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IgG, IgA and IgE Reactivities to Sperm Antigens in Infertile Women

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Abstract

Nowadays, World Health Organization (WHO) appoints infertility as a disease. Still more couples are diagnosed with unexplained infertility, it reaches almost 20 % of all cases. Immune system plays an important role: iso-immunization of women is possible by human sperm cells. For better understanding, it is necessary to detect and characterize antigens recognized by women immune system. Our study is focused on female infertility and is based on the ejaculate sample from four healthy donors. Antigens analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis and isoelectric focusing (followed by IgG, IgA and IgE immunoblotting) were detected by forty-five sera from women who were diagnosed with fertility failure. Ten children's sera were used as negative controls. The immunodominant sperm antigens detected by IgG antibodies were 68 and 123 kDa proteins with pI from 6.60 to 6.62, respectively. Concerning mucosal immunity the most frequently recognized sperm IgA-binding protein reached 130 kDa and pI 6.9, 7.4, 9.5. This study did not prove the association of IgE antibodies with unexplained infertility in women.

Keywords: Human sperm, unexplained infertility, polyacrylamide gel electrophoresis, isoelectric focusing, immunoblotting.

1. Introduction

The prevalence of infertile couples significantly increases. The World Health Organization (WHO) appoints human infertility as a disease and defines it as an inability to conceive a child after a period of one year of unprotected intercourse with normal frequency.

The cause of infertility could be due to women as well as men. Pathological spermiogram represents 40 % of the cases; problems of women genital tract also 40 % and 20 % of the reasons are still unknown. The immune system dominates in the last mentioned case. Although the relative importance of immunological factors in human reproduction remains controversial, substantial evidence suggests that human leukocyte antigens (HLA), antisperm antibodies (ASA), integrins, cytokines, antiphospholipid antibodies, endometrial adhesion factors or e.g. mucins (MUC1) contribute to reproductive failure (Choudhury and Knapp, 2001). Some cases of infertility considered inexplicable were found to have an immunologic basis. Spermatozoa are highly antigenic cells. When the state of immune tolerance is interfered, iso-immunization in women can occur. Some studies have mentioned and shown that immune infertility could be caused by iso-immunization of female organism by antigens from human semen (Verpillat *et al*., 1995; Ulcova-Gallova, 2006; Ulcova- Gallova, 2003).

Semen proteins represent potential antigenic and immunogenic structures for female immune system. Iso-immunization evokes strong immune response that prevents successful pregnancy. This phenomenon could be seen in connection with natural tolerance failure or with possible consequence of recurrent inflammation of the genital tract or susceptibility to allergic reactions (Dimitrov *et al*., 1994; Ulcova-Gallova, 2006). ASA are significantly connected with immunological infertility and they can be detected in female blood serum, also in cervical mucus. The character of ASA is most apparently sperm-agglutinating, sperm-cytotoxic and sperm-immobilizing (Shetty *et al*., 2006; Sedlackova *et al*., 2010). Unfortunately, there is no definitive global treatment known. For successful treatment of unexplained infertility it is necessary to reveal and characterize particular antigens coming from sperm cells (Jones, 1991; Doherty and Clark, 2006).

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The aim of this study was to define sperm antigens implicated in unexplained infertility in association with IgG, IgA and IgE antibodies from sera of infertile women: IgG as a representative immune reaction, IgA as a potential mucosal immune response and IgE as a potential local allergic reaction. Collected data should be used as preliminary for thorough two-dimensional analysis and further identification.

2. Materials and Methods

2.1. Sample Preparation

Semen samples from four healthy donors with normal semen characteristics (WHO, 1992) were obtained by masturbation after 3 – 5 days abstinence under informed written consent. Ejaculates were let to liquefy and a mixture of protease inhibitors (0.05 M ε-aminocaproic acid, 5 mM ethylene glycol tetraacetic acid **(**EGTA) and 0.01 M benzamidine) was added.

