Intracellular changes in Ca^{2+}, K^{+} and pH after sperm motility activation in the European eel (*Anguilla anguilla*): preliminary results

V. Gallego[a,b], F. Martínez-Pastor[b], I. Mazzeo[a], D.S. Peñaranda[a], M.P. Herráez[b], J.F. Asturiano[a] and L. Pérez[a*]

[a] Grupo de Acuicultura y Biodiversidad. Instituto de Ciencia y Tecnología Animal. Universitat Politècnica de València. Camino de Vera s/n, 46022 Valencia, Spain.

[b] INDEGSAL and Molecular Biology. Universidad de León. Campus de la Vegazana s/n, 24071 León, Spain.

[*] Corresponding author:
Dra. Luz M. Pérez Igualada
Grupo de Acuicultura y Biodiversidad
Instituto de Ciencia y Tecnología Animal
Universitat Politècnica de València
Camino de Vera s/n 46022 Valencia (Spain)
email: mlpereig@dca.upv.es
Phone: +34 96 387 9752
Fax: +34 96 387 7

Abstract

Although it is widely accepted that osmolality and ion fluxes are the main factors triggering sperm motility in fish, a complex universal mechanism for sperm motility activation does not exist in fish, and studies of marine fish species are even more scarce. Therefore, the main goal of this study was to estimate the intracellular variations in the main ions involved in sperm activation for the first time in European eel, in order to provide additional new data about this little-known process.

It was observed that levels of intracellular Ca^{2+} and K^{+} sperm ions increased significantly 30 s after the hyperosmotic shock compared to baseline levels, and remained at this level until 120 s post-activation. In contrast, the intracellular pH remained constant during the first 30 s, and decreased gradually at 60 and 120 s post-activation. Our data agree with the current main theory for explaining motility activation in marine fish, in which internal fluctuations of Ca^{2+} and K^{+} seem to participate in sperm activation. In addition, fluorescent images showed that both Ca^{2+} and K^{+} were concentrated in the apical area of the sperm head, which corresponds to the location of the eel sperm mitochondria, suggesting this organelle plays an important role in sperm motility activation.

Keywords

Sperm motility; ion flux; calcium; potassium, pH, flow cytometry

1. Introduction

In marine teleosts, the spermatozoa are quiescent in isotonic solutions, such as seminal plasma, and become motile when the sperm is diluted in hypertonic solutions, suggesting that motility is suppressed by the osmolality of the seminal plasma, and initiated by exposure to hypertonic seawater at spawning (Morisawa and Suzuki, 1980, Cosson, 2004, Morisawa, 2008). The osmotic shock faced by the spermatozoa when they are released into the marine environment leads to a rapid flux of ions and water between intracellular compartments and external medium (Oda and Morisawa, 1993; Zilli et al., 2009). It has been proposed that Ca^{2+} and K^+ are the main ions involved in sperm motility activation in marine fish (see reviews of Morisawa, 2008, Cosson, 2008), but the exact mechanism through which this happens is still unknown. Although both in marine and freshwater fish species an increase in intracellular Ca^{2+} has been observed after osmotic sperm motility activation, it is not clear if that increase comes from an influx of Ca^{2+}, as has been proposed in seawater tilapia (*Oreochromis mossambicus*; Linhart et al., 1999), if it comes from intracellular stores, as it has been proposed in the case of puffer fish (*Takifugu niphobles*) and salmonid sperm (Krasznai et al., 2003; Takei et al., 2012), or from a decrease in cell volume due to the water efflux, as has been proposed by Zilli et al. (2008) in sparids species sperm. The first step to elucidate this mechanism in European eel sperm is studying intracellular Ca^{2+} variations in quiescent sperm and then hyperosmotic activated motility.

Changes to intracellular potassium levels after sperm activation have been measured in a limited number of freshwater fish species: two salmonids (Tanimoto et al., 1994), and common carp (Krasznai et al., 2003) sperm, where a K^+ efflux or a decrease in $[K^+]_i$ after hypoosmotic activation was observed. In marine fish species, $[K^+]_i$ changes after sperm activation have only been studied in the case of the pufferfish, and an increase in $[K^+]_i$ was observed after hyperosmotic activation (Takai & Morisawa, 1995, Krasznai et al., 2003). It is unknown whether hyperosmotic activation in the sperm of other marine fish causes an intracellular increase in K^+, like in pufferfish, or a decrease, like in trout and carp.

