Study of immunological properties of sperm and seminal plasma antigens : anti-seminal and anti-sperm antibodies in female immune infertility : characterization of targeted proteins

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Immunopathologie et immunointervention thérapeutique

Etude des propriétés immunologiques des antigènes de sperme et de liquide séminal : L'infertilité féminine due à des anticorps anti-protéines de liquide séminal et de spermatozoïdes. Caractérisation des protéines cibles.

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Jointly supervised thesis

STUDY OF IMMUNOLOGICAL PROPERTIES OF SPERM AND SEMINAL PLASMA ANTIGENS: ANTI-SEMINAL AND ANTI-SPERM ANTIBODIES IN FEMALE IMMUNE INFERTILITY. CHARACTERIZATION OF TARGETED PROTEINS.

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Declaration

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I hereby declare that I have worked out this thesis independently while noting all resources used, as well as all co-authors. I consent to publication of this thesis under Act No. 111/1998, Coll., the Higher Education Act of the Czech Republic, as amended by subsequent regulations. I have been informed of all duties and obligations applicable under Act No. 121/2000, Coll., the Copyright Act, as amended by subsequent regulations.

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Summary

The World health Organization reports infertility as a disease and a failure of reproductive tract to achieve pregnancy after 12 months or more of regular unprotected sexual intercourse. Nowadays, infertility has become a common life phenomenon affecting 1 out of 5 couples at reproductive age. Idiopathic cause is mostly associated with active immune system which may produce high levels of anti-seminal and/or anti-sperm antibodies. Auto-immunization as well as iso-immunization has a significant role in up to 30% of reported cases of infertility. Semen that is defined as a complex fluid containing sperm, cellular vesicles and other cells and components, could immunize the female genital tract.

This thesis is related to female immune infertility, in particular to female iso-immunization. The better understanding of this pathophysiological event consists of (1) the determination of antibody isotype playing a significant role in this disease, then (2) the characterization and identification of semen antibody-binding proteins, seminal and/or sperm, (3) the proposal of potential diagnostic markers to adapt specific therapy and, in addition, the design of miniaturized diagnostic tool based on the selected markers, (4) the suggestion of potential immuno-intervention.

Based on the distribution of seminal/sperm-specific antibody isotypes, we suggest that immunoglobulins E, M, $A_{1,2}$, G_3 are not involved in the primary pathophysiological female sensitization. IgG_4 appears to be the major subclass interacting with sperm proteins. On a contrary, IgG_1 seems to be the one mainly involved in the reactivity towards seminal proteins. We have also extended the existing group of IgG-binding sperm proteins, among which heat shock protein 70 1A/1B, heat shock cognate protein 71 kDa and alpha-enolase have been shown, for the first time, to be related to female iso-immunization. We have put the emphasis on the role of seminal proteins in iso-immunization and not only in the IgE-mediated semen hypersensitivity as known so far. In particular, prostate-specific antigen, prostatic acid phosphatase and zinc finger protein 778 have been determined as immunodominant among IgG-binding seminal proteins.

The determination of female serum seminal/sperm-specific IgG subclasses could make the patient diagnoses more comprehensive. Anti-seminal/sperm $IgG_{1,4}$ might be of interest for immunotherapy. Furthermore, the herein described proteins could be useful biomarkers of such pathology. The miniaturized chip could be a lateral flow immunoassay-based device acting on the immunochemical detection of specific antibodies. The intended immuno-intervention could consist of the effect of intravenous immunoglobulins.

Résumé

L'Organisation Mondiale de la Santé définit l'infertilité comme une maladie et un échec de l'appareil reproducteur à parvenir à une grossesse après 12 mois ou plus de rapports sexuels réguliers non protégés. De nos jours, l'infertilité est devenue un phénomène commun affectant 1 couple en âge de procréer sur 5. Une origine idiopathique est le plus souvent associée à un système immunitaire actif qui pourrait produire des niveaux élevés d'anticorps anti-liquide séminal ou anti-sperme. L'auto-immunisation, aussi bien que l'iso-immunisation, joue un rôle significatif dans jusqu'à 30% des cas signalés. Le liquide séminal, qui est défini comme un fluide complexe contenant le sperme, les vésicules cellulaires et autres cellules et composantes, pourraient immuniser l'appareil génital féminin.

Cette thèse est liée à l'infertilité féminine immune, en particulier à l'iso-immunisation féminine. Une meilleure compréhension de cette manifestation physiopathologique consiste en (1) la détermination des isotypes d'anticorps jouant un rôle significatif dans cette maladie, puis en (2) la caractérisation et l'identification des antigènes de liquide séminal ou de sperme reconnus par ces anticorps, (3) la proposition de marqueurs diagnostiques potentiels afin d'adapter des thérapies spécifiques, et, en outre, la conception d'un outil de diagnostic miniaturisé basé sur les marqueurs sélectionnés, (4) la suggestion d'une éventuelle immuno-intervention.

En se fondant sur la distribution des isotypes d'anticorps spécifiques au liquide séminal/sperme, nous suggérons que les immunoglobulines E, M, $A_{1,2}$, et G_3 ne sont pas impliquées dans la sensibilisation physiopathologique chez les femmes. Les IgG_4, semblent constituer la sous classe majeure interagissant avec les protéines de sperme. A l'inverse, les IgG_1 semblent être principalement impliquées dans la réactivité vis-à-vis des protéines séminales. Nous avons également élargi le groupe déjà existant d'IgGs liés aux protéines de sperme à d'autres protéines, parmi lesquelles la protéine de choc thermique 70 1A/1B, la protéine apparentée aux protéines de choc thermique 71kDa et l'alpha-énolase ont été reconnues, pour la première fois, être liés à l'iso-immunisation féminine. Nous avons mis en évidence le rôle des protéines séminales dans l'iso-immunisation et pas seulement dans l'hypersensibilité au sperme par l'intermédiaire d'IgE. En particulier, l'antigène spécifique de la prostate, la phosphatase acide prostatique et la protéine à doigt de zinc 778 ont été décrites comme immuno-dominants parmi les protéines séminales reconnues par des IgGs liés aux protéines de sperme.

La détermination des sous classes d'IgG de sérum féminin, spécifiques au liquide séminal/sperme, pourrait rendre le diagnostic des patients plus complet. Les IgG_1 et IgG_4 anti-liquide séminal/sperme pourraient présenter un intérêt pour l'immunothérapie. Par ailleurs, les protéines décrites, dans notre étude, pourraient se révéler être des bio marqueurs utiles pour de telles pathologies. Le dispositif miniaturisé pourrait être de type LFIA (Lateral Flow Immuno Assay), se basant sur la détection immuno-chimique d'anticorps spécifiques. L'immuno-intervention envisagée pourrait reposer sur l'effet d'immunoglobulines intraveineuses.

Aims of the thesis

1. Determine the distribution of serum semen-specific antibodies, which immunoglobulin classes, in particular which subclasses, play a significant role in female immune infertility within the patient group selected
    a. Quantify the anti-seminal/sperm antibodies in the sera of infertile female patients
    b. Compare the observed distribution of anti-seminal/sperm antibodies in the sera of fertile healthy women

2. Propose a method for the characterization of semen antigens associated with antibody formation involved in the pathophysiological iso-immunization
    a. Characterize the immunodominant seminal and sperm patterns in order to keep as much as possible detectable epitopes of potential seminal/sperm antigens
    b. Identify the immunodominant antigen repertoire detected by the sera of female patients diagnosed with the selected fertility disorder

3. Verify suggested protocols
    a. Verify the results in order to generalize the knowledge of antigens detected by the sera of infertile women
    b. Detect both seminal and sperm antigenic structures by the sera of fertile women in order to confirm the relevance of previous results

4. Propose the potential use of obtained results
    a. Suggest potential diagnostic markers to adapt specific therapeutic treatments
    b. Based on the selected markers, design a miniaturized diagnostic tool that could refine, simplify and hasten the diagnosis in order to make patient profiling more precise and to improve the diagnosis that may differentiate between seminal- and sperm-sensitivity
    c. Propose a therapeutic immuno-intervention

CONTENT

1 INTRODUCTION

The World Health Organization (WHO) defines infertility as a disease of the reproductive tract characterized by the failure to achieve pregnancy after 12 months or more of regular unprotected sexual intercourse. Infertility has increased over the last 30 years, WHO announced that the number of infertile couples has been increasing worldwide, up to 2 million per year. In 2003, infertility was reported as the most prevalent chronic health disorder concerning couples regardless of age. The causes of infertility are diagnosed at side of a man as well as a woman, about 40% of the time per each. The rest, about 20% of the time, the fertility problems are equally shared by both of them. An undeniable fact is the reproductive age, to which infertility is related. The maximal female fertility is reached at the age of 19 to 25. On a contrary, male fertility potential is not limited. However, the quality of semen decreases. Besides, the issues of conceiving are associated with worsened state of the environment and stress as well. The background of infertility is based on congenital, hormonal, morphological and immunological disorders. It may also be related to other diseases. Nevertheless, a specific, 100% sure, cause has never been diagnosed. In 15% of all cases, the idiopathic cause is suggested, further linked to immune infertility. The failure of natural tolerance leads to local/systematic immune system response resulting in the immune rejection of male sperm. Female immune infertility has become a serious health issue at the level of reproductive immunology.

The presented thesis is focused on female immune infertility. The better understanding of this pathophysiological event consists of the characterization and identification of semen proteins, seminal and/or sperm, which are involved in the iso-immunization avoiding the fertilization. At the same level, the experimental part reveals which antibody isotype, even which antibody subclass plays a significant role in this disease. Despite the fact that female immune infertility is not, nowadays, considered as an auto-immune disease, the effect of intravenous immunoglobulins could be of therapeutic significance.

The results of this doctoral thesis contribute to a detailed patient diagnosis and an improved therapy. The data shown suggests the potential diagnostic markers that might be of value for the following treatment and might lead the design of a diagnostic tool that could refine, simplify and hasten the diagnosis to differentiate between seminal- and sperm-sensitivity.

2 MALE FACTOR IN REPRODUCTION

Human semen is produced as a concentrated suspension of spermatozoa stored in the paired epididymides. During ejaculation it is mixed with, and diluted by, fluid secretions from the accessory sex organs. It is present as a fluid conglomerate emitted in several boluses. Semen has two major quantifiable attributes: (1) the total number of spermatozoa reflecting sperm production by the testes and the potency of the post-testicular duct system, (2) the total fluid volume reflecting the secretory activity of the glands. Semen reflects a heterogeneous cell suspension that differs among individuals in protein content, sperm number, pH, volume etc. These factors defined by the World Health Organization (WHO) are crucial when judging semen quality. Semen quality (Tab. 1, 2) is taken into account to measure male fertility in clinical andrology, male fertility, reproductive toxicology, epidemiology and pregnancy risk assessments (WHO, 2010). The quality of semen is verified by the presence of several components. For instance, the abundance of proteolytic fragments, decreased seminal level of albumin, poor testicular contribution characterize the oligozoospermic ejaculates. Seminal glycoprotein deficiency, the increased level of acid phosphatase and increased prostatic secretory activity are associated with asthenozoospermia (Autiero *et al*., 1991). Since specific substances can be assigned to specific reproductive compartments, chemical substances serve as diagnostic markers. Neutral α-glucosidase, carnitine, glycerolphosphocholine represent the indicators of epididymal functions. Prostate function is evaluated according to the levels of fructose, prostaglandins, citrate, zinc and prostatic acid phosphatase (Rowe *et al.*, 1993). Semen also contains several immunoregulatory factors as well as immunogenic agents that represent the possible targets of activated inflammatory cytokines, leukocytes and complement cascade in any part of the female genital tract (Chung *et al.*, 1994; Sharkey *et al.*, 2007; Rodriguez-Martinez *et al.*, 2011). One of the most common devices to evaluate spermiogram is the Makler counting chamber. It is composed of a metallic base unit, semi-circular ring, cover glass with surface graticule (Fig. 1, Makler, 1980).

The inability to achieve penile erection or to maintain an erection until ejaculation, caused by the congenital, neurological and metabolic disorders or abnormalities, is known as erectile dysfunction (*impotentia coeundi*). In addition, if spermatogenesis is disrupted, it results in the inability to procreate (*impotentia generandi*) (Ulcova-Gallova, 2006).

Fig. 1 Makler counting chamber, metallic base tool to evaluate sperm amount. (Makler, 1980)

Tab. 1 Semen characteristics (WHO, 2010)

Semen type	**Description**
aspermia	no semen/no mature sperm in semen no/retrograde ejaculation
asthenozoospermia	percentage of progressively motile sperm below the lower reference limit
asthenoteratozoospermia	percentages of both progressively motile and morphologically normal sperm below the lower reference limit
azoospermia	no sperm in the ejaculate, activity of seminal enzymes retarded
cryptozoospermia	less than 1 million of sperm in the ejaculate or none are seen on an initial evaluation, but only after centrifugation and concentration of the sample
haemospermia	presence of erythrocytes in the ejaculate
leukospermia	presence of leukocytes in the ejaculate
necrozoospermia	low percentage of live sperm, high percentage of immotile/dead sperm in the ejaculate
normozoospermia	total number of sperm and percentages of progressively motile and morphologically normal sperm equal to/above the lower reference limit
oligoasthenozoospermia	total number of sperm and percentage of progressively motile sperm less than 20 million
oligoasthenoteratozoospermia	total number of sperm and percentages of both progressively motile and morphologically normal sperm below the lower reference limit
oligoteratozoospermia	total number of sperm and percentage of morphologically normal sperm below the lower reference limit
oligozoospermia	total number of sperm below the lower reference limit
teratozoospermia	percentage of morphologically normal sperm below the lower reference limit

Tab. 2 Reference limits for semen characteristics (Cooper *et al.*, 2010)

Semen characteristics	Reference limit
volume	>1.5 ml
pH	7.1 – 7.8
sperm concentration	> 20 mil/ml
total sperm amount	> 40 mil
vitality	> 75%
motility	> 50% motile
morphology	> 50% normal morphology
agglutination	< 2 (scale 0 - 3)
viscosity	< 3 (scale 0 - 4)
liquefaction	within 30 min
leukocytes	< 1 mil/ml

2.1 Seminal fluid

Seminal fluid (SF), also cited as seminal plasma, represents a part of the semen containing a range of organic/inorganic substances (e.g. neutral α-glucosidase, hyaluronidase, carnitine, glycerolphosphocholin, fructose, prostaglandins, citrate, zinc, selenium) that are necessary for the physiological metabolism of sperm. The seminal complex mixture of secretions originates in the testis, epididymis and accessory glands including the prostate, seminal vesicles and Cowper's gland. It also acts as a nutritive, transport and buffering medium of pH 7.35 - 7.5 that defines the main SF functions: sperm protection from the acidic environment of the vagina, metabolic support and competence, liquefaction and clot formation. SF composition is similar to blood plasma. However, it differs in saccharide content (Kumar *et al.*, 2009; Rodriguez-Martinez *et al.*, 2011; Brazdova *et al.*, 2012a).

Semen liquefaction is caused by the fibrinolytic activity of some proteinases and peptidases present in prostatic SF. The key role of seminal enzymes (kallikrein-like protease 3, prostate-specific antigen) consists of a clot digestion formed immediately after ejaculation. It is said that SF containes hundreds of proteins of molecular or catalytic activity including additional proteins classified as their regulators (Yousef and Diamandis, 2001; Pilch and Mann, 2006).

In general, seminal proteins can be divided into three different groups. The first group contains the extracellular and intracellular proteins supporting basic SF function. The second group, originating in proteasomes and membrane-enclosed structures, is mainly involved in oocyte-sperm fusion. The third group is known as the potential biomarkers of testis/prostate cancer and male infertility as they represent the abraded epithelial cells from tissue surface (Pilch and Mann, 2006).

SF is in routine examined to evaluate pathological spermiogram and to monitor the progression of either testis or prostate cancer. In particular, prostate cancer is diagnosed by the seminal level of prostate-specific antigen (PSA), prostatic acid phosphatase (PAP), prostate stem-cell antigen or glutamate carboxypeptidase II that is a prostate-specific membrane antigen (Jones, 1991; Ostrowski and Kuciel, 1994; Cao *et al.*, 2003). Prostate cancer can be diagnosed by the serum PSA level. The serum concentration of 4 ng/ml means a 20-30% risk of prostate cancer. The risk increases approximately to 60% with a PSA level higher than 10 ng/ml (Fung *et al.*, 2004). An early elevated level of serum zinc alpha-2-glycoproteins (ZAG), originally secreted by the prostate, indicates tumor growth as well. PSA, PAP and prostate-specific protein-94 (PSP-94) belong to the prostate secretion that accounts for about 30% of the total SF volume. This SF fraction is in direct contact with sperm and is the first to confront the cervical tissues. PSA (γ-seminoprotein, seminin, kallikrein-3, P30 antigen, semenogelase) is a 33 kDa member of the glandular kallikrein subfamily of serine proteases. In particular, it is a member of the kallikrein subgroup of the (chymo)-trypsin serine protease family. It differs from the prototype member of this subgroup, tissue kallikrein, by possessing specificity more similar to that of chymotrypsin than trypsin. PSA is known as a zinc-binder and the most common protease in human semen. It is mainly released in proteasomes. It hydrolyzes semenogelin-1, thus leading to the liquefaction of the seminal coagulum. Its activity is strongly inhibited by zinc ions. This inhibition is relieved by exposure to semenogelins, which are avid zinc binders (Espana *et al.*, 1991; Utleg *et al.*, 2003; Pilch and Mann, 2006). PAP belongs to histidine acid phosphatase family, is stored in lysosomes and has an acid pH optimum below 7.0. It is a non-specific tyrosine phosphatase that dephosphorylates macromolecules and inactivates lysophosphatidic acid in SF. Its isoform 2 acts as a tumor suppressor of prostate cancer through the deactivation of mitogen-activated protein kinases. Decreased PAP level exhibits poor liquefaction (Autiero *et al.*, 1991; Tanaka *et al.*, 2004).

It contains the membrane-enveloped secretory vesicles. The fructose level is a marker of seminal vesicle function. High concentration of fructose is essential for sperm survival in the female reproductive tract. Fructose and other sugars are a source of energy for mitochondria-rich sperm. A decreased fructose level means a lower intensity of fructose oxidation. It leads to a lactate accumulation, which results in reduced sperm motility (Anderson *et al.*, 2004). SF is rich in proteins secreted by seminal vesicles. Semenogelin I and II, fibronectin and fibronectin-related derivates belong to the gel-forming proteins. They are active after the cleavage by kallikrein-like protease and then have a role in entrapping sperm into a viscous

gel immediately after ejaculation. They are also involved in sperm capacitation and sperm-oocyte interaction. Another SF element is lactoferrin, thanks to which SF may have an antimicrobial activity. SF content also involves albumin, serum and testis derived, that is a predominant protein participating in cholesterol removal from the sperm membrane during capacitation. The decreased seminal level of albumin and the increased seminal level of cholesterol are found in oligozoospermic men (Lilja *et al.*, 1987; Autiero *et al.*, 1991; Kosanovic and Jankovic, 2010).

Seminal components that bind to the acrosomal sperm head region protect sperm and are then carried together with it into the higher female genital tract. SF plays an important role in moving the sperm into the female reproductive tract due to its high content of transforming growth factor beta (TGFβ) and prostaglandin E (PGE), both of which inhibit the function of natural killer (NK) cells and neutrophils that are recruited into the superficial epithelial layers of the cervical tissues. SF is rich in PGE of 19-hydroxy form that promotes an expression of chemotactic interleukin 8 (IL-8). TGFβ is synthesized in the prostate and is testosterone-dependent. This glycoprotein belongs to cell-secreted molecules and occurs in 75% in the latent form in SF. It is further activated in the female reproductive tract by either the enzymes of male/female origin, acidic vaginal pH or through conformational change after an interaction with epithelial cells. The remaining proportion of TGFβ, 25%, exists in an active form (Denison *et al.*, 1999; Robertson *et al.*, 2002). TGFβ acting may result in the immune tolerance of seminal antigens, for which TGFβI is, most likely, responsible. It is a cytokine of TGFβ family. A divergent member of this family is growth/differentiation factor 15 (GDF 15), which is highly abundant in SF. Surprisingly, its level is not related to semen quality but its expression serves as a cancer marker, often in combination with PSA. However, GDF 15 has antitumorigenic activity. In contrast, the high level of GDF 15 in female serum corresponds to spontaneous abortion as it is expressed in placenta as well. It has been suggested that thanks to the presence of seminal antigens on a conceptus, TGFβ facilitates the female immune tolerance to a fetus (Robertson, 2005; Soucek *et al.*, 2010).

SF includes a repertoire of signaling molecules interacting with epithelium in the female reproductive tract. SF may modulate the chemotactic and phagocytic response of the female reproductive tract. Phagocytes serve to filter out the morphologically abnormal sperm. Sperm selection is based on morphological or antigenic structures. Mainly, the immune modulating properties are mediated by the prostaglandins of the E series, complement inhibitors, cytokines and proteins capable to bind IgG antibodies (Tomlinson *et al.*, 1992; Kelly and Critchley, 1997). Local reactions may lead to an inflammation (Robertson, 2005).

However, SF has a built-in mechanism preventing an immunological sensitization of the female against sperm as well as seminal structures. This protective system exists due to the presence of immune inhibitors originating in the male sex accessory glands (Prakash, 1981). SF has been suggested to be the modulator of sperm-induced inflammation that leads to sperm elimination from the female genital tract (Troedsson *et al.*, 2005).

SF elicits endometrial changes by inducing pro-inflammatory cytokines and cyclooxygenase-2. Their presence leads to macrophage and dendritic cell recruitment into the female reproductive tract. Seminal components activate the income of neutrophils into the endometrial stroma (Robertson, 2005; Bronson, 2011; Morrell *et al.*, 2012). However, it has been proved that the influx of neutrophils is higher and faster when the washed sperm inseminated (Rozeboom *et al.*, 1999). This fact proves the protective and signaling activity of SF. The immuno-suppressive activity prevents the iso-immunization of the female reproductive tract and suppresses the cell-mediated cytotoxicity (Lord *et al.*, 1977). Seminal prostaglandin D2 is known for its immuno-suppressive effect, by which the anti-sperm antibody formation is avoided in the female genital tract. The immuno-modulating properties are mediated by prostaglandin E, complement inhibitors, cytokines and proteins capable of binding the Fc region of IgG. These IgG-binding proteins are Fcγ receptor-like proteins. In general, seminal antibody-binding proteins contribute to sperm protection against immune-mediated damage by enabling successful sperm passage in the female reproductive tract and by blocking an interaction with immune effectors. For instance, prolactin-inducible protein (PIP), which is a secretory glycoprotein located in seminal vesicles, binds to immunoglobulin G via its Fc fragment, it may therefore be involved in immune regulation by trapping anti-sperm antibodies (ASA) and neutralizing them (Chiu and Chamley, 2002; Chiu and Chamley, 2003).

SF has been considered to be linked to the IgE-mediated rare reaction to semen (Weidinger *et al.*, 2006). This rare phenomenon was firstly reported by James (1945). Human seminal plasma allergy (HSPA) or the so-called hypersensitivity to semen is defined by localized and/or systemic symptoms after exposure to seminal fluid. The symptoms occur immediately after contact with semen or even within several hours after intercourse. The local symptoms include vulvar/vaginal itching, burning, redness and swelling. Local reaction can appear on any semen contact site. Local symptoms can be misdiagnosed as chronic vulvo-vaginitis caused by bacteria, yeasts, viruses and other parasites. Systemic features include generalized urticaria, angioedema (face, tongue, lips, throat), dyspnea, wheezing, cough, chest tightness, rhinorrhea, nausea, vomiting, diarrhea. Generalized malaise may result in an

anaphylactic shock, which is a life-threatening reaction. The symptoms can manifest after the first time intercourse in up to 50% of cases. Response mediated by IgE antibodies is then the most common mechanism. It has been suggested (Basagana *et al.*, 2008) that female patients experiencing any allergic symptoms after/during the first time intercourse might be sensitive to other antigens/allergens that cross-react with SF. Basagana *et al.* (2008) has already proved the IgE cross-reactivity among proteins from dog epithelium and PSA. Patients diagnosed with HSPA have difficulties conceiving but infertility has not been demonstrated, so far (Shah and Panjabi, 2004; Weidinger *et al.*, 2006; Bernstein, 2011).

2.2 Spermatozoa

Mature male gamete is commonly known and cited as sperm but specifically called as spermatozoa. It was firstly described in 1677 by Anthony van Leeuwenhoek. Sperm is a male reproductive cell of approximately 55-65 µm, containing genetic information and participating in the fertilization of an ovum (150-300 µm). Sperm consists of a head, middle section (mid-piece) and tail (Fig. 2). It is characterized by a minimum of cytoplasm. The head contains a nucleus, its shape is flat and oval in order to attach and easily penetrate an oocyte. An anterior peak of sperm head carries a cap-like structure called acrosome. It is designed, thanks to its hydrolytic enzymes, to help the sperm to penetrate the oocyte. The middle section consists of mitochondrial spiral, outer dense fibers and core microtubular structures. The mitochondrial formation contains the enzymes of oxidative phosphorylation. The mid-piece is, therefore, composed of substances that propel the tail. The tail enables the sperm to be motile by rotating in a circular motion, not from side to side like a whip (Collins discovery encyclopedia, 2005). The speed of sperm in ejaculate ranges from 10 to 60 µm/s. Its movement is based on the enzymatic and microtubule components, and is calcium and magnesium dependent. The tail is able to move ten times per second. Twenty thousand movements are estimated to be needed to reach the oocyte. In vitro, the speed is positively influenced by methylxanthins, lower temperature, and negatively by proteolytic enzymes, hydrogen peroxide and human saliva as it contains amylase and lysozyme enzymes (Ulcova-Gallova, 2006).

Normal sperm is characterized by an oval head with a long tail. Abnormal sperm has the defects of any body part (Fig. 3). Defects occurring on the head cause different shapes: large (giant), small (micro), elongated, irregular, amorphous, and then involve the acrosome

deficiency and the so-called bicephalic head. The defective mid-piece is asymmetric, bent, thin, thick, irregular or with cytoplasmic droplets. The defective tail is coiled, shortened, hairpined, broken, duplicated or with terminal droplets. Any defects may impair the ability of the sperm to reach and fertilize the oocyte. In general, sperm morphological abnormalities are related to congenital background, varicocele, high fever, infection or drug use (Gilbert, 2000).

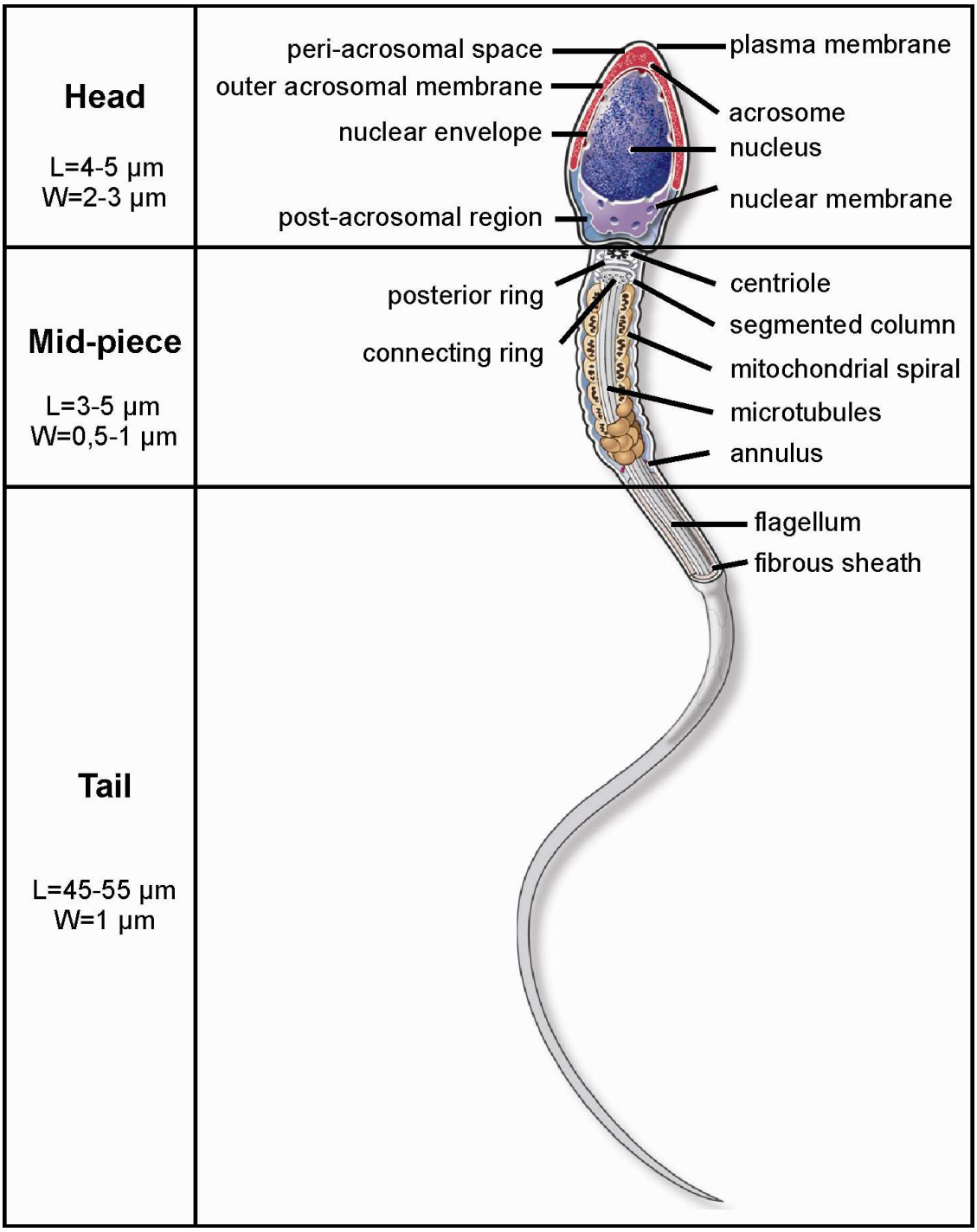

Fig. 2 Sperm description. L: length, W: width. Based on Warwick (2006), du Plessis *et al.* (2011).

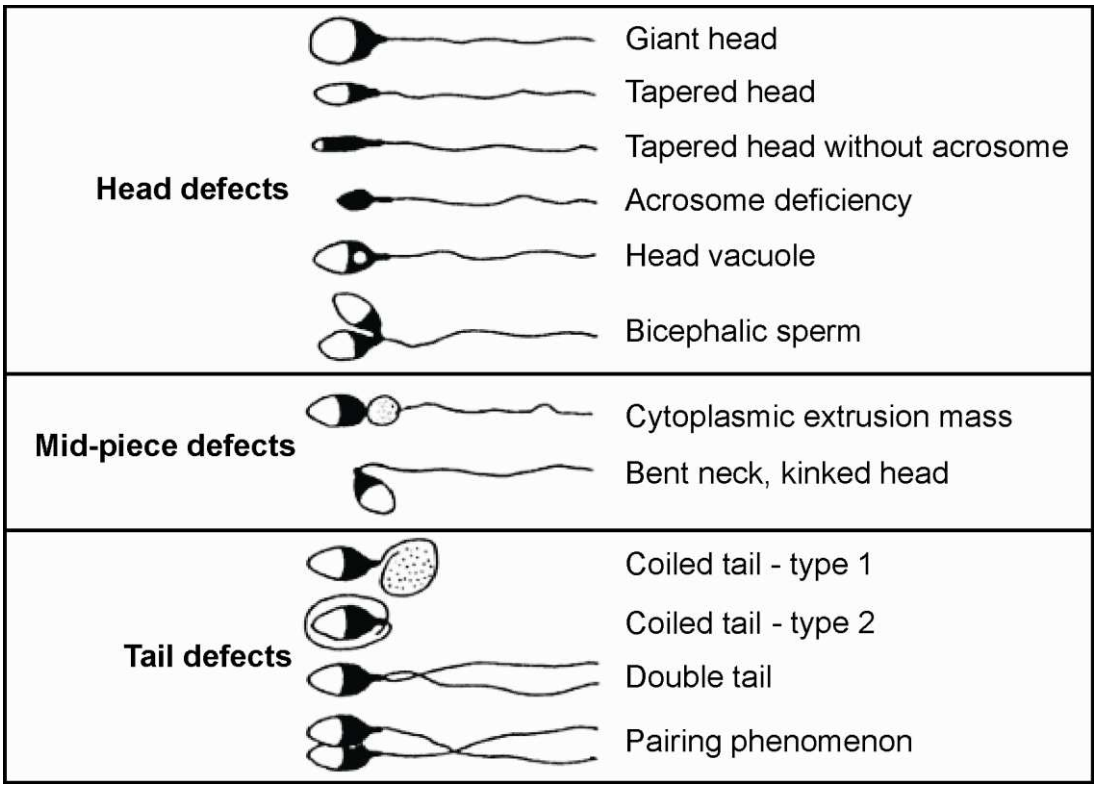

Fig. 3 Examples of sperm morphological abnormalities, head, mid-piece, tail defects. (MFMER, 2013)

2.2.1 Spermatogenesis

Spermatogenesis (Fig. 4) is a complex process creating functional sperm, starting at puberty and ending with death. It occurs in the testis, progresses to the epididymis and takes approximately 64 days for completion. Effective maturation is conditioned by temperature, 2 °C lower than body temperature. This explains its location in external genitals. Sperm cells are then stored in the epididymides. The entire process is regulated by hormones (follicle stimulating hormone, luteinizing hormone, testosterone). It consists of three major steps: (1) the multiplication of spermatogonia by mitosis, (2) meiosis to reduce the chromosome number from diploid to haploid, (3) the successful transformation of the round spermatid into the so-called spermatozoa. The spermatogonial population is created from the germ cells in the testis. The population then fuses with the Sertoli cells by creating seminiferous cords. After multiplication, several types of spermatogonia are distinguished: type A and B. The subsequent meiosis reduces the chromosome number from a diploid to a haploid form of type B spermatogonia. Type A diploid spermatogonium divides into two diploid cells called primary spermatocytes. The newly developed cells migrate into seminiferous tubules to

undergo the meiosis by creating the secondary spermatocytes. The secondary spermatocytes are haploid. The next step reflects the forming of rounded spermatids. The differentiated spermatids mature in the epididymis into functional spermatozoa. There is an evidence of post-translational modifications that are considered to be essential for efficient spermatozoa. Some of them can activate capacitation directly in the epididymis or post-ejaculatory in the female reproductive tract. The cascade of modifications includes phosphorylation, glycosylation, proteolytic cleavage and methylation. A healthy man is usually able to produce up to 100 million of sperm/day. However, the concentration of 20 million/ml reflects, nowadays, the mean amount (Ulcova-Gallova, 2006; Warwick, 2006; Vacek, 2006; du Plessis *et al.*, 2011).