1 ml of ejaculate from four individuals was centrifuged separately at 1075x g for 15 minutes at 4°C. The supernatant representing seminal fluid was removed. The pellet obtained after centrifugation of whole ejaculate represented predominantly sperm cells then small amount of epithelia. Each pellet was washed twice by PBS (0.01 M phosphate buffered saline with NaCl 0.15 M, pH 7.4) supplemented with protease inhibitors mixture and centrifuged at the same conditions. Lysis was proceeded using 1% Triton X-100 (Sigma, USA) in deionized water, containing a mixture of protease inhibitors (1:50) for 4 hours at 4 °C on ice. Insoluble parts were removed by final centrifugation at 3000x g for 10 minutes at 4°C. The supernatants of all samples were pooled.

Protein concentration of pooled samples was measured by the bicinchoninic acid protocol (BCA, Sigma, USA) (Smith *et al*., 1985) using bovine serum albumin (BSA) as standard. The unused samples were stored at -20°C until assayed.

2.2. Patients

Serum samples were obtained from forty-five women diagnosed with fertility failure and from ten eight-year-old girls. Sera were frozen immediately at -20°C until further use. Children's sera were selected as negative controls because these girls had no contacts to sperm antigens. All experiments were done under informed written consent.

2.3. One-Dimensional Polyacrylamide Electrophoresis (1D SDS-PAGE)

SDS-PAGE was performed at room temperature in Mini Protean Cell Bio-Rad (Bio-Rad, USA) according to Harlow and Lane modification (1988). Preparation required casting two different layers of acrylamide between clean glass plates, separating gel and stacking gel that includes the sample wells.

Aliquots of pooled samples were separated on 10% acrylamide gel (30% acrylamide mix, 1.5 M Tris-HCl pH 8.8, 10% SDS, 10% ammonium persulfate (APS), tetramethylethylenediamine (TEMED)–Serva, Heidelberg, Germany) with 5% acrylamide stacking gel (30% acrylamide mix, 0.5 M Tris-HCl pH 6.8, 10% SDS, 10% APS, TEMED). Slots were loaded at the protein concentration of 200 µg/ml. Sample was diluted 1:1 with nonreducing protein loading buffer (0.5 M Tris-HCl, pH 6.8, glycerol, 10% SDS, 0.1% bromophenol blue). Samples were heated for two minutes in boiling water before the loading into the gel. Pre-stained protein standard was used as known marker (Pre-stained SDS-PAGE standard, broad rang, Bio-Rad, USA). After the electrophoresis, separated proteins were either transferred onto a nitrocellulose sheet (NC, Serva, Heidelberg, Germany) or silver-stained (SilverTM Plus Stain Kit, Sigma, USA).

2.4. Immunoblotting Analysis

Mini Trans-Blot Cell Bio-Rad (Mini Protean III system, Bio-Rad, USA) was used for Western blot. Electro-transfer was performed onto 0.45 µm pore size NC membrane under an electric field of 100 V for forty-five minutes at 4°C using Tris-glycin transfer buffer (48 mM Tris, 39 mM glycin, 10% v/v methanol). The membrane was cut to three-millimeter strips then saturated by incubation for two hours at room temperature in blocking solution (PBS-Tw, 0.3% v/v Tween 20, pH 7.4). Each sheet was incubated separately with 50-fold diluted female sera at 4 °C overnight under constant rocking. Binding of the primary antibody from sera to the target protein was followed by a complex with alkaline phosphatase (AP)-linked secondary antibody formation (Goat Anti-Human IgG, Promega, USA or Goat Anti-Human IgA, Sigma, USA or Goat Anti-Human IgE, Sigma, USA). The IgG conjugate was 10000-fold diluted, IgA and IgE conjugates 5000-fold diluted. The created immune complex was visualized by chromogenic substrate NBT/BCIP (IMMUNO NBT/BCIP, Liquid substrate plus, MP Biomedicals, USA) according to the manufacture's instruction. The blots were washed three-times for ten minutes with PBS-Tw 0.1% between each incubation step.