In sea urchin (Lee et al., 1983) and mammals (Wong et al., 1981; Babcock et al., 1983), the alkalinization of intracellular pH induces sperm activation. In carp a pH increase was also observed after hypoosmotic sperm activation (Krasznai et al., 1995), but trout sperm undergo an acidification upon hypoosmotic activation (Boitano and Omoto,

1991). An increase in pH_i was observed at motility activation in the sperm of several marine fish species (Oda and Morisawa, 1993). Therefore, there is no current consensus on the intracellular pH changes related to sperm activation in fish species. We observed that extracellular pH was important for sperm motility in European eel; while acidic pH extenders (pH 6.5) induced a reversible motility inhibition, that was not observed when sperm were maintained in an extender at physiological pH (8.5) (Peñaranda et al., 2010).

Thus, this study was designed to observe the changes in intracellular Ca^{2+}, K^{+}, and pH after motility activation in European eel spermatozoa. In this study we used flow cytometry to describe the variations of these factors from the immotile stage to the motile stage, at different times after the initiation of sperm motility, with the aim of establishing the first hypothesis on motility activation in this species.

2. Materials and methods

2.1 Fish handling and sampling

Fifteen adult eel males (100±2 g; 40±5 cm) from the fish farm Valenciana de Acuicultura, S.A. (Puzol, Valencia; East coast of Spain) were moved to the aquaculture facilities at the Universidad de León (Spain). The fish were distributed in three 60-L aquaria (5 males per aquarium) equipped with separate recirculation systems, thermostats and covered to maintain constant darkness. The eels were gradually acclimatized to artificial seawater (Aqua Medic Meersalz, 37 g/l) and once a week they received an intraperitoneal injection of hCG (1.5 IU g^{-1} fish; Argent Chemical Laboratories, USA), after being anesthetized (benzocaine, 60 ppm) and individually weighed. The total treatment lasted 14 weeks, and sperm samples were obtained from the sixth week until the end of the treatment. The fish were fasted throughout the experiment and were handled in accordance with the European Union regulations concerning the protection of experimental animals (Dir 86/609/EEC).

2.2 Sperm collection and evaluation of motility

Sperm samples were collected 24 h after the administration of the hormone to obtain the highest quality sperm (Pérez et al., 2000). The eels were anesthetized, and the genital area was first cleaned with distilled water and then thoroughly dried to avoid contaminating the sperm with faeces, urine or seawater. Then, the sperm were collected

by applying gentle abdominal pressure with the help of a small modified aquarium air pump which produced a vacuum breathing force to help collect the sperm in plastic Falcon tubes. Sperm samples were kept at 4 ºC until the motility analyses, which were performed less than 1 hour after collection. Sperm motility was assessed subjectively in triplicate by a trained observer, after mixing 1 µl of sperm with 200 µl of artificial seawater (SW; Aqua Medic Meersalz, 37 g/l, with 2% BSA (w/v), pH adjusted to 8.2) and observed under a microscope in a glass slide. Samples having >50% of motile cells were selected to study intracellular ionic changes. They were diluted 1:100 in P1 medium (in mM: NaCl 125, $NaHCO_3$ 20, KCl 30, $MgCl_2$ 2.5, $CaCl_2$ 1, and pH 8.5; Peñaranda et al., 2010) and maintained at 4 ºC until the flow cytometry analysis.

2.3. Flow cytometry

The relative amounts of different ions were determined by flow cytometry using a CyAn ADP Flow Cytometer (Beckman Coulter, Brea, CA) equipped with an argon ion laser. Slightly angled scattered front light was used for the electronic gating of data collection, allowing us to exclude dead cells from the analyses.

2.3.1 Incubation protocol

For Ca^{2+}, a stock solution of 1mM Fluo-4 AM (Invitrogen F14201) in DMSO was kept at -20 ºC until use. A sample of 100 µl diluted sperm were incubated with 0.5 µl Fluo-4 AM (final concentration 5 µM) at RT (20 ºC) for 30 minutes.

For K^+, a stock solution of 1mM PBFI AM (Invitrogen, P1267) in DMSO was kept at -20 ºC until use. A sample of 100 µl diluted sperm were incubated with 0.5 µl PBFI AM (final concentration 5 µM) at RT (20 ºC) for 90 minutes.

For pH, a stock solution of 1mM Snarf-5F AM (Invitrogen, S23923) in DMSO was kept at -20 ºC until use. A sample of 100 µl diluted sperm were incubated with 0.5 µl Snarf-5F AM (final concentration 5 µM) at RT (20 ºC) for 45 minutes.

