Evaluation of the expression of sperm proteins in normozoospermic and asthenozoospermic men using monoclonal antibodies

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Recent studies have shown that infertility affects estimated 15% of all couples. Male infertility is the primary or contributory cause in 60% of these cases. Consequently, the application of assisted reproduction is increasing. These methods could benefit from an extended evaluation of sperm quality. For this reason, we analyzed sperm proteins from 30 men with normal spermograms and 30 men with asthenozoospermia. Ejaculates of both groups were tested by flow cytometry (FCM) and fluorescence with a set of well-characterized anti-human sperm Hs-monoclonal antibodies (MoAbs), which were generated in our laboratory. No statistically significant differences were found between normosperms and asthenosperms in the expression of the sperm surface protein clusterin, evaluated with Hs-3 MoAb, and semenogelin, evaluated with Hs-9 MoAb. However, FCM revealed quantitative differences in the acrosomal proteins between normozoospermic and asthenozoospermic men, namely, in glyceraldehyde-3-phosphate dehydrogenase, evaluated with Hs-8 MoAb, valosin-containing protein, evaluated with Hs-14 MoAb, and ATP synthase (cAMP-dependent protein kinase II, PRKAR2A), evaluated with MoAb Hs-36. Asthenozoospermic men displayed a highly reduced expression of intra-acrosomal proteins, with a likely decrease in sperm quality, and thus a negative impact on successful reproduction. Asthenozoospermia seems to be a complex disorder involving intra-acrosomal proteins.

Keywords: asthenozoospermia; flow cytometry; fluorescence microscopy; monoclonal antibodies; sperm proteins

INTRODUCTION

Lifestyle changes and exposure to various detrimental factors in the environment result in increased incidences of male reproductive dysfunctions. To reveal the causes of infertility in a man, classical semen analysis is carried out according to the World Health Organization (WHO) guidelines.¹ Semen parameters: sperm concentration, motility, viability, and morphology are assessed and the ejaculates are classified into four basic categories: normozoospermia (>15 × 10⁶ spermatozoa ml⁻¹, >40% motile spermatozoa and >32% spermatozoa with progressive motility, >4% spermatozoa with normal morphology), oligozoospermia (<15 × 10⁶ spermatozoa ml⁻¹, motility and morphology the same as normosperms), asthenozoospermia (<40% motile spermatozoa and <32% spermatozoa with progressive motility), and/or teratozoospermia (<4% spermatozoa with normal morphology), and their combinations, e.g., oligoasthenozoospermia, oligoasthenoteratozoospermia.

This evaluation provides only rough data and does not allow a more precise determination of the causes underlying infertility, especially in subfertile men and in men with idiopathic infertility.

Recently, new diagnostic tools – analysis of semen using antibodies to sperm proteins and proteomic analysis²⁻⁴ – have been introduced, and sperm assessment has definitely advanced to a molecular level. A number of antibodies have been prepared and proved to be useful in monitoring sperm processes and the role of individual proteins.⁵⁻¹¹

We generated monoclonal antibodies (MoAbs)¹²⁻¹⁶ and used them to test the expression of relevant proteins on spermatozoa. This approach can reveal changes in protein expression in men whose spermatozoa are not able to fertilize the egg in a natural way.

For sperm evaluation using antibodies, the method of choice is flow cytometry (FCM). FCM is a reliable, objective technique allowing evaluation of a large number of cells and a variety of parameters and functions.¹⁷⁻²⁰ In previous experiments, we used FCM for sorting the cell stages of spermatogenesis in infertile mice with chromosomal translocation²¹ and for the study of boar sperm capacitation.²² In this study, we applied MoAbs against human sperm proteins for the evaluation and comparison of the expression of these proteins in normospermic and asthenospermic men. Asthenozoospermia, the reduction of sperm motility, represents common sperm pathology in men. The concentration of sperm in the ejaculate and their morphology corresponds to normozoospermia, but the movement of sperm is changed, and their speed is reduced. The fertilizing capacity of asthenozoospermic men is restricted, and they seek help in centers for assisted reproduction.