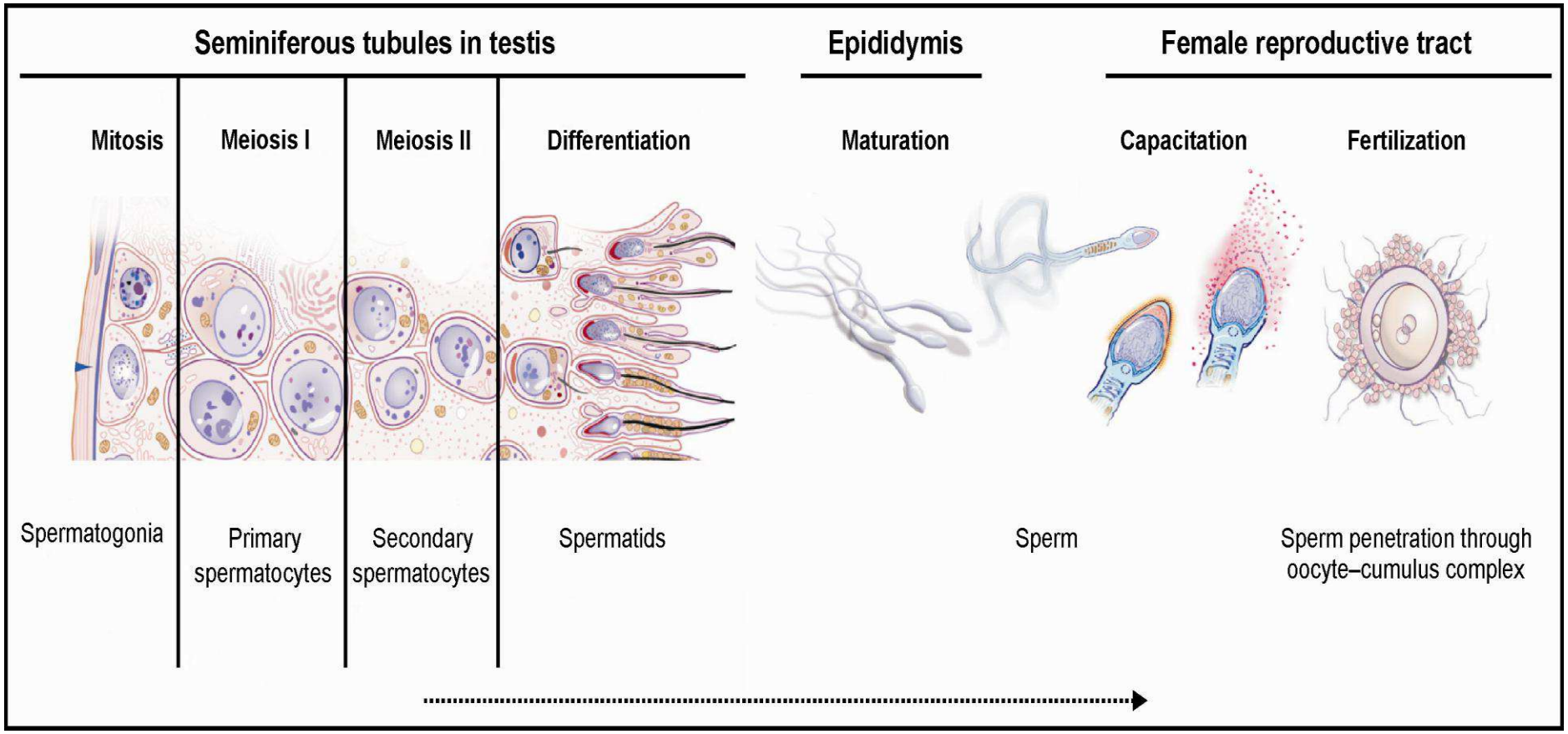

Fig. 4 Schematic view of complex spermatogenesis and following processes - maturation, capacitation, fertilization. Based on du Plessis *et al.* (2011).

2.2.2 Capacitation

Sperm capacitation was independently discovered by Austin (1951) and Chang (1951). Capacitation is defined as the process, by which the sperm becomes able to undergo an acrosome reaction in the uterus, then to penetrate and fertilize the oocyte.

The main purpose of sperm capacitation (Fig. 5) consists of hyperactivation in order to reach the oocyte, particularly in the remodeling of sperm membrane with regards to the fusion with the oocyte (Sutovsky, 2011). Capacitation involves a sperm surface remodeling, protein phosphorylation, hyperpolarization, an increased membrane fluidity, internal Ca^{2+} concentration and pH. A number of different seminal factors have been shown to act as the initial factors of capacitation: fertilization promoting peptide, adenosine, calcitonin, heparin binding proteins and angiotensin II. Other participating messengers are derived from sperm-oocyte interaction. Other factors originate in the female reproductive tract such as leukocytes, progesterone, fertilization promoting peptide, cholesterol, sialic acid binding proteins, capacitation-associated tyrosine-phosphorylated proteins, heparin binding proteins and atrial natriuretic peptide (Storey, 1995; Kumar *et al.*, 2008).

Capacitation requires a period of sperm incubation in the female tract, approximately 1 hour. It involves an increased metabolic activity, higher motility and the removal of the decapacitation factors from the sperm followed by the destabilization of the acrosomal sperm head membrane. This change involves the removal of steroids and seminal glycoproteins from sperm membrane. It allows greater binding between sperm and oocyte. Thanks to Ca^{2+} permeability, the binding is strengthened. An influx of Ca^{2+} results in sperm hyperactivation, which induces the higher cAMP level. Ca^{2+} and HCO_3^- play a critical role in the regulation of sperm function, most likely by acting as the enzyme effectors involved in signal transduction (Yanagimachi, 1994). Furthermore, the sperm adenylyl cyclase is significantly stimulated by HCO_3^-. The bicarbonate anion increases an intracellular pH and has been suggested to act as an anion antiport with respect to Ca^{2+} (Okamura *et al.*, 1985).

The major sperm sterol is cholesterol, among others e.g. desmosterol, desmosterol sulphate, cholesterol sulphate, cholesterol esters, cholestadienol. Additional cholesterol may be obtained from seminal plasma. Cholesterol moves from the sperm membrane to the acceptors and phospholipids into the sperm membrane. The ratio of cholesterol/phospholipids in a freshly ejaculated sperm is about 0.8. Albumin acts as a sink for the removal of cholesterol from the sperm plasma membrane. The loss is initially linear and leads to the exposure of a mannose receptor. Zona pellucida (ZP), an oocyte membrane composed of proteins (70%) and

saccharides (30%), contains mannose that interferes with sperm receptors. Cholesterol also inhibits the responsiveness and Ca^{2+}/H^{+} exchange in the ionophores. It has been suggested that the increased cholesterol level has a role in male unexplained infertility since the concentration is about twice as high as in the fertile subjects. Cholesterol efflux corresponds to an increased level of tyrosine as well as proline protein phosphorylation. The sperm cholesterol level varies among individuals (Mitra and Shivaji, 2005; Jha *et al.*, 2006).

Sperm protein tyrosine phosphorylation (PTP) has been considered to be the key signaling pathway. It is thought that it acts as a signal to alter mitochondrial function. Sperm PTP is dependent on the presence of calcium and bicarbonate ions. It is supported by ATP from glycolysis and is regulated through a protein kinase A (PKA) pathway. However, glucose is thought to inhibit PTP, which could be up-regulated by free radicals, most likely by superoxide anion. Moreover, the superoxide anion generation is also related to sperm lipid peroxidation, hyperactivation and viability (de Lamirande and Gagnon, 1993, Visconti *et al.*, 1998).

Capacitation also involves the membrane hyperpolarization caused by K^{+} permeability. It has been speculated whether K^{+} throughput independently allows the recruitment of Ca^{2+}. Subsequently, the membrane potential is increased in tens of mV (Zeng *et al.*, 1995; Visconti *et al.*, 1998).

An essential capacitation factor is a fertilization promoting peptide (FPP), a tripeptide (Glu-Glu-Pro) synthesized in the prostatic glands. FPP is present in SF and comes into contact with sperm after ejaculation. It becomes less active in the female genital tract due to vaginal acidic pH. It has a synergic stimulatory effect with adenosine that increases adenylyl cyclase activity in the sperm. Another seminal protein, semenogelin, appears to block sperm capacitation (Fraser and Osiguwa, 2004).

Sperm proteasome, located on the inner acrosomal membrane, is also involved in capacitation thanks to its proteolytic activity. It assists in protein removal during membrane remodeling, acrosome exocytosis by the degradation of membrane proteins in order to release the acrosomal matrix and to create the so-called acrosomal ghost. The sperm is then prepared to penetrate and fertilize the metaphase II-arrested oocyte (Sutovsky *et al.*, 2004; Zimmerman and Sutovsky, 2009).

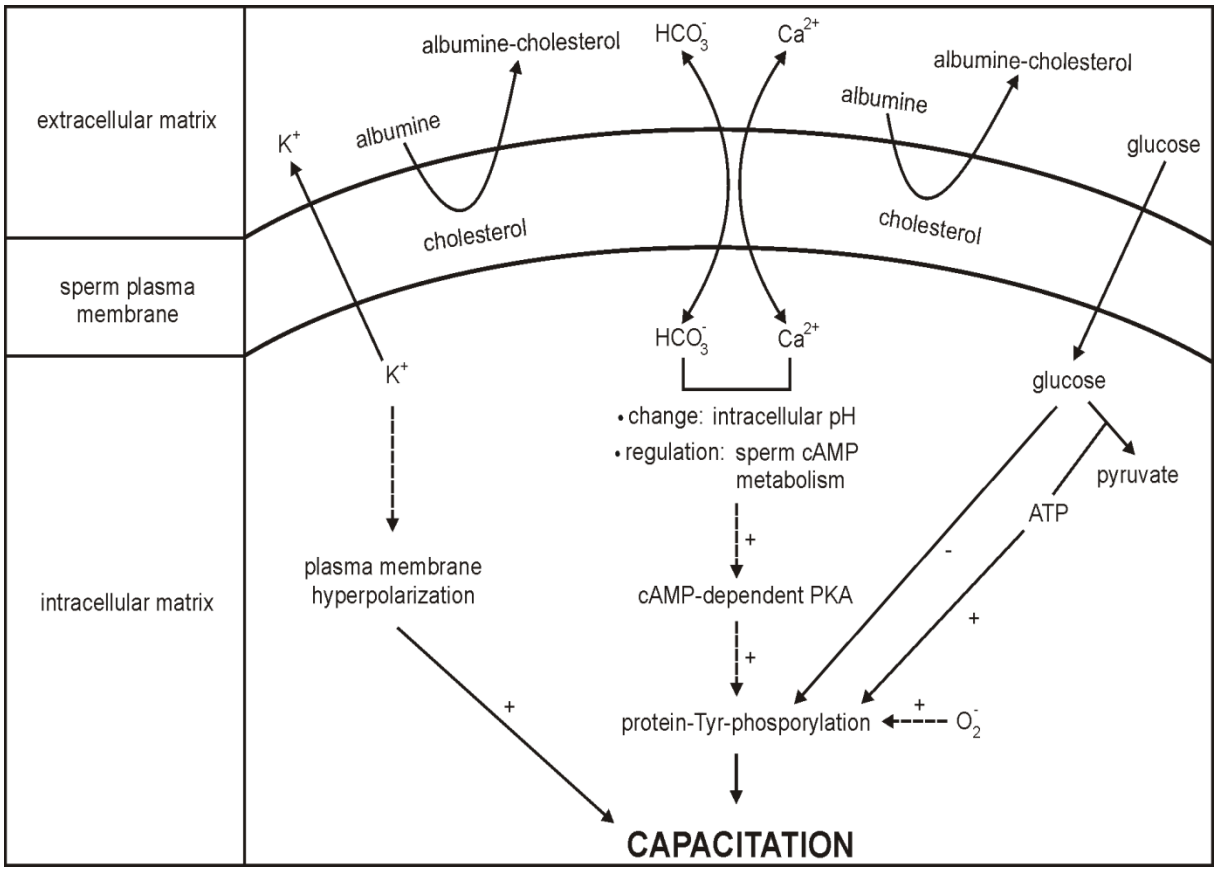

Fig. 5 Theoretical trans-membrane and intracellular signaling of sperm capacitation. PKA: protein kinase A, +/-: stimulating/inhibiting pathways, ⇢ consequence, → reaction, ion exchange. Based on de Lamirande and Gagnon (1993); Zeng *et al.* (1995); Visconti *et al.* (1998).

2.2.3 Acrosome reaction

Capacitated sperm becomes hyperactivated with regards to motility. Then, it is able to recognize the oocyte. The sperm membrane is destabilized after capacitation. It can be attached to ZP and undergo an acrosome reaction (AR). Gradual steps are mediated by an osmo-sensitive signal transducing mechanism. An oocyte-sperm fusion is coordinated by the carbohydrate-protein interaction. The carbohydrate-binding site on the sperm interacts with the oligosaccharide ligands of ZP (Topfer-Peterson *et al.*, 2000).

AR (Fig. 6) is defined as the physiological release of acrosomal content. It occurs in the female genital tract following sperm capacitation. It is a precondition to penetrate and fuse the oocyte ZP and to undergo the fertilization event. The cholesterol removal, elevated internal levels of Ca^{2+} and increased pH during capacitation are thought to trigger the acrosomal exocytosis that reduces the sperm membrane and enables to perceive the oocyte. Acrosomal

disruption involves the intracellular/membrane modifications and hydrolytic/proteolytic enzyme release. The most involved enzymes are acrosin and hyaluronidase that are required for oocyte penetration. AR activation and acrosomal exocytosis display the new membrane domains representing the new antigenic targets of the female immune system (Breitbart and Spungin, 1997; Patrat *et al.*, 2000).

AR inducing factors are zona pellucida glycoprotein 3 (ZP3) and progesterone, which is produced by the cumulus cells surrounding the oocyte. It has been shown that ZP binds at least to two sperm plasma membrane receptors, receptor activating phospholipase C (PLC) and tyrosine kinase receptor (TKR), which is coupled to PLC. Binding to a receptor activating PLC influences cAMP elevation and further PKA activation. Binding to TKR leads to the phosphorylation of the calcium transporter that activates phospholipase C (PLCγ). Activated PLCγ catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphoshate (PIP2) into inositol-triphosphate (IP3) and diacylglycerol (DAG). IP3 binds to a receptor on an outer acrosomal membrane, resulting in Ca^{2+} release. DAG stimulates phospholipase A (PLA) resulting in fusiogenic lipids (FL) and fatty acids (FA). These substances or their derivates, e.g. arachidonic acid, modulate the AR. The depolarization of plasma membrane by H^+/Na^+ ionophores increases intracellular pH and activates adenylyl cyclase, which stimulates cAMP elevation and PKA activation. H^+/Na^+ exchange also alkalizes the cytosol. The increased concentration of Ca^{2+} and increased pH allow membrane fusion, followed by the AR (Breitbart and Spungin, 1997; Patrat *et al.*, 2000).

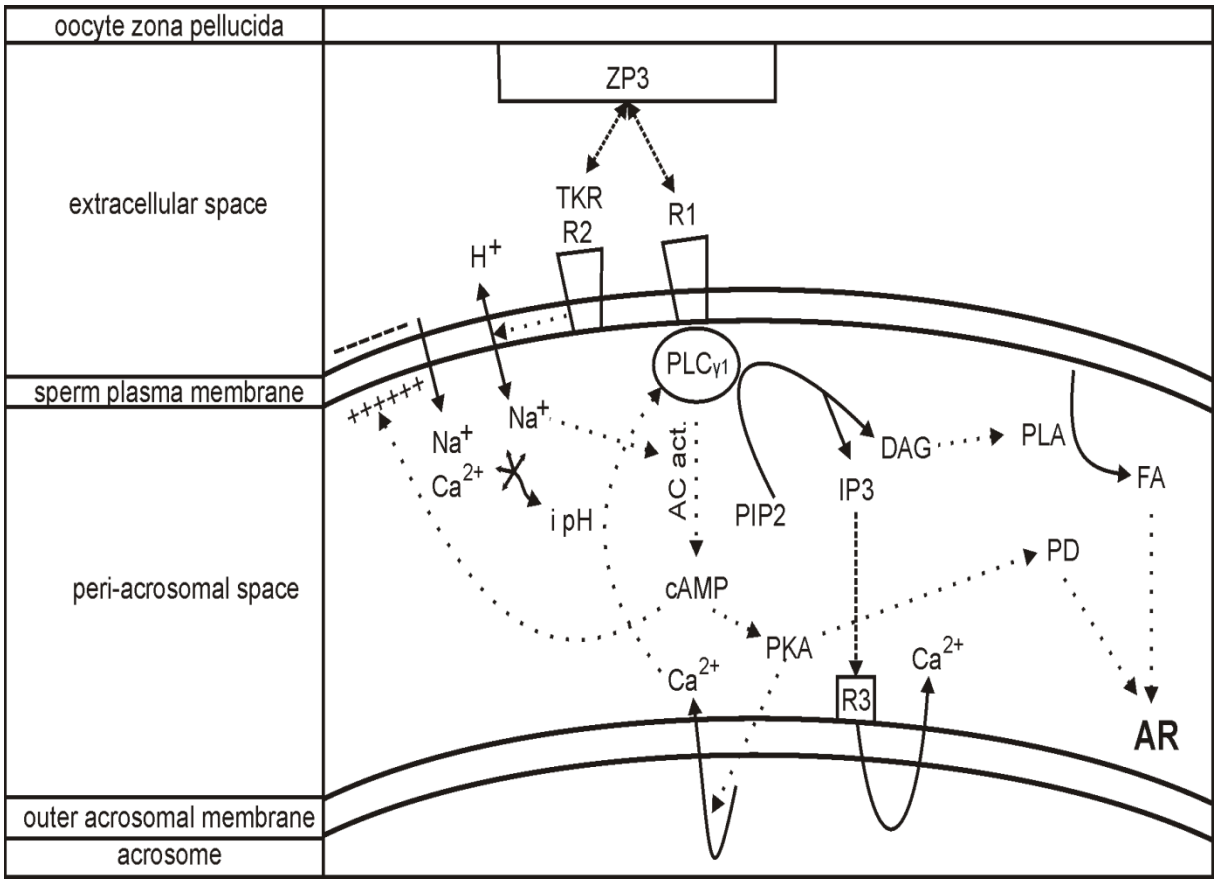

Fig. 6 Theoretical trans-membrane and intracellular signaling of sperm acrosome reaction. ZP3: zona pellucida glycoprotein, R1: coupled binding receptor, TKR R2: tyrosine kinase receptor, PLCγ_1: phospholipase C, i pH: increased intracellular pH, AC act.: activated adenylate cyclase, PKA: protein kinase A, PIP2: phosphatidylinositol 4,5-bisphoshate, IP3: inositol-triphosphate, DAG: diacylglycerol, PLA: phospholipase A, R3: IP3 binding receptor, PD: protein derivates, FA: fatty acids, AR: acrosome reaction, ⇢ binding, ⟶ reaction, ion exchange, ⇢ stimulation. Based on Breitbart and Spungin (1997), Patrat *et al.* (2000).

2.2.4 Fertilization

During sexual intercourse, millions of sperm are deposited in the vagina. In approximately 8 seconds after ejaculation, acidic vaginal pH is changed into to basic. SF immediately cleaves cervical mucus to enable sperm to move. The speed of sperm depends on the menstrual cycle and varies from 0.2 to 0.4 mm/min. Sperm is able to survive for up to 12 h in the vagina. It has been proposed that 1 sperm out of approximately 5000 reaches the fallopian tube in 10 min (Ulcova-Gallova, 2006).

Fertilization is defined as the union of male and female gametes to form a zygote (Fig. 7). Sperm is able to fertilize only a secondary oocyte at metaphase II, thus an oocyte after the meiotic division. The crucial step of fertilization consists of ZP penetration. Sperm has to pervade the oocyte-cumulus complex to reach ZP. Sperm binds to the oocyte thanks to components such as fukoidin and lectins. Low pH is required for dissolving this plasma membrane, the so-called envelope that protects the oocyte against any physical damage. The penetration of approximately 10 µm layer depends on the activity of sperm acrosomal proteases and glycohydrolases. ZP is composed of three glycoprotein layers (ZP1-3) that differ in their length. ZP1-3 layers represent a heterogeneous complex of asparagine (N)- and serine/threonine (O)-linked oligosaccharides, creating a fibrogranular layer. Sperm interacts with ZP either through spermadhesin, which is a lectin containing galactose, mannose or mannose-6-phosphate, or through proacrosin, which binds to sulphates. Sperm also binds to an oviductal epithelium through spermadhesin, thus it participates in the formation of the sperm oviductal reservoir. Once the signaling mechanism is initiated, ZP receptor kinases and fertility antigen A1 are autophosphorylated. With regards to the hyperactivity of flagellating sperm tail and its acrosomal enzymes, ZP is digested. Oocyte and sperm membranes fuse. After ZP penetration, sperm interacts with a vitelline membrane, which implies the development of an embryo. As soon as the fusion has been started through the receptors, the electrical block is set up. The polarity of the oocyte membrane is reversed and the polyspermy is blocked. The oocyte then releases its cortical granules to initiate the zona reaction, thereby preventing multiple sperm penetration. Cortical follicles/granules fuse with the plasma membrane. The cortical enzymes dissolve and remove the sperm binding receptors, thanks to which the vitelline envelope separates from the plasma membrane. Perivitelline space is created to form the fertilization membrane. When the sperm head penetrates the ovum, the tail separates from the rest of the sperm. Only a sperm nucleus enters the cytoplasm to fuse with an oocyte nucleus. After the fusion, a zygote is created. The diploid zygote undergoes division

to the 2-, 4- and then to 8-cell stage. The next stage is a morula, from which a blastocyst is formed. The blastocyst is then implanted. To summarize, the above-described process depends on sperm motility, sperm survival in acidic vaginal pH, cervical mucus viscosity and the activated female immune sytem (Yanagimachi, 1994; Topfer-Peterson, 1999; Topfer-Peterson *et al.*, 2000).

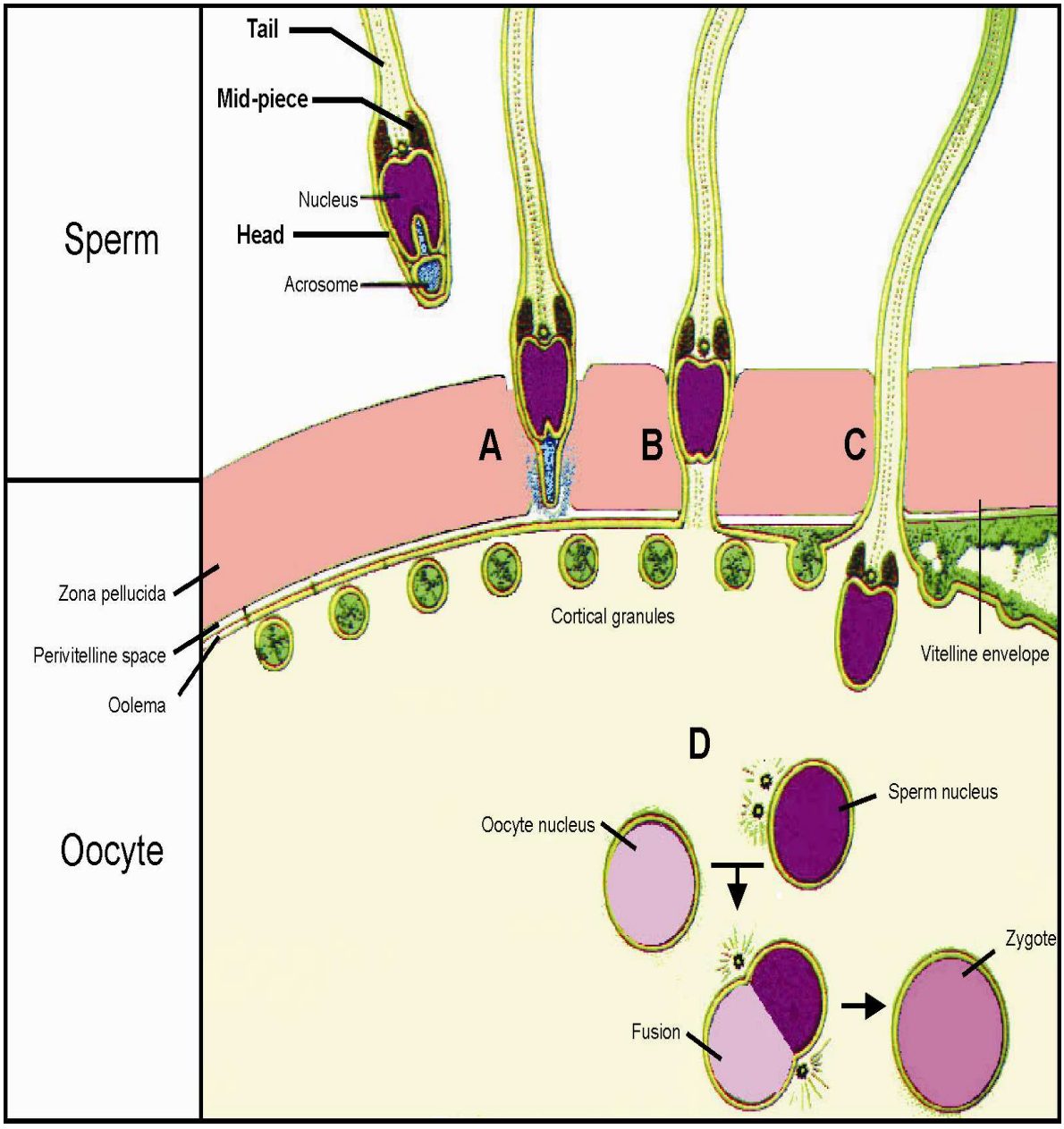

Fig. 7 Schema of fertilization. A: acrosome reaction, B: fusion of oocyte-sperm membrane, C: cortical reaction, D: fusion of oocyte/sperm nuclei, A, B, C phases last 20-30 s. Based on Alberts *et al.* (2002).

3 FEMALE FACTOR IN REPRODUCTION

3.1 Oocyte

The oocyte (Fig. 8) is a circular cell that was first found and described by Karl Ernst Ritter von Baer in 1827. The oocyte is a female gametocyte also defined as a developing female germ cell. It is 80-200 µm in diameter with a viability of 12-24 h. It is protected by ZP and surrounded by the cumulus and corona cells. It is formed in an oogenesis process. It is rich in cytoplasm, containing a lot of yolk granules to nourish the cell at an early development (Mandelbaum, 2010). In comparison with sperm, the male gametocyte, the oocyte differs in many aspects (Tab. 3).

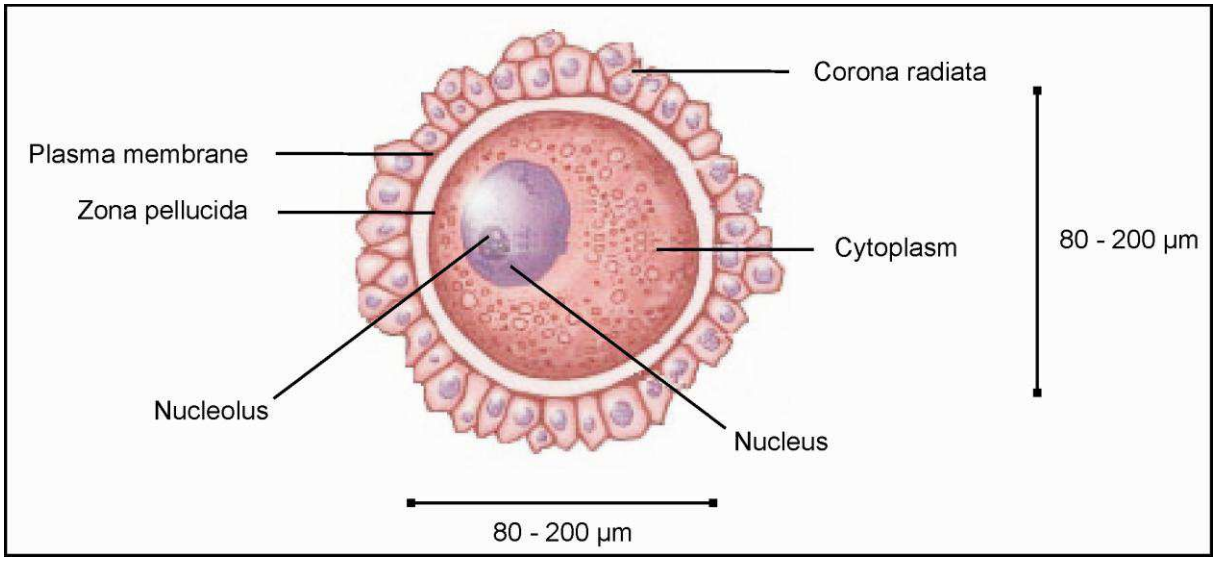

Fig. 8 Oocyte description. (http://ldysinger.stjohnsem.edu)

Tab. 3 Comparison of sperm and oocyte (Gilbert, 2000; Vacek, 2006)

Aspect	Sperm	Oocyte
size	55-65 µm	80-200 µm
size in body	one of the smallest cell	one of the largest cell
shape	straight, flagellated	spherical
body	parts – head, mid-piece, tail	uniform
cytoplasm	small amount	large amount
nucleus	condensed with no nucleoplasm	bloated with nucleoplasm
centriole	present	absent
mitochondria	compactly arranged	scattered in cytoplasm
motility	motile (2 mm/min)	immotile
protection	plasma membrane	several envelops

Tab. 3 Comparison of sperm and oocyte (continuation; Gilbert, 2000; Vacek, 2006)

Aspect	Sperm	Oocyte
viability	72 h	12-24 h
genesis	spermatogenesis	oogenesis
site of creation process	testis	ovary
chromosomes	23	23
genesis beginning	puberty	fetal period
genesis end	death	menopause
limitation of production	millions in 3 days	1 cell in 28 days
parent cells	unlimited number can become sperm	limited number can become oocyte
optimal temperature	2 °C less than body temperature	body temperature

3.1.1 Oogenesis

Oogenesis is the female equivalent of male spermatogenesis, and describes the development of an immature ovum (egg cell). The egg cells are produced in the ovary (Fig. 9) in several stages (Fig. 10). Primary follicles contain a primary oocyte and begin to produce the sex hormone estrogen. A primary oocyte carries two sets of chromosomes. In the first meiotic division, the primary oocyte is unevenly divided into a secondary oocyte (daughter cell) and the first polar body. The first polar body is an apoptotic cell that cannot be fertilized by sperm and does not contribute to zygote, embryo or fetus development. The secondary oocyte contains most of the original cytoplasm, is haploid and is bigger than the first polar body. The secondary follicle contains a secondary oocyte and produces estrogen and progesterone. As a result, the Graafian follicle develops. This follicle is named after its discoverer, Regnier de Graaf. It contains the secondary oocyte with a formed ZP. The secondary oocyte undergoes a second meiotic division into an ovum and a second polar body. In reality, the secondary oocyte is arrested at a metaphase of secondary meiotic division (metaphase II) until fertilization (chapter 2.2.4 Fertilization) takes place. Arrested at metaphase II, the secondary oocyte is released during ovulation by follicle rupture and is trapped by the linings of fallopian tube, which travels toward the uterus. The ruptured follicle undergoes transformation into a corpus luteum, a structure of approximately 2 cm (diameter). The corpus luteum is formed during the luteal phase of menstrual cycle, contains carotenoids and acts as an endocrine structure by producing progesterone. The secreted progesterone is necessary for the decidualization of endometrium. Decidualization involves several significant changes in the endometrium to implant the embryo. As the corpus luteum degenerates, a corpus albicans is developed (Gilbert, 2000; Vacek, 2006; Gellersen *et al.*, 2007).

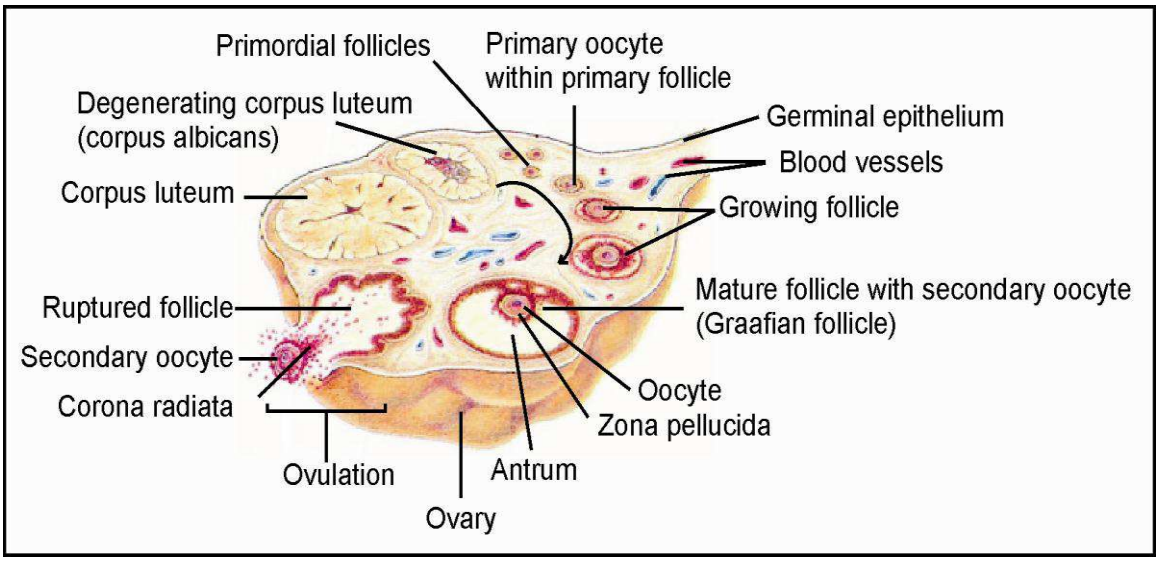

Fig. 9 Oogenesis, ovarian surface cut. (http://ldysinger.stjohnsem.edu)

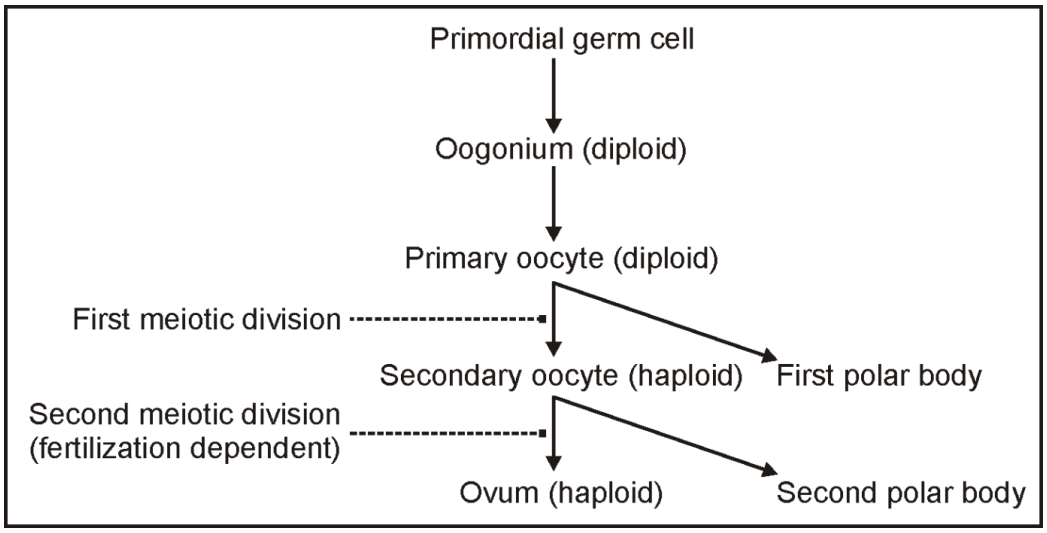

Fig. 10 Schema of oogenesis, particular division. (Gilbert, 2000; Vacek, 2006)

3.2 Embryo development

Sperm is able to fertilize the metaphase II-arrested oocyte (chapter 2.2.4 Fertilization) within 12-24 h after ovulation. After their fusion, a zygote is formed. Two-, four- and then eight-cell stages turn into a morula that represents the earliest phase of embryo development (Tab. 4). The journey of morula to uterus lasts approximately 4 days. The following step consists of a blastocyst formation. The trophoblast differentiates, then digests the uterine cells and burrows into the wall of the uterus. The blastocyst thus tries to attach itself to the uterine wall, and is implanted into the higher part of uterus. Implantation is conditioned by numerous endometrial changes (decidualization) that enable the acceptance of the blastocyst into uterus. Endometrial decidualization is a post-ovulatory and pregnancy-independent process. It remodels the endometrium and is based on the secretory transformation of the uterine glands, influx of uterine NK cells, vascular remodeling and stimulated glycogen accumulation in stromal cell cytoplasm. The decidualized stromal cells regulate trophoblast invasion and moderate potential oxidative stress and maternal immune response. Maternal macrophage activity is suppressed based on progesterone regulation and anti-idiotypic antibodies. Implantation also activates placenta development. The placenta produces progesterone and estrogen to maintain pregnancy. It serves as a vessel for the exchange of various compounds. Carbon dioxide, urea, water, and hormones are all transferred from fetus to mother. In the reverse direction, oxygen, nutrients, water, hormones, vitamins, and minerals are transported from mother to fetus. During the second trimester of pregnancy, the fetus is formed and its development continues up to the end of the third trimester (Gilbert, 2000; Vacek, 2006; Gellersen *et al.*, 2007).

