Original Article

Female Serum Immunoglobulins G, A, E and Their Immunological Reactions to Seminal Fluid Antigens

(human seminal plasma / infertility / immunoblotting)

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Abstract. One in five couples of reproductive age has been diagnosed with infertility. Some diagnoses indicate an immunological basis for this disorder. Female immune infertility may be caused by iso-immunization by seminal components. We focused on the characterization of seminal proteins to illustrate the IgG, IgA and IgE immune responses of 31 infertile women. The biochemical characterization was performed by one-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis and isoelectric focusing, both of which were followed by immunoblotting analyses. IgG mainly recognized the antigens with relative molecular masses (Mr) 95 and 183 kDa and isoelectric points ranging from 6.9 to 7.0. The immunodominant antigens recognized by IgA had the Mr of 35 kDa and isoelectric points ranging from 6.2 to 7.2. The reactivity of IgE was not confirmed within our group of patients. The seminal IgG- and IgA--binding patterns were analysed immunochemically to determine the characteristics of possible seminal proteins associated with female immune infertility.

Received February 19, 2012. Accepted September 19, 2012.

This work was supported by the grant projects of the Ministry of Education, Youth and Sports of the Czech Republic VZ MSMT 0021620812 and VZ MSMT 6046137305 and from specific university research (MSMT No. 21/2012).

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Abbreviation: 1D SDS-PAGE – one-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis, ASA – antisperm antibodies, BCA – bicinchoninic acid, BSA – bovine serum albumin, EGTA – ethylene glycol tetraacetic acid, HSP – human seminal plasma, IEF – isoelectric focusing, IgA, IgE, IgG – immunoglobulin A, E, G, Mr – relative molecular mass, NC – nitrocellulose membrane, NCa – activated NC, PGE – prostaglandin E, pI – isoelectric point, TGF-β – transforming growth factor β.

Introduction

One in five couples of reproductive age experience infertility. Twenty % of these couples suffer from infertility of unexplained aetiology. Some of the diagnoses indicate that the infertility is caused by an immunological disorder (Verpillat et al., 1995; Chiu and Chamley, 2003). Although the relative importance of immunological factors in human reproduction remains controversial, it has been suggested that human leukocyte antigens, antisperm antibodies, antiphospholipid antibodies, integrins, cytokines or endometrial adhesion factors contribute to the fertility failure (Choudhury and Knapp, 2001). Some studies (Ulčová-Gallová, 2003; Brazdova et al., 2012) have shown that immune infertility could be caused by auto-immunization in men and by iso-immunization in women by antigens from sperm cells and/or human seminal plasma (HSP) components.

Seminal plasma contains a wide range of cellular components and organic/inorganic substances, such as neutral α-glucosidase, carnitine, glycerolphosphocholine, fructose, prostaglandins, citrate, zinc, selenium (Zöpfgen et al., 1999; Rodriguez-Martinez et al., 2011). This complex mixture of secretions coming from the testis, epididymis and accessory glands serves as a medium that transports ejaculated sperm into the female genital tract (Kumar et al., 2009). Seminal components that bind to the acrosomal region of the sperm head protect it and are then carried together with sperm into the higher genital tract. HSP plays an important role in moving the sperm cells into the female reproductive tract thanks to its high content of transforming growth factor β (TGF-β) and prostaglandin E (PGE), both of which inhibit the function of NK cells, and neutrophils that are recruited into the superficial epithelial layers of the cervical tissues (Robertson, 2005; Bronson 2011; Morrell et al., 2012). HSP may modulate the chemotatic and phagocytic response of the female reproductive tract. Mainly, the immune modulating properties are mediated by the prostaglandins of the E series, complement in-

hibitors, cytokines and proteins capable of binding IgG antibodies (Kelly and Critchley, 1997). HSP allergy or hypersensitivity mediated by IgE antibodies is defined by systemic and/or localized symptoms after exposure to seminal plasma. The symptoms can manifest after the first time intercourse in up to 50 % of cases. Patients often have difficulty to conceive; however, infertility has not been demonstrated in association with HSP hypersensitivity (Weidinger et al., 2005; Bernstein, 2011).

