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Theriogenology 78 (2012) 102–109

Theriogenology

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# Regulation of spermatozoa motility in response to cations in Russian sturgeon *Acipenser gueldenstaedtii*

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Received 1 September 2011; received in revised form 14 January 2012; accepted 19 January 2012

**Abstract**

The aim of this study was to investigate the response of Russian sturgeon (*Acipenser gueldenstaedtii*) sperm to external cations ($Na^+$, $K^+$, $Ca^{2+}$, and $Mg^{2+}$) and their susceptibility on the induction of motility and swimming behavior. An *in vitro* spermatozoa motility assay was used by a computer-aided Motion-Analysis system. Sperm motility was inhibited by 60 mM NaCl (~140 mOsm/kg) and 0.7 mM KCl solutions (~ 21.4 mOsm/kg). The $Ca^{2+}$ and $Mg^{2+}$ ions were not able to inhibit spermatozoa motility. By contrast, $Na^+$ within a limited concentration range (between 45 and 55 mM) was able to reverse the inhibitory effect of $K^+$ at the critical concentration (0.7 mM). $Ca^{2+}$ and $Mg^{2+}$ were also able to reverse the $K^+$-mediated spermatozoa motility restriction at concentrations starting at 0.01 and 0.1 mM, respectively. These results provide evidence for the role of $K^+$ in suppressing spermatozoa motility, and suggest that $Ca^{2+}$, $Mg^{2+}$, and possibly $Na^+$ trigger motility in Russian sturgeon sperm.


*Keywords:* Synergistic effect; Spermatozoa behavior; Endangered species

## 1. Introduction

It is widely accepted that fish sperm are quiescent in the testes, because the osmolality of the testicular fluid is isotonic. The aquatic environment contains several cations, such as $Na^+$ and $Ca^{2+}$, and their combinations are expressed as osmolality that increases from fresh water to seawater. In freshwater teleost species, spermatozoa motility is initiated by the hypo-osmotic shock (<300 mOsm/kg). In contrast, the sperm of marine teleosts are initiated by hyperosmotic shock [1,2]. It is likely that the osmolality and ion composition of the swimming medium affect the regulatory mechanisms of spermatozoa motility [3,4].

Sturgeons and paddlefishes, which are in the order Acipenseriformes, have attracted the century-long attention of developmental biologists [5,6], but studies of their reproductive physiology are limited because of the lack of cultured animals. However, despite several limitations, they still offer an opportunity for studying aspects of reproductive physiology which play important roles in such endangered species.

Sturgeon spermatozoa, like that of freshwater teleost fish, are quiescent in the isotonic testicular fluid, and spermatozoa motility is initiated when they are released to an outer environment, such as hypotonic fresh water [7]. Sturgeon spermatozoa are motile in the range of 0–120 mOsm/kg [8,9], but the osmotic level of seminal plasma is lower than 100 mOsm/kg [10], which suggests that spermatozoa motility is controlled by both

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doi:10.1016/j.theriogenology.2012.01.025

osmotic and ionic content. The inhibition of spermatozoa motility is mainly because of osmotic pressure in most species [11], but $K^+$ plays an important role in salmonids [12] and in sturgeons [13,14]. According to these studies, a $K^+$ concentration of more than 0.5 mM can inhibit the spermatozoa motility of sturgeon. Moreover, Scheuring [15] first reported the "synergistic effects" of cations on salmonids sperm, where cations, such as $Na^+$, $Ca^{2+}$, and $Mg^{2+}$ reduced the inhibitory action of $K^+$ ions. In addition, the bivalent cations ($Ca^{2+}$ and $Mg^{2+}$) were more effective at reducing the inhibitory action of $K^+$ ions than $Na^+$. The discovery of synergistic effects between ions led to several studies that demonstrated a possible control mechanism of spermatozoa motility by the membrane potential that resulted from the combined effect of several ions [16–18].