2.5. One-Dimensional Acrylamide Gel Isoelectric Focusing (1D IEF)

IEF separation of pooled samples was carried out in a polyacrylamide gel (CleanGel™ IEF, GE Healthcare) containing 5% v/v Servalyt® pH 3-10 (Serva Electrophoresis GmbH, Heidelberg, Germany) in Milli-Q water. Sample was loaded on the anode side. The flat bed electrophoretic chamber (Multiphor II, GE Healthcare) was cooled at 15 °C. After separation, the proteins were either transferred to cyanogen bromide (CNBr) activated nitrocellulose membrane (Demeulemester *et al*., 1987) for Western blotting or Coomassie Blue stained. Isoelectric point standards in the range from 3.5 to 10.65 (Serva, Heidelberg, Germany) were used as a reference.

Passive transfer was performed onto CNBr-activated NC membrane for 1 hour. The membrane was dried and then cut into three-millimeter wide strips. The following steps were identical for those described before *(2.4 Immunoblotting analysis)*.

2.6. Gel Scanning

Coomassie blue or silver stained gels were scanned with EPSON Perfection 4990 Photo equipment. The figures were drawn using Corel PHOTO PAINT 12.

3. Results

Pooled samples of ejaculate with normal characteristics from four healthy donors were used for sperm protein extract. The protein concentration was in the range from 0.8 to $3x10^3$ µg/ml. Antibodies-binding proteins were examined by antisperm antibodies coming from forty-five serum samples of women with fertility failure. Ten control samples from sexually inactive girls were negative in immunoblotting analysis.

Prepared sample of sperm proteins was separated by 1D SDS-PAGE (Fig. 1A) and 1D IEF (Fig. 1B). Figure 1 showed the 1D sperm protein profile and IgG-binding proteins using patient and control sera. The range of molecular masses (Mr) is wide but values of pI are very close. The Mr range of sperm antigens reached the scale from 31 to 200 kDa. Immunoblotting analysis allowed us to find out the most frequent antigens, in meaning of 68 and 123 kDa proteins (Fig. 1A). 68 kDa antigen was intensely recognized by 93 % of all sera and 123 kDa antigen by 84 % of them. IgGs from serum S1 interacted also with 50, 86 and 90 kDa antigens, serum S2 interacted with Mr 36 and 48 kDa, serum S3 recognized 50 kDa antigen and serum S4 recognized 43, 50, 64 and 86 kDa antigens. However, their presence was minor. No IgG-binding activity was detected using control sera.

IEF offers resolution of sperm proteins according to isoelectric points (pI). Collected data from IEF displayed IgG reactivity to protein of pI 6.6 and 6.62 (Fig. 1B). It was recognized by 82 % of all forty-five female sera. No other one was detected individually. No IgG-binding proteins were found by using controls.

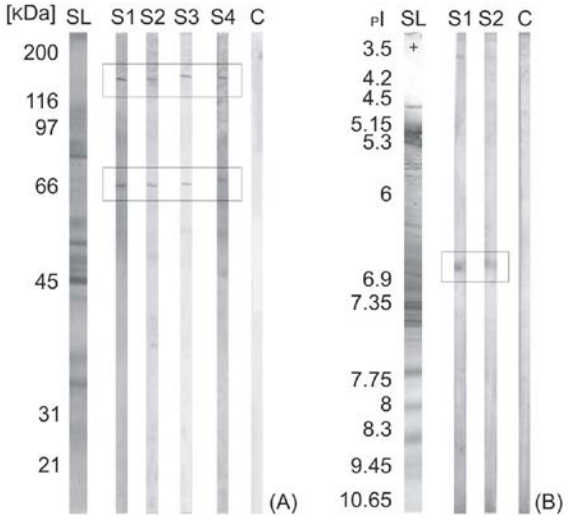

Figure 1. IgG reactivity of sperm antigens separated by 1D SDS-PAGE (A) and 1D IEF (B) followed by immunoblotting. SL: proteins of pooled sperm extract, silver and Coomassie blue stained; s1 – 4: screening of sera interacting with sperm antigens; C: negative control serum.