The aim of our study was to characterize seminal proteins recognized by the IgG, IgA and IgE antibodies from infertile women. Our results describing seminal proteins may provide basic information for future projects associated with female immune infertility.

Material and Methods

Sample preparation

Semen samples from four normospermic healthy donors were obtained by masturbation after 3–5 days of abstinence. One ml of liquefied semen from four individuals was separately centrifuged at 1075 *g* for 15 min at 4 °C. The supernatants, representing seminal fluid, were pooled and a mixture of protease inhibitors was added (0.05 M ε-aminocaproic acid, 5 mM ethylene glycol tetraacetic acid (EGTA) and 0.01M benzamidine). Protein concentration of the pooled sample was measured by the bicinchoninic acid (BCA, Sigma, St. Louis, MO) protocol (Smith et al., 1985) using bovine serum albumin (BSA) as a standard. The samples were stored at -20 °C until assayed. All experiments were performed after obtaining informed written consent.

Patients

Sera were obtained from 31 women with fertility disorder. Ten sera of eight-year-old girls (virgins) were selected as negative controls. Sera were frozen at -20 °C until further use. All experiments were performed after obtaining informed written consent.

One-dimensional gel electrophoresis (1D SDS-PAGE)

SDS electrophoresis was carried out as previously described (Brazdova et al., 2012). After electrophoresis, the separated proteins were either transferred onto a nitrocellulose membrane (NC, Serva Electrophoresis GmbH, Heidelberg, Germany) or silver stained (SilverTM Plus Stain Kit, Sigma).

Immunoblotting analyses

Western blot analyses were performed as previously described (Towbin et al., 1979). The three millimeters wide NC membrane strips were blocked with TBS (TBS-Tw 0.1%, pH 8; 0.02 M Tris, 0.14 M NaCl, 2 mM $MgCl_2$, 0.1% v/v Tween 20) containing 10 % defatted milk powder. Each membrane was individually incubated with sera overnight at 4 °C. Sera were diluted to 1 : 1000 for IgG and IgA detection, 1 : 10 for IgE detection with TBS-Tw 0.1% supplemented by 5 % defatted milk powder. NC membranes were incubated with alkaline phosphatase (AP)-conjugated anti-human IgG (Promega, Madison, WI) diluted to 1 : 10,000, anti-human IgA diluted to 1 : 5000 (Sigma) or anti-human IgE diluted to 1 : 5000 (Sigma) for 2 h at 20 °C. The AP activity was detected by NBT/BCIP (IMMUNO NBT/BCIP, Liquid substrate plus, MP Biomedicals, Santa Ana, CA).

Isoelectric focusing (IEF)

Isoelectric focusing was performed in a polyacrylamide gel as previously described (Desvaux et al., 1990). After IEF, the separated proteins were either transferred onto a cyanogen bromide-activated nitrocellulose (NCa) membrane (Demeulemester et al., 1987) or Coomassie blue stained. Passive transfer was performed for one hour at room temperature. Immunodetection was performed as described in the above section.

