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The optimization of the protocol for immunofluorescence on fish spermatozoa

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Abstract

In comparison with mammals, the fertilization of fish occurs predominantly outside the organism in a water environment, where fish spermatozoa require specific conditions to interact with oocytes. It is evident that optimal conditions for fish and mammalian spermatozoa are quite different. This paper describes a special approach to handling fish (common carp and Siberian sturgeon) spermatozoa in comparison with the samples originating from mammals (boar). This approach concerns not only the differences in the composition of the media applied but also primarily emphasizes the concrete parts of the immunofluorescence protocol determining accurate results. Individual parts of the protocol for indirect immunofluorescence of mammalian sperm were changed step by step and modified protocols were applied to immunofluorescence experiments with carp and sturgeon spermatozoa. By evaluating the changes in the integrity of the fish sperm head and flagellum, we selected the steps and corresponding conditions that are crucial for handling the fish spermatozoa. Based on our results, it may be concluded that when working with fish spermatozoa, the cells attached to the microscopic slides must not desiccate prior to the fixation, which is a usual step when working with mammalian sperm. The second crucial step is the necessity to fix the fish spermatozoa, especially when the research is focused on the structure of the flagellum. The impact of the temperature conditions is rather low, but working at low temperatures, except for the period of incubation with antibodies, leads to a higher number of unaffected cells.

Keywords: Carp sperm; Sturgeon sperm; Boar sperm; Immunofluorescence

1. Introduction

To understand the processes occurring in the course of fertilization and to be able to determine the fertilization competence, it is necessary to study all the components participating in the gamete development and fusion. For proper evaluation of the function and quality of these components, it is essential to use the appropriate approaches in biological methods.

In our previous research, we especially studied sperm cytoskeleton [1], capacitation [2,3], and seminal plasma proteins [4]. We focused mainly on mammalian models (human, boar, mouse); however, we have also recently started to pay attention to fish. Two main fields of our activity concentrate on the study of proteins that participate in the motility of chondrostean and teleostean fish and on the usefulness of the acrosome for the spermatozoa of chondrostean fish. Immunofluorescence experiments with newly prepared monoclonal antibodies (MoAbs) can be a useful tool for the

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study of these proteins, as we found in our experiments on mammalian models [5–8]. However, when dealing with fish spermatozoa, we faced many difficulties as the mammalian protocols were followed.

The structure of mammalian and fish spermatozoa differs in its complexity and organization. While the fish spermatozoa are rather simple, the mammalian sperm contain various additional structures, such as typical acrosome in the sperm head and fibrous sheath and outer dense fibers in the flagellum [9]. In our experiments we used spermatozoa of the common carp (*Cyprinus carpio*) and Siberian sturgeon (*Acipenser baerii*) as representatives of teleostean and chondrostean fish, respectively. The structure of the spermatozoa of chondrostean fish is more complicated in comparison to the teleostean species which are even phylogenetically farther from mammals.

The commonly used protocols for immunocytochemical experiments on mammalian spermatozoa [7,8] failed to meet our requirements for both undamaged sperm flagellum and intact head. After following special immunocytochemical protocols for fish spermatozoa [10–12], the number of affected cells was reduced; however, variances according to the individual protocols were still observed. Therefore, our study was focused on the optimization of those steps that are crucial for the preservation of unaffected fish spermatozoa and on the improvement of the protocol for handling fish sperm during immunofluorescence experiments. To evaluate the changes in the integrity of the fish sperm head and flagellum with respect to the various experimental conditions we used labeling with the monoclonal antibody (MoAb) against α-tubulin and DAPI staining.

2. Materials and methods

2.1. Sperm preparation

Common carp (*Cyprinus carpio*) and Siberian sturgeon (*Acipenser baerii*) sperm (provided by Prof. Linhart, D.Sc., Research Institute of Fish Culture and Hydrobiology, Vodnany, CR) were washed twice (centrifugation at $200 \times g$ for 30 min at 4 °C) in carp washing solution (CWS, 200 mM KCl, 30 mM Tris–HCl, pH 7.0, 402 mOsmol/kg) [13,14] and sturgeon washing solution (SWS, 2 mM KCl, 30 mM Tris–HCl, pH 7.5, 59 mOsmol/kg), respectively.

Boar (*Sus scrofa*) sperm (ejaculates were obtained from the Insemination Station, Klimetice, CR) were washed three times (centrifugation at $400 \times g$ for 15 min at RT) in PBS (20 mM phosphate buffer, 0.15 M NaCl, pH 7.4, 319 mOsmol/kg) [6,7]. The suspensions of washed spermatozoa were diluted to the concentration of 5×10^6 sperm cells/mL.

2.2. Immunocytochemistry

The protocol for indirect immunofluorescence labeling of boar sperm proteins [6,7] was divided into nine steps. We obtained 15 variations by the subsequent omission of selected steps and by changing the temperature conditions during the individual parts of the protocol (five variants (A–E) were multiplied by three variants (1–3), Table 1). Modified protocols were applied to immunofluorescence experiments with boar, carp and sturgeon spermatozoa.

Table 1
The protocol variations

The steps of immunofluorescence protocol		Protocol variations (according to the combinations of steps)					Protocol variations (according to the temperature conditions)		
		A[a]	B	C[b]	D	E	1[a]	2[b]	3
Step 1	Attachment	+	+	+	+	+	RT	4 °C	4 °C
Step 2	Desiccation	+	+	−	−	−	RT	4 °C	4 °C
Step 3	Fixation	+	−	+	−	+	RT	4 °C	4 °C
Step 4	Desiccation	−	−	−	−	+	RT	4 °C	4 °C
Step 5	Permeabilization	+	+	+	+	+	RT	4 °C	4 °C
Step 6	Blocking	+	+	+	+	+	RT	4 °C	4 °C
Step 7	Incubation with primary ab.	+	+	+	+	+	1 h/37 °C	1 h/37 °C	16 h/4 °C
Step 8	Incubation with secondary ab.	+	+	+	+	+	1 h/37 °C	1 h/37 °C	8 h/4 °C
Step 9	Mounting	+	+	+	+	+	RT	RT	4 °C

+/− the step was/was not included in the protocol variation.
[a] Original protocol for immunofluorescence on mammalian sperm.
[b] Variation that causes the lowest damage to fish sperm during the immunofluorescence experiment.