Despite the great importance of Russian sturgeon *Acipenser gueldenstaedtii* as a producer of highly valuable caviar, almost no information is available about gamete biology of this species [5]. In addition, information on gamete biology is needed to establish genetic conservation programs for this species, because the regulation of sperm motility is diverse and the regulatory mechanisms are suited to the inhabiting environments. Therefore, in the present study, the sperm of Russian sturgeon were used to study the extracellular effects of cations ($Na^+$, $K^+$, $Ca^{2+}$, and $Mg^{2+}$) on the regulation of spermatozoa motility and the biosensitivity of sperm to each cation. Furthermore, previous studies mostly focus on spermatozoa motility either velocity or percentage motile spermatozoa, sometimes on both but calculate separately. To judge the spermatozoa motility comprehensively, the relative spermatozoa motility represented as the mean value of spermatozoa velocity ($\mu$m/sec) $\times$ the mean value of percentage of motile spermatozoa (%) was first used and described in this study.

## 2. Materials and methods

### 2.1. Induction of spermiation and sperm collection

From March to May, the breeding and culture of Russian sturgeon were carried out at an experimental station of the Faculty of Fisheries and Protection of Water, University of South Bohemia, Vodnany, Czech Republic. Four males (10.55 $\pm$ 1.32 kg) were used. Before hormonal stimulation, fish were kept in tanks where the water temperature varied between 15 and 16 °C. Spermiation was stimulated by intraperitoneal injection of carp pituitary powder dissolved in 0.9% (w/v) NaCl solution at doses of 4 to 5 mg/kg of body weight, 48 h before sperm collection. Sperm were collected from the urogenital tract by catheter directly into 250-mL cell culture containers to avoid contamination by mucus, feces, or water. The samples were stored at 4 °C before taking the measurements.

### 2.2. Treatment medium

To test the effects of $Na^+$, $K^+$, $Ca^{2+}$, and $Mg^{2+}$ on sperm motility, sperm samples were suspended with 20 mM Tris–HCl buffer, pH 8.0, containing 10, 20, 30, 40, 50, and 60 mM NaCl, 0.2, 0.3, 0.4, 0.5, and 0.6 mM KCl, 10, 20, 30, 40, and 50 mM $CaCl_2$, or 30, 40, 50, 60, and 70 mM $MgCl_2$. To study the effect of $Na^+$, $Ca^{2+}$, and $Mg^{2+}$ on triggering spermatozoa motility inhibited by $K^+$, the sperm were suspended with 0.7 mM KCl and 20 mM Tris–HCl buffer, pH 8.0, containing 45, 50, and 55 mM NaCl, 0.01, 0.1, 2, 5, and 10 mM $CaCl_2$, or 0.1, 1, 10, and 30 mM $MgCl_2$. The osmolality of each medium was approximately calculated as the number of osmoles of solute particles per kilogram of solvent (mOsm/kg).

### 2.3. Spermatozoa motility assessment

The percentage (%) and the velocity ($\mu$m/sec) of motile spermatozoa were determined after triggering spermatozoa motility under dark-field microscopy using 20× objective magnification (Olympus BX 50, Japan). Spermatozoa motility was stimulated by diluting them in the medium mentioned above at a ratio of 1:50 for fresh spermatozoa ($6 \times 10^6$). To prevent spermatozoa from sticking to the microscope slide, 0.25% (w/v) of pluronic (Sigma-Aldrich) was added to the solution. Spermatozoa motility was recorded with a CCD video camera (SONY SSCDC50AP, Japan) mounted on a microscope. The successive positions of the heads of spermatozoa were measured from five successive frames using a video recorder (SONY S-VHS, SVO-9500 MDP, Japan), and analyzed with a micro image analyzer (Olympus Micro Image 4.0.1. for Windows). Meantime, the trajectory of sperm movement was recorded and traced by overlapped five successive frames. Measurement of spermatozoa motility for each sample was performed in triplicate.

### 2.4. Data analysis

The motility was recorded for 1 min and the motility in each time of post-activation (10, 20, 30, 40, 50, and 60 s) was calculated as a mean. Finally, the relative spermatozoa motility was represented as the mean value of spermatozoa velocity ($\mu$m/sec) $\times$ the mean value of percentage of motile spermatozoa (%). The