Our objective was to determine by our MoAbs various sperm proteins and their differences between normozoospermia and asthenozoospermia to assess changes in protein detection in

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pathological sperm, and thus determine their importance in the reproduction process.

**MATERIALS AND METHODS**

**Human ejaculates**

Ejaculates were obtained with the donors’ consent from the Clinical Center ISCARE IVF (Prague, Czech Republic). The Institutional Review Board gave their consent to the proposed experiments. The average age of the men was 38 years old in the normospermic group (range 30–45 years) and 35 years old in the asthenospermic group (range 25–42 years). Semen samples were collected from men after 48–72 h of sexual abstinence and assessed according to WHO rules.1 Ejaculates of thirty normozoospermic (N) and thirty asthenozoospermic (A) men were used for the examination. The experiments included only men whose spermograms that repeatedly (in three consecutive tests) demonstrated normospermic or asthenospermic characteristics and whose ejaculate(s) contained <1 × 10⁹ ml⁻¹ lymphocytes.

Viability of sperm was assessed twice: after ejaculation, and before cytometric and immunofluorescence analysis. Average values before cytometric, and immunofluorescence measurement were: 78% live cells in normosperms and 75% live cells in asthenspermics. Sperm of the donors was used for in vitro fertilization (IVF). According to the physicians’ decision, IVF was carried out with the sperm of asthenspermics and normosperms by intracytoplasmic sperm injection (ICSI).

**Antibodies**

Monoclonal antibodies of the Hs-series, which were established in our laboratory against human sperm proteins, were used. Briefly, BALB/c mice were immunized with human spermatozoa or their extract. After immunization, fusion of immune spleen cells with myeloma cells followed. Positive clones were selected by enzyme-linked immunosorbent assay with human sperm extract. Specificity of the antibody was tested by immunofluorescence and immunodetection after electrophoresis and Western blotting of the human sperm extract. Preparation of MoAbs and their characterization are described in Capková et al. 2002,15 Peknicova et al. 2005,23 Capkova et al. 2009,15

The Hs-8, Hs-14, and Hs-36 antibodies detect sperm proteins localized intra-acrosomally. The Hs-3 and Hs-9 antibodies detect seminal plasma proteins that secondarily bind to the sperm surface. The characteristics of MoAbs are summarized in Table 1.

As secondary antibodies, we used Alexa Fluor 555 goat anti-mouse IgM (μ chain specific) and Alexa Fluor 488 goat anti-mouse IgG (H + L) (both Molecular Probes, Eugene, USA).

**Procedures**

The reaction of Hs-antibodies with human spermatozoa was visualized by indirect immunofluorescence (IIF) and evaluated under an IF microscope and by FCM. Before both procedures, the sperm samples were washed twice with phosphate-buffered saline (PBS, pH 7.4) and centrifuged at 200 g for 10 min.

**Flow cytometry analysis**

Each sperm sample was divided into parts A and B. Samples A were processed with a Fix and Perm Cell Permeabilization Kit (Grub Bio Research, Kaumberg, Austria) according to the manufacturer’s instructions. Briefly, cells were incubated for 20 min with each reagent of the permeabilization kit. Between applications of individual reagents the sperm were centrifuged, twice washed with PBS and after the last washing, each sample was diluted with PBS to a final volume of 1 ml. Permeabilized cells were used for the detection and evaluation of intra-acrosomal sperm proteins. Samples B were not permeabilized. These samples were used for the diagnostics of sperm membrane integrity and surface proteins. The sperm concentration in both samples was determined by a hemocytometry chamber and suspensions were distributed by 5 × 10⁶ per well into a 96-well plate, centrifuged at 200 g for 10 min and then the supernatant was removed. Two hundred microliter of MoAbs (diluted in PBS with 1% BSA to a final concentration of 5 μg Ig ml⁻¹) was added per well and samples were incubated overnight at +4°C in an orbital shaker.

