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Increased expression of secretory actin-binding protein on human spermatozoa is associated with poor semen quality

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BACKGROUND: Antibodies to human sperm are useful diagnostic reagents for detection of changes in sperm protein expression and their relationship with sperm defects and male infertility. The specificity of Hs-16 monoclonal antibody (mAb) and the localization and frequency of the occurrence of Hs-16-recognized protein on human spermatozoa were investigated. METHODS: Samples from 30 fertile men with normal spermiograms and 30 men with pathological spermiograms were studied. The specificity of Hs-16 mAb was analysed by the western blotting technique and matrix-assisted laser desorption/ionization mass spectrometry. Indirect immunofluorescence with Hs-16 antibody was used to test sperm ejaculates. RESULTS: The Hs-16 antibody detected a human sperm and seminal plasma protein, which was determined to be secretory actin-binding protein (SABP). This specificity was also verified by co-localization of SABP and actin on spermatozoa with Hs-16 and anti-actin antibodies, and partial co-localization of these proteins was found. SABP was localized on the sperm tail, mainly in the midpiece of the tail. Other parts of spermatozoa were labelled with lower frequency. A significant difference was found in SABP labelling between men with normal spermiograms and donors with asthenozoospermia or oligoasthenoteratozoospermia (both $P < 0.01$), and asthenozoospermia versus oligoasthenoteratozoospermia ($P < 0.05$). Increased expression of SABP was observed in men with pathological spermiograms. CONCLUSIONS: Hs-16 antibody reacts specifically with SABP. SABP can serve as a marker of defective sperm and may be associated with fertility failure.

Key words: human spermatozoa/male fertility/monoclonal antibody/secretory actin-binding protein/semen quality

Introduction

Quality assessment of human semen is an integral part of the diagnostic procedure in all laboratories that are engaged in the assisted reproductive technology and solve problems of infertile couples. Basic semen analysis, which gives a picture of semen parameters, is represented by the World Health Organization (WHO) classification (World Health Organization, 1999). According to the guidelines, semen may be classified into four main categories that—with the exception of normozoospermia—can be further more precisely divided with respect to the sperm damage. Normal values for human semen established by WHO are sperm concentration 20×10^6 spermatozoa ml^{-1}, motility 50% or more motile spermatozoa with forward progression and morphology 30% or more spermatozoa with normal morphological forms. Besides this approach, modern tools for more exact definition of decreased fertility or complete infertility of men are employed (Bohring *et al.*, 2001; Starita-Geribaldi *et al.*, 2001; Shibahara *et al.*, 2002; Bohring and Krause, 2003; Rajeev and Reddy, 2004; Pixton *et al.*, 2004). A good result was achieved with antibody to sperm antigen in determining the exact cause of the disorder (Chiu *et al.*, 2004). Antibodies can define the sperm disorder at a qualitatively higher level—at the level of molecules that are specifically recognized by them.

Monoclonal antibodies (mAbs) to sperm antigens, primarily generated to study the cell processes in sperm and to investigate the role of molecules recognized by antibodies in egg fertilization, have acquired important practical significance. They make it possible to prepare kits which allow many defects of spermatozoa to be found simultaneously.

The mAb Hs-16, which was newly generated in our laboratory, is able to recognize human ejaculates of poor quality by increased binding on spermatozoa and thus belongs to the practically employed antibodies. Determination of the Hs-16 mAb specificity, immunofluorescence localization of the Hs-16-detected protein on spermatozoa and frequency of the Hs-16-labelled spermatozoa in human sperm samples of various quality are reported in this paper.

Materials and methods

Semen samples

Human sperm ejaculates were obtained with the donors' consent from the Institute of Sexuology (Prague, Czech Republic) and from the Pronatal

Ltd (Prague, Czech Republic). In all samples, semen parameters—sperm concentration, motility, viability and morphology—were assessed according to WHO guidelines (World Health Organization, 1999) and the ejaculates were classified as normozoospermic, oligozoospermic, asthenozoospermic and/or oligoasthenoteratozoospermic.

The set of human sperm samples consisted of 30 samples from fertile men with normal spermiograms and 30 samples from men with pathological spermiograms.

Production of Hs-16 mAb

The Hs-16 mAb was prepared by immunization of BALB/c mice with crude extract of human spermatozoa and following fusion of immune spleen cells with Sp2/0 myeloma cells. The immunization procedure and hybridoma technology were described in detail by Peknicova *et al.* (1986) and Geussova *et al.* (1997).

Electrophoresis, western blotting and immunodetection

Ejaculated human or boar spermatozoa were washed three times in phosphate-buffered saline (PBS) and extracted. Dry sperm pellet (5×10^7 cells) was resuspended in 500 μl nonreducing 2× sodium dodecyl sulphate (SDS) sample buffer for SDS polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970). After boiling in water bath (3 min), cooling at 4°C and centrifugation (23 000 *g*, 3 min, 4°C), supernatant was divided into aliquots and kept at −80°C until electrophoresis. Before electrophoresis, aliquots of SDS sperm extract were once more diluted 1:1 with 1× SDS sample buffer for SDS–PAGE.