Results

The pooled seminal fluid served as the source of possible antigens. The protein concentration ranged from 20 to 30 mg/ml. Fig. 1 shows the protein profile of HSP that was separated by SDS-PAGE (Fig. 1A SF) and IEF (Fig. 1B SF), and then detected by immunoglobulin G from the female patient sera (Fig. 1A, B S1–S6). The seminal proteins were visualized within the relative molecular mass (Mr) range of 23–203 kDa. IgG-binding proteins defined by Mr of 95 and 183 kDa were the most frequently recognized antigens (Fig. 1A S1–S6). Ninety-one % of the tested sera interacted with Mr 95 kDa and 88 % with Mr 183 kDa. In addition, some sera, e.g. S1, S2, showed reactivity to 40 kDa, other sera (S3, S4) to 42 and 156 kDa, and S5, S6 to 45 and 100 kDa proteins. HSP proteins were focused within pI ranging from 4 to 9. The pI range of 6.9–7.0 was immunodominant (Fig. 1B S4–S6). Up to three antigens could have been covered in this area. No IgG reactivity was revealed by immunoblotting using the control sera. We then determined the Mr and pI of HSP antigens that interacted with IgA antibodies. By blotting, two antigens of Mr 34 and 35 kDa were found (Fig. 2A). The 34 kDa antigen was detected in 42 % of the tested sera and 35 kDa antigen in 84 % of them. Ten % of female sera recognized the 66 kDa protein (Fig. 2A S3). Fig. 2 part B illustrates patient sera interacting with HSP antigens whose pI ranged from 4.3 to 5.5 (Fig. 2B S1–S5); in particular, pI ranging from 5.1 to 5.5 was clearly recognized by 95 % of all 31 sera. Another antigen presenting pI 4.2 was revealed in 75 % of cases. Antigen of pI 4.6 was detected in the presence of 20 % of sera. Eighty % of female sera faintly recognized up to four possible IgA-binding proteins at pI ranging from 6.2 to 7.2. These antigens were not found using the control sera. HSP proteins were separated by either SDS-PAGE (Fig. 3A) or IEF (Fig. 3B). No antigen was recognized by the IgE from any female patients as well as the control sera tested.

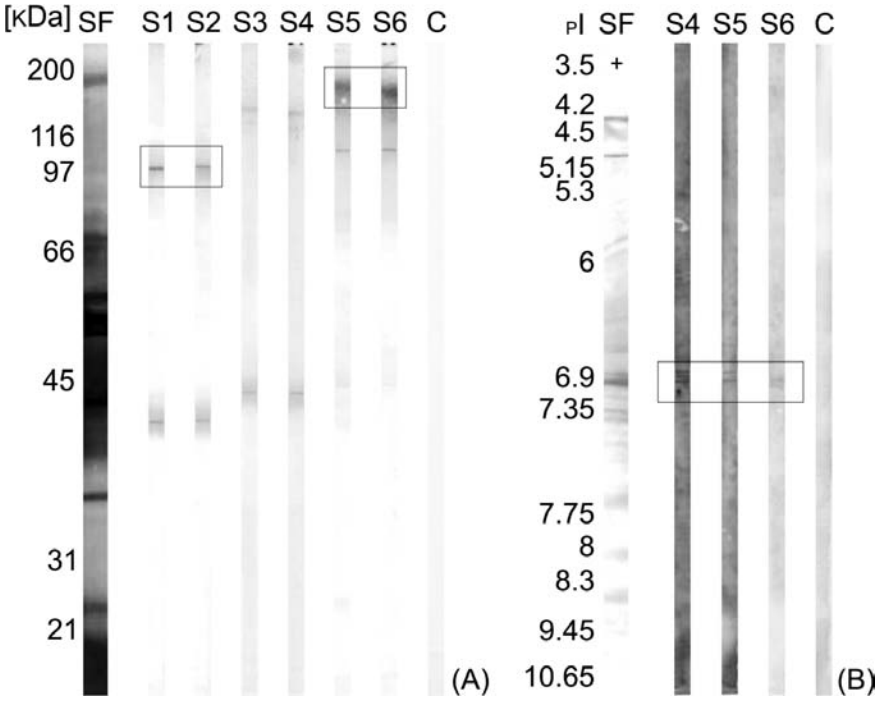

Fig. 1. IgG immunoblotting of seminal proteins separated by 1D SDS-PAGE (A) and IEF (B). SF: pooled seminal proteins, silver and Coomassie blue stained; S1–6: screening of female sera; C: negative control serum.