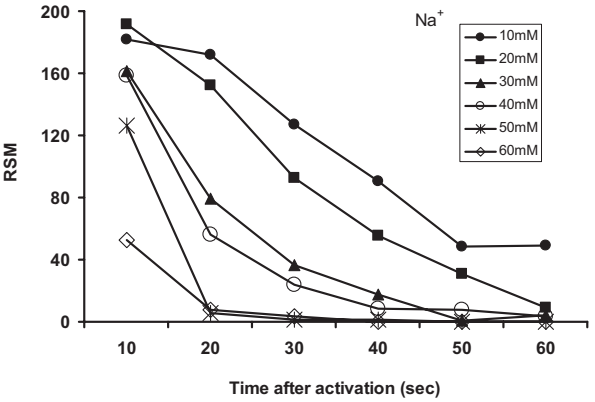


Fig. 1. The effect of extracellular $Na^+$ on RSM in Russian sturgeon (*Acipenser gueldenstaedtii*). RSM is calculated as spermatozoa velocity ($\mu$m/sec) × percentage of motile spermatozoa (%).

descriptive data analyses were carried out with the software package Statistica 2008 (StatSoft, Inc., Tulsa, USA).

## 3. Results

### *3.1. Motility of spermatozoa diluted in swimming medium without ion components*

When Russian sturgeon sperm were diluted by 20 mM Tris-HCl (pH 8) buffer, the spermatozoa was immediately activated and displayed the following characteristics: (i) 100% of spermatozoa were motile 10 s after activation with a velocity of 182.51 ± 21.38 $\mu$m/sec; (ii) after 1 min, 65.35 ± 14.96% of motile spermatozoa decreased the velocity to 150.46 ± 8.19 $\mu$m/sec; and (iii) motility duration was maintained for up to 5 min.

### *3.2. The effect of extracellular ions ($Na^+$, $K^+$, $Ca^{2+}$, and $Mg^{2+}$) on spermatozoa motility and behavior*

The motility of Russian sturgeon spermatozoa was investigated after dilution in various concentrations of electrolytes (NaCl, KCl, $CaCl_2$, and $MgCl_2$). As shown in Fig. 1, spermatozoa were motile in NaCl solution up to a concentration of 60 mM (~ 140 mOsm/kg). Moreover, spermatozoa movement was completely inhibited in 0.6 mM KCl (~ 21.2 mOsm/kg) (Fig. 2).

The trajectory of spermatozoa swimming behavior showed a straightforward pattern when sperm were immediately diluted with both NaCl and KCl solutions in all treatment concentrations (Fig. 3A). Moreover, swimming behavior became cycled when spermatozoa movement was close to the end (Fig. 3B). Spermatozoa flagellum bent at the proximal region, similar to a cane, when the spermatozoa became quiescent (Fig. 3C), while the spermatozoa that did not receive stimulation of movement exhibited a straight pattern (Fig. 3D).

In addition, when sperm were diluted in a $CaCl_2$ solution from 10 to 50 mM (~ 50–170 mOsm/kg), the sperm started to stick to each other at the flagellum, and consequently were beating and moving together. As the concentration of $CaCl_2$ increased, the sperm were bound together like a net and shook. When sperm were diluted in an $MgCl_2$ solution from 30 to 70 mM (~ 110–230 mOsm/kg), the spermatozoa moved forward with spasticity, which is a state where the head moves forward with the same wave propagation of flagellum for several seconds (Fig. 4A). When the concentration of $MgCl_2$ was increased, the head of spermatozoa began to stick to the surface of the glass slide and the flagellum kept beating (Fig. 4B).

### *3.3. The effect of extracellular $Na^+$, $Ca^{2+}$, and $Mg^{2+}$ on triggering spermatozoa motility inhibited by $K^+$*

The various concentrations of $Na^+$, $Ca^{2+}$, and $Mg^{2+}$ were used to trigger spermatozoa motility that was inhibited by a critical concentration of $K^+$ (0.7 mM). We found that spermatozoa motility of sperm inhibited by the critical $K^+$ concentration were only activated by $Na^+$ in the range of 45 to 55 mM (~ 91.4–131.4 mOsm/kg), and the duration of spermatozoa motility increased to 45 s in case of the treatment by 45 mM $Na^+$ (Fig. 5). After triggering spermatozoa motility with 0.01 mM $Ca^{2+}$ (~ 21.43 mOsm/kg), spermatozoa became motile. Furthermore, spermatozoa expressed a similar level of motility when different concentrations

