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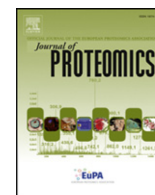

Motility of carp spermatozoa is associated with profound changes in the sperm proteome

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ABSTRACT

In freshwater cyprinids, spermatozoa are quiescent in seminal plasma and sperm motility is initiated by a decrease in osmolality (hypo-osmotic shock) after discharge into the aqueous environment. However, it is unknown at present if and to what extent changes in proteins are involved in carp sperm motility. Therefore, the aim of our study was to identify proteins related to carp sperm motility through a comparison of immobilized and activated carp spermatozoa using a 2D-DIGE approach. Our results, for the first time indicated that carp sperm motility is associated with changes in protein content. Seventy-two differentially expressed proteins were identified. These proteins are mainly involved in ubiquitin–proteasome pathways, glycolysis, the TCA cycle, remodeling and are putatively related to sperm energy metabolism and motility. Moreover proteins associated with oxidative stress responses, signal transduction by Ca^{2+}-dependent MAPK cascades, and PKC and protein folding have been identified. The proteins involved in carp sperm motility were localized to the cytoplasm, mitochondria, cytoskeleton, nucleus and sperm membrane. The identification of a high number of proteins involved in carp sperm motility would contribute to current knowledge about the molecular mechanisms of sperm motility in freshwater fish.

Biological significance: To the best of our knowledge, few changes in proteins involved in the initiation of fish sperm motility have been identified. This is a limited number of proteins compared with the 80 recently identified proteins involved in human sperm motility. However, no proteomic studies of sperm motility have yet been performed on freshwater fish. Our present study allowed for the first time a comprehensive characterization of the proteins associated with carp sperm motility and a better understanding of the molecular mechanisms underlying sperm motility activation and maintenance. The application of 2D-DIGE facilitated the identification proteins crucial for sperm structural organization and motility. The identification of a high number of proteins involved in carp sperm motility would contribute appreciably to the presently limited information available on the mechanisms of sperm motility in freshwater fish. Moreover the identified list of proteins will create a platform for future studies designed to assess the functional significance of specific proteins in sperm motility.

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1. Introduction

The spermatozoa of most teleost fish are quiescent in the testes and spermatic duct [1]. The seminal plasma, the osmolality and composition of which, depending on the species, protects and immobilizes spermatozoa until they are released into the aqueous environment and their motility is induced (sperm activation). Changes in the ionic and osmotic environment of the sperm cells are critical external factors responsible for sperm motility [2]. In salmonids [3–5] and sturgeon [6,7], the reduction of the external K^+ concentration upon the dilution of semen initiates sperm motility. Hypertonic exposure initiates sperm motility in many saltwater fish [8], while in freshwater cyprinids flagellar motility is initiated by hypo-osmotic shock [8,9]. Aside from potassium ions and osmolality, calcium ions also play a key role in the initiation of sperm motility in many teleost fish species, including carp [10,11].

After the receipt of extracellular signals from specific ion channels or receptors in the spermatozoa, axoneme is activated by signal transduction across the plasma membrane. Second messengers, such as cAMP and Ca^{2+}, play key roles in the initiation of sperm motility in fish [12, 13]. The contribution of cAMP-dependent signaling pathway has been reported to regulate the motility of sperm in salmonid fish [13,14], paddlefish [15], and sea bream [16]. This mechanism involves a complex series of phosphorylation and dephosphorylation events, such as cAMP-dependent phosphorylation of the 15-kDa movement initiating phosphoprotein [17], protein kinase A [14] and a 22-kDa dynein light chain [18]. The involvement of calcium/calmodulin-dependent protein phosphorylation in the regulation of flagellar axoneme was also demonstrated in

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puffer fish, tilapia and carp sperm [9,12,19]. In addition, it has been demonstrated that proteasomes and aquaporins play important roles in fish sperm motility [18,20,21]. Nonetheless, it should be underlined that a complex, universal mechanism for sperm motility has not been identified in fish; rather, different species-specific mechanisms have been described.

To the best of our knowledge, few changes in the proteins involved in the initiation of fish sperm motility have been identified. A proteomic approach was introduced to study the activation of sperm motility in saltwater fish, such as gilthead and stripped sea bream, and allowed for the identification of three proteins in gilthead sea bream (A-kinase anchor protein, acetyl-CoA synthetase, a protein similar to phosphatase and actin regulator 3) and two proteins in stripped sea bream (myotubularin-related protein 1 and dual-specificity tyrosine phosphorylation-regulated kinase 3) that play key roles in the initiation of sperm motility [16]. This is a limited number of proteins compared to the 80 recently identified proteins involved in human sperm motility [22]. To the best of our knowledge, no proteomic studies of sperm motility have yet been performed on freshwater fish.

Recently, we characterized the proteome of carp and rainbow trout spermatozoa and found that the majority of these proteins were functionally related to sperm motility [23,24]. However, it is unknown at present whether protein changes occur during the sperm motility period. Therefore, the aims of the present study were to examine the changes in carp sperm proteome between the immobilized and activated spermatozoa using a two-dimensional difference gel electrophoresis (2D-DIGE) approach, in order to identify proteins that undergo abundant changes during carp sperm motility activation and maintenance and to predict the biological functions and cellular pathways of the proteins involved in the regulation of carp sperm motility.