To exclude dead cells from the analysis, the spermatozoa were also incubated with TO-PRO®-3 (Invitrogen T7596) to reach a final concentration of 2 µM. Final DMSO concentrations in sperm were less than 0.05 % in all the cases, and therefore a DMSO effect on motility could be discarded.

2.3.2 Determination of intracellular Ca^{2+}, K^+ and pH

After the incubation time, 5 µl of sperm sample was added to a tube containing P1 medium (500 µl) to measure the fluorescence emitted by the specific ion in the quiescent stage. Later, 5 µl of sperm sample was added to a tube containing activation medium (seawater, 500 µl) and the fluorescence emitted by sperm cells at 30, 60 and 120 s after activation was recorded

Fluo-4 AM and Snarf-5F AM were both excited by the blue laser (488 nm), and their fluorescence was read with the FL1 (530/40BP filter) and FL4 (680/30BP filter) photodetector, respectively. PBFI AM was excited by ultraviolet light (340 nm) and its fluorescence was read with the FL6 photodetector (450/50BP filter). TO-PRO-3 was excited by the use of red laser (635 nm), and its red fluorescence was read with the FL8 photodetector (665/20BP filter). The fluorescence data was displayed in logarithmic mode. Ten thousand events were collected per sample, with a flow rate of 200 cells/s, using a gate in forward and side scatter to exclude debris and aggregates from the analysis.

2.4 Location of Ca^{2+} and K^+ in quiescent spermatozoa

An aliquot of sperm samples incubated with Fluo-4 AM or PBFI AM (see section 2.3.1) was used to obtain microphotographs through a Nikon Eclipse E600 microscope. UV-2A (ultraviolet excitation and blue emission) and B-2A (blue excitation and green emission) filters were used to obtain Ca^{2+} and K^+ images, respectively.

2.5 Statistical analysis

Weasel software (WEHI, Victoria, Australia) was used to analyze the data obtained by flow cytometry. After removing dead spermatozoa (TO-PRO®-3) from the analysis, the mean fluorescence intensity (MFI, arbitrary units) was obtained from each sample. Statistical analyses were performed using the statistical package SPSS version 19.0 for Windows software (SPSS Inc., Chicago, IL, USA). Shapiro-Wilk and Levene tests were used to check the normality of data distribution and variance homogeneity, respectively. One-way analysis of variance (ANOVA) was used to analyze data with normal distribution. Significant differences were detected using the Tukey multiple range test ($P<0.05$). For non-normally distributed populations, Kruskal-Wallis one-way ANOVA on ranks was used.

3. Results

3.1 Intracellular variations of Ca^{2+}, K^+ and H^+

The fluorescence emitted by intracellular Ca^{2+} ($[Ca^{2+}]$), K^+ ($[K^+]$) and H^+ (pH_i) were estimated for quiescent sperm and after hyperosmotic motility activation. Intracellular $[Ca^{2+}]$ increased significantly 30 s after sperm activation compared to the baseline levels, and remained at this level until the end of activation (120 s) (Figure 1A). A similar pattern was observed with $[K^+]_i$ (Figure 1B), which increased significantly after sperm activation and remained constant thereafter. However, the $[Ca^{2+}]_i$ increase was higher than the $[K^+]_i$ increase. In contrast to Ca^{2+} and K^+, pH_i (Figure 1C) level remained constant after sperm activation (30 s), only showing a significant decrease at 60 and 120 s post-activation.

3.2 Intracellular distribution of Ca^{2+} and K^+

Figure 2 shows the fluorescence emitted by the intracellular calcium and potassium located in quiescent eel spermatozoa. High fluorescence intensity for both Ca^{2+} and K^+ can be observed in the apical zone of the sperm head, which corresponds to the location of the eel mitochondria.

4. Discussion

There is little understanding of the molecular mechanism which happens in marine sperm cells after hyperosmotic shock, and ion fluxes or variations in the concentration of several ions could act as triggers of sperm motility.

We have shown for the first time that intracellular calcium and potassium levels increased with the hyperosmotic activation of sperm motility in European eel. Such increases after osmotic shock have previously been observed in another marine fish species, the pufferfish (Oda and Morisawa, 1993), but in salmonid species (Tanimoto et al., 1994) and carp (Krasznai et al., 2003), an increase in $[Ca^{2+}]_i$, but a decrease in $[K^+]_i$ was observed after hypoosmotic sperm activation. So, internal Ca^{2+} and K^+ fluctuations seem to participate in the initiation of motility in European eel sperm, agreeing with the Morisawa's (2008) hypothesis which explains motility activation in marine fish.