Pregnancy may be complicated by pre-eclampsia (PE), a disorder affecting both the mother and the unborn child. This life-threatening syndrome is the fatal culprit of perinatal morbidity/mortality, abortions and premature births. It is suggested that an inadequate trophoblast invasion leads to a reduced placental perfusion at the end of the first trimester, which is followed by endothelial dysfunction and clinical manifestation by the end of the second/during the third trimester. So far, the etiology is unknown and the diagnosis is still very complicated. The clinical manifestation varies since PE is associated with several risk factors such as obesity, molar pregnancy (a non-viable fertilized ovum is implanted into the uterus), hypercoagulation, formation of clots or diabetes mellitus. PE includes the manifestation of symptoms such as hypertension, proteinuria, edema, hyperuricemia. Additional PE symptoms including hemolysis, elevated liver enzymes and low platelet count are referred to as HELLP syndrome (Brazdova et al., submitted 2014a). PE syndrome is

considered to be a two-stage disease. The reduced placental permeability is associated with the first stage, causing abnormal implantation and fetal placental release into the maternal circulation. As a result, the maternal immune system creates antibodies against the trophoblast. The second phase consists of the maternal reaction to this condition. It is characterized by inflammation and the endothelial dysfunction of parental cells (Redman and Sargent, 2000).

Tab. 4 Embryo development (Vacek, 2006)

Day	**Process**	**Product**	**Localization**
0	ovulation	released secondary oocyte	fallopian tube
0	fertilization	zygote	fallopian tube
1-2	cleavage	2-cell stage	fallopian tube
3-4	cleavage	4-cell stage	fallopian tube
	uncompact morula formation	8-cell stage	uterus
5	early blastocyst formation	blastocyst – trophectoderm, blastocoel, inner cell mass	uterus
6-7	hatching	last stage blastocyst	uterus
8-9	implantation	blastocyst – epiblast, hypoblast	uterus
16	development	embryo	uterus

4 INFERTILITY

The World Health Organization defines infertility as a disease of the reproductive system by the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse (Rowe *et al.*, 1993). Infertility has been reported to be one of the most prevalent chronic health disorders regarding couples of any age (Smith *et al.*, 2003).

Over the last three decades, the number of documented infertility cases has increased. Decreased fertility is associated with other health issues, age, life style and environment. The agent of infertility for a couple is the male partner about 40% of the time, the female partner about 40% of the time , and about 20% of the time the fertility problems are shared by both the man and the woman. Factors contributing to infertility involve congenital, hormonal, morphological and immunological disorders. Infertility may be also related to other diseases such as severe avitaminosis, severe renal impairment, cancer and cachexia due to malnutrition or tumor (Doherty and Clark, 2006).

The main disorders involved in infertility include pathologic spermiogram, ovulation problems/anovulation, tubal diseases, pelvic adhesion/endometriosis, cervical factors and idiopathic reasons – i.e. unexplained infertility. However, a single specific cause of infertility is never certainly diagnosed (Gleicher and Barad, 2006; Siristatidis and Bhattacharya, 2007).

4.1 Unexplained infertility

Unexplained infertility (UI) is diagnosed to a couple when the standard investigations including semen analyses and tests of ovulation and tubal potency do not provide specific results or do not provide evidence of any abnormality (Crosignani *et al.*, 1993). Several reports (Crosignani *et al.*, 1993; Gleicher and Barad, 2006; Siristatidis and Bhattacharya, 2007) suggest that the diagnosis of UI is subjective and that UI is often misdiagnosed for endometriosis, tubal infertility, premature ovarian ageing and immune infertility. The prevalence of UI reaches up to 30% of infertile couples with regards to standard investigation.

Severe endometriosis affects the fertility potential. Mild endometriosis is not, however, associated with infertility in the absence of secondary organic disruption. It has been reported that approximately 20% of infertile females suffer from tubal disease, either distal or peritubal (Smith *et al.*, 2003; Gleicher and Barad, 2006). Follicle number is genetically dependent.

Female subfertility caused by poor ovarian reserve is declared when the remaining follicle amount represents a fraction of the original value. This state becomes crucial at the age of 30. The critical point of ovaries is reached at the age of 38-40 when the follicle number decreases to approximately 25 000. At the age of 50, the amount reduces to approximately 1000 follicles (Nikolaou and Templeton, 2003). In some women, the so-called poor ovarian response has been noticed when the ageing ovary produces fewer follicles, follicles growth is poor, and follicular atresia occurs (Siristatidis and Bhattacharya, 2007).

Immunological tolerance plays a key role in UI. Molecular and cellular endometrial deficiency resulting in an implantation failure can be related to UI since the natural immunosuppression does not prevent maternal immune rejection. T regulatory cells (Treg) are believed to protect the fetus from an immune attack. Treg cells function in immune tolerance. The diminished production of Treg from naïve T helper (Th) cells is determined based on the expression of transcription factor Foxp3 (Agnello *et al.*, 2003; Sumerset *et al.*, 2004). UI is not only linked to Treg differentiation, and thus to immune suppression failure, but also to its recruitment into an implantation site. This is caused by the reduced expression and insufficient function of lymphocyte and chemotactic agents present in uterus. Since Treg differentiation is regulated by TGFβ, the idiopathic infertility may be related to a reduced availability of this factor. A lack of TGFβ results in insufficient Treg induction. Diminished CD4+CD25+ Treg population, the lower expression of Foxp3 and the failure of lymphocyte adherence and chemotaxis seem to play a negligible role in primary cause of UI (Bommireddy and Doetschman, 2004; Jasper *et al.*, 2006).

4.2 Immune infertility

During the early phase of the primary immune response after exposure to an antigenic agent, IgM antibodies are produced. The so-called antibody switch into IgG and IgA is induced at the late phase of primary immune response or after repeated exposure to the same antigen (Batard *et al.*, 1993). After chronic antigen exposure, IgG_1 and IgG_4 become the predominantly produced subclasses of IgG isotype. IgG_4 is a unique antibody acting as an anti-inflammatory immunoglobulin and a blocking antibody toward IgE. It yet remains unclear whether IgG_4 is a protective or a sensitizing antibody (Aalberse and Schuurman, 2002; Guma and Firestein, 2012). Some studies (Schroeder and Cavacini, 2010; Tamayo *et al.*, 2012) proposed that IgG_1 and IgG_3 are, in general, induced in response to antigens of a protein

nature while IgG_2 especially and IgG_4 to antigens of a polysaccharide character. Furthermore, the IgA_1 subclas may generally recognize the antigens of a protein and glycoprotein nature. Antibodies directed against antigens of a polysaccharide character may occur in the IgA_2 subclass (Kutteh *et al.*, 1994; Schroeder and Cavacini, 2010; Tamayo *et al.*, 2012).

Immune/immunological infertility is diagnosed when spontaneously produced antibodies bind to the antigens occurring on either the male or female gametocyte. In particular, antibodies bind to seminal proteins or structures present on the sperm or oocyte. So far, ASA have been observed more frequently than anti-oocyte antibodies (AOA) and are related to some cases of unexplained infertility (Bohring and Krause, 2003).

4.2.1 Anti-sperm antibodies

Sperm antigenicity within the animal kingdom was first described in 1899. In 1932, Baskin observed circulating antibodies against sperm and finally in 1954, Rümke observed and described the first type of anti-sperm antibodies. ASA have cytotoxic, immobilizing and agglutinating (Fig. 10) functions. The sperm-agglutinating type is most abundant among the European population and causes the so-called shaking phenomenon, while the sperm-immobilizing type is more prevalent in the Asian population. ASA are detectable on the systematic (blood and lymph) as well as the local level (SF, cervical-vaginal mucus). In general, the IgG isotype of ASA is mainly related to blood circulation and the IgA isotype to mucosal immunity in women. In men, IgG and IgA fractions are most prevalent in SF, while IgG and IgM isotypes prevail in serum (Ulcova-Gallova, 2006).

Sperm carries the paternal genome further displayed on an oocyte and has a very heterogeneous antigenic content. Since sperm has auto-antigenic (auto-immunization) as well as iso-antigenic (iso-immunization) potential, it is capable of inducing the production of sperm-reactive T-cells in men as well as in women. It is not a single ASA that influences fertility, but more likely an ensemble of ASA that causes infertility. It has been postulated that a single sperm antigen is not able to cause infertility. Further, it has been reported that not all ASA, regardless of being produced in women or men, influence the fertility potential since the cognate antigen is not necessarily involved in the fertilization process (Wang *et al.*, 2009; Haidl, 2010; Sedlackova *et al.*, 2010; Bronson, 2011).

A highly heterogeneous sperm antigenic content could even be modified during maturation and ejaculation based on antigen sequestration. Newly expressed antigens could be then in contact with any immunocompetent cells. For example, a sperm membrane-incorporated fibronectin exhibits changes in regional antigenic expression during sperm maturation, whereas secreted fibronectin is a product of male accessory sex glands and can be attached to sperm tails during ejaculation (Kosanovic and Jankovic, 2010). Considering gastrointestinal exposure, ASA formation may be operative (Bohring and Krause, 2003).

In men, sperm germ cells are protected in the testis from an auto-immune attack by the blood-testis barrier. When the barrier is disrupted, ASA are detectable in blood serum, seminal plasma or directly attached to sperm surface membrane. An increased risk of ASA formation may follow the congenital absence of reproductive tract components. ASA are mostly associated with genital inflammation or infection (e.g. orchitis), epididymis trauma, genital surgery, cryptorchidism and varicocele (Kosanovic and Jankovic, 2010). The theory of auto-immune disease was supported by Omu *et al.* (1999) by proving that ASA formation is related to certain human leukocyte antigen (HLA) classes.

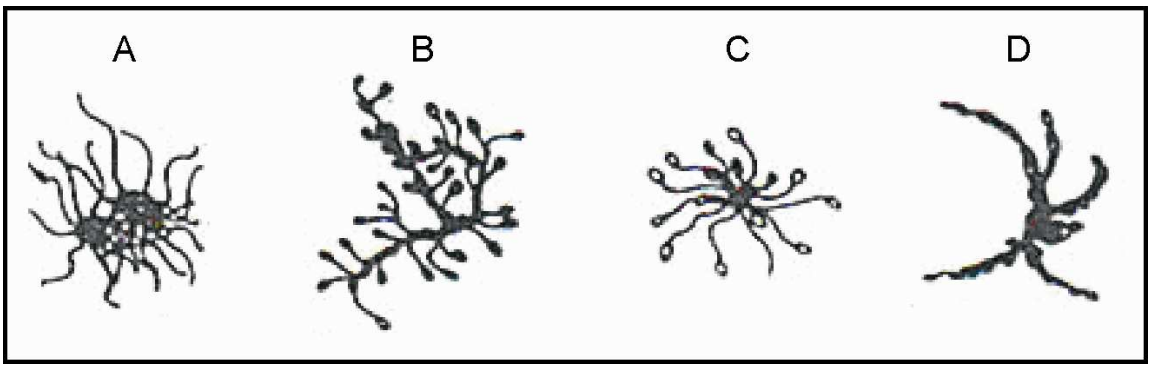

Fig. 11 Spermagglutinating effect of ASA. Sperm agglutination by head (A), tail (B), tail end-piece (C) and simultaneous agglutination by head and tail (D). (Ulcova-Gallova, 2006)

In women, the failure of natural tolerance may lead to sensitivity resulting in sperm elimination. ASA can induce antibody-mediated infertility through various pre/post-fertilization processes, such as sperm motility and agglutination, cervix mucus penetration, capacitation, acrosome reaction, zona pellucida binding and penetration, oolema binding, sperm-oocyte fusion and embryo implantation (Brazdova *et al.*, 2012b; Brazdova *et al.*, 2013b). The active local immuno-regulatory mechanism is based on vaginal and cervical tissues having an active and sensitive mucosal immune system, by which the fertility potential is affected. This explains the rather high percentage of infertile women in association to the local reactions leading to an inflammation as well as to high levels of serum anti-semen antibodies. Serum ASA are related to the long-term exposure of female to sperm and seminal deficiency in immuno-suppressive factors (Mazumdar and Levine, 1998; Bohring *et al.*, 2001).

As it was previously mentioned, sperm is able to induce the production of sperm-reactive T-cells, and thus is opsonized and then targeted by the leukocytes (sperm-cytotoxic effect) (Kurpisz and Kamieniczna, 2009; Sedlackova *et al.*, 2010; Bronson 2011). Nevertheless, there is evidence of ASA occurrence in fertile women and men. Some fertile individuals are positive in serum sperm agglutinins. It is suggested that these ASA are not clinically significant, but rather a physiological effect without a pathologic background. In this case, they might be considered as "natural" ASA (Chamley and Clarke, 2007; Kurpisz and Kamieniczna, 2009). The natural antibodies are produced in the absence of immunization, antigen exposure. They are produced by auto-reactive B-cells that were stimulated to grow. The correlation remains constant from early childhood to adulthood. In an adult serum, the prevalent percentage of natural antibodies belongs to autoantibodies of IgM and IgG isotype. This implies that auto-reactive T-cells contribute to a repertoire of auto-reactive B-cells under physiological conditions. Autoantibodies are polyreactive, help remove senescent molecules and cells, participate in immune auto-treatment of cancer and, hypothetically, may be considered as precursor antibodies (Bernabe *et al.*, 1981; Avrameas, 1991; Kazatchkine and Kaveri, 2001). The polyreactive character of such precursor antibodies may also play a part in cytotoxic reaction during early fertilization associated with infertility.

On the other hand, ASA are of great interest concerning immuno-contraceptive effect. The immuno-contraceptive potential of ASA has already been studied (Krause and Naz, 2009; Naz, 2011). It is limited by its specificity and possible role in the fertilization events. Of course, this potential immuno-contraception is of risk with regards to the induced high titers

of sperm specific T-cells. An antigenic complex prepared from the whole semen carries the risk of hypersensitivity to seminal fluid antigens or the risk of mimicry of various somatic cells. Direct ASA targeting has not yet been reported. Currently, ASA-mediated infertility is treated with systematical immunosuppression. The treatment of female immune infertility by use of monoclonal antibodies might be applicable but simultaneously complicated (Domagala and Kurpisz, 2004). Not only are sperm antigens considered in an immuno-contraceptive research, but ZP antigens are also taken into account forming a complex antigen mixture. A fertility vaccine may also operate on the level of hormones. Antibodies precisely directed to a β-subunit of human chorionic gonadotropin (hCG) may block the early gravidity stages (Ulcova-Gallova, 2006).

4.2.2 Association of seminal components with female sensitization

It has been shown that SF, a nutritious transport buffering sperm medium, has a built-in mechanism for preventing an immunological sensitization of the female. This protective system exists due to the presence of immune inhibitors originating in the male sex accessory glands (Prakash, 1981). Some seminal constituents, such as cathepsin D, are able to degrade vaginally exposed proteins that may be involved in antibody formation related to immune infertility. Furthermore, the low molecular weight proteins are of need in oocyte and sperm fusion as degradation products (Pardesi *et al.*, 2004). Seminal ZAG, a 40 kDa multidisciplinary protein, has been reported as a novel adipokine playing a significant role in fertilization, lipid mobilization, and peptide/antigen/ligands binding. ZAG may participate in the expression of female immune response since the fold is similar to major histocompatibility complex (MHC) I antigen-presenting cells. ZAG has proved to be an IgG-binding protein related to a pathophysiological iso-immunization. It may also have a protective character, as it belongs to the immunoglobulin gene superfamily and thus blocks the newly created female anti-semen antibodies (Hassan *et al.*, 2008; Brazdova *et al.*, 2013b). An antibody fraction interacting with a seminal antigen targets most of seminal proteins adsorbed on sperm. However, SF induces the recruitment of macrophages and dendritic cells into cervical and endometrial tissues. SF has been heavily involved in the rare IgE-mediated reaction to semen (Weidinger *et al.*, 2006; Brazdova *et al.*, 2012a).

4.2.3 Auto-immune aspects in infertility

Auto-immune phenomena have been previously associated with an increased prevalence of female immune infertility. This finding names anti-phospolipid, anti-nuclear, anti-thyroid, anti-annexin V, anti-protombin, anti-laminin, anti-zona pellucida antibody formation, as well as a high level of NK cells as the risk factors but not as those pathognomonic (Gleicher and Barad, 2006).

ZP, as the protective layer, is composed of glycoproteins. It represents a broad antigenic content. Antibodies against ZP prevent sperm from penetrating it. Anti-ZP autoantibody concentration can be elevated if the shape of ZP is abnormal (deformed, thickened, or thinned). These antibodies interfere with the implantation process since ZP protects the fertilized oocyte up to the 7^{th} day after fertilization and until embryo hatching. ZP is, during this time, thickened. ZP-specific antibodies are detectable in follicular and peritoneal fluid and cervical mucus in IgG, IgA and IgM isotypes (Ulcova-Gallova, 2006).

Anti-phospholipid antibodies (APA) were first found by Wasserman in 1906 and since that time have been associated with miscarriage, intrauterine fetal death and placental thrombosis. These components of the female immune system are autoantibodies directed against the negatively charged parts of phospholipids, and in particular phosphatidylserine, phosphatidylinositol, phosphatidylethanolamine, annexin V, cardiolipin, and β_2-glycoprotein. APA are mostly produced in the IgG fraction accompanied by IgA and IgM. Phosphatidylserin-specific APA cause fetus hypotrophy as a consequence of placental vascular damage. However, mother protection results in the higher production of anti-coagulating factors. Furthermore, the risk of spontaneous abortion is higher in the presence of anti-coagulating antibodies. Antibodies specific to annexin V and placental anti-coagulating protein are risk factors in reproductive failure. These IgG antibodies are detectable in 5-6% of women diagnosed with pregnancy loss, in 8-10% of women after unsuccessful in vitro fertilization (IVF), in 1% of not pregnant and healthy women and in 0% of pregnant women without pathophysiologic aspect. Complex complication is called anti-phospholipid syndrome (APS), also known as Hughes syndrome. It may cause hyper-coagulation leading to rapid organ failure. Since APA have high affinity to phosphatidylserin present on trophoblasts, APS seems to be involved in the mental retardation of newborn children (Ulcova-Gallova, 2006; Ruiz-Irastorza *et al.*, 2010).

The trophoblast expresses the major histocompatibility antigens on its surface. They are of maternal and paternal origin. Paternal patterns induce allo-immunity. Formed anti-paternal

cytotoxic antibodies usually implicate spontaneous abortion. Other factors involved in this complicated immune reaction are NK cells, alteration in Th1 and Th2 ratios and abnormal HLA-G expression (Shetty and Ghosh, 2009).

Endometrium-specific antibodies are inter alia associated with the polycystic ovary syndrome (PCOS) that is mainly classified as an endocrine genetic disorder. Women suffering from PCOS have usually problems getting pregnant. PCOS is known as Stein-Leventhal syndrome described in 1935. It is characterized by enlarged ovaries caused by cysts, irregular ovulation, irregular or no menstrual periods and increased androgen level. With regards to androgen level, PCOS is associated with hirsutism. On the other hand, it is associated with obesity, type 2 diabetes and high cholesterol level (Ulcova-Gallova, 2006; MFMER, 2013).

Pregnancy is complicated by a serious gynecological complication called endometriosis. It affects up to 10% of women in reproductive age and 25% of women diagnosed with endometriosis are infertile. Its etiology is unknown. This disorder occurs when the lining of the uterus grows in other areas outside of uterus. Ectopic bearings react on the hormone level, thus are subject to the menstrual cycle. Created cysts occur on ovaries, fallopian tubes, peritoneum, cervix and in vagina. Peritoneal endometriosis is characterized by retrograde menstruation causing secondary inflammation. Factors typical for such a condition are the high level of autoantibodies, presence of T-lymphocytes in peritoneal fluid and elevated level of NK cells (Nisolle *et al.*, 1990; Bulletti *et al.*, 2010).

4.3 Mucosal immunity of the female genital tract

The mucosal immune system operates on a local level and is represented by lymphoid tissues in mucosae and external secretory glands. It limits the access of environmental antigens, by which the fertility potential is significantly regulated as well. Any inert antigen is attacked by these secreted agents of protection. It restricts and/or avoids penetration in the systematic compartment. The female genital tissues and secretion (vaginal washes and cervical mucus) provide protection that differs from systematic reactions by the cell types involved and by their products, antibodies. However, it is the initial antigen exposure to mucosae that leads to the systematic T-cell hypo-responsiveness (Woof and Mestecky, 2005; Mestecky *et al.*, 2007).

Mucosal immunity in the female genital tract is influenced by the level of antibodies, cytokines and hormones. Humoral immune defense displayed in musocal tissue surfaces is comprised of the IgG, IgA and IgM antibody isotypes. IgG, IgA and IgM levels are dependent

on the menstrual cycle, and so are influenced by hormones. IgA and IgG reach maximum concentration before ovulation, which is linked to an increased level of interleukin 1 component β (IL-1β). Particularly, estrogen causes a higher expression of secretory IgA (S-IgA), thereby increasing the rate of selective transport. This method of regulation is responsible for antibody-isotype distribution and the characteristics thereof, including their individual properties and the transport of immunoglobulin-containing cells, antigen-presenting cells (APC), $CD4^+$ and $CD8^+$ cells in the vagina, uterus and fallopian tubes (Kutteh *et al.*, 1996). In addition, it has been shown (Franklin and Kutteh, 1999) that oral contraception influences IgA as well as IgG populations in cervical mucus. It is around one third larger than in the cervical mucus of naturally cycling women. Further, the vaginal washes of women on oral contraception present an elevated level of IgG in comparison to IgA. Several observations showed (Kutteh *et al.*, 1996; Mestecky and Fultz, 1999) that the concentration of these antibodies decreases as IgG>IgA>IgM. This relationship is linked to the presence of IgG/A/M-producing cells. The uterine endocervix contains the highest amount of IgG- and IgA-secreting cells. Cervical mucus contains higher levels of IgG than IgA, both of which are locally produced. By contrast, women on oral contraception have IgA as the predominant antibody present in cervical mucus. Among the mentioned three isotypes, IgM is the least efficiently transported antibody. The mucosal IgA antibodies are selectively transported for external secretion based on a receptor-associated mechanism. The distribution of IgG subclasses in mucosal secretion displayed concentrations proportional to that of plasma. IgD occurs rarely or in very low concentration in external mucosae. The level of IgE depends on the allergenic nature of presented antigen (Woof and Mestecky, 2005).

Despite the low IgA affinity, the avidity is high regarding multi-binding sites. Proteolytic enzymes usually degrade environmental antigens leading to a relatively low mucosal absorption rate. IgA itself is resistant to enzymes of proteolytic character. It has been suggested that certain amounts of non-eliminated antigens circulate in the complex with IgA, which further activates the systematic immune response. It is less probable that an antigen only just entering the mucosal tissue could circulate on its own (Mestecky and Fultz, 1999; Mestecky *et al.*, 2007; Corthesy, 2007).

IgA is a multivalent antibody existing in two subclasses, IgA_1 and IgA_2. While IgA_1 responds to viral (protein) antigens, IgA_2 binds to lipopolysaccharide or polysaccharide structures. IgA has an anti-inflammatory activity evidenced by the inhibition of complement activation and by a diminishing effect on NK cells. These properties might inhibit an early and precise diagnosis as no inflammatory marker may be detected. In cervical mucus as well

as vaginal washes, IgA_1 concentration is equal to that of IgA_2. S-IgA is locally produced by sub-epithelial plasma cells. Most of the time, it is a polymeric molecule that corresponds with IgA_2 since IgA_1 is rather monomeric. It has been suggested that cervical mucus contains about 80% of the polymeric form and vaginal wash about 50% (Kutteh and Mestecky, 1994; Kutteh *et al.*, 1996; Woof and Mestecky, 2005). The IgA antibody can degranulate eosinophils that cover mucosal surfaces. This pathology is observed when natural immune tolerance is disrupted. (Further reaction may evoke allergic reaction to the presented antigen such as seminal and/or sperm structure. Semen rejection at the level of mucosal immunity does not have to be displayed on the systematic level (Woof and Mestecky, 2005).

Woof and Mestecky (2005) discussed the protective character of IgA. Its protective role is observed in an antibody-dependent, cell-mediated cytotoxicity, opsonization, the activation of innate humoral factors, and the removing and further elimination of already formed immune complexes within epithelial cells and lamina propria. It is known that IgA is able to diminish the partial and total absorption of the antigen on mucosal tissues. In comparison with IgG, which after antigen-recognition activates complement resulting in inflammation, IgA acts as an inhibitor to directly avoid the adherence of antigen (Russell *et al.*, 1997). S-IgA in a complex with antigen is not able to efficiently activate the complement pathway. Exclusivity of IgA is related to its natural occurrence and polyreactivity. Another characteristic of its protective capacity is related to the cell line that produces the IgA subclasses. IgA_1 originating in B1 cells are of low affinity but wide specificity. In contrast, S-IgA derived independently from B1 and B2 cells do not differ in antigen specificity (Quan *et al.*, 1997).

4.3.1 Cervical Mucus

The uterine cervix participates in the local immune reaction by the application of immunoglobulin-producing cells in a complex mixture known as cervical mucus/fluid/plasma, which is located in and around the cervix. Cervical mucus is composed of up to 90% water, depending on the menstrual cycle, which determines the quantity as well as the quality of cervical mucus. Its composition is based on a glycoprotein web filled with mucus rich in immune-competent proteins, electrolytes (calcium, sodium and potassium), simple sugars such as fructose and glucose, amino acids, C3 and C4 complement components, Th1 and Th2 cytokines, the prostaglandins of E series, and trace elements (zinc, copper, iron, mangan,

selenium). An imbalance is frequently associated with immune infertility and spontaneous abortion (Moghissi, 1972; Schumacher, 1988; Cibulka *et al.*, 2005; Ulcova-Gallova, 2010).

Optimal pH ranges from 7.4 to 9.6. The pH of cervical mucus is alkaline during ovulation to allow sperm survival due to elevated levels of water and electrolytes. After menstruation, cervical mucus becomes rather acidic. Acidic pH is characteristic for vaginal mucus as well, ranging from 3.7 to 4.5 (Gruberova *et al.*, 2006).

The basic role consists of a barrier that prohibits anything from entering the uterus. It is associated with "stick and thick" properties. Cervical mucus acts as a natural lubricant due to its glycerol content. The apparent amount of cervical mucus is not hormone-dependent. The mucus also functions as a transport and nourishing medium for sperm by being less concentrated, transparent with a lower amount of immune-competent agents and a high fructose level, which is essential for an efficient sperm metabolism. The sugar level is progesterone dependent. On the other hand, the cervix always acts as a reservoir for sperm after sexual intercourse. Regarding iso-immunization during the entire menstrual cycle, cervical mucus contains the antibodies directed toward sperm. Their amount is then crucial for sperm-cervical mucus penetration and following fertilization. ASA-positive female patients have been commonly diagnosed with immune infertility. Approximately 5-10% of infertile female patients are positive in cervical ASA. An iso-immunization rate is observed by probing the local ASA level, which will determine an appropriate treatment (Moghissi, 1979; Schumacher, 1988; Cibulka *et al.*, 2005). It has been shown (Ulcova-Gallova, 2010) that ASA present in cervical mucus are of agglutinating character. These locally produced ASA do not differ from those systematically produced thus they affect sperm capacitation, acrosome reaction, and may interfere with zona pellucida penetration as well as embryo implantation (Livi *et al.*, 1991). The peak of ASA in IgA and IgG fraction is reached at the luteal and follicular phases of the menstrual cycle. In contrast, their levels are lowest during ovulation. This peak in ASA concentration is related to the highest level of estradiol, usually observed one day before ovulation (Franklin and Kutteh, 1999). However, Schumacher (1973) demonstrated that local immunoglobulin production could be diminished in women on oral contraception. The study demonstrated the impact of oral contraception with respect to estrogen-dependent, decreasing antibody level in cervical mucus and then with regards to progestin-dependent (a synthetic progestogen of progestational effects similar to progesterone), increasing antibody level (Schumacher, 1973). This finding supports the fact that cervical immunity before ovulation is influenced by estrogen and after by progesterone.

4.4 Treatment of female infertility

Modern era progress in medicine allows highly individualized and efficient access to infertility treatment. Despite the fact that the cause of female infertility can be difficult to diagnose, many diagnostic methods and treatment approaches are available. Effective treatment of reproductive failure depends on its cause. Concerning precise diagnosis, reproductive immunologists attempt treatment to restore fertility usually by medication, surgery or reproductive assistance by sophisticated techniques. Prior to determining the nature of an individual infertility disorder and choosing a suitable way of its treatment, a couple has to be thoroughly examined. A non-invasive method, such as an evaluation of sperm quality, represents the major male examination. Based on volume, color, consistency, sperm motility, sperm number, the number of defective forms, additive cellularity (leukocytes and lymphocytes), pH and ASA level, the semen type is specified (chapter 2, Tab. 1, 2). Women are examined by ultrasound, laboratory and cytology tests in order to determine ovulation disorders, hormonal imbalances, and external and internal genital abnormalities. Basal hormone levels in the blood are examined between the 2nd and 5th day of the menstrual cycle. This screening includes follicle-stimulating hormone (FSH), prolactin (PRL), triiodothyronine (T3), thyroxine (T4), thyreotropin (THS) and estradiol (E2). The state and patency of fallopian tubes can be tested by ultrasonography or when necessary by laparoscopy. To examine uterine cavity, hysteroscopy may be suggested. In addition, bacteriological culturing from the canal of uterine cervix is performed to exclude any infection of internal genitals. In any case, the treatment is psychologically and physically arduous. It also involves considerable financial commitments (Ulcova-Gallova, 2006; Rezabek, 2008; Pandey *et al.*, 2012).

The treatment of women diagnosed with immune infertility can be described in three steps, where each proceeding step follows the failure of previous one. Fertility can be restored by stimulated ovulation with fertility drugs, surgery or reproductive assistance, all of which carry several risks. Women of ages less than 30 are advised to use the so-called condom therapy for 3-6 months in order to avoid the contact of female immune system and sperm. This basic approach is combined with other methods of treatment. As a result of an appeased immune system, success reaches up to 40% (Ulcova-Gallova, 2006.)

Since the majority of locally produced ASA belongs to IgA antibodies - particularly to IgA_1, another type of infertility treatment might be suggested (Kutteh *et al.*, 1994). The intravaginal administration of selectively cleaving IgA proteases would block present ASA

and further sperm agglutination would not happen. A proteolytic IgA_1 cleavage is specific to prolin-serin or prolin-threonin bonds within the IgA_1 hinge region. The generated noncovalent Fab fragments would avoid an antigen binding to Fc fragments of cleaved IgA. This theory speculates about the invasion of other ASA isotypes, mostly the IgG portion, present in cervical mucus and vaginal washes, as well. Based on the IgA predominance, most of the semen epitopes may be blocked by the cleaved Fab/Fc fragment of IgA. However, the question of ASA in IgA_2 isotype and its inactivation remains unanswered (Bronson *et al.*, 1987; Russell and Kilian, 2005).

4.4.1 Fertility drugs

Immunotherapy is often chosen to modulate the organism to decrease the level of pathophysiological antibodies such as ASA and AOA. Nonspecific local or systematic approaches consist of corticosteroid administration. An application of cervical pessary with corticosteroids is appropriate for female patients having ASA only in the cervical mucus and not circulating ASA. Plasmapheresis is another option to decrease the concentration of antibodies potentially related to infertility. The advantage of this technique is high efficiency, while the chief disadvantage is short-term effect. Plasmapheresis has to be, therefore, combined with immunosuppression (Haas *et al.*, 1980; Ulcova-Gallova *et al.*, 1990).

Fertility drugs aim to regulate or trigger ovulation. Ovulation is commonly induced by anti-estrogens, e.g. clomiphene citrate. Clomiphene citrate is orally administered and stimulates the release of gonadotropin releasing hormone (GnRH). The cascade continues by a triggering of hypophysis to secrete FSH and luteinizing hormone (LH), both of which finally stimulate the growth and maturation of follicles in the ovaries. When clomiphene citrate fails and/or hypophysis cannot produce LH and FSH on its own, a gonatropin-injected treatment follows. Gonadotropins, FSH and LH, affect follicular maturation directly in the ovaries. Hormone hCG may replace LH as it mimics LH effect. Fertility drug utilization carries the risk of multiple pregnancies that increases the possibility of premature labor, low birth weight and later developmental problems. A gonatropin-injected treatment may cause the so-called ovarian hyperstimulation syndrome, wherein women suffer from swollen ovaries and associated pain, bloating, nausea, vomiting and diarrhea. All symptoms or even individual symptom can last for weeks. Long term hormonal treatment lasting 12 or more months has been linked to ovarian cancer later in life. However, it has been shown that women who have

never been pregnant are exposed to an elevated risk of ovarian cancer. Ovulation and/or conception may be negatively affected by any anatomy abnormalities such as an abnormal uterine shape, endometrial polyps, the deformed shape of uterine cavity, pelvic or uterine adhesions, fallopian tube blockage, and hydrosalpinx (distally blocked fallotropian tube, filled with water). Laparoscopy, hysteroscopy, tubal ligation, reversal micro-surgery and tubal surgery are the most used diagnostic techniques. If the blockade is not able to be corrected, the techniques of artificial insemination are recommended (Doherty and Clark, 2006; Rezabek, 2008; MFMER, 2013).

4.4.2 Reproductive assistance

The assisted reproductive technologies (ART) represent an immense step forward in infertility therapy. ART have in common the manipulation of stem cells to successfully achieve pregnancy. It includes in vitro fertilization (IVF), gamete intra-fallopian transfer (GIFT), zygote intra-fallopian transfer (ZIFT), from which IVF is the most frequent approach of ART. GIFT treatments are chosen in less than 2% of all ART procedures, ZIFT in less than 1.5%. IVF was developed by Robert Edwards who was awarded the 2010 Nobel Prize for the development of human IVF therapy. The famous date of the world's first "tube baby" is July 25th, 1978. IVF is combined with fertility drug therapy in order to evoke ovulation. The stimulated ovaries produce oocytes that are further aspirated. These oocytes are treated under special conditions with sperm until fertilization takes place. A certain amount of embryos is then placed into the uterus. It has been shown that women undergoing IVF produce the antibodies against zona pellucida. Their level increases with the repeating rate of unsuccessful IVF. These antibodies are detected in the patient sera but not in the cervical mucus. Isotypes occur following the relationship: IgG>IgA>IgM>IgE. An extracorporeal conception competes with an intra-uterine insemination (IUI) that represents less arduous methods for the female patient. IUI involves placing millions of washed sperm (normozoospermic type) into the uterus close an ovulation time, by which it is avoided the contact with cervix and its immunocompetent cells. It is commonly used in the case of unexplained infertility and may be appropriate procedure for sperm donor conception (Doherty and Clark, 2006; Rezabek, 2008; MFMER, 2013).