Samples of human seminal plasma were obtained after centrifugation (200 *g*, 15 min) of liquified ejaculates. The seminal plasma supernatant was centrifuged once more (2600 *g*, 20 min) and then divided into aliquots and kept at −80°C. Before electrophoresis, seminal plasma was diluted 1:1 with PBS, and an equal volume of 2× SDS sample buffer was added to the diluted seminal plasma.

To observe the migration of proteins on SDS PAGE under reducing conditions, samples were supplemented with β-mercaptoethanol (5% final concentration) and heated 1 min in a boiling water bath prior to electrophoresis.

SDS PAGE was carried out according to the method of Laemmli (1970). Ten microlitres of nonreduced or reduced SDS sperm extract corresponding to 0.5×10^6 cells and/or 10 μl of seminal plasma sample were loaded per lane on 15% slab gel. Electrophoresis was run for 75 min at constant current 30 mA. Electrophoretically separated proteins were then transferred onto nitrocellulose sheets as described by Towbin *et al.* (1979) and immunodetection was performed. The blot was blocked overnight in PBST [0.05% Tween-20 (v/v) in PBS] supplemented with 5% gelatin and then incubated with Hs-16 hybridoma supernatant or with the supernatant of Sp2/0 myeloma cells (negative control) or with Hs-3 hybridoma supernatant (positive control). The Hs-3 mAb specifically recognizes apolipoprotein J (Capkova *et al.*, 2002), which is secondarily incorporated into the sperm membrane. After washing, secondary horse-radish peroxidase-conjugated goat anti-mouse antibody (1:10 000, BioRad) was applied for 1 h. Then the blots were washed intensively with PBST and the reaction of the antibody was visualized with an enhanced chemiluminescence kit (ECL, Amersham, M.G.P. Zlín, Czech Republic). Relative molecular weights of proteins detected by mAb was estimated by comparison with the mobility of calibrated low- and highmolecular weight protein standards (LMW Pharmacia, Prague, Czech Republic).

After one-dimensional (1D) electrophoresis of SDS human seminal plasma extract in 12% polyacrylamide gel, a small part of the gel was cut off and subjected to the standard western blot procedure (Towbin *et al.*, 1979) with Hs-16 as primary antibody. The immunoblot was fitted in the rest of Coomassie Brilliant Blue (CBB R 250) stained gel and the Hs-16-corresponding band was further analysed by matrix-assisted laser desorption/ionization (MALDI).

Proteolytic digestion and sample preparation for MALDI

Unless otherwise indicated, all chemicals for sample preparation and mass spectrometric (MS) analysis were purchased from Sigma-Aldrich (St Louis, MO, USA).

CBB-R 250-stained protein bands were cut from the gel and decolourized in sonic bath at 60°C several times with 10 mM dithiothreitol, 0.1 M 4-ethylmorpholine acetate (pH 8.1) in 50% acetonitrile (ACN). After complete destaining, the gel was washed with water, shrunk by dehydration with ACN and reswollen in 60 mM iodoacetamide, 0.1 M 4-ethylmorpholine acetate (pH 8.1). The gel was incubated for half an hour in the dark at room temperature. After cysteine alkylation, the gel was washed with water, shrunk by dehydration with ACN and reswollen in water. The rehydration and dehydration of the gel was repeated three times. Next, the gel was reswollen in 0.05 M 4-ethylmorpholine acetate (pH 8.1) in 50% ACN and then the gel was partly dried using a SpeedVac concentrator (Savant, Holbrook, NY, USA). Finally, the gel was reconstituted with cleavage buffer containing 0.01% 2-mercaptoethanol, 0.05 M 4-ethylmorpholine acetate, 10% ACN and sequencing grade trypsin (Promega, 50 ng μl^{-1}). Digestion was carried out overnight at 37°C, the resulting peptides were extracted with 30% ACN/1% acetic acid and subjected to MS analysis.

MS analysis

The mass of individual peptides obtained after tryptic digestion of Hs-16-detected protein was determined by the MALDI method. Mass spectra of peptides were measured using a MALDI- Time-of-Flight (MALDI-TOF) mass spectrometer, a peptide map was established and mass spectra were searched against the database using Profound software. Mass spectrometer BIFLEX II (Bruker-Franzen, Bremen, Germany) was equipped with a nitrogen laser (337 nm) and gridless delayed extraction ion source. Ion acceleration voltage was 19 kV and the reflectron voltage was set to 20 kV. Spectrum was calibrated internally using the monoisotopic $[M + H]^+$ ions of trypsin autoproteolytic products. A saturated solution of α-cyano-4-hydroxy-cinnamic acid in 50% ACN/0.2% trifluoroacetic acid was used as a MALDI matrix. One microliter of matrix solution was mixed with 1 μl of the sample on the target and the droplet was allowed to dry at ambient temperature.