2. Materials and methods

2.1. Semen collection

The milt of common carp (*Cyprinus carpio* L) was obtained from fish maintained at the Institute of Ichthyobiology and Aquaculture of the Polish Academy of Sciences in Gołysz, Poland. Twenty-four hours before the collection of the carp semen, the males were injected intradorsally with Ovopel (one pellet containing of 18–20 μg of GnRH analog and 8–10 mg of metoclopramide per kilogram of fish bw; Interfish Ltd., Hungary). The milt was obtained at the midpoint of the spawning season (July 2nd 2014) from 5 to 7 year old carp (n = 6) by gentle abdominal massage, taking care not to pollute it with blood, feces or urine. Samples with a spermatozoa content $>10 \times 10^9$ mL^{-1} and >80% motile spermatozoa were selected for proteomic analysis.

Approval (53/In/IRZiBŻ PAN/2011) by the Animal Experiments Committee in Olsztyn, Poland was gained before starting any experiments.

2.2. Sperm activation and preparation of sperm extract

Semen from six carp males was diluted with immobilizing solution 1:4 (94 mM NaCl, 27 mM KCl, 50 mM glycine, 15 mM Tris–HCl, pH 7.5; [25], then one part was activated with hatchery water at a 1:19 ratio while the other was diluted at the same ratio with immobilizing solution. After 30 s, because it has been established that carp sperm motility is very short in fresh water (30-40 s [26]), samples were centrifuged at 3000 g for 10 min at 4 °C. After the removal of the supernatant, the pellets (containing spermatozoa) were suspended in protein extraction buffer (immobilizing solution with 0.1% Triton) and sonicated on ice 6 times for 5 s at 30% amplitude using a VCX-130 Ultrasonic Processor (Sonics & Materials, Inc., Newtown, CT, USA). After sonication, the solution was kept on ice for 1 h and centrifuged for 10 min at 14,000 *g* at 4 °C. Protein lysates were stored at −80 °C until analysis.

Before the proteomic analysis, aliquots containing approximately 800 μg of sperm proteins were processed using a Clean-Up Kit (GE Healthcare, Uppsala, Sweden) according to the manufacturer's protocol. Samples were resuspended in 80 μL of lysis buffer to a protein concentration of 5–10 mg/mL. The protein concentration prior to and after the cleaning procedure was measured by a Coomassie (Bradford) Protein Assay Kit (Thermo Scientific, Waltham, MA, USA).

2.3. Determination of sperm motility and concentration

Immediately after sperm activation, 0.7 μL of sperm subsamples were placed into a well of a 12-well multitest glass slide and covered with a coverslip. The sperm motility parameters were as follows: MOT — percentage of motile sperm (%),VCL — curvilinear velocity (μm s^{-1}), VSL — straight line velocity (μm s^{-1}), VAP — average path velocity (μm s^{-1}), LIN — linearity (%, 100 × VSL/VCL), and ALH — amplitude of lateral head displacement (μm) were analyzed for a 15–30 s post-activation period using the Hobson Sperm Tracker (Hobson Vision Ltd., Baslow UK) with settings as described by Wojtczak et al. [27]. Each sample was analyzed in duplicate. Sperm concentration was measured using a spectrophotometric method [28].

2.4. Fluorescent labeling of immobilized and activated spermatozoa samples with CyDyes and 2D-DIGE

The sperm from the same individual was used for both immobilized and activated sperm. Aliquot of 50 μg of protein from each sample (immobilized and activated spermatozoa extract) were dissolved in a labeling buffer (7 M urea, 2 M thiourea, 4% *w*/*v* CHAPS, 30 mM Tris, pH 8.0) and labeled with CyDye DIGE Fluor minimal dyes (GE Healthcare, Uppsala, Sweden; reconstituted in fresh 99.8% anhydrous DMF) at a concentration of 400 pmol dye/50 μg of protein. Cy 2 was used to label the internal standard, and Cy3 and Cy5 to label individual immobilized and activated sperm samples. A dye swap (Cy3/Cy5) was performed between activated and immobilized sperm samples (Table 1) to exclude dye bias. An internal standard was created by mixing equal amounts of protein from the immobilized and activated spermatozoa. The labeling reaction was performed in the dark on ice for 30 min.

Differentially labeled samples (50 μg each of Cy2-, Cy3-, and Cy5-labeled samples) were mixed together according to the scheme presented in Table 1. In each gel therefore extract from immobilized (Cy3 or Cy5) and activated spermatozoa (Cy5 or Cy3) from the same individual male and internal standard (Cy2) were separated. Rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 130 mM DTT, 2% Pharmalyte, pH 3–10 NL and 0.002% bromophenol blue) was then added to each sample mixture to reach a final volume of 350 μL and loaded onto IPG strips (18 cm; pH 3–10 NL; GE Healthcare) with passive rehydration (18 h). Then, proteins were separated by isoelectric focusing on an Ettan IPGphor apparatus (GE Healthcare) operating at 20 °C with the current limited to 50 μA per strip and the following voltage program: 100 V/1 h, 500 V/2 h, 500 V/4 h, a linear gradient to 1000 V over 1 h and a linear gradient to 8000 over 3 h, then an 8000 V constant for 2.5 h. After isoelectric focusing, the strips were equilibrated in SDS equilibration buffer (6 M urea, 75 mM Tris–HCl, pH 8.8, 29.3% glycerol,

Table 1
Mixing and Cy dying scheme of immobilized and activated carp spermatozoa. Sperm from the same individual was used for both immobilized and activated sperm (n = 6 for each group).

Gel no.	Cy2 (50 μg)	Cy3 (50 μg)	Cy5 (50 μg)
1	Pooled standard	Immobilized sperm 1	Activated sperm 1
2	Pooled standard	Immobilized sperm 2	Activated sperm 2
3	Pooled standard	Immobilized sperm 3	Activated sperm 3
4	Pooled standard	Activated sperm 4	Immobilized sperm 4
5	Pooled standard	Activated sperm 5	Immobilized sperm 5
6	Pooled standard	Activated sperm 6	Immobilized sperm 6

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