4.4.3 Intravenous immunoglobulins

Intravenous immunoglobulins are defined as plasma pools obtained from thousands, and sometimes hundreds of thousands, of healthy blood donors. Such a large set of individuals increases the potential high variety of antibodies present in plasma with specificity against bacterial, viral, and parasitic antigens. On the other hand, it carries the risk of diluting the amount of specific antibodies of rare activity. IVIg is a substitutive treatment in primary and secondary immunodeficiencies. It is used in several auto-immune as well as inflammatory diseases, and was put into medical practice 25 years ago (Bayry *et al.*, 2003; Kaveri, 2012).

IVIg was, for the first time, successfully demonstrated as a valuable substitutive treatment in auto-immune disease, particularly in idiopathic thrombocytopenic purpura. The range of immune disorder has been enlarged since that time, as it has been established in the treatment of Guillain-Barré syndrome, Kawasaki disease, chronic inflammatory demyelinating polyneuropathy, myasthenia gravis, multifocal neuropathy, corticosteroid-resistant dermatomyositis, antineutrophil cytoplasmic auto-antibody-positive vasculitis, auto-immune uveitis, multiple sclerosis, anti-factor VIII auto-immune disease, polymyositis, anti-phospholipid syndrome, recurrent spontaneous abortion, systemic lupus erythematosus (SLE), insulin-dependent diabetes mellitus, Crohn's disease and in the prevention of graft-versus-host disease and others. Usually, IVIg is administered in the dose of total 2 g/kg over 2 or 5 consecutive days. Despite the promising effect of IVIg, precise disease diagnoses and a detailed knowledge of immunology, the mechanism of IVIg action remains unexplained (Kazatchkine and Kaveri, 2001; Bayry *et al.*, 2003; Bayry *et al.*, 2011).

The mode of IVIg action is complex. It operates on several levels: Fc receptors, inflammation, B-cells and antibodies, T-cells, and cell growth. The immunoregulatory effect regarding Fc receptors consists of their blockade on macrophages as well as on effector cells. IVIg induces antibody-dependent cellular cytotoxicity. It reduces inflammation by the induction of anti-inflammatory cytokines, inhibition of activated endothelial cells, attenuation of complement-mediated damage and neutralization of microbial toxins. In general, the anti-inflammatory effect depends on interaction with endothelial cells and cytokines. IVIg triggers the activation and effect of B and T lymphocytes and interferes with APC. It expands Treg. Another attribute is comprised of the provision of anti-idiotypic antibodies. IVIg neutralizes T-cell superantigens, pathologic antibodies and then circulating auto-antibodies by anti-idiotypes. It selectively down/up-regulates antibody production, apoptosis and the production of Th cell cytokines. Specifically, it decreases tumor necrosis factor α (TNFα), IL-1β, and it

increases interleukin 1 receptor agonist (IL-1Ra). It acts also as an inhibitor of lymphocyte proliferation. The immune system interacts with IVIg through its Fc receptors, T and B lymphocytes, NK and dendritic cells (DC), complement, cytokines and granulocytes. In detail, DC differentiation and maturation are inhibited in vitro by high IVIg dosage. IVIg also blocks the secretion of IL-12 by matured DC while elevating IL-10 secretion. The results of IVIg utilization in therapy depends on a synergic effect with the immunomodulatory components of individual patients. In order to understand the IVIg operation, attention is paid to the sialylated Fc fragment of IgG (Bayry *et al.*, 2003; Galeotti *et al.*, 2009).

Nowadays, IVIg is routinely and commercially prepared. This product contains mostly polyspecific IgG antibodies. The subclass distribution mirrors the normal human serum. The trace components are immunoglobulin A and M, soluble CD4, CD8, HLA molecules. The half-life of IVIg infused to an immuno-competent person is approximately 3 weeks (Kazatchkine and Kaveri, 2001).

IVIg therapy is relatively safe. High doses may evoke some side effects such as headache, fever, nausea, emesis, hypertension, and muscle cramps. Severe reactions include throat/chest tightness, chills, dizziness, breathlessness and in rare cases also collapse or anaphylactic shock. The third generation of IVIg, nowadays present in the market, has been immensely improved. After hepatitis C contamination in 1993, IVIg therapeutics avoid the risks of prion disease since intact IgG antibodies are isolated with particular steps of viral inactivation/elimination thanks to low pH and caprylic acid treatment, pasteurization, and nanofiltration (Radosevich and Burnouf, 2010; Bayry *et al.*, 2011).

4.4.3.1 IVIg and infertility related disorders

IVIg may represent a potential therapeutic strategy in fertility disorders related to an immune system with an auto-immune background. SLE is considered as the most serious disorder related to infertility. It is a multifactorial auto-immune disease characterized by the presence of autoantibodies linked to nuclear antigens. It is characterized by system failure that can affect any part of the body. Created immune complexes precipitate. This action further activates an immune response. Since SLE is associated with fetal death or miscarriage, treatment by IVIg might be of potential interest. Unfortunately, sufficient randomized studies to prove the positive effect of IVIg, as has been done for previously mentioned diseases, are few in number (Ulcova-Gallova 2006; Smyth *et al.*, 2010; Bayry *et al.*, 2011). Pregnancy

complication and pre-eclampsia may be related to anti-phospholipid syndrome. Anti-phospholipid and antiapolipoprotein antibodies provoke thrombosis. Spontaneous abortion can be prevented by IVIg dose of 1g/kg per day for 2 consecutive days every month until birth. As the study of Coulam and Acacio (2012) showed, IVIg treatment did not bring any benefit in comparison with combined therapy involving heparin and aspirin. It has been speculated whether or not the advantage of IVIg administration profits from a blockade of the neonatal IgG-Fc receptor. It may lead to the catabolism of pathologic antibodies. Anti-cardiolopin antibodies could be then neutralized by anti-idiotypic antibodies (Bayry *et al.*, 2011; Coulam and Acacio, 2012).

5 RESULTS

5.1 Publications

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5.1.1 Anti-sperm antibodies

Sperm has a very heterogeneous antigenic content, auto-antigenic as well as iso-antigenic potential, and is capable to induce the production of sperm-reactive T-cells in men and women. Some studies suggested that it is not a single anti-sperm antibody (ASA) that influences fertility but is rather multiple ASA. On the other hand, a single sperm antigen is not able to cause infertility. There is evidence in fertile individuals reacting spontaneously to sperm sensitization by ASA formation, in particular 10% of fertile men and 2% of fertile women but fertility is not threatened. It could be concluded that not all produced ASA, in men and women, influence fertility potential and/or inhibit fertilization process in vitro/vivo. Several theories have been postulated, e.g. the cognate antigen is not necessarily involved in the fertilization process. Understanding of ASA formation and its mechanism could improve the treatment in cooperation with modern diagnosis. Then, it would be worthy to distinguish among individuals producing ASA without/with a fertility disorder. Furthermore, ASA are of interest for their contraceptive effect. The recent advance could contribute to the finding of antigen appropriate for such an acceptable, effective and safe vaccine. It has to reflect an immune response that blocks a key point in the reproductive process. Anti-sperm antibody-mediated immune infertility provides a naturally occurring model to indicate which antigen would be suitable.

The detailed mechanisms of ASA production leading to infertility are described in this review article. ASA are of great interest to actual research since they have been proved to be related to immune infertility, both male and female.

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PROTILÁTKY PROTI SPERMIÍM

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Obsah

1. Úvod

V současné době se asi jedna pětina párů ve vyspělých zemích včetně České republiky potýká s neplodností. Neplodnost je podle Světové zdravotnické organizace definována jako neschopnost páru počít dítě během jednoho roku nechráněného pohlavního styku.

Příčiny neplodnosti jsou nejrůznějšího charakteru. Snížená plodnost může být způsobena např. nesprávným životním stylem, konzumací návykových a omamných látek, hormonálními poruchami, genetickými odchylkami a souvisí též s psychickým stavem. Také nesprávná funkce imunitního systému může vést ke snížení plodnosti. Mezi nejrozšířenější poruchu imunitního systému patří tvorba protilátek proti lidským pohlavním buňkám, zejména pak proti spermiím. Jde o tzv. protilátky proti spermiím (antisperm antibodies – ASA)[1,2].

Již na počátku 20. století bylo zjištěno, že zvířecí spermie mohou být imunogenní. Experimentální zvířata skutečně po imunizaci spermiemi produkovala protilátky. Mnohé studie se poté zaměřily na určení orgánové a druhové specifity antigenů na spermiích[1]. Byla také popsána přítomnost protilátek, které aglutinovaly a imobilizovaly spermie v krevních sérech žen, které nemohly z nevysvětlitelných důvodů otěhotnět. Rovněž bylo zjištěno, že v krevních sérech některých neplodných mužů se vyskytovaly protilátky proti spermiím a jejich neplodnost korelovala s hladinou těchto protilátek[3].

ASA lze nalézt jak u mužů, tak u žen. Jejich výskyt byl zaznamenán u 9–36 % neplodných párů[4–7], přičemž u neplodných mužů se vyskytují asi v 8–21 % a u neplodných žen asi v 6–23 % celkové populace[4,6–8]. Zároveň se však vyskytují asi u 12,5 % plodných mužů a u 1,4 % plodných žen[5,7]. Nelze tedy říci, že všechny ASA, které se vyskytují, způsobují neplodnost[5–7,9]. Jsou známy jak monoklonální, tak polyklonální protilátky, které se vážou na spermie, ale neinhibují proces fertilizace ani *in vitro*, ani *in vivo*[5].

V současné době lze pomoci neplodným párům s takovou poruchou metodou intracytoplasmatické injekce (ICSI – intracytoplasmatic sperm injection), kdy je jediná spermie zavedena mikroinjekcí do cytoplasmy oocytu. Z tohoto důvodu se poněkud vytratil zájem o další výzkum ASA. Přesto však zůstává několik důvodů, proč ASA dále studovat. Existuje totiž široká škála mechanismů, kterými ASA působí. Porozumění těmto mechanismům spolu s moderní diagnostikou by mohlo umožnit lepší vytipování pacientů, u kterých ASA skutečně způsobují neplodnost, od pacientů, u kterých je sice výskyt ASA potvrzen, ale není pro jejich neplodnost významný. Navíc se již od 30. let 20. století systematicky testují kontracepční vakcíny pro ženy. Prohloubení našich znalostí o ASA by mohlo přispět k nalezení takového antigenu, s jehož pomocí by mohla být vyvinuta taková vakcína, která by byla bezpečná a navodila by reverzibilní, ale spolehlivou neplodnost[10].

2. Vznik a působení protilátek proti spermiím

2.1. Původ protilátek proti spermiím u mužů

Protože tolerance k autoantigenům vzniká časně a spermie je buňka, která se do puberty nevyvíjí, nerozpozná dospělý imunitní systém sobě vlastní antigeny, které jsou na spermii, a vyvolá proti spermiím imunitní odpověď. Proto musí anatomické a fyziologické mechanismy spermie před vlastní imunitní odpovědí aktivně chránit[6].

Takovou ochranou je pro spermie hematotestikulární bariéra. Ta je složena z kontinuální vrstvy Sertoliho buněk uvnitř semenotvorných kanálků. Sertoliho buňky jsou velmi těsně propojeny a vytváří tak prostředí v centrální části semenotvorných kanálků, kam se nedostanou velké proteiny a buňky imunitního systému. Tato těsná spojení se u savců do puberty netvoří. Touto vrstvou se oddělí spermie a jejich prekurzory od imunitního systému. Není to

však jediný mechanismus ochrany spermií. U muže je mnohem větší množství spermií uloženo v nadvarleti a chámovodu než v semenotvorných kanálcích. Tyto části mužského reprodukčního traktu ale nemají kompletní anatomickou bariéru, a proto nemohou zabránit případnému vstupu buněk imunitního systému či úniku specifických antigenů spermií do mikrocirkulace, což může navodit imunitní odpověď. Proto existují imunosupresivní faktory, které brání rozvoji imunitní reakce proti antigenům spermií. Přesné mechanismy imunosuprese však ještě nebyly zjištěny[7,10]. Předpokládá se však, že výskyt určité subpopulace CD8 lymfocytů (supresorové buňky) a CD8 aktivátorů ve spermatu brání destrukci spermií imunitním systémem[11]. Uvažuje se např. i to, že malý únik specifických spermiových antigenů může navodit pozdní autotoleranci[12].

K poškození hematotestikulární bariéry může dojít při testikulárním traumatu, kongenitální absenci chámovodu[8,10] nebo při jejím porušení při vasektomii[3,4,8,10,13], dále při varikokéle (rozšíření žilní pleteně v oblasti varlat), příušnicích, které jsou komplikované zánětem varlete[5,8] a při úrazu páteře[8]. ASA byly detegovány také u pacientů s cystickou fibrózou[7]. V některých případech však může dojít k tvorbě ASA z nezjištěných příčin.

2.2. Původ protilátek proti spermiím u žen

Už v průběhu 20. a 30. let minulého století bylo zjištěno, že po intramuskulární imunizaci spermiemi u žen, které byly v minulosti plodné, u nich došlo k produkci potenciálně spermatoxického faktoru, který je chránil před těhotenstvím a byl měřitelný v séru. Proto lze spermii považovat za buňku, která může být pro ženu potenciálně imunogenní[10].

Vagina a děložní hrdlo jsou vybaveny velmi aktivním slizničním imunitním systémem[10]. Je také dobře známo, že pohlavní styk stimuluje velký příliv leukocytů, neutrofilů a makrofágů do oblasti cervixu a dělohy. Tato zánětlivá imunitní odpověď pomáhá eliminovat abnormální spermie nebo spermie, které neoplodnily, zdá se však také, že tato imunitní odpověď je důležitá v přípravě ženského reprodukčního traktu na implantaci embrya, čímž zlepšuje plodnost[14]. Faktory obsažené v semenné plasmě spermatu, zvláště transformující růstový faktor β (transforming growth factor-β, TGF-β) a prostaglandiny, brání ve vývoji paternálně specifické imunitní odpovědi. TGF-β inhibuje proliferaci B-buněk, ale zároveň indukuje jejich přechod k produkci IgA. Tento efekt tedy může podporovat tvorbu ASA v ženském systému. To, jak se TGF-β projeví, závisí na jeho množství a také na koncentraci ostatních cytokinů a signálních molekul v lokálním prostředí. Množství aktivního a celkového TGF-β je různé v různých ejakulátech. Proto jeho množství a přítomnost ostatních cytokinů nebo imunoregulačních molekul v ejakulátu může hrát roli v tom, zda žena začne produkovat ASA či nikoliv. Zatím ale nebylo ověřeno, zda produkce ASA u žen koreluje s množstvím TGF-β, prostaglandinů a ostatních imunoregulačních buněk v partnerově ejakulátu[10].

2.3. Tekutiny obsahující protilátky proti spermiím

ASA lze u mužů detegovat v krevním séru[1–3], semenné plasmě[3,10] a také na povrchu spermie[5,15]. U mužů, kteří ASA tvoří z nezjistitelných příčin, tj. neutrpěli trauma varlat, atd., není jejich původ zcela jasný, ale předpokládá se, že jsou produkovány lokálně a nejde tedy o krevní transudát[10].

U žen je produkce ASA lokálního charakteru. Zpočátku je lze nalézt v hlenu děložního hrdla. Jestliže je žena dlouhodobě a opakovaně imunizována stykem s antigeny spermií, začnou se pak ASA tvořit i v dalších částech reprodukčního traktu. Lze je pak najít v děložní dutině, vejcovodech, břišní dutině folikulární tekutině a krevním séru[10].

2.4. Povaha protilátek proti spermiím

ASA obsažené v tekutinách neplodných jedinců jsou často ze třídy IgG, dále se vyskytují i IgA a IgM. U IgA se předpokládá, že by mohlo jít o lokálně produkovanou protilátku[16], protože je součástí slizniční imunity. IgA koluje také v krvi a jeho poměr k IgG (nejčastější typ protilátek) 1:5 je zachován jak v séru, tak v ostatních tekutinách, např. cervikálním hlenu[17].

2.5. Potenciální mechanismy působení protilátek proti spermiím

Bylo zjištěno několik možných mechanismů, kterými mohou ASA působit na spermie – mohou spermii imobilizovat a znemožnit tak její transport, mohou ovlivnit kapacitaci a akrosomovou reakci, a tím i průnik spermie do vajíčka. ASA mohou také aktivovat komplement a působit proti embryu.

Spermie musí po ejakulaci překonat překážku v podobě cervikálního hlenu. Ten je v době ovulace hojný a řídký, proto je v této době průnik spermie nejjednodušší. Cervikální hlen zároveň působí jako filtr, který nepustí do vyšších pasáží ženského reprodukčního traktu abnormální spermie a oddělí je tak od zdravých nepoškozených spermií, které mohou dále postupovat. ASA mohou poškodit běžnou interakci spermie s cervikálním hlenem[18–20] tím, že inhibují průnik spermií cervikálním hlenem, i když jsou spermie zcela nepoškozené[21], a to především lokálně produkované ASA typu IgA[22,23]. Pravděpodobně sekretorní komponenta IgA vázaného k povrchu spermie se váže také ke glykoproteinům cervikálního hlenu a způsobuje tak kývavý pohyb spermie v hlenu[24], rovněž Fc-oblasti IgA spermie mohou způsobit tento jev[25]. ASA mohou zpomalit nebo inhibovat pohyb spermií v děložní dutině a vejcovodech[26].

Aby mohla spermie oplodnit vajíčko, musí po ejakulaci projít mnoha změnami, které se souhrnně nazývají kapacitace. Tento proces ještě není plně objasněn, sestává ale z fosforylace proteinů a redistribuce lipidů a vyústí v destabilizaci membrány[27,28] a je důležitý pro usnadnění akrosomální reakce, která pak vede ke splynutí spermie

a oocytu. ASA mohou svou vazbou bránit ve změně fluidity membrány a tím znemožnit proces kapacitace[29]. Názory na ovlivnění akrosomové reakce prostřednictvím ASA se však různí: např. procento spermií, u kterých proběhla akrosomová reakce je vyšší u těch, na které byly navázány ASA[30], naopak ASA nemají žádný efekt na akrosomovou reakci[31].Tyto příklady ukazují, že ASA mají na kapacitaci a akrosomovou reakci různý vliv; některé mohou tyto děje nepříznivě pozměnit, jiné ne.

Jako komplement se označuje soustava zhruba 30 sérových a membránových proteinů, které kooperují jak mezi sebou, tak s dalšími imunitními mechanismy. Základními složkami komplementu jsou sérové proteiny označované C1-C9. Po různých podnětech dochází ke kaskádovité aktivaci jednotlivých složek. Meziprodukty této kaskádovité reakce mají výrazné biologické funkce (opsonizace, chemotaxe). Terminální produkt komplementové kaskády perforuje membrány cizorodých mikroorganismů nebo buněk, působí jejich lysi, a tím je zabíjí[32]. Interakce komplement-aktivujících ASA s normálními spermiemi vede k významnému snížení pohyblivosti spermií a také k morfologickým změnám na spermii a následné lysi *in vitro*[33]. Bylo dokázáno, že ASA vázající se na spermie získané z ejakulátů mužů s ASA jsou schopné aktivovat komplement[34].

Studie na různých zvířecích modelech ukázaly asociaci mezi ASA a post-implantační degenerací embrya[35], v jedné studii u králíka byla nalezena křížová reakce tekutin obsahujících ASA s králičí morulou a blastocystou, která měla embryotoxický efekt v *in vitro* kultuře[36]. Existují však i některé důkazy, které spojují imunitu proti spermiím s postfertilizačními účinky u lidí, např. se spontánními potraty v prvním trimestru u žen s ASA[10]. Důvodů, proč by ASA měly reagovat s embryem, je několik. Za prvé je membrána spermie integrována jako mozaika do membrány zygoty při oplodnění, a tak jsou antigeny spermií obsaženy ve vyvíjejícím se embryu, i když pouze v jen velmi nízké hustotě[37]. Exprese genů v embryu může vést k syntéze antigenů, se kterými mohou ASA křížově interagovat. Tudíž při vývoji embrya, zejména pak při rýhování blastocysty, mají ASA příležitost k vazbě na křížově reagující antigen a potenciálně tak mohou způsobit degeneraci embrya nebo případně znemožnit jeho implantaci[38].

3. Závěr

Problém neplodnosti se v posledních letech stává stále více aktuálním tématem. Žijeme v moderní rychlé době s velkým množstvím stresových situací, vzhledem k současnému životnímu stylu neustále přibývá neplodných párů. Snahou a cílem současného vědeckého úsilí je pokud možno co nejméně invazivním způsobem těmto lidem pomoci a zároveň popsat faktory, které neplodnost způsobují a mechanismus, jakým k tomu dochází. Těchto faktorů je celá řada, od nejrůznějších chorob a genetických vlivů až po životní prostředí, psychický stav a antikoncepci. Mnohé z faktorů spolu souvisí, jejich negativní účinek se ovlivňuje a může vyústit právě v neschopnost početí.

Vznik protilátek proti spermiím (ASA) jak u mužů, tak u žen může souviset s řadou vnějších i vnitřních faktorů a stává se proto častou příčinou neplodnosti. Existuje velmi mnoho mechanismů, jakými mohou ASA negativně působit. Důkladný popis a pochopení těchto mechanismů, které je předmětem vědeckého bádání, by v budoucnu mohlo vést ke spolehlivému sledování poruch a později k navržení účinné terapie neplodnosti.

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Infertility affects about 20 % of the population in advanced countries including the Czech Republic. The reasons are hormonal, genetic, mental or immunological abnormalities. The most common immunological disorder is the production of antibodies against oocytes and sperm cells. The sperm cell has a potentially immunogenic structure for both women and men. Anti-sperm antibodies can be used for monitoring infertility. Several antigens on sperm cells have been characterized.

5.1.2 Disintegration of human sperm and characterization of its antigen

The active and sensitized immune system may affect fertility. Immunological background has been studied in association with sperm antigens. A change in cellular immunity against sperm may cause the antibody-mediated rejection of semen. Our paper represents the first part of research that deals with the immunological properties of sperm concerning female immune infertility. Despite the progress in molecular genetics and proteomics, we consider sperm disintegration to be a key step in sperm antigen characterization. Regarding post-translational modifications that seem to be essential for efficient spermatozoa, it is necessary to prepare the protein extract in order to keep as many conformational and linear epitopes on the sperm antigens as possible. On the other hand, not all processes of sperm disintegration meet these conditions. Then, several epitopes or entire patterns might be lost or damaged.

In this study, we focused on the mechanical and chemical disintegration in order to compare the detected particular sperm antigens and antigenic patterns recognized by immunoblotting. The sperm samples were processed by sonication, hypo-osmotic shock and utilization of detergents, urea and benzalkonium chloride, to process the sperm disintegration concerning the above mentioned conditions. The source of antibodies was the sera of female patients diagnosed with fertility failure. Sera were collected according to the level of sperm-agglutinating antibodies using the Fridberg test and antibody class using indirect MAR test. A negative control was required as well. A serum of 10-year-old girl was apparently appropriate since it had been presumed that ASA might not have been present. The complex repertoire of sperm antigens was detected in the sperm protein extract that was prepared using Triton X-100. The most frequent sperm antigens had 68 and 123 kDa.

DEZINTEGRACE LIDSKÝCH SPERMIÍ A CHARAKTERIZACE JEJICH ANTIGENŮ

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Klíčová slova: neplodnost, protilátky proti spermiím, imunoblot

Úvod

Neplodnost je již v dnešní době definována jako civilizační nemoc. Bezprostředních příčin je mnoho, např. zhoršující se stav životního prostředí, rychlé životní tempo, stres. Z lékařského hlediska se na neplodnost nahlíží jako na neschopnost početí potomka během jednoho roku nechráněného pohlavního styku s běžnou frekvencí. Porucha může být na straně ženy, muže, anebo obou partnerů. V případě ženy je neplodnost důsledkem chromosomálních defektů, poruch podmíněných anatomicky (srůsty, endometrióza) nebo hormonálně. Jako nejčastější příčiny ženské neplodnosti jsou uváděny nepřítomnost ovulace, nedostatečná funkce žlutého tělíska, syndrom polycystických vaječníků, zvýšená hladina prolaktinu. Také intenzivní chemoterapie ovlivní plodnost. U muže může být plodnost omezena v důsledku poranění varlat nebo díky vrozeným dispozicím[1–3].

Příčina neplodnosti bývá také imunologického charakteru. U ženy dochází ke změně buněčné imunity proti spermiím a následné rejekci spermatu, u muže k porušení hematotestikulární bariéry a vytvoření autoprotilátek (antisperm antibodies, ASA). ASA spermie aglutinují, znemožňují jejich pohyb, brání splynutí spermie a vajíčka, zvyšují riziko potratu[1,4]. Imunitní systém ovlivňuje i průběh těhotenství. Neznámým příčinám, tzv. idiopatickým[5], dominuje imunitní systém.

Chceme-li studovat antigeny spermií, je nezbytné připravit vhodný proteinový extrakt spermií, čehož je možné dosáhnout mechanickou a chemickou cestou lyze. V rámci naší experimentální práce byla mechanickým způsobem sonikace, při které dochází ke kavitaci. Během kavitace vznikají v kapalině působením ultrazvukových vln oblasti zředění a stlačení. V místech zředění se nacházejí dutiny. Při zániku dutin je uvolněna tlaková vlna, která je považována za destrukční prvek. Chemické metody zastupovalo působení povrchově aktivních látek, pro naši studii jsme vybrali deoxycholát sodný (DOC), 3-[(3-cholamidopropyl)dimethylamonio]-1-propansulfonát (CHAPS), Triton X-100 a X-114. Dále byla zahrnuta lyze pomocí chloridu benzalkonia, účinné látky spermicidních prostředků, jedná se o bezvodou směs alkylbenzyldimethylamonných chloridů s alkylovými řetězci C_8–C_{18} (cit.[6]). Poslední zvolenou látkou byla močovina jakožto chaotropní činidlo[7] a lyze navozením hypotonického prostředí.

Experimentální část

Metody dezintegrace spermií

Vzorky ejakulátu byly získány od čtyř dobrovolných dárců masturbací po 3–5 dnech sexuální abstinence. Dárci byli vybráni na základě výsledku spermiogramu (normospermiogram podle manuálu světové zdravotnické organizace, WHO 1999). Lyze byla prováděna vždy ze 4 ml suspenze ejakulátu. Každý ml suspenze byl samostatně centrifugován při 600x g, 4 °C, 10 minut. Seminální plazma ve formě supernatantu byla odstraněna a peleta tvořená spermiemi byla následně třikrát promyta 0,5 ml PBS s inhibitory proteas (Protease inhibitor cocktail, Sigma, USA) 1:100. Dezintegrace každé pelety byla provedena 50 µl lyzovacích roztoků s danou účinnou látkou 4 h na ledu za stálého kývání. Účinné látky představoval 1% DOC (Sigma, USA), 1% Triton X-114 (Sigma, USA) a X-100 (Sigma, USA), 13M CHAPS (Sigma, USA), 6M močovina (Sigma, USA), 3M chlorid benzalkonia (Sigma, USA) a destilovaná voda. Po proběhnutí lyze byla suspenze 10 min centrifugována 4300x g při teplotě 4 °C. Lyze za použití destilované vody probíhala přes noc při teplotě 4 °C na ledu za stálého kývání. Kratší doba dezintegrace využitím hypotonického prostředí neposkytovala vhodný proteinový podíl. Výsledný supernatant představoval proteinový extrakt spermií. V tomto kroku se provedlo smísení čtyř lyzátů ze vzorku čtyř vybraných dárců. Ve směsném vzorku byla měřena koncentrace bílkovin pomocí BCA™ Protein Assay Reagent A (cit.[8]) (BCA, Pierce, USA) užitím hovězího sérového albuminu (BSA) jako standardu.

Testovaná séra

45 krevních sér žen s prokázanou poruchou plodnosti bylo využito k imunodetekci. Séra byla vybrána dle výše titru spermaglutinujících protilátek (Fridbergův test, též TAT)[9] a třídy protilátek (nepřímý MAR test)[10]. Jako negativní kontrola bylo užito krevní sérum osmileté dívky. Alikvoty byly uchovány při teplotě –20 °C.

Jednorozměrná polyakrylamidová elektroforéza v přítomnosti SDS (1D SDS-PAGE) následovaná imunoblotem

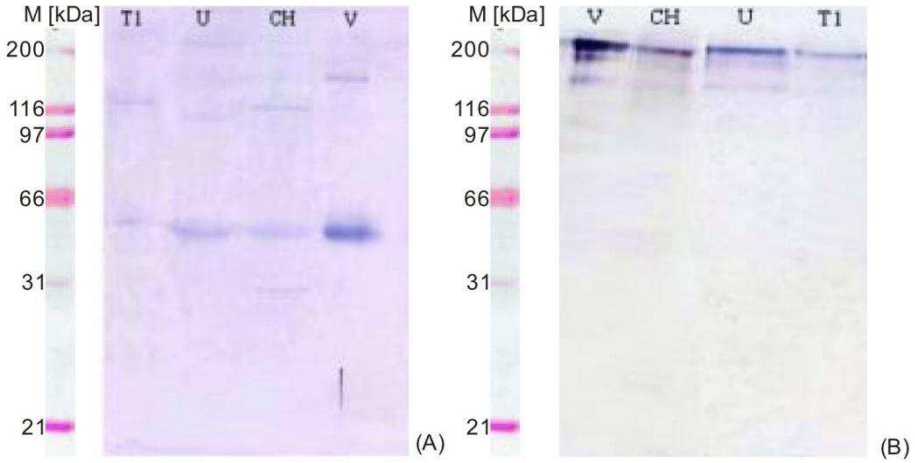
SDS-PAGE byla povedena při pokojové teplotě v Mini Protein Cell Bio-Rad dle modifikace Harlow a Lane (1988). Alikvot směsného lyzátu byl separován na 10% polyakrylamidovém gelu (30% akrylamidový mix, 1,5M Tris-HCl pH 8,8, 10% SDS, 10% persíran amonný – APS, TEMED – Serva, Německo) a 5% akrylamidovém zaostřovacím gelu pH 6,8. Vzorek byl nanesen v koncentraci 25 μg proteinů. Před nanesením byl smíchán v poměru 1:1 s tzv. vzorkovacím pufrem neredukujícím (protein loading buffer non-reducing, PLB-N; 0,5M Tris-HCl pH 6,8; glycerol, 10% SDS; 0,1% bromofenolová modř) nebo redukujícím (protein loading buffer reducing, PLB-R; 0,5M Tris-HCl pH 6,8; glycerol, 10% SDS; 0,1% bromofenolová modř, 5% β-merkaptoethanol). Směs byla 3 min vařena a aplikována do jamek v gelu. Jako proteinový standard byl použit širokospektrý molekulový marker (SDS-PAGE standard, broad rang, Bio-Rad, USA). Separované proteiny v gelu byly detegovány stříbrem užitím kitu Silver^{TM} Plus Stain Kit (Sigma, USA).

Aparatura Mini Trans-Blot Cell Bio-Rad (Mini Protean III system, Bio-Rad, USA) byla použita pro Western blot. Přenos proteinů byl proveden pomocí stejnosměrného elektrického proudu (elektroblot) na 0,45 μm pórovou nitrocelulosovou membránu (NC, Serva, Německo) při 100 V, teplotě 4 °C, 45 min, užitím Towbinova pufru (Tris-glycinový pufr, 48 mM Tris, 39 mM glycin, 10% methanol, pH 9,2). Membrána byla saturována 2 h blokovacím roztokem – 10% odtučněné sušené mléko (Laktino, Promil, ČR) v TBS-Tw (Tris buffered saline tween; 0,02M Tris,14M NaCl, 4,5M $MgCl_2$, 0,1% Tween 20, pH 8,0) při laboratorní teplotě. Poté byla membrána inkubována s krevními séry pacientek přes noc při teplotě 4 °C. Séra byla ředěna 1:1000 v 5% odtučněném sušeném mléku v TBS-Tw. Membrána byla třikrát promyta TBS-Tw a inkubována s konjugátem Goat AntiHuman IgG-AP (Promega, USA) ředěným 1:15 000 v 5% odtučněném sušeném mléku v TBS-Tw 2 h při laboratorní teplotě. Nitrocelulosová membrána byla třikrát promyta TBS-Tw. Imunokomplex byl detegován pomocí aktivity konjugovaného enzymu užitím chromogenního substrátu (IMMUNO NBT/BCIP, Liquid substrate plus, MP Biomedicals, USA). Po zvolení nejvhodnějšího způsobu dezintegrace spermií byl postup opakován s obměnou, kdy byla membrána krájena na 3mm proužky a každý odděleně saturován a inkubován s krevním sérem pacientky. Navazující postup se shodoval s postupem pro celou membránu. Na základě metody lineární regrese byla pomocí molekulového standardu určena molekulová hmotnost nalezených antigenů.

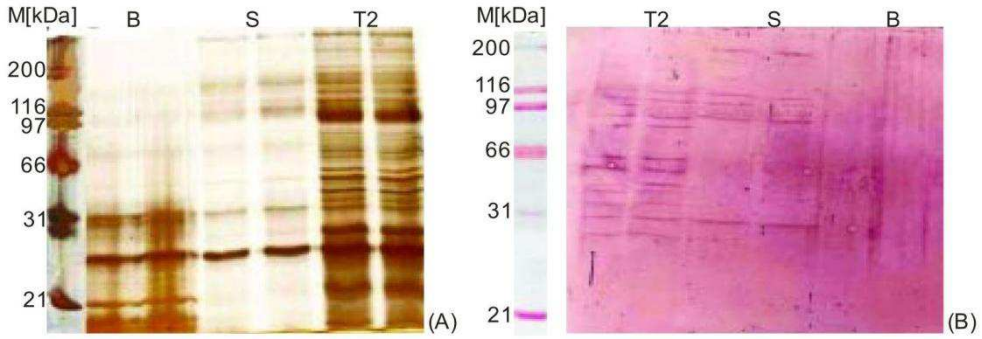
Výsledky a diskuse

Čtyři vzorky spermatu byly využity k přípravě proteinového extraktu spermií. Koncentrace bílkovin v lyzátech dosáhla rozsahu 0,4–3 mg ml^{-1}. Směsný vzorek proteinového extraktu spermií byl separován metodou SDS-PAGE v redukujícím (obr. 1A, 2) i neredukujícím (obr. 1B) prostředí. V obou případech následoval imunoblot (obr. 1−3).