Indirect immunofluorescence

Liquified human ejaculates were centrifuged at 200 *g* for 15 min. The seminal plasma supernatant was discarded, cell pellet resuspended and three times washed in PBS, pH 7.4. Washed spermatozoa were diluted in PBS to a final concentration of 1×10^6 cells ml^{-1} and small drops smeared onto glass slides. Dried smears were fixed with 3.6% formaldehyde (60 min, room temperature), rinsed with PBS and after blocking with bovine-serum albumin (1 h, 3% bovine-serum albumin in PBS) incubated with Hs-16 mAb (undiluted hybridoma supernatant, immunoglobulin (Ig) concentration <20 μg ml^{-1}) for 60 min at 37°C. In controls, the supernatant of Sp2/0 myeloma cells was applied as the first antibody instead of the Hs-16 antibody. After three washes in PBS, the smears were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG immunoglobulin (γ-chain specific, Sigma, Prague, Czech Republic), diluted 1:64 in PBS and incubated for 60 min at 37°C, washed in PBS, rinsed with dH_2O and mounted in Vectashield with 4′,6-diamidino-2-phenylindole H-1200 (Vector Laboratories, Inc., Burlingame, CA, USA).

To compare Hs-16 staining of smeared, air-dried and live spermatozoa, immunofluorescence was also carried out with sperm

suspension in a tube. Washed sperm pellet (1×10^6 cells ml^{-1}) was resuspended in 100 μl of Hs-16 mAb and cells were incubated with the antibody for 60 min at 37°C. After three washes, sperm suspension was dropped onto glass slides, smeared and the immunofluorescence procedure followed as described.

The immunofluorescence was observed under a Nikon Labophot 2 microscope equipped with a Nikon Plan 40/0.65 objective, and 300 sperm cells were evaluated from each donor. Photographs were taken with a COHU 4910 CCD camera (Cohu Inc. Electronics Divisions, San Diego, CA, USA) with the aid of the LUCIA imaging software (Laboratory Imaging, Ltd, Prague, Czech Republic).

Co-localization of Hs-16-recognized protein and actin

Sperm smears were incubated with the mixture of mouse Hs-16 mAb (undiluted hybridoma supernatant) and polyclonal rabbit anti actin antibody (1:80, Sigma, cat. no. A 2668). After 2 h of incubation at 37°C, slides were washed with PBS and incubated (1 h, 37°C) with cyanine dye (Cy3)-conjugated goat anti-mouse IgG (1:1500, Sigma) and FITC-conjugated goat anti-rabbit IgG (1:160, Sigma). Samples were then washed and processed as described. In controls, PBS solution was used instead of Hs-16 and anti-actin antibody mixture. To exclude reactivity between antibodies, rabbit anti-actin antibody was combined with goat anti-mouse Cy3-conjugated IgG and mouse Hs-16 mAb was combined with goat anti-rabbit FITC-conjugated IgG as the secondary antibody, and no labelling was observed.

Statistical analysis

The statistical differences among the compared groups were analysed by one-way analysis of variance and Student–Newman–Keuls' test, and P-values of <0.01 and <0.05, respectively, were considered significant.

Results

Specificity of the Hs-16 mAb

The Hs-16 mAb reacted on western blot with SDS extracts of human spermatozoa and human seminal plasma and the reaction pattern was the same with both extracts.

The Hs-16 mAb labelled a protein band of 16 kDa and this reaction was identical under non-reducing and reducing conditions (Figure 1). No reaction of Hs-16 mAb was found on western blots with SDS extracts of boar and mouse spermatozoa (data not shown). Thus, the Hs-16 mAb is specific for human spermatozoa.

The Hs-16-relevant protein band (found using Hs-16 immunoblot on CBB R250-stained gel with electrophoretically separated SDS human seminal plasma extract) was subjected to mass fingerprinting analysis (MALDI MS). According to the peptide map, the 16-kDa protein was identified as a secretory actin-binding protein (SABP): seven out of nine peptides that were obtained by tryptic digestion of the 16-kDa protein matched with SABP and two remaining peptides were trypsin autoproteolytic fragments (Figure 2). The sequence coverage of the total SABP sequence was 62%. Peptide mapping was evaluated by using the ProFound searching algorithm (see web address in references).

Co-localization of actin and SABP in sperm cells

As was shown by MALDI-MS, the Hs-16 mAb recognizes SABP. This finding was tested in a co-localization experiment.

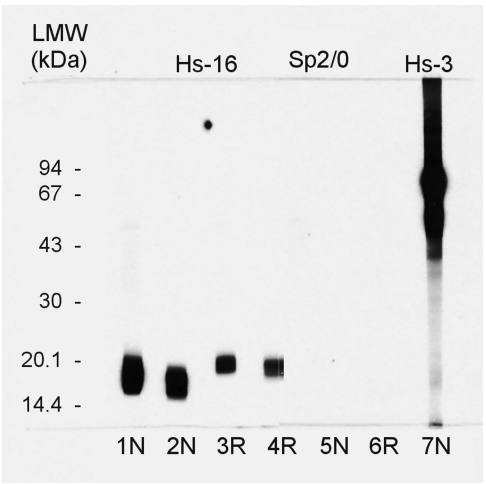

Figure 1. Reaction of the Hs-16 monoclonal antibody (mAb) on western blot with sodium dodecyl sulphate extracts of human sperm and human seminal plasma. Nos 1, 3, 5, 7: sperm extract; nos 2, 4, 6: seminal plasma extract. Immunodetection with Hs-16 mAb (nos 1, 2, 3, 4), supernatant of Sp2/0 cells (nos 5 and 6): negative control; Hs-3 mAb (no. 7): positive control. N, nonreduced sample; R, reduced sample.