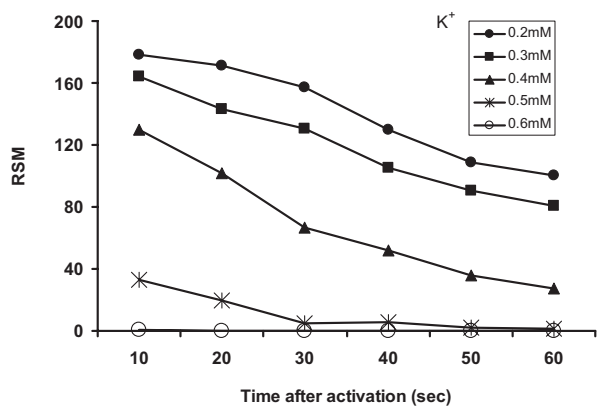


Fig. 2. The effect of extracellular $K^+$ on RSM in Russian sturgeon (*Acipenser gueldenstaedtii*). RSM is calculated as spermatozoa velocity ($\mu$m/sec) × percentage of motile spermatozoa (%).

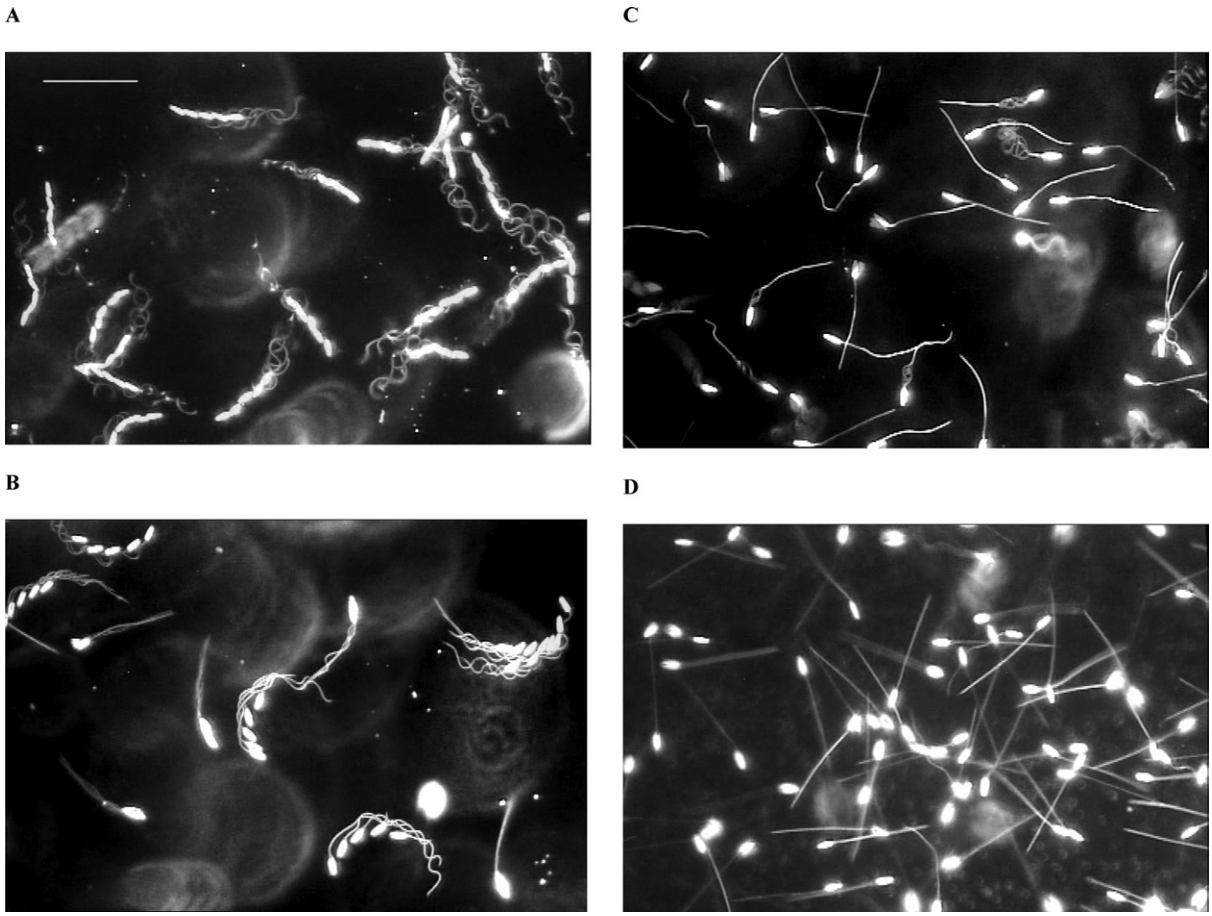


Fig. 3. Representative trajectory types of sturgeon spermatozoa. (A) Type of straightforward movement; (B) circulated movement in the time close to the stop; (C) quiescent status of activated sperm after movement with a bent flagellum at the proximal region; (D) inactivated sperm with straight flagellum. Bar = 50 $\mu$m.

of $Ca^{2+}$ (0.1–10 mM $Ca^{2+}$; ~ 21.7–51.4 mOsm/kg) were used (Fig. 6). Moreover, spermatozoa started to stick together when the concentration of $Ca^{2+}$ was over 10 mM. Spermatozoa motility was triggered at a concentration of 0.1 mM (~ 21.7 mOsm/kg) for $Mg^{2+}$, and the motility was similar for concentrations up to 10 mM (~ 51.4 mOsm/kg) (Fig. 7). Moreover, spermatozoa motility decreased at 30 mM $Mg^{2+}$ (~ 111.4 mOsm/kg). Interestingly, the spermatozoa swimming behavior was similar to that in NaCl and KCl solutions when the sperm were diluted in concentrations of $Ca^{2+}$ <2 mM and $Mg^{2+}$ <1 mM (Fig. 3B, 3C).

## 4. Discussion

The present study focused on the osmo- or ion-regulatory mechanism of spermatozoa motility in Russian sturgeon. The osmolality of seminal plasma in sturgeons is much lower than most teleosts, and in the case of Russian sturgeon, is approximately 67 mOsm/kg [19]. However, the effects of electrolytes on spermatozoa motility initiation and suppression vary among species, indicating that the mechanisms of initiation of spermatozoa motility are different [20].