Regarding Ca^{2+}, it has been reported that this ion plays an important role in the control of the axonemal movement in some marine species (Zilli et al., 2012). The flagellum

can change its beating pattern in response to Ca^{2+} concentrations (Cosson et al., 2008), and thus, Ca^{2+} fluctuations could regulate the spermatozoa's kinetic features (Brokaw, 1991; Cosson et al., 2008). Oda and Morisawa (1993) reported that in the case of pufferfish (*Takifugu niphobles*) the addition of Ca^{2+} ionophore to quiescent spermatozoa induced motility activation in the same manner as in the seawater, suggesting that an increase in $[Ca^{2+}]_i$ is sufficient for the activation of sperm motility.

Regarding K^+, there are not many studies about the effect of this ion on sperm motility in marine fish species. In Atlantic croaker (*Micropogonias undulatus*), K^+ channel blockers reduced the percentage of motile cells (Detweiler and Thomas, 1998). In puffer fish it has been recently demonstrated that $[K^+]_i$ increased after sperm activation even after activation in potassium-free activation media (Gallego et al, 2013). Therefore, the increase of $[K^+]_i$ at the initiation of eel sperm motility could be one of the triggers for sperm motility activation, as occurs in puffer fish, while in carp a decrease in intracellular K^+ was observed after hypoosmotic activation (Krasznai et al., 2003).

In relation to intracellular pH (pH_i), the baseline levels remained constant after 30 s post-activation and a gradual decrease was observed after 60 s post-activation. Our results agree with the data published by Oda and Morisawa (1993) about two marine fish species, in which after an initial increase in intracellular pH, the levels return to the baseline levels 30 s post-activation. Our first pH_i reading was taken 30 s post-activation. Therefore, we do not know if a pH increase occurs in the first few seconds after activation in eel sperm, as is the case with pufferfish. Also, our results agree with those of Boitano & Omoto (1991), regarding rainbow trout sperm, where an intracellular acidification upon sperm hypoosmotic activation was observed. These authors considered that changes in intracellular pH do not regulate trout sperm motility, contrarily to what happens in other species. The slow decrease in pH over time observed in the present study suggests that this change could be a consequence of motility activation, rather than a trigger for sperm motility initiation. The observed pH_i decrease might simply be the result of the mitochondrial sperm respiration during the active movement.

Finally, regarding the ion distribution in quiescent European eel sperm, fluorescence images showed that Ca^{2+} and K^+ were located mainly in the mitochondrion, which in this species is a single, small and round organelle, located in the apex of the spermatozoa head, opposite the axoneme (Marco-Jiménez et al., 2006). The accumulation of calcium stores in the mitochondrion has previously been observed in

human sperm (Costello et al., 2009), but potassium accumulation has not been reported in any other species studied so far. However, the existence of specific potassium channels in the mitochondria of several animal tissues (liver, heart, brain, kidney, skeletal muscle, human T lymphocytes and amoeba; review in Szewczyk et al., 2009) indicates that a differential concentration of K^+ between this organelle and the cytoplasm is possible in these tissues. It is not known whether sperm mitochondria possess such K^+ channels, but the accumulation of the ion in this organelle therefore suggests an important role of the eel sperm mitochondria in the function of the sperm.

In conclusion, intracellular concentrations of Ca^{2+} and K^+ ions in sperm increase after hyperosmotic sperm activation in European eel, with a progressive decrease in intracellular pH. Fluorescence images suggest an accumulation of both ions in the mitochondrion, which could represent ion stores. However, further studies including the use of ion channels blockers, activation media with/without specific ions, as well as changes in cell volume, may be necessary to determine the fluxes of these ions and their role in motility activation on the European eel sperm.

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Figure legends

Figure 1. Intracellular ion concentrations on pre- and post-activation times (30, 60 and 120 s) in European eel spermatozoa: a) Ca^{2+}; b) K^{+} and c) pH. Asterisks indicate significant differences with baseline pre-activation levels.

Figure 2. Pictures show (a) Ca^{2+} and (b) K^{+} distribution on quiescent European eel spermatozoa. Arrows indicate mitochondrion.