In the immunofluorescence assay with human spermatozoa, the mixture of Hs-16 mAb and anti-actin antibody as the primary antibody was applied, and labelling was visualized with two secondary antibodies conjugated with different fluorochromes. The staining with both primary antibodies was not completely identical, but certain co-localization was obvious in some parts of spermatozoa (Figure 3). Anti-actin antibody stained the whole sperm tail and weakly the sperm acrosome. Hs-16 mAb most often stained the sperm tail and predominatly its midpiece part. Thus, staining with both antibodies was co-localized in the sperm tail, especially in the midpiece of the tail.

The labelling of spermatozoa with anti-actin antibody and Hs-16 mAb was also evaluated quantitatively. Semen of six donors was screened in the co-localization experiment and the results are summarized in Table I. The frequency of spermatozoa staining with anti-actin antibody and the Hs-16 antibody varied. The anti-actin antibody stained spermatozoa with higher frequency than Hs-16 mAb. In one of our samples (Figure 4, no. 53), there was a complete identity in

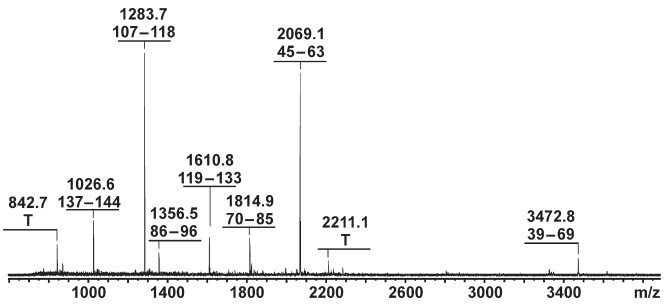

Figure 2. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometer spectrum of secretory actin-binding protein tryptic fragments. The mass and positions of peptides are shown. Trypsin autoproteolytic fragments are labelled with T.

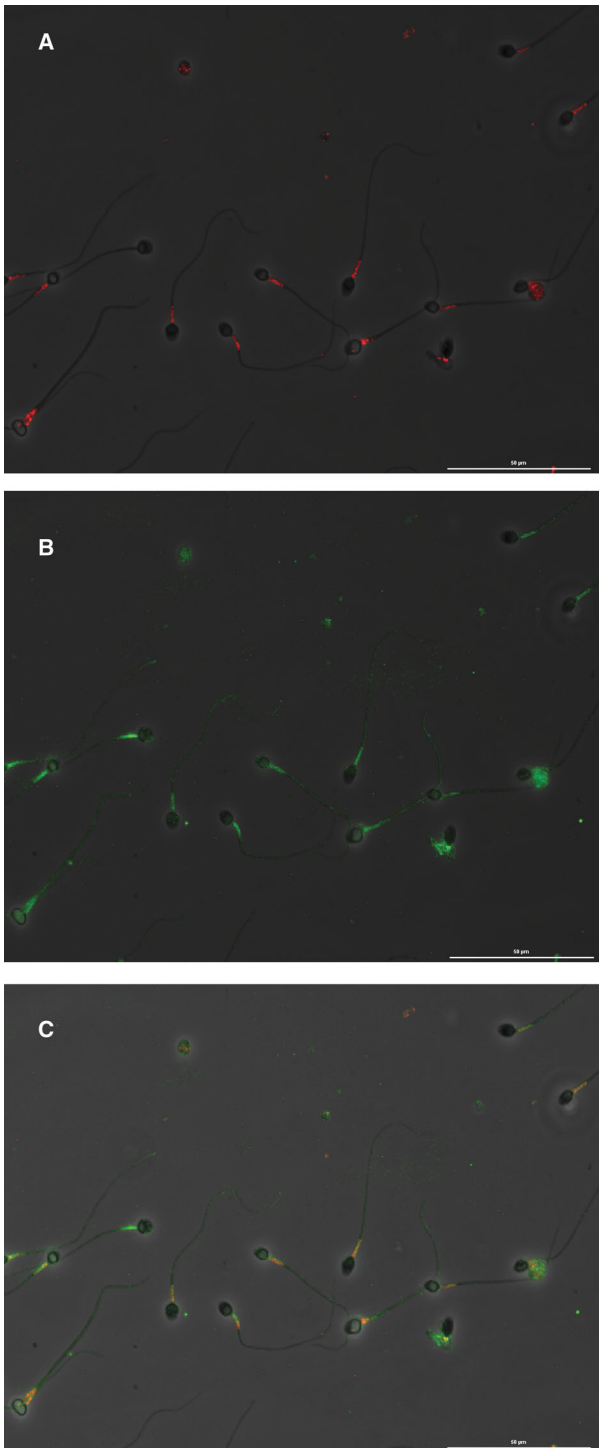

Figure 3. Co-localization of actin and Hs-16-detected protein (secretory actin-binding protein) in human spermatozoa. A mixture of Hs-16 mAb and anti-actin antibody as the primary antibody was applied, and labelling was visualized with two secondary antibodies conjugated with different fluorochromes. (**A**) SABP staining, secondary GAM IgG was conjugated with cyanine dye (Cy3). (**B**) actin staining, secondary GAR IgG was conjugated with fluorescein isothiocyanate (FITC). (**C**) co-localization of SABP and actin.

staining with both primary antibodies, as well as in the frequency of staining. Both antibodies stained the midpiece of the sperm tail and all spermatozoa were labelled. In this case, a complete immotility of spermatozoa was found.