Toth, et al. [21] reported that the activation of spermatozoa motility in lake sturgeon (*Acipenser fulvescens*) is inhibited by $Na^+$ at concentrations of 40 mM and higher. In the case of Persian sturgeon (*Acipenser persicus*), spermatozoa are sensitive to $Na^+$ when the concentration reaches 50 mM or higher, and an optimal duration of spermatozoa motility and percentage of motile spermatozoa were reported at a concentration of 25 mM $Na^+$ [22]. In the present study, we have found that the presence of $Na^+$ ions in activation medium had mostly osmotic effects on sperm cells, since the spermatozoa motility decreased fol-

lowing the increasing concentration of NaCl solution, and was completely terminated when the osmolality of the NaCl solution (~140 mOsm/kg) was close to double the osmolality of seminal plasma of Russian sturgeon.

According to the Nernst equation, the ratio of $K^+$ ions outside of a cell and $K^+$ inside a cell denotes membrane potential. Furthermore, the membrane potential is hyperpolarized when the concentration of $K^+$ inside of a cell decreases because of the current of $K^+$ ions outside of a cell through $K^+$ ion channels. It is also speculated that $K^+$ efflux does not occur when a large amount of $K^+$ ions outside of a cell is present compared to the concentration of intracellular $K^+$ ions. In this study, the concentration of $K^+$ ions over 0.7 mM completely suppressed spermatozoa motility in Russian sturgeon, which was still lower than the $K^+$ concentration in seminal plasma (~2.29 mM) [19]. These results suggest that the concentration of intracellular $K^+$ in these sperm cells is low. Moreover, the $K^+$ current between the intracellular and extracellular regions may be another main factor associated with spermatozoa motility initiation. The $K^+$ efflux was also reported to be able to induce spermatozoa motility in common carp (*Cyprinus carpio*) and Marble goby (*Oxyeleotris marmorata*) sperm [11,23].