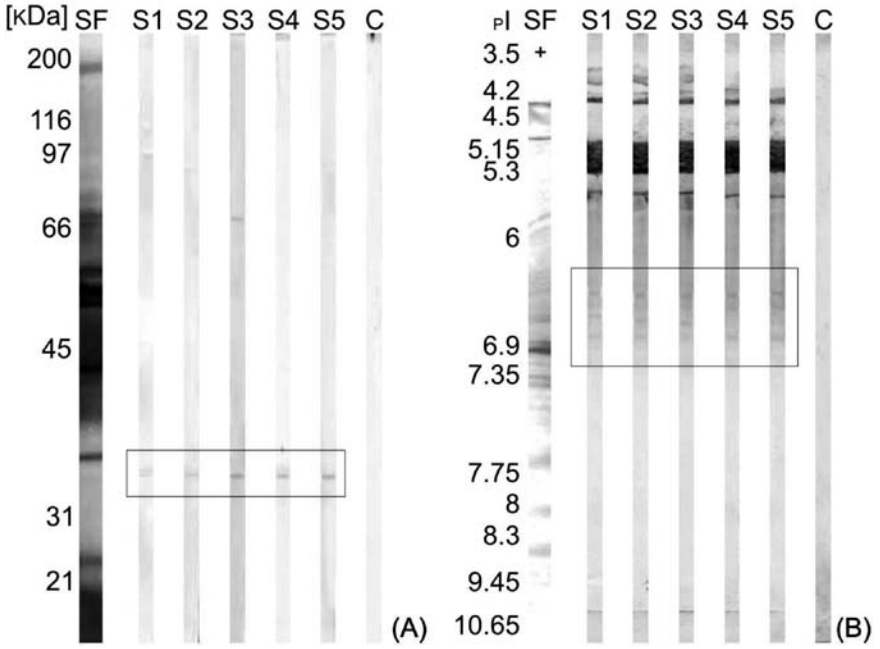

Fig. 2. IgA immunoblotting of seminal proteins separated by 1D SDS-PAGE (A) and IEF (B). SF: pooled seminal proteins, silver and Coomassie blue stained; S1–5: screening of female sera; C: negative control serum.

Discussion

Semen is defined as a complex fluid containing sperm cells, cellular vesicles and other cells, e.g. migrating leucocytes or spermatogenic cells (Rodriguez-Martinez et al., 2011). Each component could immunize the female genital tract.

Our results represent a pilot study dealing with female immune infertility in association with seminal proteins. We compared the immune response of infertile women and the control group (virgins) to HSP proteins. We chose this type of control group since the presence of anti-semen antibodies is not expected in the sera of virgins (Blum et al., 1989). In order to confirm the complex repertoire of common antigens, we decided to prepare the pooled seminal fluid from four healthy normospermic donors (WHO, 2010). We used non-reducing (Fig. 1A) and hydrophilic conditions (Fig. 1B) to keep