Table I. Co-localization of secretory actin-binding protein and actin in human spermatozoa: quantitative evaluation.

Donor number	Labelled spermatozoa (%)		Classification of ejaculates (World Health Organization, 1999)	Co-localization
	Actin[a]	SABP[b]		
56	79	21	OAT	Partial (T-MP)
31	40	11	O	Partial (T-MP)
57	70	34	OAT	Partial (T-MP)
13	50	4	N	Partial (T-MP)
14	85	15	N	Partial (T-MP)
53[c]	~90	~90	A	Identical T-MP

MP, midpiece; T, tail; N, normozoospermia; OAT, oligoasthenoteratozoospermia; O, oligozoospermia; A, asthenozoospermia.
[a]Anti-actin antibody.
[b]Hs-16 antibody (anti-SABP).
[c]Complete immotility of spermatozoa was found.

Immunofluorescence staining of human spermatozoa with Hs-16 mAb

There was no significant difference in Hs-16 staining between 3.6% formaldehyde-fixed and nonfixed spermatozoa. Therefore, immunofluorescence assays were carried out only with smeared, dried, 3.6% formaldehyde-fixed spermatozoa. For each analysed sample, 300 cells were examined. The number of stained spermatozoa in individual ejaculates ranged from 1.8 to 42.5% of cells. Localization of Hs-16-detected protein (SABP) on the sperm surface was not uniform. The antibody labelled various parts of sperm cells—midpiece of the sperm tail, whole tail, whole sperm, acrosome, equatorial segment (Figure 5). However, the sperm tail and mainly its midpiece part were labelled most brightly and most often (70% of labelled cells), whereas the other parts of spermatozoa were labelled with lower frequency (30% of labelled cells) and staining was weaker.

Hs-16 staining of human sperm samples with normal and pathological semen parameters

According to the basic WHO guidelines, all sperm samples were assessed as normozoospermic, oligozoospermic, asthenozoospermic and/or oligoasthenozoospermic and SABP expression in sperm samples with different parameters was tested using Hs-16 mAb. All data are shown in Figure 4.

The percentage of Hs-16-stained ejaculated spermatozoa was the lowest in sperm samples from normozoospermic fertile donors ($n = 30$, mean value 8.96%). A similar result was obtained in sample from a man with oligozoospermia (value of Hs-16 staining 11.4%), but this sample was not included in statistical analysis because only one case of pure oligozoospermia was evaluated.

Hs-16 staining was higher in sperm samples defined as asthenozoospermia ($n = 21$, mean value 19.8%; one sample no.53 with about 90% labelled sperm was excluded, see above) and oligoasthenoteratozoospermia ($n = 7$, mean value 28.9%). However, there were a limited number of samples in the oligoasthenoteratozoospermia group.

SABP expression in sperm samples with different parameters was statistically evaluated and the results are summarized in Figure 6.

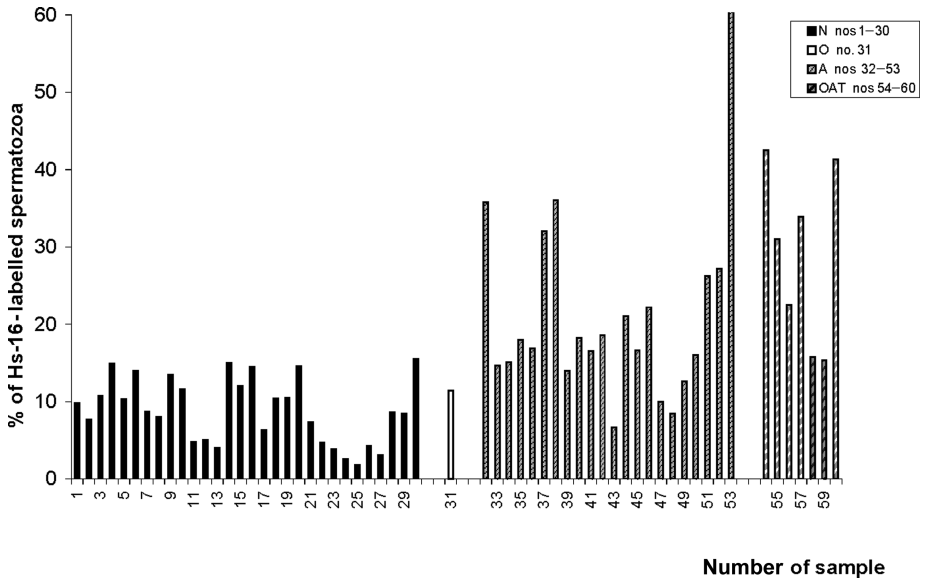

Figure 4. Frequency of the Hs-16 mAb-labelled spermatozoa in normospermic and non-normospermic semen. N, normozoospermia; A, asthenozoospermia; OAT, oligoasthenoteratozoospermia; O, oligozoospermia.