Sperm control samples were also diluted in PBS with 1% BSA to a final volume of 200 µl per well. After incubation, the samples were centrifuged (200 g, 10 min, +4°C), washed twice with 200 µl of PBS, and 200 µl of 1000x diluted secondary antibody Alexa Fluor 555 IgM (for Hs-8, Hs-14, Hs-36) or Alexa Fluor 488 IgG (for Hs-3, Hs-9) was added to each well. As a control, we used samples without primary and secondary antibodies (evaluation of autofluorescence) or without primary antibodies with secondary antibodies only (negative control). Re-suspended samples were incubated for 1 h at 37°C in the dark, washed twice with PBS and diluted to a final volume of 150 µl per well. FCM data acquisition was performed on a BD LSR II instrument (Becton Dickinson and Company, NJ, USA), excitation lasers 488 nm (Coherent Saphire 488-20 DPSS, filter 525/50, DM 505LP) and 561 nm (Melles Griot 85-YCA-25, filter 585/15, DM 565LP) to measure the fluorescent intensity in the Alexa Fluor 488 and Alexa Fluor 555 channels. Analysis was performed using FlowJo 7.5.4. software (TreeStar Inc., Ashland, OR, USA). The differences among individual samples in the percentage of cells (incidents) above the set threshold level of fluorescence intensity were assessed and statistically compared. Ten thousand cells were analyzed per well with a flow rate of 3000 cells s⁻¹.

**Indirect immunofluorescence**

Washed cells were diluted in PBS to a final concentration of 1 × 10⁹ cells ml⁻¹ and 10 µl drops were placed on glass slides. Drops were air-dried and permeabilized for 10 min at room temperature (RT) with acetone. Slides were rinsed in PBS, blocked in PBS-0.05% Tween + 1% BSA (Serva, Heidelberg, Germany) + 10% normal goat serum.
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between N and A (Figure 1).

The percentage of the sperm stained by individual antibodies in N and A donors are shown in Supplementary Table 1.

Figure 2

Flow cytometry detection of relevant proteins on fixed (permeabilized) and nonfixed cells

The fluorescent intensity of Alexa 555 (or Alexa 488)-conjugated secondary antibody in normospermic (N) and asthenospermic (A) sperm samples is shown in FCM histograms (Supplementary Figure 1).

The percentage of the sperm stained by individual antibodies in N and A donors are shown in Figure 2.

The most significant differences in the percentage of labeled spermatozoa were found in fixed sperm cells with antibodies against acrosomal proteins. Statistical analysis showed a significantly reduced expression of proteins detected with Hs-8 (P < 0.01), Hs-14 (P < 0.001), and Hs-36 (P < 0.05) antibodies in asthenozoospermics compared to normozoospermics (Figure 2a).

Furthermore, Hs-3 and Hs-9 antibodies against sperm surface proteins labeled a lower percentage of fixed sperm in asthenozoospermics than in normozoospermics, but the differences were not statistically significant for any of these two antibodies (Figure 2a).

The expression of the relevant proteins was also investigated in nonfixed cells. No statistically significant differences were found in the percentage of cells labeled with Hs-anti-acrosomal or Hs-anti-sperm adhesive antibodies between N and A (Figure 2b). As expected, the Hs-8, Hs-14, Hs-36 antibodies labeled a lower percentage of nonfixed sperm (about 20%–30%) than the fixed ones. The Hs-3 antibody labeled an approximately equal percentage of fixed and nonfixed cells and Hs-9 labeled more fixed cells (roughly 40%–70%) than the nonfixed ones (roughly 15%–30%).

Correlation of Hs-8, Hs-14 and Hs-36 reactions with sperm of the same donor

The correlation between the percentage of sperm cells stained by individual anti-acrosomal antibodies was analyzed in the individual donors (Figure 3).

A statistically significant correlation was found between the percentage of sperm labeled by Hs-8 and Hs-14 in the normospermics (r = 0.67, P < 0.001) and the asthenospermics (r = 0.63, P < 0.001), but no significant correlation was found between the percentage of the cells stained by Hs-8 and Hs-36 in the normospermics and the asthenospermics, and between Hs-14 and Hs-36 in the normospermics. In these cases, no statistically significant differences between the individual Pearson correlation coefficients in normospermic and asthenospermic groups were detected (delta r test). The only difference between r close to the significant level was found in the correlation between Hs-14 and Hs-36 antibodies (P = 0.0614) in the asthenospermics (Figure 3 and Supplementary Table 1).