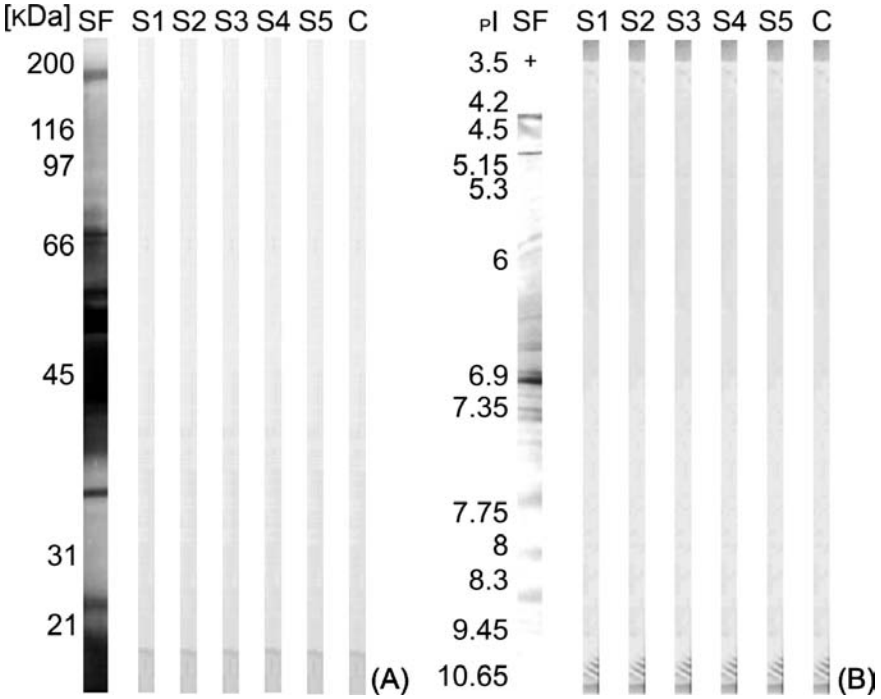

Fig. 3. IgE immunoblotting of seminal proteins separated by 1D SDS-PAGE (A) and IEF (B). SF: pooled seminal proteins, silver and Coomassie blue stained; S1–5: screening of female sera; C: negative control serum.

as much as possible conformational and linear epitopes on the seminal proteins (Brázdová et al., 2011). Chiu and Chamley (2002) studied antibody-binding HSP proteins using reducing conditions. In comparison, our results showed IgG-binding proteins of higher molecular masses (Fig. 1A). Until mass spectrometry is performed with the HSP antigens, we cannot identify them. Another explanation for the differences in molecular mass may consist in the utilization of fresh ejaculates (Autiero et al., 1991), while our experiments were dependent on frozen samples. Freezing and thawing may have altered the protein structure (Cutler, 2004).

Using female sera, we proved that the specificity of IgA antibodies differs from IgGs (Fig. 2). IgA-binding proteins have a lower Mr and more acidic pI than those interacting with the IgG isotype. The various Mr and pI of seminal IgG- and IgA-binding patterns may be explained by different roles of each class. The immunoglobulin isotypes have been suggested (Wolf et al., 1995) to have a specific activity in the fertilization process. However, we cannot attribute the various antigenic characteristics (Mr and pI) to a particular immunoglobulin class. Although IgG antibodies are present mostly in the serum, IgA antibodies were thought to be secreted locally (Davajan et al., 1972; Clifton et al., 1992).

Lee et al. (2008) reported a case of hypersensitivity of female serum to seminal plasma. This serum showed strong IgE reactivity to HSP proteins ranging from 30 to 45 kDa further used for desensitization. The 40, 42, and 45 kDa antigens revealed by IgG in our analyses may be responsible for another type of immune infertility in women. Allergy to seminal fluid is thought to be a rare phenomenon based mainly on IgE-seminal antibodies that mediate dangerous anaphylaxis in women (Lee et al., 2008). We found no IgE reactivity to common seminal proteins within our group of patients (Fig. 3). Ohman et al. (1990) observed IgE-mediated reaction to seminal proteins but detected no IgG, unlike we did in our experiments. In both previously cited observations the patients commonly suffered from the allergic symptoms of HSP hypersensitivity. In contrast, our patients did not suffer from the symptoms of immediate hypersensitivity caused by allergen-specific IgE antibodies.

In conclusion, the seminal proteins recognized by serum IgG and IgA antibodies as immunodominant may be relevant to female immune infertility by causing cytotoxic events at early fertilization. Our results suggest that the anti-seminal IgG and IgA antibodies from the selected group of patients exhibit different specificities. Since no IgE reactivity was found against proteins from the male seminal fluid in our work, we assume that the selected infertile female patients may not have an inherent allergic mechanism linked to the seminal components. Further studies of seminal proteins should contribute to a better understanding of female immune infertility.

Acknowledgement

The authors would like to gratefully thank Mr. Anthony McMahon for proofreading their article. We thank all donors.

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