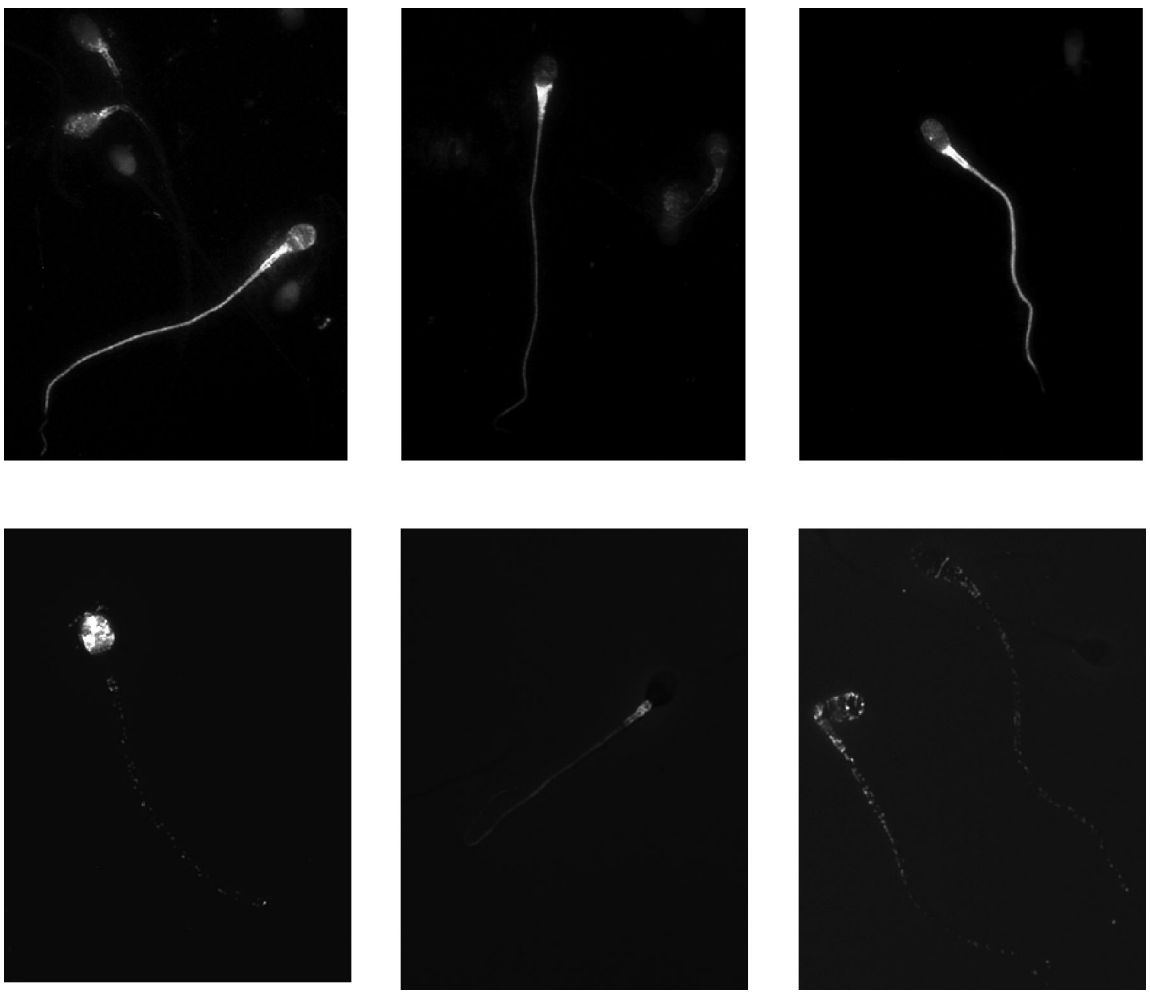

Figure 5. Immunofluorescence staining of human spermatozoa with Hs-16 mAb. First row, smeared, air-dried spermatozoa; second row, live spermatozoa.

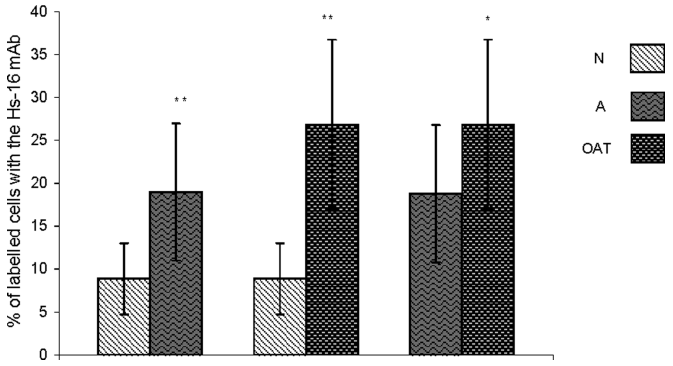

Figure 6. Statistical analysis of Hs-16 expression (secretory actin-binding protein) on human spermatozoa of different quality. Each column represents the mean ± SEM. Significant differences in Hs-16 staining were found between the normozoospermia (N) group and the asthenozoospermia (A) group, the N group and the oligoasthenoteratozoospermia (OAT) group and the A group and the OAT group. $*P < 0.05$; $**P < 0.01$.

Significantly higher SABP expression was found in men with asthenozoospermia ($P < 0.01$) and oligoasthenoteratozoospermia ($P < 0.01$) compared to men with normal spermiograms, and in men with oligoasthenoteratozoospermia ($P < 0.05$) compared to asthenozoospermic donors.