Correlation of anti-acrosomal and anti-surface protein reactions with sperm of the same donor

The correlation between the percentages of sperm cells stained by individual anti-acrosomal and anti-surface protein antibodies was analyzed in the individual donors (Supplementary Table 1).

A statistically significant correlation was found between the percentage of sperm labeled by Hs-8 and Hs-3 (r = 0.46, P < 0.01) and Hs-36 and Hs-3 (r = 0.61, P < 0.001) in the normospermics. In asthenospermics, the statistically significant correlation was found between the
percentage of sperm labeled by Hs-8 and Hs-9 antibodies ($r = -0.37$, $P < 0.05$) and Hs-14 and Hs-9 antibodies ($r = -0.38$, $P < 0.05$).

Correlation of flow cytometry and indirect immunofluorescence results
Sperm labeling in individual donors was assessed by FCM. In the case of Hs-8 and Hs-14 antibodies, which displayed the largest differences in the number of labeled sperm cells between normospermics and asthenospermics, immunofluorescence microscopy was also used (Figure 4).

The percentage of cells stained by Hs-8 or Hs-14 obtained by fluorescence microscopy and the percentage of Hs-8 or Hs-14 positive cells analyzed by FCM correlated almost perfectly, as indicated by the squared Pearson correlation coefficient (Hs-8 $r^2 = 0.9389$, Hs-14 $r^2 = 0.9397$).
Fertilization, pregnancy, and implantation rates (%) for normospermic and asthenospermic sperm and their correlation with flow cytometry results

Sperm of the donors was used for IVF. According to the physicians’ decision, IVF was carried out with asthenospermic and normospermic sperm by ICSI.

Data about fertility treatment are summarized in Supplementary Figure 3. The fertilization and transfer rates were not different between the two groups. The pregnancy rate and the implantation rate were somewhat higher in healthy normozoospermic men, but without statistical significance.

**DISCUSSION**

The search for differences between the sperm capable of fertilizing eggs in a natural way and sperm that are not capable of fertilization is a long-lasting task. Recently, the possibilities of finding differences between normal and pathological sperm cells at a molecular level have greatly increased.

Two-dimensional electrophoresis of sperm proteins combined with computer analysis was used to find the differences between the sperm of fertile and infertile men.\(^{24-26}\) The advantage of this method is the rapid assessment of a large number of proteins and the identification of changes typical of a particular category of sperm.

Another approach is to find differences between fertile and infertile men using defined antibodies against sperm proteins.\(^{27,28}\) In the past, we applied immunofluorescence microscopy and described the changes in the protein expression in men with various sperm pathologies.\(^{29-31}\)

Our set of MoAbs against human sperm (abbreviated Hs-antibodies) comprises of antibodies against sperm proteins and seminal plasma proteins that adhere to the sperm surface during their passage through the epididymis (Table 1).

Using FCM, various sperm features were measured such as sperm concentration,\(^{32}\) sperm viability and acrosomal integrity,\(^{33-35}\) mitochondrial function,\(^{36}\) and DNA integrity.\(^{37-38}\) Fluorescence-activated cell sorting applied in FCM is used for sorting sperm population with defined properties, e.g., with a reduced number of spermatozoa with fragmented DNA\(^{39}\) and a selection of spermaticogenic cells from testicular biopsies.\(^{40}\)

Using FCM, we evaluated the expression of sperm proteins and compared data between normozoospermic (N) and asthenozoospermic (A) men. Asthenozoospermia is characterized by reduced motility (<40% motile spermatozoa), and one would expect that the sperm tail would be affected in asthenospermics as it is the organ of the movement. However, experimental approach showed that asthenozoospermia is a complex, multifactorial damage, which interferes with cellular structures in different cell compartments. Low sperm motility seems to be accompanied by diminished sperm genomic integrity, abnormal DNA condensation and also by defects of the sperm midpiece.\(^{41}\) The complex nature of asthenozoospermia was also confirmed by our data obtained with anti-acrosomal antibodies. In comparison with the sperm of normal healthy men, the sperm of asthenospermics had a reduced amount of acrosomal proteins.