Another task of our study was to find out the potential mucosal immune response of IgA antibodies from female patient sera against sperm proteins which were separated by 1D SDS-PAGE (Fig. 2A) and 1D IEF (Fig. 2B). 130 kDa antigen turned out to be the dominant one and it was claimed in 71 % of all sera (Fig. 2A). Other antigens interacting with IgA antibodies were not detected. No IgA-binding at all was observed by using control sera.

Three antigens (pI 6.9, 7.4 and 9.5) were recognized by IgA antibodies in Western blot of 1D IEF gel (Fig. 2B). pI 6.9 was proved in 44 % of used sera, pI 7.4 in 51 % and 9.5 in 66 %. No IgA-binding was detected using control sera.

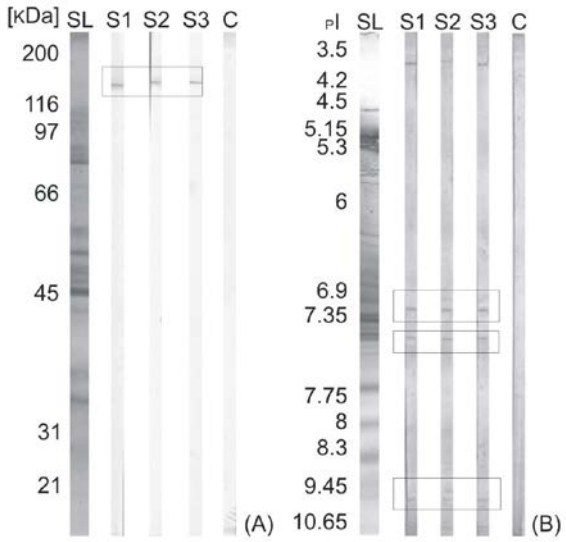

Figure 2. IgA detection of sperm antigens separated by 1D SDS-PAGE (A) and 1D IEF (B) followed by immunoblotting. SL: proteins of pooled sperm extract, silver and Coomassie blue stained; s1 – 3: screening of sera interacting with sperm antigens; C: negative control serum.

Figure 3 illustrates anti-human sperm reaction type IgE. Sperm proteins were not recognized by IgE antibodies from sera of women with fertility failure either after SDS-PAGE (Fig. 3A) or IEF (Fig. 3B).

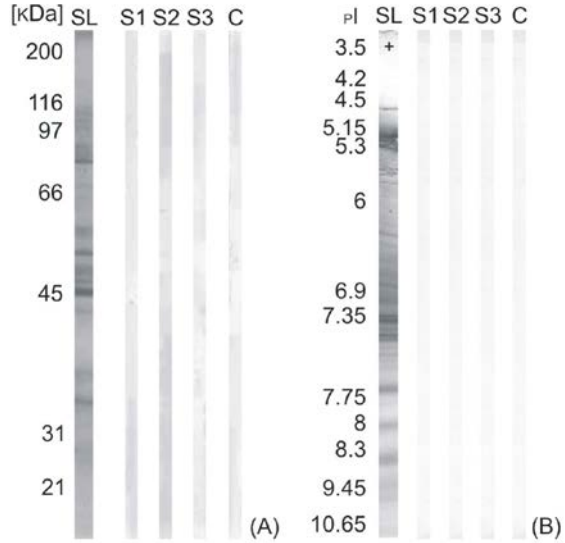

Figure 3. IgE detection of sperm antigens separated by 1D SDS-PAGE (A) and 1D IEF (B) followed by immunoblotting. SL: protein of pooled sperm extract, silver and Coomassie blue stained; s1 – 3: screening of sera interacting with sperm antigens; C: negative control serum.

4. Discussion

More and more couples with fertility failure are diagnosed with unexplained infertility. For better understanding, it is necessary to look upon sperm as a

potential immunogenic agent. The characterization of sperm antigens plays an important role in successful treatment of immunological infertility.