Figure 1

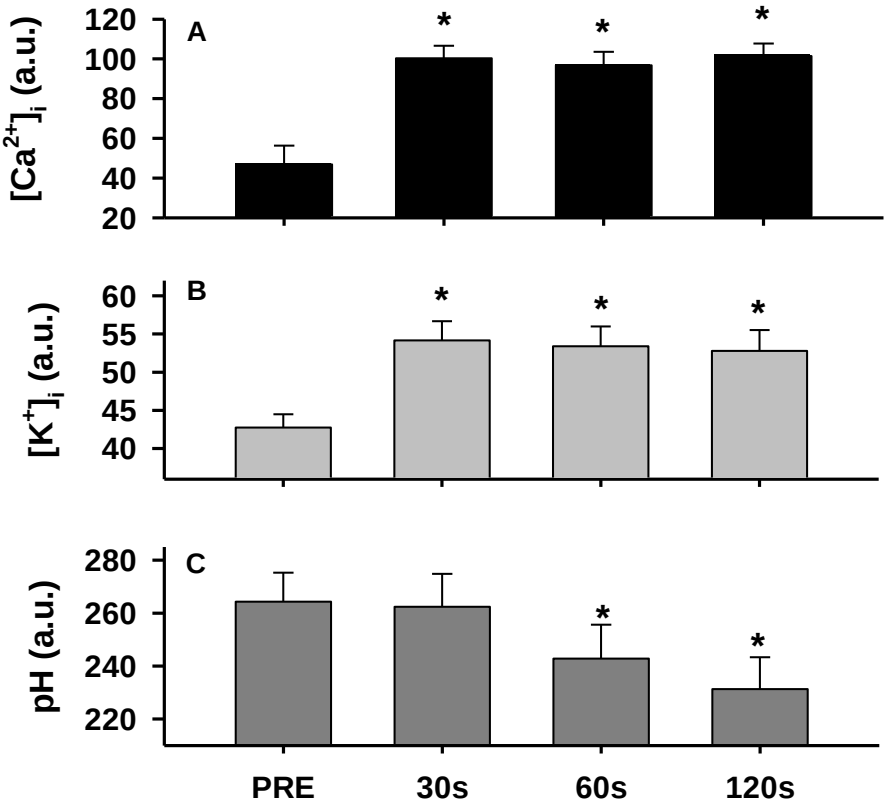
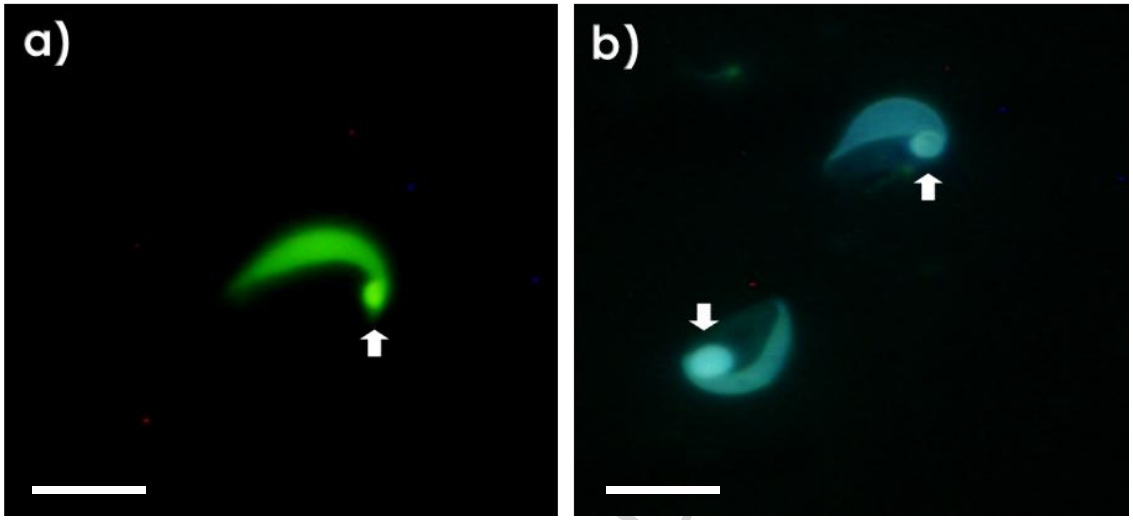


Figure 2



Highlights

- ✓ Main ions involved in sperm activation were analysed for the first time in European eel.
- ✓ Intracellular Ca^{2+} and K^{+} increased significantly after activation.
- ✓ Intracellular pH remained constant during the first 30 s, decreasing at 60 s.
- ✓ Fluorescent images showed that both Ca^{2+} and K^{+} were concentrated in mitochondria.