The largest differences in protein expression between normozoospermics (N) and asthenozoospermics (A) were found in the proteins detected by Hs-8 (\(P < 0.01\)) and Hs-14 (\(P < 0.001\)) antibodies. Therefore, we also assessed both of these groups under an immunofluorescence microscope and checked not only the expression of proteins, but also their localization on spermatozoa. A very high correlation of the results obtained by these two methods was confirmed by the regression curves and Bland–Altman analysis (Figure 4 and Supplementary Figure 2).

We also observed differences in the expression of seminal plasma proteins on spermatozoa. Unlike our Hs-16 antibody, which more frequently detects SABP (sperm actin binding protein) on pathological sperm,\(^{42}\) we did not find a statistically significant difference in the expression of clusterin (detected by Hs-3 MoAb) and semenogelin (detected by Hs-9 MoAb) between the group of normal healthy men and the group of asthenosperms.

In addition, we correlated the number of cells stained by our anti-acrosomal antibodies in the groups of normosperms and asthenosperms. We found positive correlations between the number of stained cells using almost all antibodies, but only some of them were statistically significant. The Hs-8 and Hs-14 antibody staining patterns were very similar, but those of Hs-36 were different (Figure 3). This may reflect the fact that individual patients express the appropriate acrosomal proteins differentially, and the statistically significant differences between the normosperms and asthenosperms were caused not only by a lower amount of protein in the acrosome, but also by differential expression of individual proteins during spermatogenesis.

The most statistically significant differences in protein expression between N and A were found by the Hs-14 antibody. This MoAbs reacts with valosin-containing protein (VCP), and apparently nonspecifically binds to beta tubulin (unpublished results). However, the significant difference in the expression of Hs-14-detected protein(s) between N and A could not be influenced by an unspecified reaction with beta tubulin, because Hs-14 labeling of the sperm tail in both A and N groups was only exceptionally observed.

In general, we observed a decrease of the levels of proteins detected using our antibodies in the acrosomal area. This result is in agreement with our previously obtained results when we evaluated the fertilization rate of spermatozoa with various pathologies (oligoasthenoteratozoospermia, oligoasthenozoospermia, oligoospermia) after ICSI. In this sperm, we found reduced levels of acrosome proteins in relation to the pathology and fertilization rate.\(^{43}\) Data about the fertilization treatment obtained after ICSI with sperm of the normosperms and asthenosperms tested in this study showed the same fertilization rate in N and A. This is probably due to the fertilization of eggs by the ICSI method, which eliminated the disadvantage of asthenospermic sperm cells with their reduced motility. Pregnancy and implantation rates were increased in normosperms compared with asthenosperms, but the differences were not statistically significant (Supplementary Figure 3).

As we also observed differences in the expression of individual epitopes detected by appropriate antibodies, we assume that the lower expression of individual proteins was caused by their impaired production during spermatogenesis. This idea is supported by experiments in which the experimentally induced pathology in mice could influence the expression of genes having a role in spermatogenesis and presence of sperm proteins.\(^{44}\)

We may also speculate that, conversely, the production of some other proteins may be up-regulated under pathological conditions, and the asthenosperms would consequently have higher levels of these other proteins in the acrosomes of their sperm population.

**CONCLUSION**

We found that intra-acrosomal sperm proteins VCP, glyceraldehyde-3-phosphate dehydrogenase, and PRKAR2A are
differentially expressed in normal healthy men and asthenozoospermics, with a reduced expression in asthenozoospermics. These proteins are involved in energy metabolism and apoptosis of cells. Our results indicate the possibility of evaluating sperm quality in reproductive medicine by MoAbs against selected sperm proteins.

AUTHOR CONTRIBUTIONS
JC conceived the study, participated in its design and coordination and helped to draft the manuscript. AK carried out the immunooassays, LD participated in the design of the study and performed the statistical analysis, OT prepared and characterized the human sperm, JP prepared the MoAbs against human sperm and helped to draft the manuscript. All authors read and approved the final manuscript.

COMPETING INTERESTS
All authors declare no competing interests.

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Supplementary information is linked to the online version of the paper on the Asian Journal of Andrology website.

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