In this paper, we analyzed the reactivity of IgG, IgA and IgE present in the sera of women with fertility failure with human sperm antigens separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis and isoelectric focusing. Our aim was to describe characteristics of immunodominant human sperm antigens in Western blot of 1D gel, in terms of the molecular weight and the isoelectric point of the most frequently recognized antigens. The emphasis of experimental work was on appropriate choice of the healthy donors with normal sperm parameters (WHO, 1992). Pooled sperm extract represented the antigen source. In the present study four donors of semen were chosen and forty-five sera of women with fertility failure were used for immunodetection and ten children's sera as negative control.

In general, higher levels of IgG antibodies in women with fertility failure represent the immunopathological response. Considering all results we showed that major proteins are rarely recognized as antigens, those are detected among the minor proteins in sperm protein extract.

Another study distinguished a panel of IgG-binding proteins of molecular weight (Mr) less than 70 kDa (Feng, 2008). Our results showed higher molecular masses. Feng (2008) reported immunoreactivity of sera from female infertile patients toward the 35, 40, 47, 65 kDa proteins extracted from donor sperm. 123 kDa antigen revealed in our Western blot analysis is not in an agreement with mentioned study. We explain this by using different protocol for sperm protein extract. It is possible that 1% SDS used in their study may dissolve another groups of antigens than 1% Triton-X100 used in our samples. They reported immunogenicity of antigens coming from sperm surface membrane. Potentially, antigen presenting Mr of 123 kDa could come in origin from inner part of sperm cells. Which protein it is exactly, membrane or plasmatic, and whether or not the similar Mr mean the same proteins can be judged only on the base of mass spectrometry analysis. The same applies to close values of Mr: 65 kDa reported by Feng and 68 kDa revealed in our study. IgG-binding proteins reached pI 6.6 and 6.62. It presents one or two antigens. Bohring (2001) showed approximately same isoelectric point (pI 6.5) only for one antigen but detected by male infertile patients and also not as the most frequently recognized one. It means that IgG-binding protein with pI close to 6 could be universal for both sexes. We would like to remark the sample deposit in the part of pI 3.7. Overall, collected data demonstrated that IgG antibodies presented in female patients interfere with infertility.

Kuttech (1995) determined the immunoglobulin A distribution of antisperm antibodies in the sera and cervical mucus of infertile women. Our results are in agreement with the presence of IgA in female patient sera. In comparison with study of Bohring (2001) involving characterization of IgA-binding proteins from sera of men subjects, our results differ from those described in the literature: Bohring (2001) found Mr not higher than 85 kDa and pI value more basic than 6 while we displayed Mr of 130 kDa and pI 6.9, 7.4, 9.5, maybe representing isoforms. Although, these three bands were not always detected simultaneously by patient sera therefore cannot be marked as isoforms. IgA reaction to possible protein of pI 3.7 means a sample deposit and to evaluate it as the real IgA-mediated antigen would be very doubtful. It is well-known that antibodies in the genital track are predominantly IgG and not IgA (Johansson and Lycke, 2003). Our findings indicate that mucosal immunity represented by IgA reactivity to sperm proteins and their local and circulating distribution provide important and valuable knowledge about association with infertility in women.

Our study in contrast with other (Marthur *et al*., 1981) did not prove anti-IgE reactivity from female sera. The absence of IgE-mediated response could be explained by the very low level of IgEs in patient sera, antibodies possibly bound antigens very faintly or shortly after immediate contact with sperm cells, or it is possible to explain this by the fact that no women suffered from the typical allergic reactions after sexual intercourse like itching, swelling, burning etc. even anaphylaxis to individual semen components. Our results did not document the occurrence of local allergic reaction mediated by IgE antibodies which was however published elsewhere (Ferre-Ybarz *et al*., 2006). It is also well-known that the allergic reaction to semen components is not always associated with infertility (Ebo *et al*., 1995; Weidinger *et al*., 2005).

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