Jestliže byl vzorek před separací metodou SDS-PAGE inkubován ve vzorkovacím pufru neredukujícím, pak nedošlo k dostatečnému rozdělení proteinů. Vzorky lyzované roztokem chloridu benzalkonia (bezvodá směs alkylbenzyldimethylamonných chloridů s alkylovými řetězci C_8–C_{18}), Tritonem X-100 a získané lyzí pomocí ultra-



Obr. 1. **Imunodetekce proteinového extraktu spermií v redukujících (A) a neredukujících podmínkách (B) užitím krevního séra pacientky s poruchou plodnosti.** M: molekulový standard pro SDS-PAGE, T1: lyze Tritonem X-114, U: močovinou, CH: CHAPS, V: destilovanou vodou



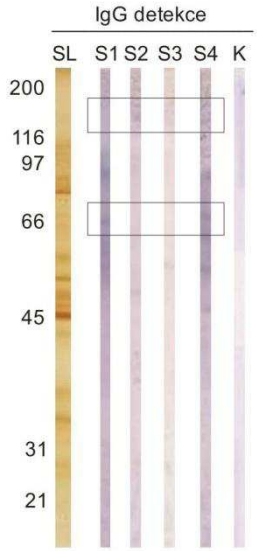
Obr. 2. **Jednorozměrná polyakrylamidová elektroforéza v přítomnosti SDS (A) a imunodetekce (B) proteinového extraktu spermií získaného lyzí.** M: molekulový standard, B: lyze spermií pomocí chloridu benzalkonia, S: lyze sonikací, T2: lyze Tritonem X-100

zvuku (sonikace) byly inkubovány pouze v prostředí redukujícím a následně děleny pomocí SDS-PAGE (obr. 2A). Separaci následovala imunodetekce (obr. 2B).

Při porovnání metod dezintegrace lidských spermií na základě SDS-elektroforézy a imunoblotu byl zvolen Triton X-100 jako nejvhodnější účinná látka lyze. Nalezené molekulové hmotnosti (M_r) dosahují širokého rozpětí od 31 do 200 kDa (obr. 3). Jako nejčetnější byly určeny molekulové hmotnosti 68 a 123 kDa. Antigen o velikosti 68 kDa byl rozpoznán 93 % použitých sér, antigen o velikosti 123 kDa 84 %. Minoritně byly zastoupeny i další, sérum S1 dále interaguje s antigeny molekulové hmotnosti 50, 86 and 90 kDa, sérum S2 s 36 kDa, sérum S3 s 50 kDa a sérum S10 s 43, 50, 64 a 86 kDa. Nebyla prokázána interakce IgG protilátek osmileté dívky (negativní kontrola) s antigeny spermií.

Vzhledem k stále vyrovnanějšímu poměru ženského a mužského faktoru neplodnosti, nesmíme ženu vyjímat z partnerského celku[1]. Pokud zmiňujeme ženu s poruchou plodnosti, jedná se jen o vstupní parametr naší studie. Séra žen byla podrobena spermaglutinačním testům[9] na minimálně dvou různých vzorcích spermií zdravých dárců s parametry normospermiogramu[10]. Pozitivní séra způsobovala aglutinaci (shlukování) spermií, snižovala jejich pohyblivost nebo měla na spermie účinek toxický. Pomocí nepřímého MAR testu byla specifikována třída antispermatozoidálních protilátek[11]. MAR test (mixed antibody reaction) je reakce, kdy se na spermie naváží polystyrenové částice s protilátkami proti lidským imunoglobulinům. Pozitivní výsledek je nežádoucí. Pro potřeby detekce a s ohledem na plánované použití konjugátu (Goat Anti-Human IgG-AP) byla vybrána séra s vysokým titrem protilátek třídy IgG.

Naše práce reprezentuje jednu z etap výzkumu imunitních vlastností lidské spermie a jejího vztahu k imunitnímu systému ženského pohlavního traktu. Zdrojem protilátek byly v našem případě séra pacientek s poruchou plodnosti. Tento poměrně frekventovaný pojem je v literatuře vysvětlován různě. Velmi často je definován právě použitím negativních kontrol, např. dětských sér,



Obr. 3. **Imunodetekce antigenů spermií separovaných jednorozměrnou polyakrylamidovou elektroforézou v přítomnosti SDS.** M: molekulový standard proteinů, SL: barvený proteinový extrakt spermií, S1 – S4: screening sér interagujících s antigeny spermií, K: negativní kontrola

kde se nepředpokládá přítomnost protilátek proti spermiím. Nedávné práce ukazují na možnost zkřížené reaktivity protilátek alternativní etiologie reagujících se spermiemi. Mnozí autoři upozornili na možnost zkřížené imunoreakce antigenů spermií s protilátkami vyvolanými např. mikrobiálními antigeny nebo dokonce jen zánětlivými procesy[12–16]. Naše negativní kontrola však evidentně takový druh předchozí stimulace nevykazovala.

I přes pokrok molekulární genetiky a proteomiky považujeme přípravu proteinového extraktu spermií pomocí lyzovacích metod za přínosný postup. Vzhledem k posttranslačním modifikacím možných antigenních struktur na povrchu spermie, si lze jen těžko představit charakterizaci

antigenů spermií bez základních lyzovacích postupů[17]. U každého lyzovacího postupu je dobré mít na paměti charakter lyzovacích látek a možnost ztráty jednotlivých konformačních epitopů, ne-li celých antigenů přítomných v nativním stavu. Zvážíme-li všechna výše uvedená fakta, jeví se jako nejvhodnější neionogenní tenzid, kterým byl v naší studii Triton X, obzvláště pokud bychom chtěli získat membránové proteiny[18]. Na základě škály separovaných proteinů metodou SDS-elektroforézy a odezvy na imunoblotu byl vybrán Triton X 100. Nicméně, tato studie je brána spíše jako úvodní, nejen z hlediska výtěžnosti potenciálních antigenů, ale i z hlediska metodologického. Jak bylo již zmíněno, nelze zcela přesně předvídat zachování přítomnosti všech jednotlivých epitopů buněčných antigenů a jejich imunogenicity v procesu lyze, a proto musíme o to větší pozornost věnovat výběru detekčního systému[9,11].

V kontrastu naší studie s jinými[19,20], jež se také zabývaly charakterizací antigenů spermií, jsme nalezli podobné molekulové hmotnosti detegované pomocí IgG protilátek. Nicméně jsme detegovali i antigeny o vyšší molekulové hmotnosti, např. 123 kDa. Je možné, že právě tento antigen pochází z vnitřního prostředí spermie, a proto není ve shodě se zmíněnými výsledky z literatury. Zda se jedná o identické proteiny, membránové nebo plazmatické, bude možno rozhodnout až na základě probíhajících experimentů hmotnostní spektrometrie. Chceme-li porozumět mechanismu imunologicky podmíněné neplodnosti, je třeba studovat antigeny spermií podrobně[21]. Výsledky naší studie by mohly přispět k úspěsné léčbě a zlepšení diagnostiky neplodnosti.

Závěr

Dnešní doba přináší stále vyšší riziko neplodnosti. Světová zdravotnická organizace ji definuje již jako nemoc. Je-li neplodnost ženy imunologicky podmíněna, je třeba najít antigeny spermií, na které reaguje imunitní systém tvorbou protilátek a brání tak úspěšnému početí. Pro získání proteinové frakce spermií byly použity chemické i fyzikální způsoby dezintegrace. Chemické metody byly zastoupeny lyzí buněk v hypotonickém prostředí (destilovaná voda) a použitím povrchově aktivních látek (DOC, CHAPS, Triton X-100 a X-114), močoviny a chloridu benzalkonia, který je součástí spermicidních látek. Fyzikální metodou byla sonikace. Jako nejúčinnější metoda se jeví dezintegrace Tritonem X-100. Za použití Tritonu X-100 bylo dosaženo nejlepšího rozdělení proteinů metodou SDS-PAGE a následná imunodetekce na nitrocelulosové membráně byla nejvýraznější. Vzorek před dělením proteinů bylo vhodnější inkubovat ve vzorkovacím pufru redukujícím. Nejčastěji byly detegovány antigeny spermií o molekulové hmotnosti 68 a 123 kDa. Podrobnější charakterizace a případná izolace antigenů bude předmětem našeho dalšího studia.

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A. Brázdová[a] , J. Zídková[a], J. Cibulka[b], M. Vališová[a], V. Škop[a], and Z. Ulčová-Gallová[b] *([a] Department of Biochemistry and Microbiology, Institute of Chemical Technology, Prague, [b] Department of Gynecology and Obstetrics, Faculty of Medicine, Charles University and Faculty Hospital, Plzeň):* **Disintegration of Human Sperm and Characterization of Its Antigens**

For treatment of female immunological infertility and of sensitivity to sperm it is necessary to characterize particular sperm antigens recognized by antibodies. For disintegration of sperm, sonication and chemical or related methods (detergents, benzalkonium chloride, urea, hypoosmotic shock) were used. The characterization was performed by SDS-electrophoresis followed by immublotting using IgG antibodies from 45 sera of infertile females. The serum of an eight-year-old girl was used as a negative control.

The use of Triton X-100 seems to be the best way for preparation of sperm protein extract. The interactions of serum IgG antibodies with 68 and 123 kDa sperm proteins were shown to be the most frequently recognized.

5.1.3 IgG, IgA and IgE reactivities to sperm antigens in infertile women

Still more couples have been diagnosed with the unexplained infertility associated with immune infertility. Immune infertility is commonly related to anti-sperm antibody formation. Higher levels of IgG antibodies in women with fertility failure represent the immunopathological response. For better understanding, it is necessary to detect and characterize antigens recognized by the female immune system.

The aim of this pilot 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 allergic reaction. The emphasis of experimental work was on an appropriate choice of the healthy individuals having normal sperm parameters. The pooled sperm extract represented the antigen source. We analyzed the specificity of female serum IgG, IgA and IgE to human sperm antigens separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis and isoelectric focusing. IgG, IgA and IgE immunoblottings were performed using forty-five sera from women who were diagnosed with fertility failure. Sera of 10 children were used as negative controls. The immunodominant sperm antigens detected by IgG antibodies were 68 and 123 kDa proteins, 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 an association of IgE antibodies with unexplained infertility in women. Collected data were used as preliminary for thorough two-dimensional analysis and further identification.

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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).

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

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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 immuno 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.

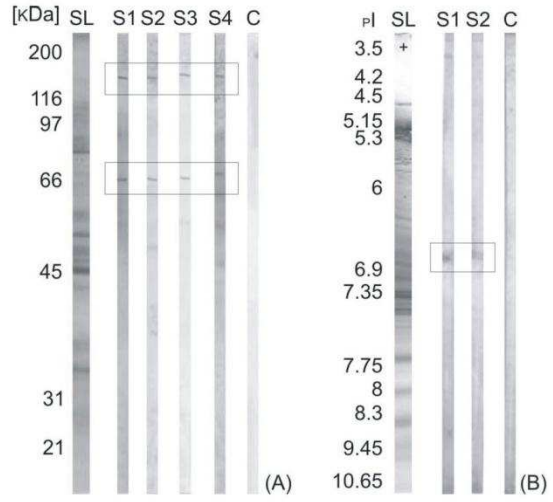

Figure 1. IgG reactivity of sperm antigens separated by 1D SDS-PAGE (A) and 1D IEF (B) followed by immunoblotting. SL: proteins of pooled sperm extract, silver and Coomassie blue stained; s1 – 4: screening of sera interacting with sperm antigens; C: negative control serum.

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.

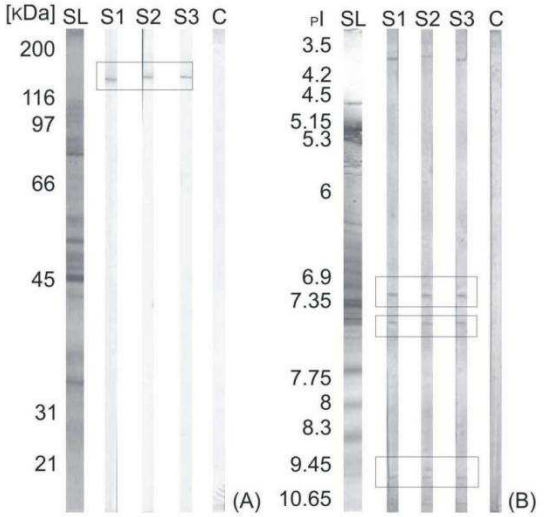

Figure 2. IgA detection of sperm antigens separated by 1D SDS-PAGE (A) and 1D IEF (B) followed by immunoblotting. SL: proteins of pooled sperm extract, silver and Coomassie blue stained; s1 – 3: screening of sera interacting with sperm antigens; C: negative control serum.

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).

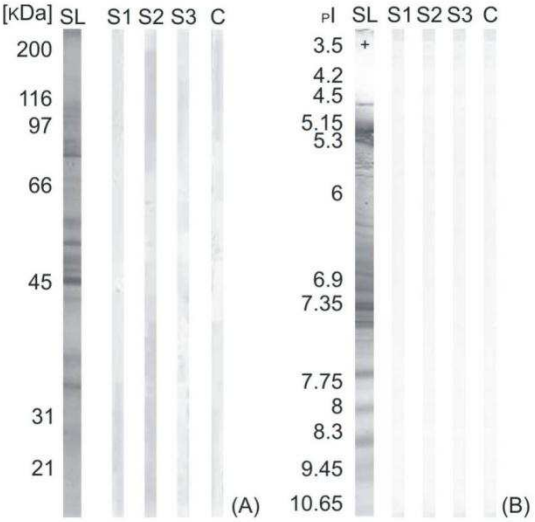

Figure 3. IgE detection of sperm antigens separated by 1D SDS-PAGE (A) and 1D IEF (B) followed by immunoblotting. SL: protein of pooled sperm extract, silver and Coomassie blue stained; s1 – 3: screening of sera interacting with sperm antigens; C: negative control serum.

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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5.1.4 Female serum immunoglobulins G, A, E and their immunological reactions to seminal fluid antigens

One in five couples of reproductive age has been diagnosed with infertility. Some diagnoses indicate an immunological basis. Female immune infertility can be caused by semen iso-immunization. Seminal fluid contains a range of cellular and a-cellular components that represent the antigenic structure evoking the response of the female immune system. Seminal components protecting sperm usually bind to the acrosomal region of the sperm head and are then carried together with sperm into the higher genital tract. Thus, seminal fluid may modulate the chemotactic and phagocytic response of the female reproductive tract. On the other hand, these seminal components might be the first targets of cytotoxic events at early fertilization. Moreover, the female immune system reacts by the producing of prostaglandins, complement inhibitors, cytokines.

This study represented an ongoing research on female immune infertility. Actually, we pointed out for the first time, that to best of our knowledge, female iso-immunization is related to seminal fluid components as well as to sperm itself and not only to IgE-mediated rare allergic reaction leading to the so-called semen-hypersensitivity. The particular aim of our study was to prove and illustrate an association of seminal proteins with female immune infertility based on the female serum IgG, IgA and IgE antibodies.

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. Mainly, IgG 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 analyzed immunochemically to determine the characteristics of possible seminal proteins associated with female immune infertility. Our results suggested that the anti-seminal IgG and IgA antibodies from the selected group of patients exhibit different specificities. We assumed that the selected infertile female patients may not have had an allergic built-in mechanism linked to seminal components since no IgE reactivity was found against proteins from the male seminal fluid. Based on our experiments, we proved, that immunoglobulins G as well as A were involved the female reaction of iso-immunization.

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.

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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.

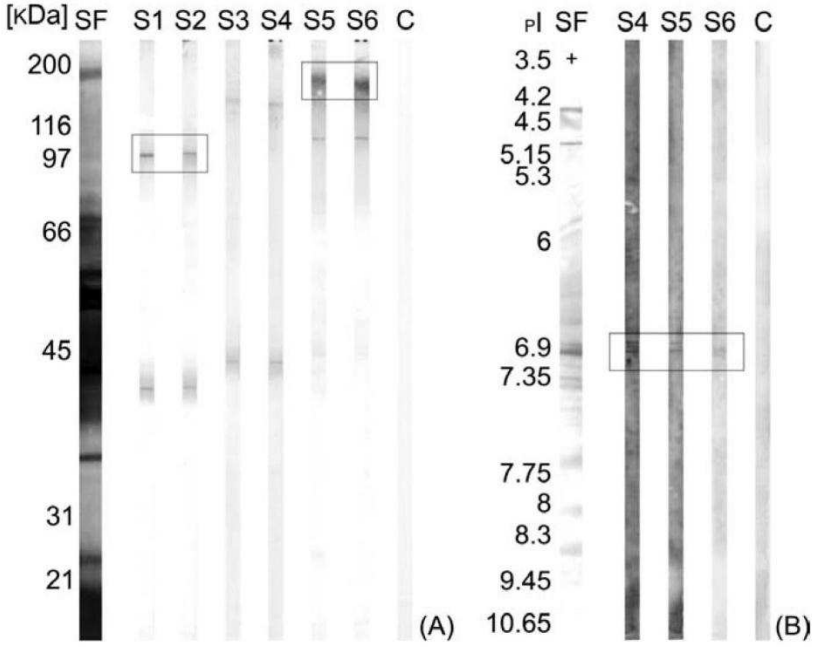

Fig. 1. IgG immunoblotting of seminal proteins separated by 1D SDS-PAGE (A) and IEF (B). SF: pooled seminal proteins, silver and Coomassie blue stained; S1–6: screening of female sera; C: negative control serum.

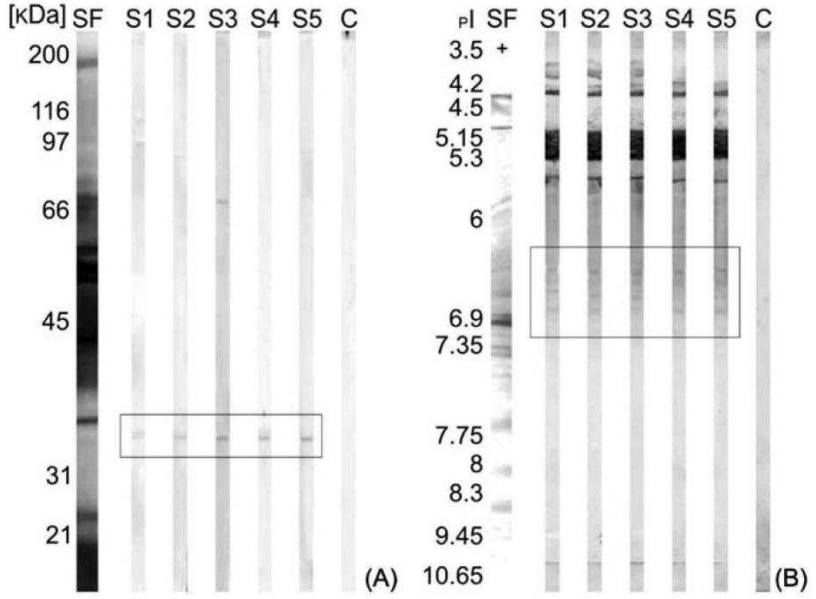

Fig. 2. IgA immunoblotting of seminal proteins separated by 1D SDS-PAGE (A) and IEF (B). SF: pooled seminal proteins, silver and Coomassie blue stained; S1–5: screening of female sera; C: negative control serum.

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

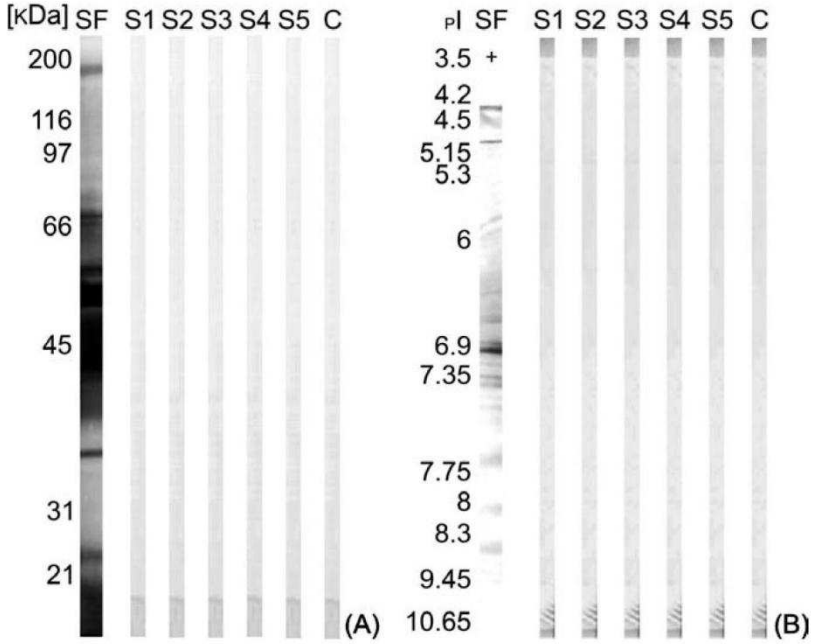

Fig. 3. IgE immunoblotting of seminal proteins separated by 1D SDS-PAGE (A) and IEF (B). SF: pooled seminal proteins, silver and Coomassie blue stained; S1–5: screening of female sera; C: negative control serum.

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.

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5.1.5 Immunodominant semen proteins I: New patterns of sperm proteins related to female immune infertility

Infertility affects approximately 10% of couples at reproductive age. Idiopathic causes indicate an immunological origin. Immune reactions may be responsible for up to 30% of reported cases of infertility. Semen constituents may represent potential immunogenic structures for women. Since vaginal and cervical tissues have an active and sensitive mucosal immune system, the active local immunoregulatory mechanisms in the female reproductive tract affect fertility. This explains the rather high percentage of infertile women in relation to local reactions leading to inflammation and high levels of serum anti-semen antibodies.

Our research reflected the association of sperm proteins with iso-immunization in women based on the IgG-mediated reaction. The aim of our study was to identify the new patterns of sperm proteins recognized by serum IgG antibodies obtained from women diagnosed with fertility failure, and to compare them to antigens recognized by serum IgGs obtained from fertile women. Using a mass spectrometry-based proteomic approach, we characterized and determined eight sperm proteins interacting with patient IgG antibodies. HSP 70 1A/1B, HSP cognate 71 kDa, alpha-enolase were, for the first time, shown to be associated with female iso-immunization. We pointed out that sperm alpha-enolase contributes to the female immune infertility mediated by IgG antibodies and not only to either auto-immunization in men or the rare cases of semen hypersensitivity mediated by IgE in women, as has been posited so far. The immunodominant sperm proteins seemed to be related to antibody formation as a pathologic response to iso-immunization. Our results might be useful in the diagnoses of female immune infertility and provide potential targets for further therapeutic treatment as well as contribute to a better understanding of this pathologic reaction that threatens fertilization.

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Immunodominant semen proteins I: New patterns of sperm proteins related to female immune infertility

Research Article

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Abstract: Infertility affects approximately 10% of couples at reproductive age. Semen constituents may be potential immunogenic structures for women. The aim of our work is to detect and identify sperm proteins interacting with serum IgG antibodies from women with fertility disorders. The biochemical characterization of sperm antigens was performed using one and two dimensional gel electrophoresis, both of which were followed by immunoblotting. The IgG-binding proteins of interest were identified using mass spectrometry. From the serum pool of 30 infertile women, we detected sperm antigens within a relative molecular mass range between 30–80 kDa with an isoelectric point of 4–7. No antigens were detected using the serum pool from 10 fertile women (control group). Heat shock proteins (HSP 70) were identified as major sperm antigens associated with female immune infertility. Additionally, we report for the first time that alpha-enolase is a significant sperm antigen from the serum pool of infertile women. We suggest that the IgG-binding proteins identified in our study are related to immune infertility in the case of certain women with abnormally high levels of IgG antibodies linked to sperm proteins. Our results might be useful in the diagnoses of female immune infertility and may provide potential targets for further therapeutic treatment.

Keywords: *IgG antibodies • Alpha-enolase • Immunoblotting*

1. Introduction

Ten percent of couples at reproductive age suffer from infertility. Idiopathic causes indicate an immunological origin. Immune reactions may be responsible for up to 30% of reported cases of infertility [1,2]. Since vaginal and cervical tissues have an active and sensitive mucosal immune system, the active local immunoregulatory mechanisms in the female reproductive tract have an effect on fertility. This explains the local reactions leading to an inflammation and the high level of serum antisemen antibodies in infertile women [3,4].

Sperm has a highly heterogeneous antigenic content that can be modified during maturation and ejaculation, e.g. the attachment of secreted fibronectin to the sperm tail [5]. The female genital tract generally tolerates the male antigens commonly presented on sperm. However, semen has a built-in mechanism preventing the immunological sensitization against sperm in female. The failure of this natural tolerance may lead to sensitivity resulting in sperm elimination. Sperm is opsonized and targeted by the leukocytes [6-8]. Sperm also has both autoantigenic [9,10] as well as isoantigenic [11,12] potential. It induces the production of sperm-reactive T-cells, thus the formation of antisperm antibodies (ASA) may follow from exposure to the immune system. In men, autoimmunity to their own sperm has already been reported particularly with respect to heat shock proteins (HSPs) [9] and alpha enolase [10]. ASA are related to some cases of unexplained infertility. It has been suggested that ASA block fertilization by inhibiting sperm motility,

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capacitation, acrosome reaction, impairment of sperm penetration into the cervical mucus and by preventing oocyte binding and penetration. ASA's also affect pre-implantation embryonic development and may have a contraceptive effect [13,14]. Nevertheless, there is the evidence of their occurrence in both fertile women and men. Since ASA may not lead to infertility, they are believed to be the so-called natural ASA in these fertile individuals. In addition, it has been suggested that seminal fluid (SF), a nutritious transport buffering sperm medium, acts as a modulator of sperm-induced inflammation [8,15,16].

Our research reflects the association of sperm with isoimmunization in women based on the IgG-mediated reaction. The aims of our study were to identify new repertoire of sperm proteins recognized by serum IgG antibodies obtained from women diagnosed with fertility failure, and to compare them to antigens recognized by serum IgGs obtained from fertile women. Several antigens are described, eight of which are identified as immunodominant when using a mass spectrometry-based proteomic approach. The presented results might contribute to a better understanding of the pathologic reaction that threatens successful fertilization.

2. Experimental Procedures

2.1 Sample preparation

Semen samples from four normozoospermic healthy donors [17] (average age =27 years) were obtained by masturbation after 3–5 days of abstinence. A mixture of protease inhibitors (Sigma Cocktail, USA) was added to ejaculates. In order to separate SF and sperm, the semen samples were centrifuged at 1075x*g* for 15 min at 4°C. The pellets (predominantly sperm cells) were washed and treated as previously described [11] to process the sperm disintegration. Each pellet was briefly washed twice by PBS (0.01 M phosphate buffered saline with NaCl 0.15 M, pH 7.4) supplemented with protease inhibitors and centrifuged as above. Lysis was proceeded by using 1% Triton X-100 (Sigma, USA) in deionized water, containing a mixture of protease inhibitors (1:50) for 4 h at 4°C on ice. Insoluble parts were removed by final centrifugation at 3000x*g* for 10 min at 4°C. To increase the amount of potential antigens in a protein extract, individual sperm lysates (L) were pooled. Protein concentration was measured according to Smith *et al*. [18]. The samples were stored at -20°C until assayed. All experiments were performed after obtaining informed written consent.

2.2 Patients

This study has been approved by the institutional ethical committees and informed written consent has been obtained by patients. Patient sera were obtained from 30 women with fertility disorder (average age = 35 years) and ten sera from fertile women (control group, average age = 33 years) with proven fertility (one or two children). An equal volume of each serum in each group was pooled to obtain sufficient volume of patient and control serum for repetitive analyses. The aliquots were frozen at -20°C until assayed.

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

SDS electrophoresis was carried out as described in Brazdova *et al*. [12]. After electrophoresis, the separated proteins were either silver-stained (SilverTM Plus Stain Kit, Sigma, USA) to visualize the complex spectrum of sperm proteins or transferred to a nitrocellulose sheet (NC, 0.45 µm pore size, Serva, Germany) for immunoblotting.

2.4 Two-dimensional polyacrylamide gel electrophoresis (2D SDS-PAGE)

Isoelectric focusing (IEF) was performed in the ReadyPrep IPG Strips pH 3-10 (Bio-Rad, USA) containing equal amounts of protein. After IEF, the strips were treated in accordance with the manufacturer's instructions (ReadyPrep 2-D Starter Kit, Bio-Rad, USA), then put on 10% acrylamide SDS gel and overlaid with melted agarose (ReadyPrep 2-D Starter Kit, Bio-Rad, USA). The separated proteins were either transferred onto a nitrocellulose sheet (NC, 0.45 µm pore size, Serva, Germany), silver-stained (SilverTM Plus Stain Kit, Sigma, USA) or Coomassie stained (Brilliant Blue R-250, Sigma, USA) to excise the spots of interest for mass spectrometry.

2.5 Immunoblotting analyses

Western blot analyses were performed as described in Tobwin *et al*. [19]. The membrane was saturated with PBS-Tw 0.3% (0.14 M NaCl, 8.1 mM $Na_2KPO_4.12\ H_2O$, 1.8 M KH_2PO_4, 2.7 mM KCl, 0.3% v/v Tween 20; pH 7.4). Each sheet was incubated with serum overnight at 4°C. NC sheets were then incubated with horseradish peroxidase (HRP)-conjugated anti-human IgG (Sigma, USA) for 2 h at 20°C. The HRP activity was detected by 3-amino-9-ethylcarbazole substrate (AEC, Sigma, USA).

2.6 Mass spectrometry determination

The protein spots of interest were analyzed by the MALDI-TOF method. IgG-binding proteins revealed by immunoblotting were excised from Coomassie stained

gels. Two pieces of each gel that did not contain visible proteins were analyzed as negative controls. The spots were destained by 100 mM ammonium bicarbonate/acetonitrile (NH_4HCO_3/ACN, 1:1, v/v). The proteins were reduced (10 mM dithiothreitol, 45 min, 56°C), alkylated (55 mM iodoacetamide, 30 min, 25°C) and digested (trypsin [Promega, USA], 3 h, 37°C). The given peptides were extracted from the gel with 35% ACN supplemented with 0.1% trifluoroacetic acid (TFA) and by 70% ACN with 0.1% TFA. The extracts were lyophilized, resolved in 0.1% TFA, desalted using ZipTip C_{18} pipette tips (Millipore, USA), mixed with the matrix solution (1:1, v/v) and placed on the steel target plate. The matrix (2, 5-dihydroxybenzoic acid, DHB) was used at a concentration of 15 mg/ml in 33% ACN supplemented with 0.1% TFA. The samples were measured using a Biflex IV mass spectrometer (Bruker Daltonics, Germany) equipped with a UV nitrogen laser (337.1 nm, Laser Science, USA). Spectra were measured in a reflection mode (mass interval 700–4000 Da). The Bruker peptide calibration standard II (Bruker Daltonics, Germany) was used for an external (next-spot) calibration. Spectra were recalibrated using tryptic peaks as an internal calibration. Raw data were processed by mMass program [20]. Proteins were identified using Mascot search engine [21] within SwissProt database [22].

3. Results

To obtain a complex spectrum of sperm proteins and potential antigens, the individual sperm lysates (L) were pooled. The L pool contained between 8 and 9 mg/ml of protein.

Figure 1 shows the sperm protein spectra that were separated by 1D SDS-PAGE (Figure 1, lane L). Sperm proteins were visualized within the molecular mass (Mr) range of 20-35 kDa. However, 80 kDa sperm proteins were also silver stained. To distinguish Mr of IgG-binding proteins, blotting analyses was performed (Figure 1, lane 1, 2). However, the major sperm proteins, when silver stained, were seldom detected as antigens on the 1D blot (Figure 1, lane L, 1). Among the major sperm proteins measuring 25, 35, and 80 kDa, only the 80 kDa protein was detected as an antigen. No IgG interactions with sperm antigens were detected using the control serum pool from fertile women (Figure 1, lane 2). Figure 2 illustrates 2D protein maps and immunoblotting analyses. The whole sperm protein repertoire was visualized with silver staining within the Mr range of 30–80 kDa with a pI of 4–7 (Figure 2 A). Only a few proteins interacted with the female serum IgG antibodies. Those detected on 2D blots were subsequently identified. The greatest reactivity was detected in the HSPs: heat shock-related 70 kDa protein 2, heat shock cognate 71 kDa protein and heat shock 70 kDa protein 1A/1B (Figure 2 B, spot No 2, 3, 4). Spots identified as the 78 kDa glucose-regulated protein, protein disulfide-isomerase A3, calreticulin, zinc finger protein 501 and alpha-enolase were also determined to be sperm antigens (Figure 2 B, spot No 1, 5, 6, 7). Another dominant sperm protein detected at Mr of 75–80 kDa with pI 5–6 was recognized by the patient serum pool but was not identified by mass spectrometry. No IgG interactions with sperm antigens were detected using the control serum pool from fertile women (Figure 2 C). The biochemical attributes and parameters of the above mentioned proteins are summarized in Table 1.

4. Discussion

Human semen contains many protein constituents that represent potential targets of the female immune system. Possible immune reactions following the interaction

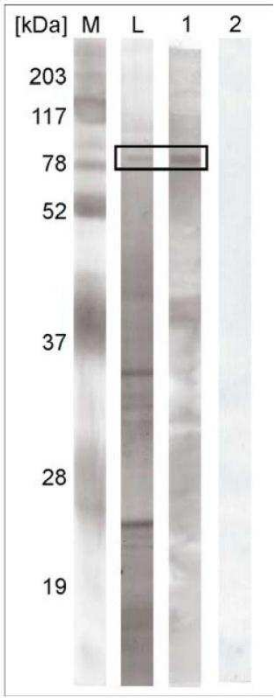

Figure 1. Sperm extract separated by 1D SDS-PAGE (silver stained) followed by immunoblotting. M – molecular standard for SDS-PAGE; lane L – sperm extract; lane 1 – immunodetection in the serum pool from infertile women, lane 2 – immunodetection in the serum pool from fertile women; frame – dominant proteins detected by blotting as dominant antigens.

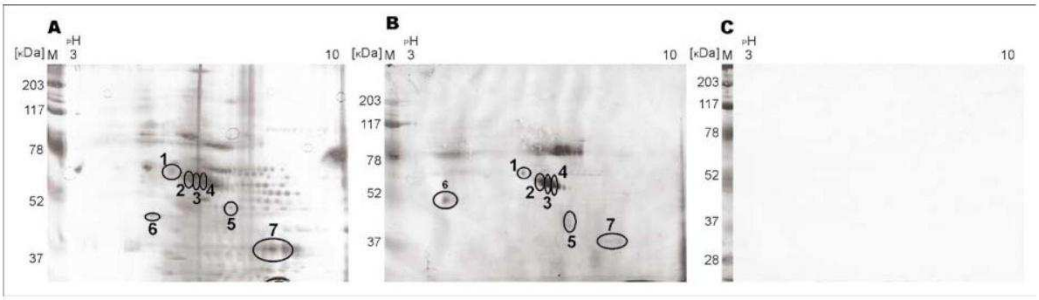

Figure 2. Sperm extract separated by 2D SDS-PAGE either silver stained (A) or followed by immunoblotting (B, C). M – molecular standard for SDS-PAGE; B – immunodetection of sperm antigens using the serum pool of infertile women, C – immunodetection of sperm antigens using the serum pool of fertile women; 1 – 7 circled marks – identified antigens by mass spectrometry.

Protein	Spot	Protein coverage [%]	Mr [kDa]	pI
78 kDa glucose-regulated protein	1	45	72	5.0
Heat shock-related 70 kDa protein 2	2	43	70	5.5
Heat shock-related 70 kDa protein 2	3	33	70	5.5
Heat shock cognate 71 kDa protein	3	26	71	5.4
Heat shock 70 kDa protein 1A/1B	3	30	70	5.5
Heat shock-related 70 kDa protein 2	4	37	70	5.5
Protein disulfide-isomerase A3	5	32	57	6.0
Calreticulin	6	42	48	4.3
Zinc finger protein 501	6	46	32	4.3
Alpha-enolase	7	36	48	7.0

Table 1. Biochemical attributes and parameters of identified proteins.

of semen with the female genital tract may prevent successful fertilization [1,11]. This study was carried out to identify as many sperm proteins recognized by the serum pool of infertile female patients as possible.