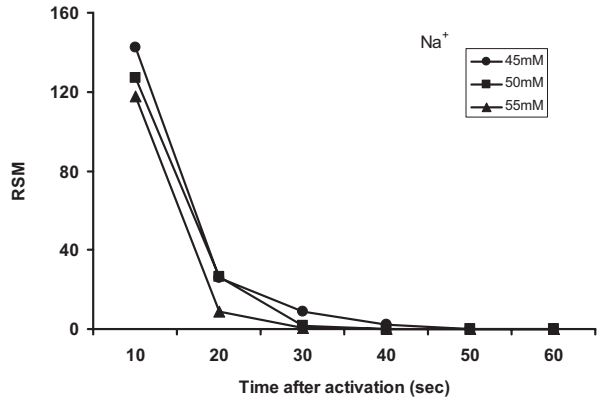


Fig. 5. The effect of extracellular $Na^+$ on triggering RSM of Russian sturgeon (*Acipenser gueldenstaedtii*) inhibited by $K^+$. Sperm were suspended in 20 mM Tris-HCl (pH 8) and 0.7 mM KCl buffer containing different concentrations of NaCl solution (45–55 mM). RSM is calculated as spermatozoa velocity ($\mu$m/sec) × percentage of motile spermatozoa (%).

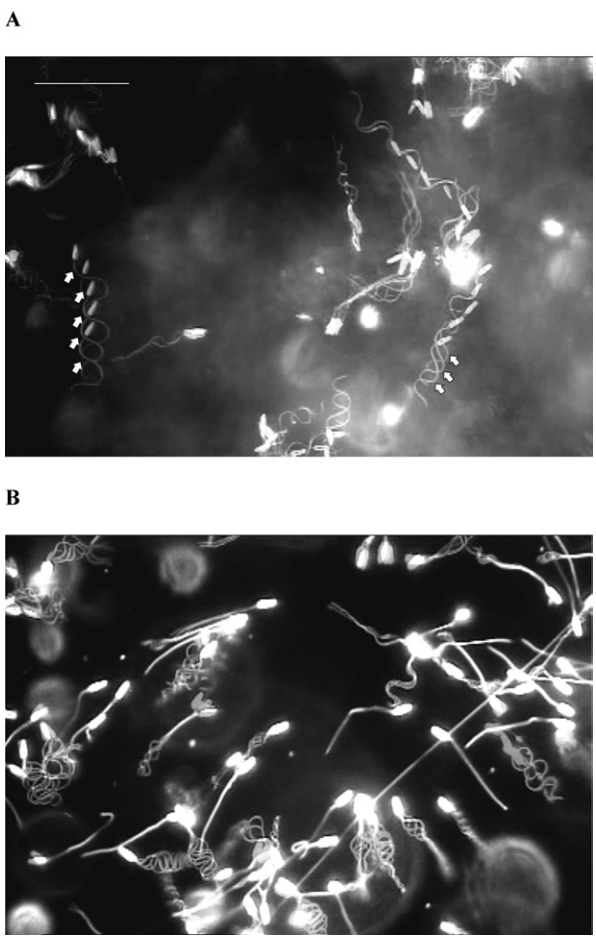


Fig. 4. Representative trajectory in Russian sturgeon (*Acipenser gueldenstaedtii*) spermatozoa after dilution in $Mg^{2+}$ solution containing from 30 to 50 mM $MgCl_2$ (A), and over 60 mM $MgCl_2$ (B). A, The head of sperm move straightforward, but the flagellum is spastic (see the arrows); B, The head of sperm affix to the glass, but the flagellum continue to beat. Bar = 50 $\mu$m.

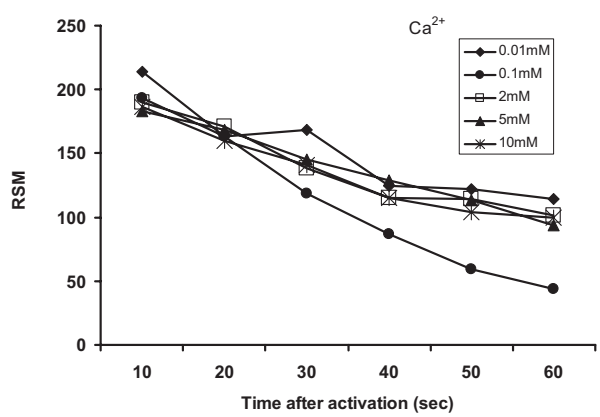


Fig. 6. The effect of extracellular $Ca^{2+}$ on triggering RSM of Russian sturgeon (*Acipenser gueldenstaedtii*) inhibited by $K^+$. The sperm were suspended in 20 mM Tris-HCl (pH 8) and 0.7 mM KCl buffer containing different concentrations of $CaCl_2$ solution (0.01–10 mM). RSM is calculated as spermatozoa velocity ($\mu$m/sec) × percentage of motile spermatozoa (%).