(**A**) SABP staining, secondary GAM IgG was conjugated with cyanine dye (Cy3) (**B**) actin staining, secondary GAR IgG was conjugated with fluorescein isothiocyanate (FITC) (**C**) co-localization of SABP and actin.

We also compared Hs-16 staining in air-dried and native sperm samples. The test showed a small (but not significant) increase in SABP expression (Hs-16 mAb stained ~20% more spermatozoa) in sperm smeared and air-dried onto glass slides in comparison with suspension of live sperm both in normospermic (control) and asthenospermic samples (Table II). It is obvious that SABP was already bound to live cells and did not bind only to spermatozoa that could have been damaged by air-drying. We have also noticed that there was a higher variability in Hs-16 labelling of live spermatozoa. Labelling was more often observed in the head or part of the head (acrosome, eqatorial segment, post-acrosomal region) than in air-dried spermatozoa and it was rather finely granulated (Figure 5).

Discussion

The Hs-16 antibody generated in our laboratory is a species-specific mAb, which reacted only with human spermatozoa.

Table II. Immunofluorescence staining of air-dried and live spermatozoa with Hs-16 monoclonal antibody

Donor number	Labelled spermatozoa (%)		Classification of ejaculates (World Health Organization, 1999)
	Sperm suspension in tube (live sperm)	Air-drying smears of sperm	
70	12.6	16.1	N
71	14.6	17.2	N
73	21.7	25.7	A
74	16.1	19.6	A
75	24.3	27.7	A

No reaction of the antibody was found on western blots and in immunofluorescence assays with spermatozoa of boar and/or mouse sperm.

The exact specificity of the Hs-16 was determined by the recently introduced method MALDI-TOF analysis. On the basis of the peptide map of the Hs-16-detected protein (Figure 2), four candidate proteins were designated: prolactin-inducible protein (PIP) and gross cystic disease fluid protein 15 (GCDFP 15), both isolated from human breast tissue, extraparotid glycoprotein (EP-GP) from submandibular gland and SABP, which occurs in human seminal vesicles and seminal plasma. All these proteins have a high degree of similarity, but the tissue origin of individual candidate proteins made it possible to explicitly identify the Hs-16-detected protein as SABP.

SABP, a glycoprotein, was originally isolated from human seminal plasma, and its interaction with actin was detected by agar gel immunoelectrophoresis (Akiyama and Kimura, 1990). The complete amino acid sequence of SABP was described immediately and sequence comparison revealed that the SABP is identical with PIP, GCDFP-15 and EP-GP (Schaller *et al.*, 1991).

The single polypeptide chain of SABP consists of 118 amino acids with calculated M_r of 13 506. The carbohydrate composition of SABP shows an unusually high amount of fucose and this is the reason of the discrepancy between calculated M_r and M_r determined from 1D SDS electrophoresis (16–20 kDa) and MALDI analysis (16 572 Da).

The biological function of this protein and its homologues is still unclear. SABP, GCDFP-15 and EP-GP were found to bind to actin and fibrinogen. However, the affinity for these proteins does not appear to have any direct physiological role. The homologue of SABP, EP-GP, binds to the surface of bacterial strains colonizing the oral tract and its role in passive mucosal immunity has been proposed (Schenkels *et al.*, 1997).

SABP is also known as gp17 factor (apparent MW 17.5 kDa). This glycoprotein was detected in the human seminal plasma, and it was shown that gp17 is capable of binding CD4 surface marker of T cells, macrophages and sperm cells (Autiero *et al.*, 1991). The binding of gp17 to CD4 was inhibited by anti-CD4 antibody, and gp17 was thought to be a CD4-masking factor in human seminal plasma that may modulate the immune response at insemination (Bergamo *et al.*, 1997; Caputo *et al.*, 1998). The gp17 binding to CD4 blocks CD4-mediated T-cell apoptosis (Gaubin *et al.*, 1999); thus, considerations about the biological role of gp17 supposed that gp17 may prevent interaction of other molecules with CD4 (e.g. CD4 interaction with the human immunodeficiency virus-1 envelope protein gp120), and in this way, it protects the surface structures of sperm in the male and female genital tracts (Autiero *et al.*, 1991; Autiero *et al.*, 1995; Autiero *et al.*, 1997; Bergamo *et al.*, 1997, Caputo *et al.*, 1998, Caputo *et al.*, 1999).

gp17 was localized by the immunofluorescence assay in the post-acrosomal region of the spermatozoon, and after *in vitro* capacitation, gp17 was detected all over the spermatozoa heads but only a minor part of the capacitated spermatozoa exhibited CD4 staining and the staining remained localized in the post-acrosomal region (Bergamo *et al.*, 1997).

Although the biological significance of SABP is not yet quite clear, it is obvious that SABP is a protein with multiple biological activities. One of the first known facts on SABP was the finding that this seminal plasma protein binds actin. Major cytoskeletal protein actin was found in the head and also in the neck and tail of spermatozoa (Eddy and ÓBrien, 1994). In the tail of human spermatozoa, actin was localized at the ultrastructural level around the connecting piece of the neck and on the surface of the fibrous sheath (Flaherty *et al.*, 1988, Escalier *et al.*, 1997).