We pooled sperm extract samples from four male donors, which should have covered a wide repertoire of proteins and potential antigens present in sperm [11,12,17,23]. We suggest that the previously mentioned antigens are specifically related to female immune infertility, as no IgG interaction was detected with semen antigens using the control pooled serum from fertile women.

As abnormal, senescent or damaged sperm increase sperm immunogenicity [3], the detection of more antigens on 2D gels might be expected. Therefore, only samples from normozoospermic donors were used. To identify internal/external sperm antigens, protein extract must be prepared with a high degree of heterogeneity. Nonionic detergents [10] in general and Triton X-100 in our study have been reported as a suitable detergent [11] to solubilize proteins, and is compatible with our proteomic approach. The determination of sperm stress proteins, such as a group of HSPs, calreticulin and 78 kDa glucose-regulated protein, shows that they might be also involved in female sensitization. Since HSPs are membrane proteins and calreticulin is originally thought to be an autoantigen with an intracellular location, we show that the sperm lysate covers both groups of potential antigens (internal/external).

Our results are in agreement with other studies [24-26] that reported HSPs, calreticulin and 78 kDa glucose-regulated protein as sperm antigens. The HSP 70 family has been identified as chaperons present on the sperm surface [27]. Particularly, HSP 70 2, a testis specific protein, has been described as an autoantigen [28] as well as an isoantigen [27], which we confirm in this study. Additionally, we are the first to report that HSP 70 1A/1B and HSP cognate 71 kDa relate

to the isoimmunization of women (possibly leading to reproductive failure). We show that these proteins, which are important for the rearrangement of sperm proteins and for the composition of the final antigen conformation [7] (such as the identified disulfide-isomerase A3), may interact with female antibodies.

Alpha-enolase was also found to be an isoantigen in women. This result confirms the results of Domagala *et al.* [10], who studied cognate sperm antigens using the sera of ASA-positive infertile men and women. In our study, alpha-enolase was, however, firstly reported as a significant sperm antigen detected by the serum pool of infertile women. It has been reported [8] that female isoimmune responses do not follow the same pattern of reactions in respect to carbohydrate recognition. Based on these observations, we suggest that alpha-enolase could be a potential universal marker for antisperm antibodies related to immune infertility. On the other hand, alpha-enolase could represent a potential target of further therapeutic treatment. Furthermore, this multifunctional enzyme stimulates immunoglobulin production and plays a part in the allergic reactions [29]. In contrast with Lopez-Alemany *et al.* [29], who suggested that alpha-enolase is an allergen-induced allergic response based on IgE antibodies, our study demonstrates that alpha-enolase is a sperm IgG-binding antigen as well. Previous work [12] has shown similar results, with no sperm IgE-binding protein detected within a selected group of 45 female patients.

We suggest that all identified IgG-binding proteins are related to an abnormality in certain infertile women presenting high levels of IgG antibodies specific to sperm proteins. By immunoblotting and mass spectrometry, we determined eight sperm proteins interacting with patient IgG antibodies. Among them, three proteins (HSP 70 1A/1B, HSP cognate 71 kDa, alpha-enolase) were shown to be associated with female isoimmunization for the first time. We point out that sperm alpha-enolase contributes to female immune infertility mediated by IgG antibodies, not only to autoimmunization in men or in rare cases of semen hypersensitivity mediated by IgE in women. as had been previously suggested. Immunodominant sperm proteins seem to be related to antibody formation as a pathological response to isoimmunization.

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5.1.6 Immunodominant semen proteins II: Contribution of seminal proteins to female immune infertility

Seminal fluid is routinely examined to evaluate semen quality and to diagnose prostate cancer. However, it may play a significant role in pathophysiological antibody formation, which then contributes to female immune infertility. Failure of natural tolerance may over stimulate the immune system towards SF as a complex or towards seminal proteins directly bound to sperm. Anti-seminal antibodies may threaten fertilization. There is a vast repertoire of reactions leading to the activation of inflammatory cytokines and leukocyte infiltration.

This study represented another part of complex mosaic dealing with seminal proteins relevant to antibody formation in infertile women. In this report, we showed that the amount of IgG antibodies directed to seminal components is worth mentioning since we quantified the anti-seminal IgG antibodies in fertile and infertile women. Based on these results, we concluded that 100% of women, selected upon immune infertility criteria, express IgG antibodies against SF proteins. We identified the isoantigens recognized by the serum pool of infertile women. They seemed to be related to the pathologic immune response associated with female sensitization. The herein determined proteins confirmed our theory that SF proteins are thus involved not only in the IgE-mediated semen hypersensitivity and are not only a protective medium for sperm but they also represents potential immunogenic structures for the female immune system. Based on our results, prostate-specific antigen, prostatic acid phosphatase, zinc-alpha-2-glycoprotein and zinc finger protein 778 might be even considered as the biomarkers of such pathology within selected infertile females.

Original Article

Immunodominant Semen Proteins II: Contribution of Seminal Proteins to Female Immune Infertility

(IgG antibodies / female immune infertility / prostate-specific antigen / prostatic acid phosphatase)

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Abstract. Seminal fluid is a protective medium for sperm, but it also represents potential immunogenic structures for the female immune system. Anti-seminal antibodies may threaten early fertilization. The aim of our work is to detect and identify seminal proteins that are related to female isoimmunization. In this report, we quantified serum anti-seminal IgG antibodies. Seminal proteins were analysed by two-dimensional gel electrophoresis followed by immunoblotting. To identify IgG-binding proteins of interest, a proteomic approach was selected. The dominant seminal antigens were detected within the relative molecular mass ranging from 25 to 85 kDa and the isoelectric point from 5 to 7. The detected proteins were further identified as prostate-specific antigen, prostatic acid phosphatase, zinc-α-2-glycoprotein and zinc finger protein 778. Since these proteins were recognized by IgGs produced by infertile women and not by fertile women, we presume that major seminal antigens may play an important role in the pathogenesis of female immune infertility. Our study suggests the pattern of seminal proteins for further therapeutic attempts in the diagnosis of female immune infertility.

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Abbreviations: 2D SDS-PAGE – two-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis, ACN – acetonitrile, AEC – amino-ethylcarbazole, AP – alkaline phosphatase, DHB – dihydroxybenzoic acid, ELISA – enzyme-linked immunosorbent assay, HRP – horseradish peroxidase, IEF – isoelectric focusing, IgE/G – immunoglobulin E/G, MALDI-TOF – matrix-assisted laser desorption/ionization-time of flight, Mr – relative molecular mass, NC – nitrocellulose membrane, PAP – prostatic acid phosphatase, pI – isoelectric point, PSA – prostate-specific antigen, SF – seminal fluid, TFA – trifluoroacetic acid, WHO – World Health Organization, ZAG – zinc-α-2-glycoprotein, ZNF778 – zinc finger protein 778.

Introduction

Seminal fluid (SF) represents a part of semen containing a range of organic/inorganic substances that are necessary for the physiological metabolism of sperm. It also acts as a transport, diluent and buffering medium (Kumar et al., 2009; Brázdová et al., 2012a). SF includes a repertoire of signalling molecules interacting with epithelium in the female reproductive tract. Local reactions may lead to inflammation (Robertson, 2005). SF has a built-in mechanism preventing immunological sensitization of the female against sperm as well as seminal structures. This protective system exists due to the presence of immune inhibitors originating in the male sex accessory glands (Prakash, 1981). SF has thus been suggested to be the modulator of sperm-induced inflammation (Troedsson et al., 2005), although in most cases SF prevents sensitization.

Some studies showed that the female fertility potential can be altered as a result of antibody formation against sperm antigens. The antibody fraction reacting with seminal antigens targets most of seminal proteins adsorbed on the sperm. SF induces the recruitment of macrophages and dendritic cells into cervical and endometrial tissues. Vaginal isoimmunization against any se-

men component can impair fertility. On the other hand, SF participates in the remodelling of cervical tissue to enable embryo pre-implantation. SF has also an important role in fertilization and embryonic development (Ulčová-Gallová, 2006; Robertson, 2007; Brázdová et al., 2012b). So far, only few seminal antigens linked to the female immune response have been characterized and determined. SF has rather been involved in the rare IgE-mediated reaction to semen (Weidinger et al., 2006). In this case of hypersensitivity, patients have difficulties to conceive, but infertility has not been demonstrated. SF is investigated with the purpose to evaluate pathological spermiograms and to monitor the progression of prostate cancer by the level of prostate-specific antigen (PSA) and prostatic acid phosphatase (PAP) (Jones, 1991; Ostrowski and Kuciel, 1994).

The aim of our study was to describe seminal antigens and to identify various patterns interacting with serum IgG antibodies obtained from women diagnosed with fertility failure. We identified four antigens using mass spectrometry based on the proteomic approach. Our results brought evidence of SF sensitization and its connection with female isoimmunization and not only with semen hypersensitivity. We provided a significant basis for the profiling of infertile female patients.

Material and Methods

Sample preparation

Semen samples from eight normozoospermic (WHO, 2010) healthy donors (the average age of the voluntary donors was 27 years) were obtained by masturbation after 3–5 days of sexual abstinence. A mixture of protease inhibitors (Sigma Cocktail, Sigma-Aldrich, St. Louis, MO) was added to the ejaculates. SF was separated from sperm by centrifugation at 1075 × *g* for 15 min at 4 °C. The SF supernatants were pooled to increase the amount of potential proteins/antigens. Protein concentration was measured according to Smith et al. (1985). The samples were stored at -20 °C until assayed. All experiments were performed after obtaining informed written consent.

Patients

Patient sera were obtained from 30 women with fertility failure (patients with repeated *in vitro* fertilization failure, the average age of the women was 35 years) and 10 from fertile women (control group, the average age of the women was 33 years) with proven fertility (one or two children). The equal volume of each serum was pooled to obtain sufficient volumes of patient and control serum. The aliquots of pools were frozen at -20 °C until assayed. This study was approved by the institutional ethical committees and informed written consent was obtained from the female infertile patients and female fertile controls.

Quantification of serum anti-seminal IgG antibodies

Female serum IgG antibodies linked to SF proteins were quantified by ELISA method. In the first protocol, to evaluate and quantify total serum IgG, the microplates (MaxiSorp™, Nalge Nunc International, Roskilde, Denmark) were coated with anti-human IgG (whole molecule, Sigma Aldrich) in 50 mM carbonate-bicarbonate buffer (0.2 M Na_2CO_3, 0.2 M $NaHCO_3$; pH 9.64) overnight at 4 °C. The plates were saturated with 0.5% gelatine (type B, Sigma-Aldrich) in PBS-Tw 0.1% (0.14 M NaCl, 8.1 mM $Na_2KPO_4.12\ H_2O$, 1.8 mM KH_2PO_4, 2.7 mM KCl, 0.1% v/v Tween 20; pH 7.4) for 2 h at room temperature. The coated and saturated wells were incubated with the individual patient or control serum or human IgG (Fc receptor specific, Sigma-Aldrich) of known concentrations in the serial dilutions for 2 h at 37 °C in PBS-Tw 0.1% and then with alkaline phosphatase (AP)-conjugated goat anti-human IgG (Fc receptor specific; Sigma-Aldrich) for 2 h at 37 °C. The AP activity was detected by p-nitrophenyl phosphate disodium kit (Sigma-Aldrich). Optical density was measured at 405 nm versus 630 nm. The wells were rinsed with 0.9% NaCl-Tw 0.1% after each incubation step.

In the second protocol, the SF reactivity of patient and control sera was tested by ELISA in an indirect non-competitive format. The micro-plates were coated with 1 μg/well of SF in a 50 mM carbonate-bicarbonate buffer overnight at 4 °C. The plates were saturated with 0.5% gelatine in PBS-Tw 0.1% for 2 h at room temperature. The coated and saturated wells were then incubated with the patient or control sera for 2 h at 37 °C in PBS-Tw 0.1% and then with AP-conjugated goat anti-human IgG for 2 h at 37 °C. The AP activity was detected by p-nitrophenyl phosphate disodium kit. Optical density was measured at 405 nm versus 630 nm. The wells were rinsed three times with 0.9% NaCl-Tw 0.1% after each incubation step. The anti-seminal IgG concentration was obtained by linear regression in comparison with the standard IgG calibration curve obtained in the first protocol.

Two-dimensional polyacrylamide gel electrophoresis (2D SDS-PAGE)

Isoelectric focusing (IEF) was performed in the ReadyPrep IPG Strips pH 3–10 (Bio-Rad, Mississauga, ON). After IEF, the strips were equilibrated according to the manufacturer's instructions (ReadyPrep 2D Starter Kit, Bio-Rad), then put on 10% acrylamide SDS gel and overlaid with melted agarose (ReadyPrep 2D Starter Kit, Bio-Rad). The separated proteins were either transferred onto a nitrocellulose sheet (NC, 0.45 μm pore size, Serva, Heidelberg, Germany) or silver-stained (SilverTM Plus Stain Kit, Sigma-Aldrich) to visualize the proteins, or Coomassie-stained (Coomassie Brilliant Blue R-250, Sigma-Aldrich) to excise the spots of interest as it is a suitable indication for mass spectrometry.

Immunoblotting analyses

Western blot analyses were performed as described (Towbin et al., 1979). The membrane was saturated with PBS-Tw 0.3% (PBS, 0.3% v/v Tween 20; pH 7.4). NC sheets were incubated with the serum pool overnight at 4 °C, then with horseradish peroxidase (HRP)-conjugated anti-human IgG (Fc specific, Sigma-Aldrich) for 2 h at 20 °C. The HRP activity was detected by 3-amino-9-ethylcarbazole substrate (AEC, Sigma-Aldrich).

2D image digitizing

2D images were digitized using the program ImageJ to evaluate the density/intensity of identified spots. The pictures were processed at 740×575 pixels, converted into 3D format and digitized at 660×600 pixels at RGB grey scale level depth. The intensity was calculated using the Micro Array Profile tool.

Mass spectrometry determination

The protein spots of interest were analysed by the MALDI-TOF method. IgG-binding proteins revealed by immunoblotting were excised from Coomassie-stained gels. The spots were destained by 100 mM ammonium bicarbonate/acetonitrile (NH_4HCO_3/ACN, 1:1, v/v). The proteins were reduced (10 mM dithiothreitol, 45 min, 56 °C), alkylated (55 mM iodoacetamide, 30 min, 25 °C) and digested (trypsin [Promega, Madison WI], 3 h, 37 °C). The particular peptides were extracted from the gel by 35% ACN supplemented with 0.1% trifluoroacetic acid (TFA) and by 70% ACN with 0.1% TFA. The extracts were lyophilized, resolved in 0.1% TFA, desalted using ZipTip C_{18} pipette tips (Millipore, Bedford, MA), mixed with the matrix solution (1:1, v/v) and placed on the steel target plate. The matrix (2, 5-dihydroxybenzoic acid, DHB) was used at the concentration of 15 mg/ml in 33% ACN supplemented with 0.1% TFA. The samples were measured using the Biflex IV mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a UV nitrogen laser (337.1 nm, Laser Science, Franklin, MA). Spectra were measured in a reflection mode (mass interval 0.7–4 kDa). The Bruker peptide calibration standard II (Bruker Daltonics) was used for external (next-spot) calibration. Spectra were recalibrated using tryptic peaks as internal calibration. The raw data were processed by the mMass program (Strohalm et al., 2008). The proteins were identified using the Mascot search engine (Perkins et al., 1999) within the SwissProt database (Swiss-Prot Protein Knowledgebase online).

Results

The individual SF samples from eight donors were pooled to cover the complex spectrum of seminal proteins/potential antigens. The protein concentration of the SF pool ranged from 20 to 25 mg/ml. Figure 1 shows the concentration of seminal-specific IgG antibodies in the individual sera of infertile and fertile women. IgG

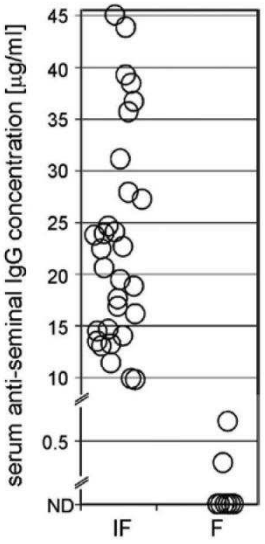

Fig. 1. ELISA quantification of female serum IgG antibodies linked to SF proteins. IF: infertile female patients, F: fertile female controls, ND: non-detectable level.

antibodies linked to seminal proteins were detected in all 30 sera of infertile females, ranging from 9.5 to 45 µg/ml. In comparison, the anti-seminal protein IgG level was detectable only in two sera of fertile controls out of 10, 0.32 and 0.66 µg/ml, respectively. Figure 2 illustrates 2D electrophoretogram (Fig. 2A) and IgG immunoblotting analyses (Fig. 2B, C). The SF profile (Fig. 2A) provided the spectrum of proteins ranging from 25 to 100 kDa and isoelectric point (pI) from 5 to 9. By comparison of 2D gels with their 2D blots, we found antigens recognized by the serum pool from female infertile patients (Fig. 2B). The most intensive spots were identified by MALDI-TOF as a zinc finger protein 778 (ZNF778, Fig. 2A, spot 1), prostatic acid phosphatase (PAP, Fig. 2B, spot 2) and prostate-specific antigen (PSA, Fig. 2A, spot 4). Another protein was weakly detected by the immunoblotting of SF and was further identified as zinc-α-2-glycoprotein (ZAG) (Fig. 2B, spot 3). Other patterns of seminal antigens showing Mr of 30–35 kDa, pI 5.6 and then Mr of 117–130, pI 5.7, were detected on blots but not identified with a sufficient mass spectrometry score. No seminal IgG-binding proteins were detected using the control serum pool (Fig. 2C). The digitized signal of seminal proteins/antigens is shown in Figure 3A and B, respectively. We conclude, based on 2D histograms, that the identified proteins are major antigens detected by the infertile patient serum pool. Interestingly, all isoforms of the immunodominant SF proteins were detected as the antigenic patterns. The biochemical attributes and parameters of the above-mentioned proteins are summarized in Table 1.

Discussion

Failure of natural tolerance may over-stimulate the immune system towards SF or sperm. Female IgG anti-

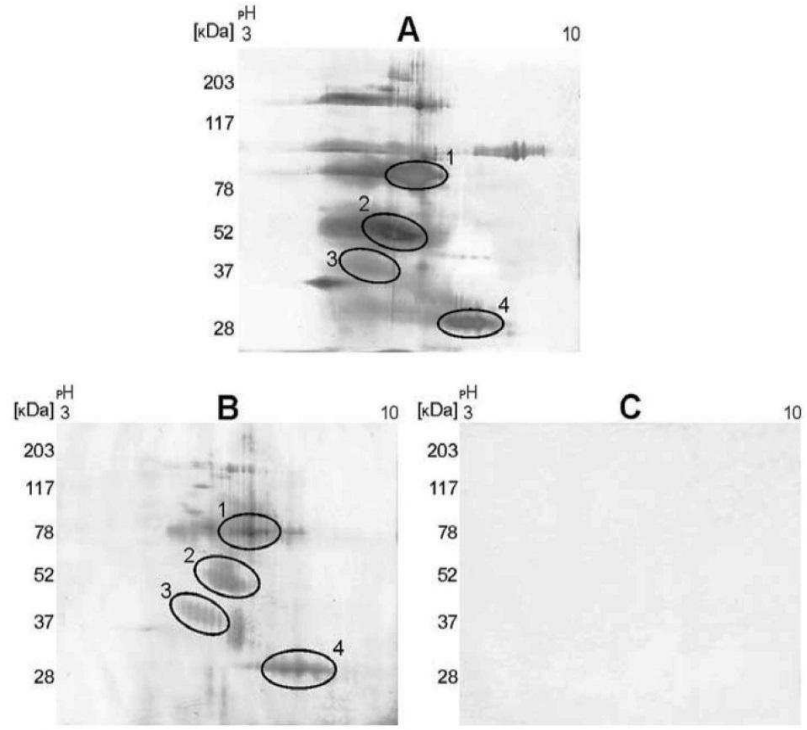

Fig. 2. Seminal proteins separated by 2D SDS-PAGE either silver-stained (A) or immunoblotted (B, C). B: immunodetection using the serum pool of infertile women, C: immunodetection using the serum pool of fertile women, 1–4: antigens identified by mass spectrometry.

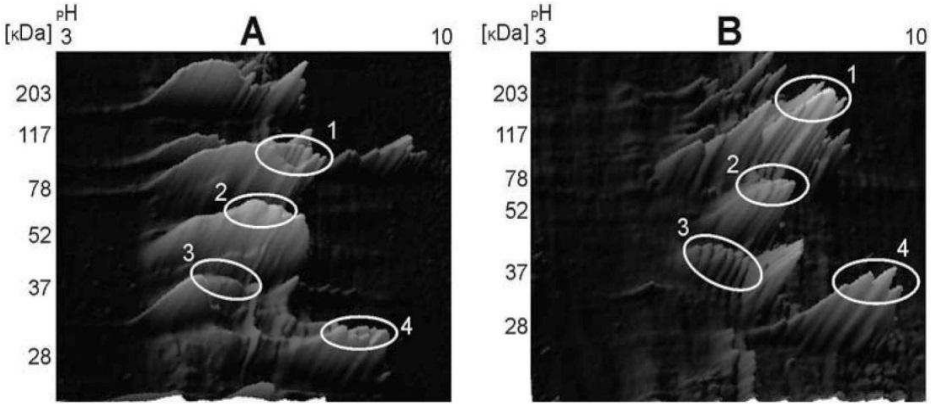

Fig. 3. Quantification of signal revealed in 2D images. A: quantified spots on silver-stained 2D gel, B: quantified spots on 2D blot, 1–4: antigens identified by mass spectrometry.

Table 1. Biochemical attributes and parameters of identified proteins

Protein	Spot	Mr [kDa]	pI	Score$_a$	Protein Coverage$_b$ [%]	Integrated density on 2D gel [%]	Integrated density on 2D blot [%]
Zinc finger protein 778 (ZNF778)	1	85	5.8	84	17	35.7	38.1
Prostatic acid phosphatase (PAP)	2	45	5.6	92	35	29.6	33.2
Zinc-α-2-glycoprotein (ZAG)	3	35	5.6	189	54	21.5	29.4
Prostate-specific antigen (PSA)	4	29	6.7	156	56	28.3	30.8

[a] Protein score was evaluated by the Matrix search engine as -10*Log(P), where the value P was the probability (Perkins et al., 1999). Protein score greater than 56 was considered as significant ($P < 0.05$).

[b] Protein coverage was calculated as the ratio of amino acid number among the detected peptides to the amino acid number of each protein.

bodies act on the SF when the ejaculate is deposited into the vagina. IgG antibodies against SF proteins have been described and were suggested to be involved in female immune infertility (Brázdová et al., 2012a). The repertoire of reactions leading to the activation of inflammatory cytokines and leukocyte infiltration is wide. Which seminal protein activates these reactions has not been pointed out yet (Starita-Geribaldi et al., 2001; Sharkey et al., 2007; Plessis et al., 2011). Our results represent ongoing study of seminal proteins (Brazdova et al., 2012a) relevant to antibody formation in infertile women. In this report, we quantified the concentration of anti-seminal IgGs in fertile and infertile women. Then, we identified the isoantigens recognized by the serum pool of infertile women.

On the basis of previous studies (Rodriguez-Martinez et al., 2011; Brázdová et al., 2012a) and the fact that men might differ in their SF content, we decided to prepare a pool of seminal fluids from eight healthy normozoospermic (WHO, 2010) donors to extend the potential repertoire of IgG-binding proteins by increasing the heterogeneity of the sample. In order to characterize individual SF proteins and to identify the located seminal antigens from a complex SF mixture, we used a proteomic approach based on 2D immunoblotting and mass spectrometry. Our experiments using two different methods – quantitative (ELISA) and semi-quantitative (immunoblotting) – show that male seminal proteins are able to trigger antibody production in the female patients diagnosed with immune infertility. Using the control serum pool from fertile women, no IgG binding with seminal antigens was detected on the immunoblot. This is in agreement with ELISA results showing that only two fertile women out of 10 show a very low level of anti-SF-reactivity. Such differences in IgG concentration and specificities between fertile and infertile women support the hypothesis of the anti-seminal IgG antibodies involved in the pro-inflammation process preventing successful fertilization.

Seminal proteins contribute to male fertility and are essential in several steps of fertilization (Qu et al., 2007). In order to characterize the proteins that could be the targets of antibodies in infertile women, we kept the seminal samples untreated to maintain, as much as possible, their native form. This fact could explain the smears on 2D images (Fig. 2A and B). The optimal solubility of the SF extract would be achieved using a mixture containing urea, thiourea and CHAPS (Starita-Geribaldi et al., 2001). This mixture is considered to keep the entire spectrum of epitopes that might be involved in the elicitation of pro-inflammatory circuits in the female reproductive tract.

SF is mostly associated with semen hypersensitivity. Particularly, PSA has been suggested to be linked to allergic reaction or anaphylaxis involving IgE antibodies. PSA and one isoenzyme of acid phosphatase, PAP, are used to monitor and assess the progression of prostate cancer and are usually also related to a pathological spermiogram (Jones, 1991; Ostrowski and Kuciel, 1994; Weidinger et al., 2006; Hassan et al., 2008a). In the current study, both markers may have indicated the activated humoral immunity of infertile females since they were identified as the immunodominant antigens. As it was previously mentioned, PSA is thought to be associated with the rare phenomenon of semen hypersensitivity inducing specific IgE antibodies; however, we have shown that PSA is also able to induce IgG antibodies in infertile women. ZNF778 is a nuclear protein that has been described at the nucleic acid level in various tissues including testis (Ota and Suzuki, 2004). ZNF778 is considered, for the first time to the best of our knowledge, as an IgG-binding protein that may be involved in antibody formation during the pathological isoimmunization. ZAG has been mentioned as a sperm antigen and suggested to have a role in the expression of the immune response (Lilja et al., 1987; Cross, 1996). Our results are in agreement with this observation and we confirmed that ZAG is an IgG-binding protein even though we found it in the SF. This localization could be explained by the fact that ZAG possibly complexes with other seminal structures (Hassan et al., 2008b).

We conclude that (1) 100 % of women, selected upon infertility criteria, express IgG antibodies against SF proteins and (2) at least three SF proteins, namely PSA, PAP and ZNF778, are related to the pathologic immune response associated with female sensitization. SF proteins are thus involved not only in the IgE-mediated semen hypersensitivity. The herein described proteins might be considered as biomarkers of such pathology.

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5.1.7 Immunodominant semen proteins III: IgG_1 and IgG_4 linkage in female immune infertility

Semen-specific immunoregulatory factors as well as immunogenic agents represent the potential targets of the female immune system. Consequentially, inflammatory cytokines are produced, leukocytes infiltrate into the female genital tract and complement cascade may be activated. The activated immunity as reflected in sperm-reactive T-cells, anti-sperm antibodies, seminal protein-binding antibodies interferes with various pre/post-fertilization processes.

In this study, attention was paid to the high levels of serum specific anti-seminal/sperm antibodies. The task consisted of the quantification of anti-seminal/sperm antibodies presented in the sera of infertile female patients and fertile women in order to determine the immunoglobulin classes and subclasses mostly involved into pathophysiological iso-immunization. The profiling of infertile female patients implied that antibody formation was not associated with allergy as IgE was not detected. Either $IgA_{1,2}$ or IgM did not significantly correlate with the systemic iso-immunization. Concerning our results, the proportionality did exist between seminal and sperm IgG_1 and IgG_4 antibody fractions. IgG_4, as a minor isotype of IgGs, appeared to be mainly produced and linked to IgG_1. Seminal antigen fraction thus seemed to be of a protein-like nature; on a contrary, sperm antigens rather of a polysaccharide-like character. The data shown were of value to a detailed patient diagnoses. Early determination of serum seminal/sperm-specific immunoglobulin G subclasses could contribute patient profiling and could be of interest for further immune-therapy.

Journal of Medical Biochemistry

IMMUNODOMINANT SEMEN PROTEINS III: IgG1 AND IgG4 LINKAGE IN FEMALE IMMUNE INFERTILITY

--Manuscript Draft--

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Abstract:	Summary: Background: Active immune mechanism in the female reproductive tract may produce high levels of anti-seminal/sperm antibodies. It seems that isoimmunization is also associated with infertility. The aim of our study consists in the profiling of specific serum immunoglobulin classes and subclasses of infertile women. We focus on the distribution of serum seminal/sperm-specific antibodies in order to find those apparently related to isoimmunization. Methods: Immunoglobulins G1-4, A1,2, M and E were measured by ELISA in serum from 30 infertile and 10 fertile females. Results: Anti-seminal/sperm IgG1 and IgG4 fractions were predominantly detected. Strikingly, the ratio between IgG1 and IgG2 was 3:1 in seminal specific and 2:1 in sperm specific antibodies. Surprisingly, IgG3 antibodies were nearly negative for both antigen fractions, seminal and sperm. Conclusions: Concerning our results, the proportionality does exist between seminal and sperm antibody fractions. Based on the poorly detectable levels of semen specific IgE, M, A1,2, the markers of pathologic female isoimmunization appear to be the serum IgG1 and IgG4. These preliminary may findings contribute to a detailed patient diagnosis and an improved therapy.
Suggested Reviewers:	Gerard Chaouat

IMMUNODOMINANT SEMEN PROTEINS III: IgG_1 AND IgG_4 LINKAGE IN FEMALE IMMUNE INFERTILITY

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Running title: Antibodies in female immune infertility

Summary:

Background: Active immune mechanism in the female reproductive tract may produce high levels of anti-seminal/sperm antibodies. It seems that iso-immunization is also associated with infertility. The aim of our study consists of the profiling of specific serum immunoglobulin classes and subclasses of infertile women. We focus on the distribution of serum seminal/sperm-specific antibodies in order to find those apparently related to iso-immunization.

Methods: Immunoglobulins G_{1-4}, $A_{1,2}$, M and E were measured by ELISA in serum from 30 infertile and 10 fertile females.

Results: Anti-seminal/sperm IgG_1 and IgG_4 fractions were predominantly detected. Strikingly, the ratio between IgG_1 and IgG_2 was 3:1 in seminal specific and 2:1 in sperm specific antibodies. Surprisingly, IgG_3 antibodies were nearly negative for both antigen fractions, seminal and sperm. **Conclusions:** Concerning our results, the proportionality does exist between seminal and sperm antibody fractions. Based on the poorly detectable levels of semen specific IgE, M, $A_{1,2}$, the markers of pathologic female iso-immunization appear to be the serum IgG_1 and IgG_4. These preliminary may findings contribute to a detailed patient diagnosis and an improved therapy.

Keywords: antibodies; ELISA; seminal fluid; sperm

List of non-standard abbreviations: AP, alkaline phosphatase; F, control sera of fertile women; IF, sera of infertile patients; L, sperm lysate; ND, nondetectable levels; SD, standard deviation; SF, seminal fluid

IMUNODOMINANTNI SEMINALNI PROTEINI III: POVEZANOST IgG_1 I IgG_4 U IMUNOJ NEPLODNOSTI KOD ŽENA

Sažetak:

Pozadina: Aktivni imuni mehanizam ženskog reproduktivog trakta može proizvesti visok nivo antiseminalnih/spermalnih antitela. Čini se da je izoimunizacija takođe povzana sa neplodnošću. Cilj naše studije je karakterizacija specifičnih klasa i podklasa serumskih imonoglobulina kod neplodnih žena. Fokusirali smo se na distribuciju serumskih seminalnih/spemalnih specifičnih antitela kako bismo pronašli one povezane sa izoimunizacijom.

Metode: Imunoglobulini G_{1-4}, $A_{1,2}$, M i E mereni su ELISA testovima u serumu 30 neplodih i 10 plodnih žena.

Rezultati: Zapažene su uglavnom anti-seminalne/spermalne IgG_1 i IgG_4 frakcije. Upadljivo je da su IgG_1 i IgG_2 u odnosu 3:1 u seminalnim specifičnim antitelima, dok taj odnos iznosi 2:1 u spermalno specifičnim. Iznenađujuće, IgG_3 antitela su skoro negativna za obe antigene frakcije, kako seminalnu tako i spermalnu.

Zaključak: Što se tiče naših rezultata, proporcionalnost između frakcija seminalnih i spermalnih antitela zaista postoje. Na osnovu slabo detektovanih nivoa seminalno-specfičnih IgE, M, $A_{1,2}$, čini se das u glavni marker patološk izoimunizacije kod žena serumski IgG_1 i IgG_4. Ovi preliminarni nalazi mogu doprineti detaljnijoj dijagnozi i poboljšanoj terapiji.

Introduction

Human semen that is defined as a complex fluid containing sperm, cellular vesicles and other cells and components, could immunize the female genital tract. Semen immunoregulatory factors as well as immunogenic agents are thus the potential targets of activated inflammatory cytokines, initiate leukocyte infiltration and complement cascade in the female genital tract. The active local immunoregulatory mechanism of the female reproductive tract is related to the fertility potential. Sperm is able to induce the production of sperm-reactive T-cells in men as well as in women. Antisperm antibodies interfere with the antibody-mediated infertility through various pre/post-fertilization processes (1, 2). Seminal antibody-binding proteins contribute to sperm metabolism, passage in the female reproductive tract and block an interaction with immune effectors. Seminal fluid (SF) induces pro-inflammatory cytokines and suppresses the cell-mediated cytotoxicity (3, 4).

In primary response to some allergens/antigens, IgE antibodies might be prevalent. The so-called switch into IgG and IgA antibodies is induced at the late phase of primary immune response and/or after the repeated exposure to the same antigen (5). After the chronic antigen exposure, IgG_1 and IgG_4 become the predominantly produced subclasses of IgG isotype. In addition, IgG_4 is unable to activate the classical complement pathway and is then known as an anti-inflammatory immunoglobulin and a blocking antibody towards IgE antibodies. It remains still unclear whether IgG_4 is a protective or rather sensitizing antibody (6, 7).

In this paper, the profile of antibody-based immune response to seminal/sperm proteins in infertile and fertile women has been studied to document which class or subclass of serum immunoglobulin might be mostly involved in the immune rejection of sperm associated with female immune infertility.

Material and Methods

Sample preparation

Semen samples from eight normozoospermic (8) donors (aged 25-30) were treated as previously described (1, 4). Sperm-free SF was processed untreated; the sperm disintegration was processed by Triton X-100. To increase the amount of potential antigens in the protein extracts, the obtained individual sperm lysates (L) were pooled as well as SF. The samples were stored at -20 °C until assayed. All experiments were performed after obtaining informed written consent.

Patients

This study has been approved by the institutional ethical committees and informed written consent has been returned by patients. Sera were obtained from 30 women with a fertility disorder (patients with repeated in vitro fertilization failure, aged 29-38) and from 10 women (control group, aged 28-37) with proven fertility (1-2 healthy children). The serum samples were frozen at -20 °C until assayed.

Quantification of serum anti-seminal/sperm immunoglobulins

Serum anti-seminal/sperm IgG_{1-4}, $IgA_{1,2}$, IgM, IgE and then the SF/L reactivity of patient and control sera were tested by ELISA.