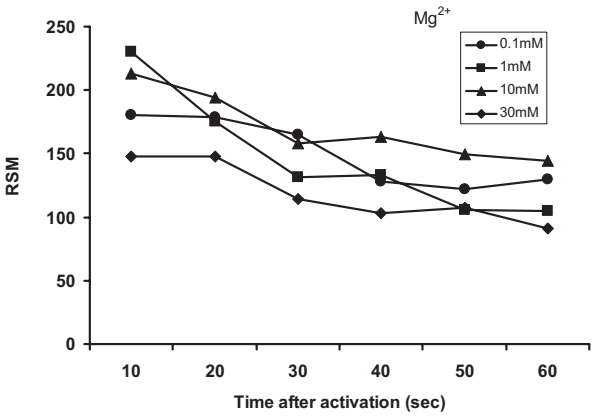


Fig. 7. The effect of extracellular $Mg^{2+}$ on triggering RSM of Russian sturgeon (*Acipenser gueldenstaedtii*) inhibited by $K^+$. The sperm were suspended in 20 mM Tris-HCl (pH 8) and 0.7 mM KCl buffer containing different concentrations of $MgCl_2$ solution (0.1–30 mM). RSM is calculated as spermatozoa velocity ($\mu$m/sec) × percentage of motile spermatozoa (%).

The initiation of spermatozoa motility by $Ca^{2+}$ ions has been reported in several fish species [24–26]. In salmonids and common carp, the $Ca^{2+}$ was thought to be able to induce hyperpolarization of the sperm membrane followed by a transient influx of $Ca^{2+}$ through $Ca^{2+}$ channels [27,28]. However, spermatozoa motility in Java carp (*Puntius javanicus*) was inhibited in the presence of $Ca^{2+}$ (5 mM), suggesting that an increase in $Ca^{2+}$ concentration because of membrane hyperpolarization seems to be harmful to spermatozoa motility, while $Ca^{2+}$ had no effect on the spermatozoa motility of walking catfish (*Clarias batrachus*) [23]. It is likely that the effect of an increase in the concentration of $Ca^{2+}$ ions on spermatozoa motility initiation is diverse among species, and the membrane potential may be associated with a regulation of $Ca^{2+}$ influx [29]. In Russian sturgeon, we observed that the spermatozoa membrane was very sensitive to $Ca^{2+}$ ions, especially the membrane of flagellum. When a higher concentration of $Ca^{2+}$ was used, the spermatozoa motility was not inhibited and spermatozoa were stuck together by flagellum. Many enzymes require calcium ions as a cofactor, including those of the blood-clotting cascade [30]. Therefore, we hypothesized that in the case of Russian sturgeon sperm, the calcium-dependent enzymes may be located around the flagellum. However, further studies are required to verify this role of $Ca^{2+}$.

There is currently a lack of information on the effects of $Mg^{2+}$ ions on spermatozoa motility in teleosts and sturgeon. In American paddlefish (*Polyodon spathula*), Linhart, et al. [14] reported that spermatozoa velocity was significantly improved by 5 mM $Mg^{2+}$. Several other studies that have focused on the intracellular mechanisms of spermatozoa motility in teleosts have confirmed a key role of $Mg^{2+}$ in the initiation of the activation of spermatozoa motility, especially in demembranated sperm [3]. In addition, Alavi, et al. [22] reported that $Mg^{2+}$ ions have a negative effect on the motility of Persian sturgeon (*Acipenser persicus*) spermatozoa when the concentrations of $Mg^{2+}$ increased to 15 mM. In our study, we observed that the Russian sturgeon spermatozoa was motile at $Mg^{2+}$ concentrations up to 70 mM, but the swimming behavior appeared spastic, and the spermatozoa head became affixed to the glass slide after the extracellular $Mg^{2+}$ concentration increased. It is well known that $Mg^{2+}$ is an essential element in biological systems. However, biological membranes are impermeable to $Mg^{2+}$ (and other ions), so transport proteins must facilitate the flow of magnesium inside and outside of cells as well as in intracellular compartments. In fish sperm, $K^+$, $Ca^{2+}$, and $Na^+$/$Ca^{2+}$ channels have been reported [2,31]. Therefore, it seems likely that there are also $Mg^{2+}$ channels located on the membrane of sturgeons sperm, and may be located on the head, since spermatozoa motility was not inhibited by any concentration of $Mg^{2+}$.

