s after the activation of spermatozoa (Kim and Tveiten, 2002).

The velocity of spermatozoa is often low in marine fishes, for example, average of velocity is 75 µm/s in Atlantic Cod (Trippel and Nilson, 1992).

Asturiano et al. (2004) reported that VCL, VSL and percentage of spermatozoa motility in *Anguilla anguilla* were equal to 40, 12 µm/s and 40% respectively. Our results showed that the maximum VCL, VSL, and Linearity were related to the application of SAS_2 equaling to 68.60, and 60 µm/s and 86.71%, respectively (Table 5). Christ et al. (1996) noted that VCL, VSL and motility percentage of common carp were 145, 60 µm/s and 60% respectively. Maximum VSL and motility percentage in A type spermatozoa of *B. sharpeyi* in present study were in accordance with findings of Christ et al. (1996) (Table 5).

Assessment methods of sperm quality based on computer-assisted sperm analysis (CASA) developed since 1980 and so far various ways for the sperm quality evaluation has been used and each of them has advantages and disadvantages, however, their settings and preparation of the software is based on human sperm quality assessment protocol (Kime et al., 2001; Pavlov, 2006; Liu et al., 2007).

Comparing to mammalian spermatozoa, fish spermatozoa has not acrosome, but has a more complex path and motility period of less than one minute and so fish sperm analysis using CASA

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methods requires adaptations for each species (Wilson Leedy and Ingermann, 2007). The most important advantage in the study of sperm quality with Image J software is its easy download way through the website http://rsb.info.nih.gov.ij.plugins.casa.html and it also can be used and planned based on conditions and characteristics of spermatozoa in different aquatic species (Wilson Leedy and Ingermann, 2007; Cabrita et al., 2008). A movie section used for computer analysis of sperm is different from 0.5 sec (Burness et al., 2005) to 1 minute (Trippel, 2003), but the best data usually will be achieved during 5 to 20 seconds after spermatozoa activation (Kim et al., 2001).

In conclusion, the experienced saline activator solutions were able to affect the different sperm quality indices and fertilization rate of *B. sharpeyi*. In this regard, application of SAS_1 and SAS_2 could be recommended for improvement of propagation of this species in south of Iran. Also, due to high cost, complexity and hard accessibility of CASA software for most researchers, assessment of fish sperm quality with Image J software is recommendable.

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