In the first protocol, to obtain the standard calibration curves, the micro-plates (MaxiSorp^{TM}, Denmark) were coated with either mouse anti-human IgG_{1-4} (Calbiochem, United States), $IgA_{1,2}$ (Abcam, United Kingdom), goat anti-human IgM (Sigma-Aldrich, United States) or IgE (Sigma-Aldrich, United States) in a 50 mM carbonate-bicarbonate buffer (9) overnight at 4 °C. The plates were saturated with 0.5% gelatine (Sigma-Aldrich, United States) in PBS-Tw 0.1% (4) for 2 h at room temperature. The wells were incubated

with the individual patient or control sera or human IgG_{1-4}, $IgA_{1,2}$, IgM, IgE (Calbiochem, United States) as standards of known concentration in serial dilution for 2 h at 37 °C in PBS-Tw 0.1% and then with alkaline phosphatase (AP)-conjugated, either mouse anti-human IgG_{1-4} (Calbiochem, United States), $IgA_{1,2}$ (Beckman Coulter, United States), goat anti-human IgM or IgE (Sigma-Aldrich, United States) for 2 h at 37 °C. The AP activity was detected by p-nitrophenyl phosphate disodium (Sigma-Aldrich, United States). Optical density was measured at 405 nm versus 630 nm.

In the second protocol, to quantify the serum anti-seminal/sperm IgG_{1-4}, $IgA_{1,2}$, IgM, IgE, the micro-plates were coated with SF/L in a 50 mM carbonate-bicarbonate buffer overnight at 4 °C. The following procedure was identical to the first protocol. The anti-seminal/sperm immunoglobulin concentrations were obtained by linear regression in comparison with the standard calibration curve obtained in the first protocol.

Results

Figure 1 shows the concentration of seminal/sperm-specific IgG_{1-4} in the individual sera of infertile/fertile females. In the sera of infertile women, the predominant anti-SF IgG subclasses were IgG_1 and IgG_4, reaching maximum concentration of 45 µg/ml (IgG_1) and 38 µg/ml (IgG_4). In particular, anti-SF IgG_1 antibodies were detected in 100% of sera tested with the mean of 22 µg/ml (SD 9), IgG_2 in 97% with the mean of 8 µg/ml (SD 6), IgG_3 in 6% with the mean of 0.1 µg/ml (SD 0.05), IgG_4 in 90% with the mean of 12 µg/ml (SD 9). In the sera of infertile women, the prevalent anti-sperm IgG subclass was IgG_4 ranging from 9 to 45 µg/ml with the mean of 23 µg/ml (SD 10). Anti-sperm IgG_{1-4} antibodies were detected within all patient sera processed with the mean of 11 (SD 5, IgG_1), 7.5 (SD 5, IgG_2), 0.3 (SD 0.1, IgG_3), 23 µg/ml (SD 10, IgG_4). Twenty percent of control sera contained seminal-specific IgG_1 and 30% contained seminal-specific IgG_2, both of which ranged from 0.2 to 0.5 µg/ml.

Sperm-specific $IgG_{1,2}$ were detected in 30% of control sera reaching the top value of 0.5 µg/ml. None of the control sera had the detectable levels of anti-seminal/sperm $IgG_{3,4}$. (Figure 1 should be inserted here.)

Figure 2 shows seminal/sperm-specific $IgA_{1,2}$, IgM, IgE concentrations in the individual sera of infertile/fertile women. Anti-seminal/sperm IgE, IgM and IgA_1 were not detected in the patient sera. Only 1 patient serum out of 30 contained anti-sperm IgA_2 (0.18 µg/ml). Then, 1 control serum out of 10 contained anti-seminal IgA_1 (1.2 µg/ml), IgA_2 (1.8 µg/ml) and anti-sperm IgA_1 (1.9 µg/ml), IgA_2 (2.1 µg/ml). (Figure 2 should be inserted here.)

Discussion

We present our essential preliminary findings concerning the distribution of serum anti-SF/L $IgG_{1,2,3,4}$, $IgA_{1,2}$, IgM and IgE as a consequence of pathophysiological female iso-immunization.

We show IgG_1/IgG_4 predominance depending on the values themselves, 2:1 in anti-seminal specific antibodies, also valid in a reverse statement between anti-sperm IgG_1 and IgG_4 levels. The IgG_1:IgG_4 ratio in anti-sperm antibodies is in agreement with the working hypothesis of Batard et al. (5) who proved that the prolonged exposure to immunogenic agents such as grass pollen allergens generates IgG_1 further shifting into IgG_4. Then, we speculate that the detected antigens might be the same since the distribution of tested antibodies follows the similar trend of IgG_1>IgG_2>IgG_3<<IgG_4. Based on barely detectable IgG_3 within the patient group, we assume that IgG_3 does not have any protective or inflammatory role in female immune infertility. Among the four IgG subclasses, $IgG_{1,3}$ activate complement cascade (10). Since patients do not suffer from the inflammatory symptoms, we suggest that complement cascade may not be activated by IgG_3 in the semen rejection. It has been specified (11) that the response to protein antigens is primarily mediated

by IgG_1 and IgG_3, whereas IgG_2 especially and IgG_4 are induced in response to polysaccharide antigens. Since seminal/sperm-specific IgG_1 and IgG_4 were inversely correlated, IgG_1 seemed to be bind to seminal antigens of a protein character and IgG_4 to sperm structures of an oligosaccharide nature. Whether or not serum IgG_4 is a blocking or sensitizing antibody in infertile females remains unexplained unlike in other studies dealing with other pathologies (6, 7). The absence of data following patients over time prevented us to better understand the role of IgG_4.

Concerning male autoimmunity, IgA antibodies were proved to be associated with a systematic factor in male immune infertility (12). The nondetectable levels of $IgA_{1,2}$ in 97% of female patient sera tested imply that either IgA_1 or IgA_2 do not correlate with systemic iso-immunization. However, we are able to affirm that only IgA_1 is not involved in the disease as it predominates in the sera (13) examined in our study. Immunoglobulin M contributes to opsonization and is involved in the primary response to antigen exposure (14). Since the patient sera are not collected nor tested immediately after the sensitization, the nondetectable level reflects the potential secondary immune response where IgM does not play a significant role. However, we cannot refute that IgM is not involved either in primary or secondary antibody response to iso-immunization. Not a single patient was diagnosed with semen hypersensitivity which is in agreement with an absence of anti-seminal/sperm IgE, usually related to the pathophysiology of allergy (4).

The described distribution of seminal/sperm-specific $IgG_{1,2,3,4}$, $IgA_{1,2}$, IgM, IgE indicates that immunoglobulins E, M, $A_{1,2}$, G_3 are not involved in pathophysiological female sensitization. IgG_4, as the minor isotype of IgGs, appears to be mainly produced and linked to IgG_1 in the 1:2 ratio in seminal and 2:1 in sperm specific antibodies. Seminal antigen fraction seems to be of a protein nature; on a contrary, sperm antigens are rather of a polysaccharide character. Early determination of serum seminal/sperm-specific immunoglobulin G subclasses

might make patient profiling more precise and complete the information for diagnosis. Anti-seminal/sperm IgG_1 and IgG_4 could be of interest for further therapy targets.

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Conflict of interest statement

We have no conflicts of interest to declare in relation to this article.

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Figure legends

Figure 1. Quantified female serum anti-seminal/sperm $IgG_{1,2,3,4}$. IF: sera of infertile patients (30), F: control sera of fertile women (10), ND: nondetectable level, ○: anti-seminal antibodies, ◺: anti-sperm antibodies, bars: arithmetic means.

Figure 2. Quantified female serum anti-seminal/sperm $IgA_{1,2}$, IgM, IgE. IF: sera of infertile patients (30), F: control sera of fertile women (10), ND: nondetectable level, ○: anti-seminal antibodies, ◺: anti-sperm antibodies.

Figure 1
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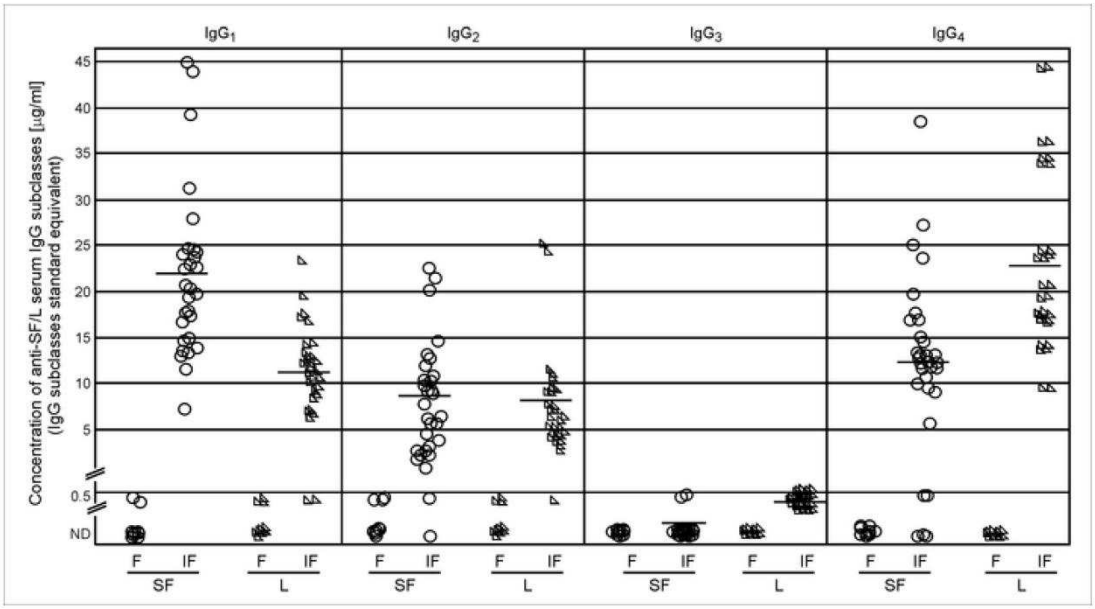

Figure 2
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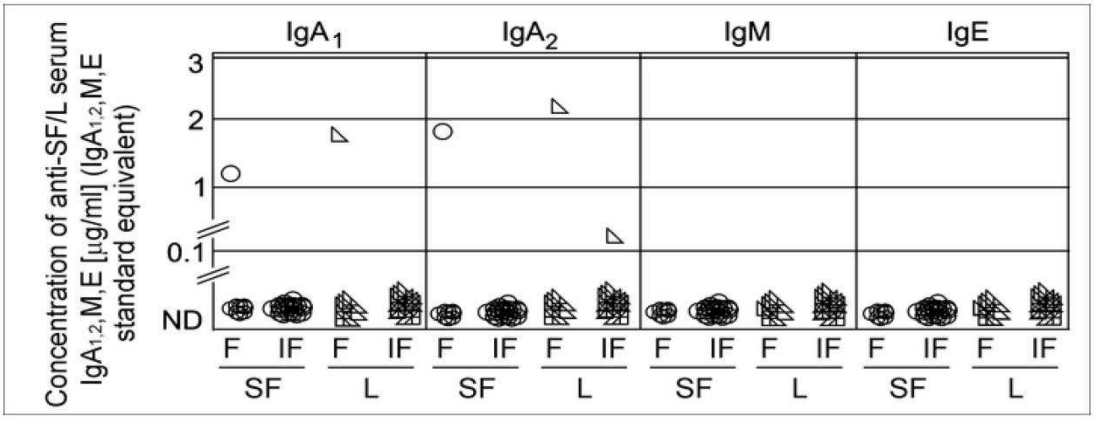

5.1.8 Pre-eclampsia: a life-threatening pregnancy syndrome

Pre-eclampsia is a serious pathological state affecting 5-10% of pregnant women. Its etiology is, so far, unknown. However, it is diagnosed in the second half of pregnancy, in particular after the 20th week. This life-threatening syndrome is the fatal culprit of perinatal morbidity/mortality, abortions or premature births.

The origin of pre-eclampsia is explained by utero-placental ischemia, immune disorders as systemic inflammatory response, genetic predispositions, the malfunction structures of protein/lipoprotein disorders as well as by the hypothesis concerning oxygen radicals. The symptoms mostly correspond to the changes of blood vessels and kidneys. An inadequate trophoblast invasion leads to a reduced placental perfusion at the end of the first trimester, which is followed by endothelial dysfunction and clinical manifestation by the end of the second/during the third trimester.

The monitoring of pre-eclampsia is, nowadays, a key point for women suffering from this disorder. The pre-eclampsia markers include blood pressure, urinal protein amount, insufficient cerebral perfusion, nausea, vomiting, epigastric pain, distorted vision. The biochemical screening includes the monitoring of malondialdehyde levels, uric acid, fibronectin, human chorionic gonadotropin, increase in hemoglobin and hematocrit and the enzyme activity of selected enzymes as the markers of oxidative stress. The complex monitoring and laboratory screening of risk pregnancies could contribute to a better understanding and be helpful for the early diagnosis of pre-eclampsia.

Pre-eclampsia: a Life-threatening Pregnancy Syndrom

Running title: Factors in pre-eclampsia

Abstract

Pre-eclampsia is a serious pathological state affecting 5-10 % of pregnant women. Currently, it is diagnosed in the second half of pregnancy, particularly after the 20th week. Symptoms mostly correspond to the changes of blood vessels and kidneys. The severity of pre-eclampsia is proportional to symptomatic manifestations, thus the more symptoms present, the higher the risk is of pre-eclampsia developing. Although there are a series of studies dealing with pre-eclampsia pathology, the complete etiology is still unknown. In this review paper, several theories are presented and discussed.

Keywords

placenta, pre-eclampsia, pregnancy, oxidative stress

INTRODUCTION

Pre-eclampsia (PE) is a disorder affecting both the mother and the unborn child. It occurs only during pregnancy and may persist until the postpartum period. Depending on the nature of the disease, it may be distinguished into the mild and severe form. In some cases, early and late type of PE is indicated and they are discussed as separate entities. Mild PE is defined by hypertension (≥140/90 mm Hg) on two occasions (at least 6 h apart) without the evidence of end-organ damage, and the presence of 300 mg of proteins in a 24 h collected urine sample but no other symptoms affecting either mother or baby. Severe PE is characterized by blood pressure over 160/110 mm Hg and may lead to liver/renal failure, disseminated intravascular coagulopathy and central nervous system abnormalities. If preeclampsia-associated seizures are experienced, the disorder has developed into eclampsia. Premature births, induced by PE, are the major cause of maternal, neonatal morbidity and the increased mortality of newborns. The incidence rate varies from 5 to 8% of all pregnancies in developed European countries (Arbogast and Taylor 1996, Pre-eclampsia foundation 2013). Eclampsia and HELLP syndrome (hemolysis, elevated liver enzymes, low platelet count) may be an outcome of PE. This complex pathologic condition is most often characterized by the rise in blood pressure, which can result in seizures, cerebral stroke, multiple organ failure and death of the mother and/or child. Swelling, sudden weight gain, headache and visual disturbance are less frequent symptoms. PE occurs after the 20th week of pregnancy and is therefore not diagnosed until the second half of pregnancy based on emerging clinical symptoms. In rare cases, it may occur before the 20th week. Although the etiology is unclear, several PE risk factors are known. The most common manifestations include hypertension, edema and proteinuria. However, the higher permeability of placenta is also associated with PE. The higher permeability of oxygen reactive forms through the placenta may lead to the development of oxidative stress (OS) (Arnostova et al. 2007, Escudero and Sobrevia 2008, Pre-eclampsia foundation 2013). Several theories have been suggested to explain the origin of PE: the hypothesis of uteroplacental ischemia (changes in the biology of placenta), immune disorders (systemic inflammatory response), genetic predispositions, malfunction structures of protein/lipoprotein disorders, and the hypothesis of oxygen radicals (Anthony and Johanson 1996). The hypothesis of uteroplacental ischemia is based on the expression of cytotrophoblastic adhesive molecules. It is suggested that the reduced penetration of substances through placenta leads to fetal damage and the release of toxic substances with some regulatory factors. A common pathologic finding is a placenta with multiple surface infarcts (Mechurova 2002). The current theory concerning the pathophysiology of preeclampsia discusses a lack of trophoblast invasion into the wall of the spiral arteries. It is linked to the hypothesis of systemic inflammatory response supported by the fact that the fetoplacental unit contains paternal antigens that are capable of slowing down the trophoblast invasion. The following consequence consists of the hepatic expression of human leucocyte antigen G (HLA-G) that may inhibit trophoblast invasion in uterine smooth muscle. Subsequently, the activated leukocytes release an elastase and attack an endothelium (Mechurova 2002, Stara 2012). Genetic hypothesis is based on the genetic predisposition of daughters whose mothers have a verifiable PE history. In this case, the risk of disease increases up to 22%, for sisters up to 39% (Mechurova 2002). The PE patients have a higher concentration of triglyceride and the lipoproteins of very low density (VLDL) in the plasma. VLDL hypothesis derives from the endothelial lining of the vascular damage effect of VLDL. The most discussed theory is that of OS. The balance between the oxygen radicals and antioxidants is disrupted during PE development. Natural antioxidant ability is unfortunately reduced, which may lead to placental OS. This hypothesis is supported by the fact that the placenta in PE patients exhibits discontinuous perfusion due to the reduced pathological trophoblast invasion into the maternal vascular system. The above mentioned OS is considered as a significant consequence causing the reduced placental permeability (Borzychowski et al. 2006).

PATHOLOGY OF PRE-ECLAMPSIA

Nowadays, the PE syndrome is considered as a two-stage disease. The first stage is defined by the reduced placental permeability (specific proteins, placental 'debris') associated with abnormal implantation and fetal placental release into the maternal circulation. The malfunction of placenta may, in some cases, induce hypertension, renal impairment, proteinuria, the damage of other organs and the HELLP syndrome. The second stage is the maternal reaction to this condition. It is characterized by

inflammation and the endothelial dysfunction of parental cells (Redman and Sargent 2000). One of the fundamental pathophysiological changes is the so-called firm connection of fetal and maternal placenta. Pathological examination of placentas has documented the placental infarction, narrowing of arteries and blood vessels, fibrin deposition and venous thrombosis. Placental ischemia induces hypertension, proteinuria, is also associated with the deposition of fibrous material in the glomerular tubules - called glomerular endoteliosis (Baumwell and Karumanchi 2007). The promising recent research deals with proteins that are produced by placenta and inhibit angiogenesis. Vascular endothelial growth factor (VEGF) and placental growth factor (PIGF) are pro-angiogenic factors that are involved in trophoblast proliferation (Grill et al. 2009). The growth factors and their receptors originate in the placenta. Placental cells also secrete the soluble isoform receptor (sFlt-1), which is generated through an alternative mRNA splicing. Soluble Flt-1 isoform has a role as an anti-angiogenic factor. It neutralizes the effect of PIGF and VEFG. The over-expression of sFlt-1 is associated with placental ischemia and hypoxia (Clark et al. 1998). Cooper et al. (1996) reported that VEGF and PIGF concentration are lower in the second trimester in women with late PE in comparison with physiological pregnancies. This fact is most significant in patients with severe PE, where VEGF and PIGF amount is reduced in comparison with women with mild PE (St-Jacques et al. 1994). Maternal immune disorders play an important role in the pathophysiology of PE. Trophoblasts contain a wide range of immunogenic agents, such as HLA-G. This glycoprotein is responsible for the low trophoblast immunogenicity along with the high resistance to the maternal immune reaction. The presence of trophoblast with an insufficient HLA-G expression can activate the incompatible decidual immune cells in the uterine cavity (Matthiesen et al. 2005). The placental vascular wall is an integrated organ, composed of endothelial cells, smooth muscles and fibroblasts. Endothelial cells contain the surface receptors for thrombin, clotting factors, angiotensin II, and many more. Cytokines (e.g. interferon-gamma) and various types of reactive oxygen species mediate an endothelial dysfunction. Clinical symptoms usually appear after the 20th week of pregnancy. Endothelial dysfunction may result in the increased permeability of endothelial cells, activation of platelets and the coagulation cascade, shifting the balance of vasoactive mediators and thus favoring vasoconstriction and the development of OS (Davidge 1998).

CLINICAL MANIFESTATION OF PRE-ECLAMPSIA

The clinical manifestation varies since PE is associated with several risk factors (obesity, molar pregnancy, hypercoagulation, formation of clots, diabetes mellitus). It is reported that the prevalence of PE increases up to 66% if there is already an existing chronic kidney disease (Baumwell and Karumanchi 2007). It was found that male seminal plasma antigens, expressed on trophoblasts, reduce the risk of PE. The major clinical manifestation includes hypertension, proteinuria, edema, hyperuricemia and HELLP syndrome. Blood pressure is a function of peripheral vascular resistance and cardiac output. According to the World Health Organization (WHO), hypertension is considered as a blood pressure of 140/90 mmHg or higher at two separate measurements within 6 h (Hajek 1999). Proteinuria means the increased amount of total proteins in the urine. Glomerular membrane damage and reduced protein absorption in the back tubules cause an increase of up to 300 mg in 24 h (Brown et al. 2001). Swelling is caused by the increased protein loss or the inadequate intake of fats and sugars. In the advanced stage of pregnancy, it may be caused by obstruction due to enlarged veins in the uterus (Felfernig-Boehm et al. 2000). Hyperuricemia is defined as the increased level of uric acid in the blood. If the level is higher than 320 μmol/l, it becomes one of the primary PE indicators. The most significant cause of hyperuricemia is the insufficient feedback resorption of uric acid in renal tubules (Cai et al. 2009). HELLP syndrome differs from PE by hemolysis, the increased activity of liver enzymes and decreased level of platelets. HELLP is diagnosed in 20% of severe PE with the so-called thrombotic microangiopathy, which is defined as a syndrome involving the occurrence of clots in the veins, acute hemolytic anemia and thrombocytopenia (Felfernig-Boehm et al. 2000, Stara 2012).

CLINICAL SCREENING FOR PRE-ECLAMPSIA

The PE markers include increased blood pressure, elevated or decreased protein in the urine, insufficient cerebral perfusion (headache, dizziness, tremor), nausea, vomiting, epigastric pain, distorted/double vision. Nowadays, the laboratory screening includes the monitoring of malondialdehyde levels, uric acid, fibronectin, human chorionic gonadotropin (hCG), increase in

hemoglobin and hematocrit and the enzyme activity of selected enzymes (aspartate aminotransferase, alanine aminotransferase and lactate dehydrogenase). Malondialdehyde, which is a lipid peroxidation product, can be used as a new PE marker (Hajek 1999). Purine level is dependent on the influence of oxygen radicals. Although the released fibronectin from damaged vascular endothelial cells could theoretically contribute to PE prediction, this marker may easily provide false-positive results (Friedman and Schiff 1995).

ASSOCIATION OF OXIDATIVE STRESS AND PRE-ECLAMPSIA

Oxidative stress is an imbalance between the free radicals and reactive oxygen species and their elimination by antioxidants. OS represents a disproportion between the oxidants and the antioxidants. This misbalance leads to cell damage that may influence the entire body (Durackova 2010). Reactive oxygen species (ROS) are described as the molecules of independent existence. Free radicals, with their unpaired electron, exhibit a high chemical reactivity towards other organic molecules. The main source of free radicals is the mitochondrial respiratory chain. It is known that 1-4% of oxygen is converted to superoxide and hydrogen peroxide during oxidative phosphorylation. Another source of free radicals is biotransformation process in the endoplasmic reticulum. The exogenous factors, which significantly increase the production of free radicals, are: UV radiation, smoking, industrial pollution, pesticides, some pharmaceuticals and many organic solvents. ROS can cause intracellular reactions with lipids, DNA and proteins. These interactions can lead to the loss of cell permeability/genome stability and also other cellular functions (Cadenas and Sies 1998). PE causes hypoperfusion, placental ischemia and hypoxia and also reduces placental permeability so that reactive oxygen compounds are intensively produced. A reduced placenta can release various mediators such as the hCG hormone, cytokines, reactive oxygen and nitrogen species (nitrous oxide, hydrogen peroxide, superoxide anion). These mediators are detected when endothelial dysfunction occurs (Llurba et al. 2001). The throughput of reactive oxygen increases along PE developing during pregnancy. This fact may lead to OS (Dennery 2010). The most important enzymatic oxidants are catalase, superoxide dismutase, glutathione-S-transferase, glutathione reductase and glutathione peroxidase (Kaur et al. 2008).

CONCLUSIONS

Albeit the vast advances in research and knowledge of PE during recent times, the basic research is unfortunately still lacking behind. This life-threatening syndrome is the fatal culprit of perinatal morbidity/mortality, abortions or premature births. It is suggested that an inadequate trophoblast invasion leads to a reduced placental perfusion at the end of the first trimester, which is followed by endothelial dysfunction and clinical manifestation by the end of the second/during the third trimester. Despite the therapeutical progress, the diagnosis is still very complicated. Currently, the diagnosis of PE is based on clinical symptoms that are noticeable at the end of the second trimester. Therefore, great attention is paid on the biochemical actions associated with the development of PE. The monitoring of biochemical markers could contribute to a better understanding and be helpful for early diagnosis. Whereas the main causes of PE are still unknown, the current research of biochemical processes associated with PE requires much more attention and financial support.

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6 FUTURE ASPECTS

6.1 Design of a miniaturized diagnostic tool

The aim of designing a diagnostic tool consists of the differentiation between sperm and the seminal sensitization of infertile women in order to make medical diagnoses more comprehensive, and to hasten the therapeutic intervention. Such expertise has to be easily and rapidly performed to provide an answer, e.g during the consultation with a reproductive immunologist. These requirements may be fulfilled by, for instance, lateral flow immunoassay-based device (LFIA, Fig. 12, 13) acting on the immunochemical detection of specific antibodies. LFIA is a widely used technology to detect pathogens (Karakus and Salih, 2013), toxins (Anfossi *et al.*, 2013), contaminants (Maiolini *et al.*, 2014) and is a reliable and important assistant for diagnostic purposes, such as pregnancy test (Posthuma-Trumpie *et al.*, 2009). However, to the best of our knowledge, our aim is the first report concerning the design of a colloidal nanometer-sized-particle-based test for the multi-analyte detection (seminal and/or sperm sensitivity of infertile women). In general, the LFIA-based test is simply and fast performed by medical or research specialists as well as any person without professional or specialized training. It also requires a very low amount of testing analyte, no reagent preparation and no specialized equipment, all of which contribute to a low-cost alternative to expensive experimental measurements (Kolosova *et al.*, 2007). The choice of a membrane-based format is in favor of a qualitative one-step test with visually interpreted results. Based on the knowledge, experience and results obtained during our experimental procedures, the cyanogen bromide (CNBr)-activated nitrocellulose (NC) membrane (Demeulemester *et al.*, 1987) seems to be the most appropriate surface layer. As the membrane is thin and fragile, it has to be attached to a solid support. The intended miniaturized diagnosis device would consist of two main parts to test the seminal as well as sperm sensitivity of female patients to immunodominant antigens. Each testing part would involve three testing wells, the first one to detect patient reaction, the second one as a positive control acting as a reference for the intensity of reaction related to the patient tested and to allow quantitative evaluation, and the third one as a control zone acting as a reference to show whether the reaction works properly or not. Briefly, the patient profiling procedure would consist of the application of a blood drop directly on the deposit place, into the input window (the first well). The CNBr-activated NC sheet serving as a capillary bed would easily transport the applied fluids. When soaked, blood led by a thin capillary would migrate to a

filter. Serum or plasma, depending on the presence or absence of coagulating compound on the filter itself, would migrate. While passing through an antibody mobile tracer, the analytes (present in serum or plasma) would bind to conjugate (IgG) coupled with nanoparticles. The antibody mobile tracer would contain purified immunoglobulins against immobilized seminal and sperm antigens, coupled with nanoparticles. The complexes of sample analytes and conjugates would migrate to the immobilized seminal and sperm antigen strips. If the complexes bind to the so-called strips, the strip-area changes color. Optical detection is based on gold nanoparticles. The migrating fluids then enter the wick, acting as a waste recipient. According to the positive control zone, it would be possible to compare the intensity of patient reaction, and according to the third window position, to confirm whether or not the reaction works properly. The LFIA set-up test may take a few minutes to be developed depending on the sensitivity (Posthuma-Trumpie *et al.*, 2009). The detection thresholds and cut-off levels have to be precisely determined. Attention has to be paid to the fact that the device has been designed based on the results obtained and corresponding to the human subject selected for this study. Eight male healthy normozoospermic individuals (the average age of the voluntary donors was 27) and female infertile patients diagnosed with repeated unsuccessful in vitro fertilization (the average age of the women was 35) were selected. As we assume that described and proposed protein markers related to female iso-immunization may have more than one epitope, the sandwich format is applicable. In this case, the optically observed response is supposed to be proportional to the amount of analytes in the applied sample.

6.2 Immuno-Intervention in female immune infertility

Therapeutic regimens have, most of the time, been developed to counter autoimmune defects. Female fertility disorders have been associated with a failure of natural tolerance to male semen, resulting in a cascade of immune reactions, the activation of the female immune system and sperm rejection. Treatments using intravenous immunoglobulins may act on the tolerance level of the female immune system. IVIg may modify inflammatory response, interfere with natural killer cell cytotoxicity, the proportion of peripheral blood cells, Th1:Th2 ratio and with Treg cells. All these facets may interact to achieve a successful pregnancy. However, the existing studies of extensive IVIg effect in patients with pregnancy failure have

not reached statistical significance. As immuno-intervention is only the theoretical aspect of this thesis, IVIg benefit is a subject of debate.

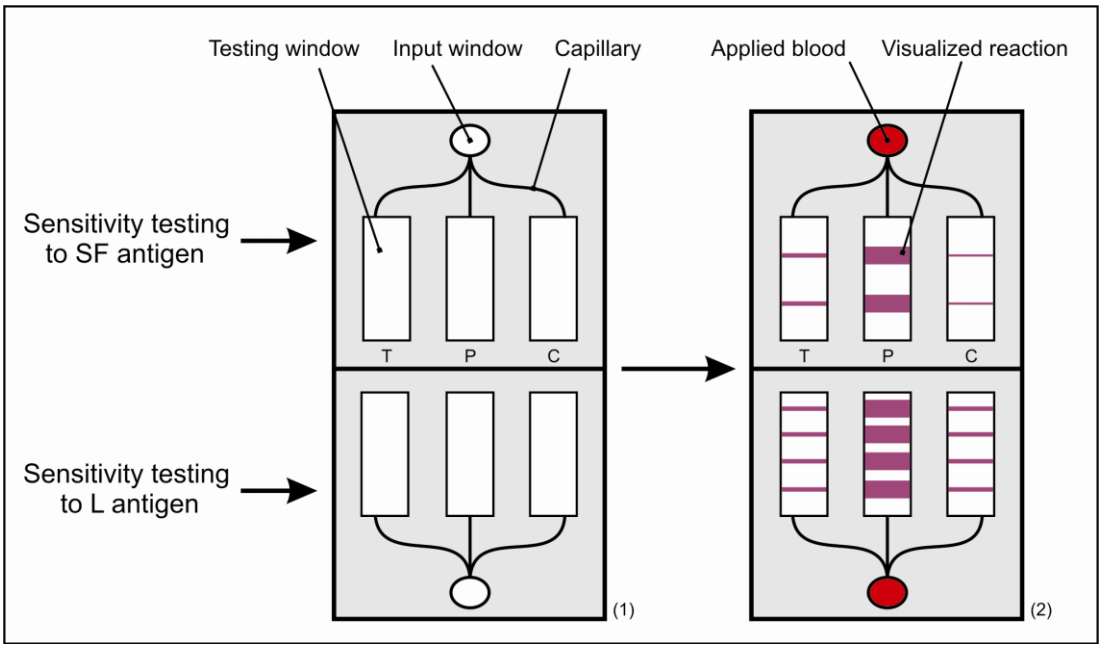

Fig. 12 External view of a colloidal gold-based lateral flow immunoassay for diagnostic purposes concerning female iso-immunization caused by seminal and/or sperm antigens. SF: seminal fluid, L: sperm lysate, T: testing zone, P: positive control zone, C: control zone of test function, (1): unused device, (2): device used to reveal the patient sensitivity to seminal and sperm antigens.

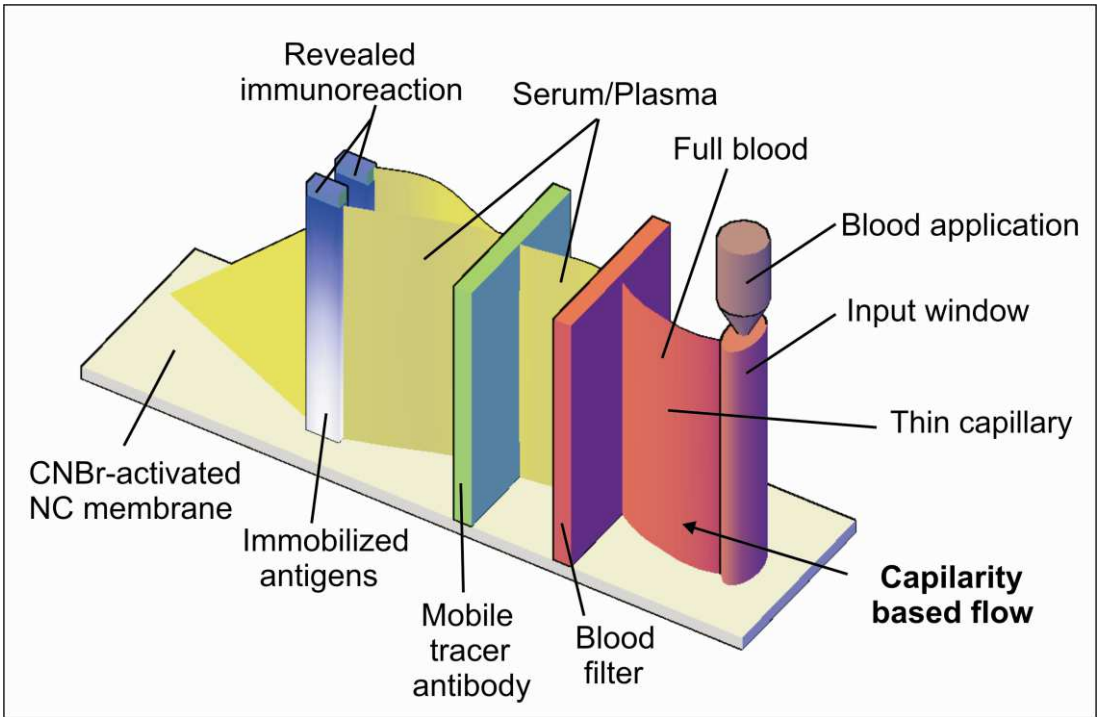

Fig. 13 Detailed view of internal composition of testing zone in a miniaturized diagnostic device. CNBr-activated: cyanogen bromide (CNBr)-activated, NC: nitrocellulose.

7 DISCUSSION

Immune infertility, in terms of reproductive failure, has become a serious and frequent problem involving a significant number of couples. Immunological aspects play an important role during many stages of reproduction.

7.1 Antibody recognition

Immunoglobulin diversity becomes apparent in certain stages of B-cell development. During the primary response to antigen exposure, IgM and IgD are co-produced. The process of IgM and IgD expression is entirely antigen dependent. A reaction associated with hypersensitivity and parasite infection provokes generation of IgE antibodies. Although IgE is produced mainly in allergic states and in response to parasite infections, it is the serum immunoglobulin of lowest concentration in healthy individuals. In addition, it has the shortest half-life (few days). Within the secondary immune response, the so-called class switch recombination is processed and IgG antibodies become prevalent. IgG is the predominant isotype present in body except mucosae, wherein IgA predominates. IgG also has the longest serum half-life (around 20 days) and exists in four subclasses that differ in specificity, function and concentration. The common distribution is as follows: IgG_1>IgG_2>IgG_3>IgG_4. Considering the totality of all the IgGs, IgG_1 comprises in approximately 60%, IgG_2 accounts for 25%, IgG_3 for 10% and IgG_4 for 5% (Svensson *et al.*, 2013). Only IgG_1 and IgG_3 can effectively activate complement, as the activation ability of IgG_2 is too low and IgG_4 completely fails to fix complement. In general, the distribution of serum antibodies follows the IgG>IgA>IgM rank order in healthy individuals regardless of gender and age (Schroeder and Cavacini, 2010; Tamayo *et al.*, 2012; Jefferis, 2012).