$Na^+$ and $Ca^{2+}$ can reduce the inhibitory effects of $K^+$ in spermatozoa of teleosts [3,26] and sturgeons [14,32]. Similar to those studies, we found that $Na^+$, $Ca^{2+}$, and $Mg^{2+}$ can activate the motility of Russian sturgeon sperm that had been inhibited by 0.7 mM $K^+$. For $Na^+$, concentrations between 45 and 55 mM induced a short period of movement. We hypothesize that those concentrations can open the $Na^+$/$K^+$ channels, and the resulting membrane hyperpolarization by $Na^+$ influx and $K^+$ efflux stimulates spermatozoa motility. Furthermore, when the concentration of $Na^+$ was higher than 55 mM, the osmotic effects suppressed spermatozoa motility.

Moreover, we have also found that when spermatozoa motility was inhibited by 0.7 mM $K^+$, the presence of $Ca^{2+}$ and $Mg^{2+}$ ions at low concentrations (0.01–0.1 mM) separately was able to stimulate spermatozoa movement. Tanimoto and Morisawa [27] have already found that divalent cations, including $Ca^{2+}$, $Mg^{2+}$, and $Sr^{2+}$, can initiate salmonid spermatozoa motility even in medium supplemented with $K^+$. Boitano and Omoto [18] proposed that divalent cations can mask the surface potential of salmonid spermatozoa membrane, leading to membrane hyperpolarization. By contrast, $Ca^{2+}$ and $Mg^{2+}$ channels may be present on the membranes of sturgeon spermatozoa, which would induce

membrane hyperpolarization through the influx of $Ca^{2+}$ and $Mg^{2+}$.

Morita, et al. [23] found that spermatozoa swam in a circular path and became quickly quiescent in the presence of $Ca^{2+}$ in Java carp, whereas the spermatozoa swam forward when extracellular $Ca^{2+}$ was removed. In contrast, $Ca^{2+}$ ions may not induce the asymmetry of flagellar beating in Marbled goby and walking catfish. In demembranated sea urchin (*Echinus melo*) sperm, the asymmetry of flagella beating increased with elevated $Ca^{2+}$ concentrations [33]. Furthermore, Brokaw and Nagayama [34] also reported that calmodulin modulates the asymmetry of sea urchin sperm flagella beating. It was recently shown that these turns occur in response to a transient increase in intracellular $Ca^{2+}$ in the flagellum [35,36]. Furthermore, in the presence of high concentrations of $Ca^{2+}$ ($10^{-4}$ M), an increase in the asymmetry of flagella leads to a "cane shape" during the quiescent state [37]. In our study, asymmetric movement was observed in the presence of all ions investigated, but this behavior appeared only at the end of spermatozoa movement, and the spermatozoa became quiescent during the cane shape state. In contrast to previous studies, we found that the asymmetry of flagellum beating did not increase with elevated $Ca^{2+}$ and $Mg^{2+}$ concentrations.

## 5. Conclusions

Based on the results presented in this study, the response of sperm to $K^+$ suggested that the osmolality is not the principal factor preventing spermatozoa motility in Russian sturgeon. This study also showed sperm biosensitivity to cations and the synergistic effects of cations on spermatozoa motility and swimming behavior. In particular, $Ca^{2+}$ and $Mg^{2+}$ ions play a significant role in spermatozoa motility activation, although the spermatozoa were able to move without extracellular $Ca^{2+}$ and $Mg^{2+}$. Those specific characters provide useful information for full understanding the regulation mechanism of sturgeon sperm motility and artificial reproduction of such endangered species.

## Acknowledgments

This work was supported by several research grants CZ.1.05/2.1.00/01.0024, IAA608030801, 523/08/0824, ME10015, QH92308, QH82119, 046/2010/Z, 047/2010/Z and KJB608030901.

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