Actin is implicated in important events such as capacitation and acrosomal reaction (Brevis and Moore, 1997; Moore, 2001; Howes *et al.*, 2001; Brener *et al.*, 2003; Dvořáková *et al.*, 2005). The involvement of actin in acrosome reaction was supported by the effect of anti-actin antibodies, which *in vitro* significantly supressed the zona pellucida-induced acrosomal reaction and motility of sperm (Liu *et al.*, 2002). In the genital tract SABP may act as anti-actin antibody, i.e. SABP might function as a natural suppressor of acrosomal reaction and sperm motility. Such a possibility is also suggested by the effect of seminal vesicle autoantigen (SVA)—mouse counterpart of SABP—on sperm motility (Huang *et al.*, 1999) and sperm capacitation (Huang *et al.*, 2000).

Recently, sperm proteome mapping of a patient who repeatedly failed at IVF revealed an altered expression of several proteins compared with fertile donors (Pixton *et al.*, 2004). Two proteins displaying increased expression in the patient's sample were conclusively identified by the MALDI-TOF MS and/or MS/MS. One of them was SABP. This observation inspired us to screen the SABP expression in sperm donors using our Hs-16 mAb.

Our study included healthy fertile men as well as men with obstructed fertility.We have assessed 60 semen samples from different donors and we observed significantly higher expression of Hs-16-detected protein on spermatozoa from asthenozoospermic and/or oligoasthenoteratozoospermic donors, in comparison with normozoospermic samples. Nevertheless, there were six samples (Figure 4, nos 4, 6, 14, 16, 20, 30) in the normozoospermic group with relatively high Hs-16 expression (~15% of labelled cells). Such samples might fail in fertilization. As it has been described, fertilization failure in IVF still occurs even with sperm without any apparent defects according to conventional semen analysis (Tournaye *et al.*, 2002).

On the other hand, there were also three samples (Figure 4, nos 43, 47, 48) among asthenozoospermic donors with low Hs-16-detected protein expression (~8% of labelled cells), although conventional sperm parameters (only 40% motile spermatozoa in the samples) suggested poor semen quality.

Our work was based on immunofluorescent screening of SABP using the Hs-16 mAb with the aim to find its localization and frequency of occurrence on human spermatozoa in sperm samples of different quality. When we compared the SABP localization with the localization of homologous proteins such as gp17 in human, SVA in mice (Huang *et al.*, 1999) and/or seminal-vesicle secretory protein II (SVS II) in rats (Seitz and Aumuller, 1992), our results were partially but not completely identical. The reason for the discrepancy may lie in the different techniques used for preparation of cells and in the immunofluorescence procedure. We used ejaculated spermatozoa and we did not select them by swim-up migration and permeabilize them before labelling with antibody in the immunofluorescence assay.

A source of the discrepancy may also be in mAbs against SABP. It was demonstrated that there are differences in staining of SABP and its human homologues with specific mAbs (Caputo *et al.*, 1998).

In the co-localization experiment, we observed labelling with mAb Hs-16 and anti-actin antibody almost exclusively in the midpiece region of the sperm flagellum. It means that only partly identical labelling with both antibodies was found and that SABP did not bind in all sites labelled with anti-actin antibody. The reason for this observation may be some damage to the sperm surface in co-localization sites.

Excepting conventional analysis, antibodies specifically detecting sperm proteins and changes in their expression during cell processes are still often used to assess spermatozoa. Up to now, the greatest interest was aimed at acrosomal proteins, and mAbs were generated that can be and have already been used for evaluation of acrosome integrity (Braun *et al.*, 1991; Gallo *et al.*, 1991; Kaplan and Naz, 1992; Fierro *et al.*, 1998; Čapková *et al.*, 2000; Pěknicova *et al.*, 2001), estimation of spermatogenesis and male infertility diagnostics (Dorjee *et al.*, 1997; Chládek *et al.*, 2000; Pěknicová *et al.*, 2005). mAbs to acrosomal proteins were also successfully used to reveal sperm damage and detect the changes in mammalian fertility due to the presence of pollutants such as endocrine disruptors in the environment (Pěknicová *et al.*, 2002; Kyselova *et al.*, 2003; Kyselova *et al.*, 2004). However, the antibodies to seminal plasma proteins still seem to be more powerful because these proteins may participate in gamete binding and may influence sperm motility. The boar seminal plasma protein that was detected by our ACR.3 mAb (Moos *et al.*, 1992) *in vitro* significantly reduced the binding of boar spermatozoa and porcine oocytes (Čapková and Pěknicová, 1997). Similarly, antibodies to boar spermadhesins changed the binding of sperm to porcine oocytes (Veselsky *et al.*, 1999). Thus, the assessment of seminal plasma proteins in human spermatozoa would represent another significant biomarker in IVF, as SABP detection shows.

The recent knowledge shows that the future evaluation of human sperm and prediction of successful fertilization in IVF will be done on the basis of sperm testing with a wide battery of suitable antibodies (Lal *et al.*, 2002). Another possibility is represented by the proteomic approach. This includes, for example, functional protein microarrays (Zhu and Snyder, 2003) or 'classical' analysis of protein expression on 2D electrophoretic gels with computer evaluation (Brewis, 1999; Shetty *et al.*, 1999; Pixton *et al.*, 2004).

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