Changes in immunoglobulin isotype levels are related to the type of disease. Preponderance of certain isotype subclasses reflects ongoing immunological processes and is specific to certain pathologies. Increased concentration of IgEs is correlated with allergies and to responses to some parasites. In the case of specific immunotherapy, prolonged exposure to antigenic stimuli induces increase levels of specific IgG_4 in allergic patients. In some autoimmune disorders, for instance acute or chronic bullous pemphigoid, specific IgG_1 and IgG_4 were revealed as dominant. Patients suffering from this skin disease are diagnosed with a deficiency of IgG_3 (Dopp *et al.*, 2000). Other autoimmune pathologies, for instance

myasthenia gravis, are specific IgG_1 and IgG_3-mediated (Liu *et al.*, 2011). With regards to our results, the anti-seminal/sperm antibody distribution of infertile female patients follows the IgG_1>IgG_2>IgG_3 ratio (Brazdova *et al.*, 2013a; Brazdova *et al.*, submitted 2014b). Suprisingly, specific IgG_4 was detected as the apparent predominant subclass and appeared to be linked to specific IgG_1. The sort of relationship also exists does exist also between specific IgG_1 and IgG_2. Unlike other studies (Yamada *et al.*, 1989; Dopp *et al.*, 2000), our patients reflected the elevated levels of specific IgG_1, IgG_2 and IgG_4. The distribution did not vary within the patients selected. As other diseases are associated only with the increased levels of some IgG subclasses, e.g. cystic fibrosis and chronic Pseudomonas aeruginosa with specific IgG_2 and IgG_3, female immune infertility, and in particular the repeated IVF failure, may be related to autoimmune phenomenona and notably to IgG_1 and IgG_4-mediated reactions. IgG subclasses may thus indicate specific immune responses and represent potential targets of immuno-therapy.

7.2 Protein markers

Iso-immunization is related to immune infertility and is caused by semen protein/s. Identification of immune-dominant semen proteins or their entire repertoires is the key point to a better understanding of female sensitivity to male semen. Provoked antibody formation may be associated with individual proteins. Such markers are needed to provide information about the stage of disease in the prior to therapy. The goal of this study is to find clear and reliable markers of female iso-immunization, i.e. specific proteins to which the immune system reacts by inducing high levels of serum antibodies preventing the fertilization. The plan consisted of utilization of obtained results as reference points to design a diagnostic device. This device should, in a timely and quickly manner, distinguish between seminal fluid and sperm sensitivity of infertile female patients. Such markers have to be specific enough to avoid incorrect analysis through recognition by natural antibodies present in sera of fertile women.

In men, auto-immunity to sperm has already been reported to heat shock 70 kDa protein, heat shock related 70 kDa protein 2, disulfide isomerase ER60, inactive form of caspase-3, component 2 and ξ chain subunits of the proteasome and alpha-enolase. These proteins were pointed out as indicators of impaired sperm function and male infertility associated with ASA (Bohring *et al.*, 2001; Bohring and Krause, 2003; Domagala and Kurpisz, 2004). Different

protocols to process sperm disintegration provide different protein mixtures, thus different antigens could be determined. To avoid exclusion of membrane or cytoplasmic antigens, it was decided to follow the sample preparation by which most of epitopes are kept (Brazdova et al., 2011). Concerning iso-immunization, the female immune system recognizes a set of proteins that differs from those interacting with male antibodies as well as another set of proteins that are in common (Brazdova *et al.*, 2013b). Sperm heat shock 70 kDa protein, heat shock related 70 kDa protein 2 and alpha-enolase are recognized as auto- as well as iso-antigens. This puts them in a class of universal markers linked to antibody-related infertility. Alpha-enolase is, in addition, an IgE-binding sperm protein as previously reported (Lopez-Alemany *et al.*, 2003). This fact supports the previous hypothesis of universal markers. Another protein, disulfide isomerase, participates in the rearrangement of sperm proteins and in the composition of the final antigen exposure (Bronson, 2011). Type ER60 is recognized by male serum IgG auto-antibodies (Bohring *et al.*, 2001; Bohring and Krause, 2003) and type A3 by female serum IgG iso-antibodies (Brazdova *et al.*, 2013b). The identification of this enzyme proves its general significance in immune infertility.

Human seminal fluid is a concept often discussed in terms of prostate cancer and allergic reactions. In the background of these two distinct immune reactions, PSA plays a key role as corresponding marker. In addition, PSA and PAP are used to monitor the pathological spermiogram and the progression of prostate cancer (Jones, 1991; Weidinger *et al.*, 2008). PSA and PAP were shown in this study to contribute to female immune infertility. They may indicate the activated humoral immunity of infertile women as they were recognized as IgG-binding immuno-dominant proteins in the presented results. Some seminal components may complex with sperm proteins that enables the effective recognition by female immune system followed by antibody formation. As it has been shown (Brazdova *et al.*, 2013a), some sperm proteins are recognized by female serum antibodies only in complex with those of seminal fluid origin. ZAG has been shown to have this ability (Brazdova *et al.*, 2013a). Based on the obtained results, heat shock protein 70 kDa, heat shock related 70 kDa protein 2, disulfide isomerase, alpha-enolase, zinc alpha-2-glycoprotein, prostate-specific antigen and prostatic acid phosphatase should constitute a comprehensive list of markers that will improve the actual diagnoses.

7.3 Immunoassay to screen female semen sensitivity

An immunoassay is defined as a laboratory technique based on the principle of specific binding between an antigen and its homologous antibody in order to identify and quantify the specific antigens or antibodies in a sample. Immunoassays rely on the inherent ability of a selected antibody to bind to the specific structure of an antigen, and thus exploit the ability to form the immuno-complex (Yetisen *et al.*, 2013).

For decades, immunoassays have been ubiquitous in research laboratories and have progressed studies of biological systems by tracking different analytes. For over 40 years, these tests have been used for biomedical purposes to improve the state of diagnoses by making them more accurate. It has enhanced the possibility of earlier diagnoses and decreased the severity of illness by identifying and assessing the progression of disease. It strengthened confidence in the choice of therapeutic approach. Nowadays, immunoassay is a routine test. In the agro-food industry, immunoassays are used to detect contaminants. They also contribute to monitor specific molecules used during product processing in quality control. Many home pregnancy tests are actually the immunoassays detecting human chorionic gonadotropin as the pregnancy marker. Other examples of clinical immunoassays include tests that measure levels of insulin to assess hypoglycemia or prostate-specific antigen to detect prostate cancer (Butler *et al.*, 2001; Chatterjee *et al.*, 2008).

Exclusively for every test, it is necessary to consider the type of assay with an impact on either qualitative or semi-quantitative approach related to basic principles. In addition, characteristics such as the potential limitation and aspects such as membrane material, material of the sample and absorbent pad, type and size of the label, recognition element, position of the test line, optimization and the last but not the least the concurrency of automated formats must be considered. Furthermore, the type of immuno-pathologic reactions (type I-IV) related to the disease has to be considered as well (Posthuma-Trumpie *et al.*, 2009).

As infertility has been becoming a civilization disease, the progress of medical and research attempts is enormous. Immune infertility is commonly associated with antibody production against male semen. Women suffering from this fertility disorder have to often undergo a long-term treatment. To make the patient information more comprehensive and to provide a complete diagnosis, the miniaturized device has become of a research interest. Infertile female patient screening for the sensitivity to sperm and/or seminal antigens might make the therapy simplified. Several choices were taken into account: enzyme-linked immunosorbent assay, memory lymphocyte immuno-stimulation assay, lateral flow test,

magnetic immunoassay, radioimmunoassay, blotting analyses and microfluidic device. LFIA may be an appropriate test to be performed within a few minutes, even at bed site, requiring only basic equipment. The results can be given in qualitative and semi-quantitative forms. This type of test is intended for primary individual screening to evaluate the presence or absence of female serum antibodies against immuno-dominant semen proteins, seminal as well as sperm. On the other hand, it could be used as a reference to monitor treatment progress of ongoing antibody generation as this test would provide semi-quantitative results as well. In addition, only limited sample pre-treatment is needed.

7.4 Immuno-Intervention strategies

The development of drugs that would intervene in the immune system is, at present, of scientific interest. Plenty of specific and unspecific immuno-stimulatory strategies have been applied. Numerous approaches agree on whole cell vaccines, peptide vaccines and dendritic cell-based immunotherapy, all of which proved their safety based on quality control and clinical trials. These mandatory issues allow dividing the patients into sub-groups depending on their immune response. The question of T- and B-cell antigen assessment is a novel subject for clinical immunotherapy (Geissler and Weth, 2002).

The aim of vaccination, either by a whole cell or peptide, is to induce protective immunity. The crucial step consists of the identification of the antigen inducing a common reaction in all individuals, then in the prediction of immune response and protective mechanism. Antigen has to be chosen to fulfil the requirement to bind to at least one allele of MHC in every human individual. The serious risk is related to T- and B-cell antigen selection that may elicit an inappropriate immune response in some subjects and fail to induce protective immune response in all subjects. Therapeutic intervention may also regulate signaling pathways concerning T- and B-cell expansion. Furthermore, regulation can operate on the ligand-receptor binding to inhibit T-cell response or it can act on the signal transduction (Lanzavecchia, 1993). In general, T-cells are the targets of therapeutic immuno-intervention. Regarding high levels of antibodies preventing early steps of fertilization, this therapeutic immuno-approach might be potentially subjected to immuno-contraception research. However, the precise effects related to immunological mediator, risks and efficacy have to be taken into account in the design of such a vaccine.

Therapeutic approaches may depend on the polyvalent character of natural antibodies. Natural antibodies are effective molecules of the immune system. They serve as the so-called first line barier to the infection. The effect of intravenous immunoglobulins is associated with natural antibodies. IVIg is a commercially produced mixture of blood immunoglobulins obtained from at least 1000 healthy donors. IgG predominates in IVIg. IVIg is suggested to display the entire antibody repertoire, notably the panorama of specificities. Despite the fact that monomeric immunoglobulins predominate, dimers are present as well. The mechanisms of action are plenty. It is mainly used as a treatment in three major disease categories, (1) primary and secondary immune deficiencies, (2) autoimmune diseases and (3) acute infections. The efficiency of the therapy is controversial as there is evidence of side effects (Nimmerjahn and Ravetch, 2005; Seite *et al.*, 2008; Kaveri, 2012). IVIg is also used as a treatment for unexplained recurring miscarriages. Regarding immune infertility, women having local immune reaction might not significantly benefit from IVIg as (1) IVIg is given as an infusion made primarily of IgG antibodies mainly and the immunity of the genital tract is associated with IgA antibodies, thus (2) the beneficial effect is related to IVIg biological activity and lifetime of at least 21 days (Yamada *et al.*, 2012). In this case, continuous iso-immunization would maintain the production of specific anti-seminal and anti-sperm antibodies. On the other hand, IVIg may induce a starting effect in cellular processes resulting in the normalization of anti-seminal as well as anti-sperm pathologic reaction. This therapeutic immuno-approach may act on idiotype regulatory network and also benefits from Treg immuno-suppressive activity or other still unknown immunoregulatory circuits, IVIg could be a valuable immuno-tool to restore physiological, non-pathologic, female reaction to male semen.

Regarding female IgE-associated reaction to male semen, IVIg might act as blocking antibodies toward generated IgE. However, anaphylactic shock provoked by seminal fluid could become severe as one of the potential IVIg side effects. Given that the affected women would be in addition IgA deficient patients who can, by definition, produce IgG antibodies, these patients would more likely produce IgG against the IVIg (Orbach *et al.*, 2005; Katz *et al.*, 2007). Nevertheless, it has not been shown, so far, that IgE-mediated allergic reaction of women to seminal fluid is related to infertility. In this case, the administration of IVIg would not be of effective value.

On the other hand, as this work is focused on the female patient diagnosed with repeated IVF failure, IVIg could be a useful treatment in some cases of women undergoing repeated IVF loss. IVF success depends on endometrial thickness, embryo quality and number

implanted, maternal age and, of course, the reactivity of maternal immune system. Most of the time, IVIg is applied in combination with Humira as a TNF inhibiting anti-inflammatory drug, heparin and aspirin (Coulam and Acacio, 2012; Li *et al.*, 2013). Studies that empirically introduced IVIg differ in duration, up to 7, 20, 28 weeks or even during the entire pregnancy. The precise mechanism of IVIg effect has not been clarified in the selected female patients, yet. IVIg therapy may thus depend on a synergic effect with the immuno-modulatory components of individual patients. This immuno-intervention may also play a role in the neutralization of pathologic antibodies and circulating auto-antibodies by anti-idiotypic network (Haller *et al.*, 2008; Jerzak *et al.*, 2010; Coulam and Acacio, 2012). The positive effect is not negligible. Unfortunately, the various ages of patients followed in numerous studies, diversity of statistical tests to evaluate patient sub-groups and various durations of IVIg administration prevent strong conclusion of the beneficial effect of IVIg in patient with previous IVF failure. Promising results and clear evidence remain to be confirmed as it has already been done in the case of autoimmune diseases. By all odds, IVIg immuno-intervention has been definitely highlightedfor its positive aspect.

8 CONCLUSION

1. The submitted work deals with female immune infertility with respect to iso-immunization and the levels of anti-seminal and anti-sperm antibodies from sera of women with fertility disorders. For this purpose, ELISA, one- and two-dimensional SDS-PAGE followed by mass spectrometry were chosen to determine the immunological properties of sperm and seminal plasma antigens involved in such pathologies.

2. The sera of infertile female patients were donated in cooperation with the Counseling and Laboratory for Reproductive Immunology - Genetics Pilsen, Czech Republic given the criteria of the positive Friberg spermagglution test and indirect mixed antiglobulin reaction test. Sera were obtained from 30 women with a fertility disorder (patients with repeated in vitro fertilization failure, aged 29-38) and from 10 fertile women (control group, aged 28-37) with proven fertility (1-2 healthy children). Normozoospermic semen samples were obtained from 8 volontary, healthy donors (aged 25-30).

3. The design of the protocol to prepare a protein sample was based on our studies published in this field. These methods used were as mild as possible in order to keep a maximum of epitopes, both of seminal and sperm origin, in their native conformations. To enrich protein sample and to increase the amount of potential antigens, individually prepared samples were pooled (Brazdova *et al.*, 2011).

4. Quantification of seminal- and sperm-specific antibodies present in the sera of infertile women was performed by ELISA. The results were compared to a control group of fertile women with poorly detectable serum seminal- and sperm-specific antibodies.

5. Thereafter, it was shown that the distribution of serum specific anti-seminal/sperm immunoglobulin G corresponds to the following trend: $IgG_1 > IgG_2 > IgG_3$ including high levels of IgG_4, all of which were observed in all 30 patient sera while the levels in sera of fertile women were barely detectable. Predominantly detected fractions were anti-seminal/sperm IgG_1 and IgG_4. Surprisingly, the levels of anti-seminal and anti-sperm

antibodies differed in IgG_2 fractions. In addition, IgG_3 antibodies were nearly negative for both antigen fractions, seminal and sperm (Brazdova *et al.*, submitted 2014b).

6. The output of the presented thesis might be of medical interest. Early determination of specific antibodies, and specifically those of anti-seminal and/or anti-sperm character, would provide detailed information related to the pathology of infertile women.

7. One-dimensional seminal and sperm protein mapping was run simultaneously by SDS-PAGE and IEF, both of which were followed by immunoblotting. IgG and IgA recognized seminal as well as sperm antigens. However, IgG and IgA specificity differs. IgA-binding seminal proteins are predominantly of low molecular mass and pI ranging from acidic to neutral while IgG-binding seminal proteins are of high molecular mass and pI neutral. On the other hand, IgA-binding sperm proteins are of high molecular mass and of pI ranging from neutral to basic. IgG-binding sperm proteins are a heterogeneous group with molecular mass ranging from 60 to 180 kDa and pI neutral (Brazdova *et al.*, 2012a, b).

8. The following essential step consisted of two-dimensional protein mapping. Immune infertility is known to be associated with sperm heat shock proteins. The known group of iso-antigens was extended by heat shock protein 70 1A/1B and heat shock cognate protein 71 kDa, which were firstly reported as related to female iso-immunization (Brazdova *et al.*, 2013b). In addition, one protein, alpha-enolase, was detected as auto- as well as iso-antigen (Domagala and Kurpisz, 2004; Brazdova *et al.*, 2013b).

9. Strikingly, seminal proteins play a notable role in female immune infertility. So far, seminal fluid has been associated with the IgE-mediated semen hypersensitivity. Such an immune response has never been found in the presented work. Based on two-dimensional characterization followed by mass spectrometry identification, a prostate-specific antigen, prostatic acid phosphatase and zinc finger protein 778, were determined as immunodominant among IgG-binding seminal proteins recognized by the sera of infertile women (Brazdova *et al.*, 2013a).

10. Three-dimensional evaluation (integrated density) of two-dimensinal electrophoresis and immunoblotting confirmed the above mentioned proteins to be immunodominant and associated with iso-immunization (Brazdova *et al.*, 2013a).

11. Potential innovation of presented results consists of their power to be used to conduct the specific immuno assay. Morover, wider applications of such immuno assay would be highly valuable to resource/equipment-poor consulting rooms. Lateral flow immunoassay seems to be the best choice used as a point of care test for primary screening as well as follow-up.

12. Immunotherapy inherently belongs to infertility treatment. The non-invasive approach afforded by IVIg may be a valuable treatment of female infertility. IVIg has already been widely used therapeutic intervention in immune-mediated disorders as it acts on various immune components. Immuno-modulating therapy, of course, requires the thorough studies including clinical trials.

9 PERSPECTIVES

To make the patient profiling more precise, it would be necessary to focus on (1) autoimmune phenomena with regards to auto-antibodies and idiotypic regulating networks, (2) the potential relationship between the pathophysiology of repeated IVF failure and high levels of IgG_4 potentially linked to "short-life" IgE, (3) blocking antibodies towards IgA, IgM, IgE and the role of cross-reacting antibodies, (4) the immuno-modulatory effect of IVIg for treatment, and (5) better understanding of local immunity.

Auto-antibodies are immunoglobulins naturally present in the human body and react with at least one self-antigen. The role of autoantibodies in immune regulation is not negligible. Potentially, they may act on the peripheral immuno-suppressive level. It is known that auto-antibodies are produced in IgM and IgA as well as the IgG isotype. The class switch causes μ-chains turning into γ-chains, which results in IgG predominance in the serum. As of yet, the detailed mechanism remains unclear. This type of antibody is characterized by low and broad affinity, high avidity and connectivity, being the first line defense against infection, clearance of aging cells, and anti-tumoral and anti-inflammatory control (Lacroix-Desmazes *et al.*, 1998). All of these aspects are necessary to be considered in auto- as well as iso-immune reaction of women to male semen samples. The study of B- and T-cells in infertile women within the selected pathology would provide complex information concerning how to exert immuno-regulation evoked by IVIg. In addition, the characterization of the idiotypic network in infertile patients in comparison with fertile individuals would contribute to the research dealing with blocking antibodies towards IgA, IgM, IgE. Given that the anti-inflammatory and blocking potential of IgG_4 (Aalberse and Schuurman, 2002; Guma and Firestein, 2012), it would be possible to specify its connectivity to IgE with respect to a local allergic reaction, later observed without obvious symptoms and complications. Cervical mucus samples subjected to more detailed analyses might be of potential interest to immuno-vaccines with regards to the presence of anti-seminal/sperm antibodies.

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11 LIST OF FIGURES

12 LIST OF TABLES

13 LIST OF ABBREVIATIONS

APA	anti-phospholipid antibodies
APC	antigen-presenting cells
APS	anti-phospholipid syndrome
AOA	anti-oocyte antibodies
AR	acrosome reaction
ART	assisted reproductive technologies
ASA	anti-sperm antibodies
ATP	adenosine-5'-triphosphate
CD	cluster of differentiation
CNBr	cyanogen bromide
D	derivates
DAG	diacylglycerol
DC	dendritic cells
E2	estradiol
ELISA	enzyme linked immuno sorbent assay
FA	fatty acids
FL	fusiogenic lipids
FPP	fertilization promoting peptide
FSH	follicle-stimulating hormone
GIFT	gamete intra-fallopian transfer
GnRH	gonadotropin releasing hormone
GDF 15	growth/differentiation factor 15
hCG	human chorionic gonadotropin
HELLP	hemolysis, elevated liver enzymes, low platelet count syndrome
HLA-G	human leukocyte antigen G
HSPA	human seminal plasma allergy
IgG, A, M, E	immunoglobulin G, A, M, E
IgG_{1-4}	subclasses 1-4 of immunoglobulin G
$IgA_{1,2}$	subclasses 1, 2 of immunoglobulin A
IL-1β	interleukin 1, component β
IL-1Ra	interleukin 1 receptor agonist

IL-8	interleukin 8
IP3	inositol-triphosphate
IUI	intra-uterine insemination
IVF	in vitro fertilization
LFIA	lateral flow immuno assay
LH	luteinizing hormone
MHC	major histocompatibility complex
NC	nitrocellulose
NK cell	natural killer cell
PAP	prostatic acid phosphatase
PCOS	polycystic ovary syndrome
PD	protein derivates
PE	pre-eclampsia
PGE	prostaglandin E
PIP	prolactin-inducible protein
PIP2	phosphatidylinositol 4,5-bisphoshate
PKA	protein kinase A
PLC	phospholipase C
PLCγ	phospholipase C γ
PLA	phospholipase A
PRL	prolactin
PSA	prostate-specific antigen
PSP-94	prostate-specific protein-94
PTP	protein tyrosine phosphorylation
R3	IP3 binding receptor
S-IgA	secretory IgA
SF	seminal fluid
SLE	systemic lupus erythematosus
TGFβ	transforming growth factor beta
TGFβI	transforming growth factor beta I
Th	T helper cells
THS	thyreotropin
TKR	tyrosine kinase receptor
TKR R2	tyrosine kinase receptor R2

TNFα	tumor necrosis factor
Treg	T regulatory cells
T3	triiodothyronine
T4	thyroxine
WHO	World Health Organization
ZAG	zinc alpha-2-glycoprotein
ZIFT	zygote intra-fallopian transfer
ZP	zona pellucida
ZP1-3	three glycoprotein layers of zona pellucida

14 ANNEXES

14.1 Publications not related to the topic of female immune infertility

Machova I., **Brazdova A.**, Fusek M., Zidkova J. (2012): Tumor markers and their use in clinical practice. *Chemicke Listy* **106**, 16-19.

Shahali Y., **Brazdova A.**, Calleja M., Charpin D., Senechal H., Poncet P. (2013): Indoor long-term persistence of cypress pollen allergenic potency: a 10-month study. *Annals of Allergy, Asthma & Immunology* **111**, 428-430.

Shahali Y., Nicaise P., **Brazdova A.**, Charpin D., Scala E., Mari A., Sutra J.P, Chollet-Martin S., Senechal H., Poncet P. (2014): Complementarity between microarray and immunoblot for the comparative evaluation of IgE repertoire of French and Italian cypress pollen allergic patients. *Folia Biologica-Prague*, submitted.

14.1.1 Tumor markers and their use in clinical practice

Tumor markers are quoted as cancer or oncological markers as well. They are substances produced either by tumor or normal cells in a response to the development of cancer. Since their level in peripheral circulation is related to the severity of disease, these markers are used for the detection, diagnosis, prognosis, progression and control of treatment. Given that there is no absolutely specific or universal cancer marker, a set of different markers for different cancer types are used in clinical practice. A problematic feature of using these as diagnostic tools is their false positive results in both physiological as well as non-cancer conditions.

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NÁDOROVÉ MARKERY A JEJICH VYUŽITÍ V KLINICKÉ PRAXI

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Klíčová slova: nádorové markery, prostatický specifický antigen, tkáňový polypeptidový antigen, α-fetoprotein

Obsah

1. Úvod

Rakovina je definována jako soubor onemocnění vycházející z poruch genomu, např. mutace, delece, amplifikace nebo nedostatečné reparace genových defektů. Tyto poruchy vedou k poruchám regulace růstu a množení poškozených buněk. V současnosti nejsou dostupné léčebné postupy, které by tyto poruchy na úrovni DNA cíleně opravovaly. Časně zachycená nádorová onemocnění mohou být úspěšně léčitelná. Důraz je kladen především na včasnou diagnostiku onkologických onemocnění a léčbu s minimálními nežádoucími účinky a náklady. Onkologická onemocnění jsou po nemocech srdce a cév nejčastější příčinou úmrtí v ČR[1]. Statistiky ukazují zvyšující se výskyt nádorových onemocnění. Léčba pacienta s nádorovým onemocněním zahrnuje často kombinaci několika postupů (léčba chirurgická, chemoterapeutická, biologická, radioterapeutická, popř. hormonální terapie, imunoterapie, hypertermie, kryoterapie, fotodynamická terapie)[2]. Stanovení hladiny nádorových markerů je nedílnou součástí vyšetřovacích metod pro stanovení diagnózy, prognózy a kontroly průběhu léčby.

Nádorovým markerem rozumíme molekuly převážně bílkovinné povahy, které se nacházejí v organismu v důsledku maligního zvratu. Lokalizace nádorových markerů je rozmanitá. Mohou být přítomny přímo ve tkáni nádoru (buněčný nádorový marker) nebo jsou nádorem produkovány (s nádorem asociované antigeny), popř. jsou produkovány hostitelem jako odpověď na přítomnost nádoru (indukované nádorové markery – proteiny akutní fáze, např. α-1-antitrypsin, C-reaktivní protein, haptoglobin)[3,4]. Mezi markery produkované přímo tumory se řadí enzymy (laktátdehydrogenasa), imunoglobuliny a jejich části (tzv. paraproteiny), hormony (choriový gonadotropin, kalcitonin), fragmenty glykoproteinů, onkofetální antigeny (α-1-fetoprotein, karcinoembryonální antigen), fragmenty cytokeratinů (tkáňový polypeptidový specifický antigen, tkáňový polypeptidový antigen), molekuly receptorové povahy (estrogenový a progesteronový typ) a cirkulující buněčné elementy (cirkulující nádorové buňky)[4]. Mezi nejnovější rakovinné markery patří i detekce genových mutací, např. tumor- asociovaných genů.

2. Charakteristika nádorových markerů

Ideální nádorový marker pro klinickou praxi by měl mít stoprocentní specifitu při co nejvyšší senzitivitě, v praxi by se jednalo o jeho „nulovou hladinu“ u lidí bez maligního onemocnění a raný záchyt rakovinného bujení. Jakýkoli orgánově specifický marker by měl korelovat se stádiem a růstem nádoru, prognózou a účinností terapie[3]. V současnosti zatím není k dispozici univerzální marker, proto se při analýzách stanovuje více rakovinných markerů, jejichž vzájemná specifita a senzitivita se překrývá.

2.1. Aplikace v praxi klinické biochemie

Výhodou biochemické diagnostiky onkologických onemocnění pomocí markerů je relativně nízký práh stanovitelnosti, díky kterému je možno odhalit přítomnost maligního bujení s nádorem již o hmotnosti 1 mg, tedy cca 10^6 rakovinných buněk. Klinická diagnóza je schopná většinou odhalit nádory obsahující cca 10^9 buněk[4]. V současnosti je většina rozpustných nádorových markerů stanovována z krevního séra pacientů. V rámci léčebné terapie se markery využívají pro primární diagnostiku, sledování konkrétního průběhu nemoci a účinnosti terapie. Důležitou úlohu mohou sehrát i ve screeningu onemocnění.

Screening je organizované, kontinuální a vyhodnocované preventivní vyšetření prováděné pro včasný záchyt zhoubných onemocnění u asymptomatických pacientů. Většina markerů vzhledem k nízké specifitě a senzitivitě není vhodná pro preventivní screening velkého počtu asymptomatických pacientů. Ke screeningu se většinou přistupuje u symptomatických pacientů nebo u pacientů s vysokým rizikem vývoje nádorového onemocnění, pře-

devším u genetických predispozic – např. vyšetřování kalcitoninu v rodinách s výskytem medulárního karcinomu štítné žlázy[4].

Pro stanovení skutečné hodnoty markerů u diagnostikovaného onemocnění a v průběhu léčby je třeba brát ohled na biologické poločasy rozpadu jednotlivých markerů. Je důležité zvolit správné intervaly vyšetření krve tak, aby byl skutečně postihnut účinek terapie, nikoliv jen tzv. lysis fenomén (prudké navýšení hladiny markeru v důsledku cytolýzy způsobené protinádorovou terapií). Z tohoto důvodu se nádorové markery stanovují nejdříve koncem 3. týdne, resp. ve 4. týdnu od zahájení terapie[4]. U stanovení hladin je nutné vyloučit případné rušivé vlivy. Je známo, že některá stanovení jsou ovlivněna postupem vyšetření. Například stanovení prostatického specifického antigenu (PSA), jakožto markeru rakoviny prostaty, je podmíněno odběrem krve až 48 h po rektálním vyšetření prostaty. Závažným bodem vyhodnocení analýzy je možnost falešné pozitivy výsledků způsobené zvýšenou hladinou markerů jako důsledek jejich produkce u nemaligních onemocnění, životosprávou a jinými vlivy nebo poruchou jejich vylučování (zejména u poruchy funkce jater a ledvin)[3,4]. Hladina markerů se posuzuje v závislosti na klinické remisi a průběhu terapie. Trend stoupající hladiny ve třech po sobě jdoucích odběrech značí recidivu, resp. progresi. Nárůst o více než 25 % značí progresi onemocnění, pokles o více než 50 % značí parciální remisi[4].

2.2. Klasifikace nádorových markerů

Jak bylo již výše zmíněno, rakovinné markery lze klasifikovat do dvou hlavních skupin: markery produkované nádorem a markery asociované s nádorem.

2.2.1. Markery produkované nádorem

Tato skupina se dále člení na antigeny (onkofetální, onkoplacentární, specifické, proliferační atd.), hormony, enzymy.

Významným onkofetálním markerem je α-fetoprotein (AFP). AFP je fyziologický glykoprotein produkovaný ve fetálních játrech a žloutkovém vaku v průběhu embryonálního a fetálního období. Jedná se o analog albuminu, jehož hlavní funkcí v organismu je transport řady látek (bilirubin, mastné kyseliny, retinoidy, atd.). Po narození jeho koncentrace klesá. V dospělém zdravém organismu je jeho syntéza minimální, pouze u těhotných žen přechází přes placentu do krve a je tedy fyziologicky zvýšen v těhotenství[4,5]. Z klinického pohledu je senzitivita AFP vysoká především u hepatocelulárního karcinomu (senzitivita u neléčeného onemocnění je až 80 %), dále pak pro germinální nádory vaječníků a varlat. U čistě embryonálních nádorů a nádorů žloutkového vaku je dosaženo až 80% senzitivity, u teratomů je to 20 % (cit.[4–6]).

Do skupiny onkoplacentárních markerů se řadí např. glykoproteinový hormon lidský choriový gonadotropin (hCG). Tento hormon se skládá z α-podjednotky, která je téměř shodná s dalšími hormony (folikuly stimulující a luteinizační hormon) a β-podjednotky, která odpovídá za biologickou funkci jednotlivých hormonů[4]. Fyziologicky je hCG zvýšen v průběhu těhotenství. V tomto případě tedy nelze hCG spojovat s rakovinnými markery[7,8]. Metody stanovení hCG jsou založeny na rozpoznání epitopů C-konce β-podjednotky specifickými protilátkami. Citlivost těchto testů je limitována afinitou protilátek k β-hCG. Zvýšená hladina slouží jako ukazatel nádoru varlat, tzv. seminomu. Ve vysokých koncentracích je hCG produkován především trofoblastickými nádory. Zvýšené hodnoty hCG se nalézají u řady jiných nádorů, včetně nádorů vaječníků, gastrointestinálních, močového měchýře, plic, pankreatu, ledvin. Screening pomocí hCG je doporučován u symptomatických pacientů, např. při podezření na germinální nádory varlat. Stanovení hladiny hCG v séru se používá především pro určení stádia nemoci, potvrzení histologického charakteru nádorů varlat a pro následné monitorování[4]. Zvýšená koncentrace hCG se vyskytuje i u patologických stavů, které nesouvisí s nádorovým onemocněním, např. při výskytu myomů nebo ovariálních cyst[4].

Prostatický specifický antigen (PSA) zastupuje skupinu specifických markerů. Jedná se o serinovou proteasu štěpící inhibitor motility spermií – semenogelin, převládající protein lidského ejakulátu[4,5,9]. Fyziologicky dochází k produkci PSA v kolumnárních a kuboidních buňkách prostaty. PSA je mimo to produkován normálními i maligními buňkami mléčné žlázy, avšak jedná se jen o buňky s progesteronovým receptorem[5]. Zvýšená hladina PSA je přednostně spojena s nemocemi souvisejícími s prostatou, tyto nemoci nemusí být rakovinného původu. Často se jedná o benigní hyperplasii prostaty nebo prostatitidu[10]. Vzhledem k tomu, že hladina PSA u mužů nad 40 let postupně s věkem vzrůstá, je třeba nastavit detekční limity podle věkových skupin pacientů[11]. Zvýšené hladiny PSA způsobují i nedávná rektální vyšetření prostaty, ejakulace, biopsie, transuretální resekce nebo jiná mechanická dráždění prostaty[4,5].

Do skupiny proliferačních markerů antigenně specifických řadíme tkáňový polypeptidový antigen (TPA). TPA je jednořetězcový polypeptid tvořený proteolytickými fragmenty cytokeratinu typu střední mikrofilamenty (cytokeratin 8, 18 a 19) (cit.[4,5,12]). Produkce TPA bývá spojena s rychlou obnovou buněk. Zvýšené hladiny v séru byly pozorovány zejména u pacientů s rakovinou[13]. Využívá se k monitorování progrese karcinomu močového měchýře, nádoru prsu, plic, ledvin a gastrointestinálního traktu[4,5]. Nicméně i některá onemocnění jater (cirhóza, hepatitida) nebo infekční choroby zvyšují hodnotu TPA (cit.[4]).

2.2.2. Markery asociované s nádorem

Markery sdružované s nádorem jsou zastoupeny určitými proteiny v plazmě a receptory (např. steroidní receptory).

Významným plazmatickým proteinem sloužícím také jako rakovinný marker je ferritin. Tento glykoprotein je fyziologicky produkován retikuloendoteliálními buňkami sleziny, jater a kostní dřeně. Snížená koncentrace bývá průvodním jevem anémie z nedostatku železa[4,5]. Patologic-

ké zvýšení ferritinu nalezneme u nádorových onemocnění, jako je akutní myeloblastická leukemie, hodgkinův a nehodgkinův lymfom, mnohočetný myelom. Nespecifické zvýšení pak provází i maligní melanomy a testikulární nádory. Koncentrace tohoto markeru hematologických malignit obvykle koreluje se závažností onemocnění[4].

Steroidní receptory jsou v současnosti nejvýznamnějším markerem rakoviny prsu[4]. Primárním účelem stanovení molekul estrogenového (ER) a progesteronového (PR) receptoru je odhadnout pravděpodobnou odpověď na endokrinní terapii u pacientů s rakovinou prsu. Jejich stanovení se provádí v současnosti nejvíce imunohistochemicky[4,6].

Další významný receptor je z rodiny guanylyl cyklas – guanylyl cyklasa C (GCC), který se fyziologicky nachází jen ve střevní sliznici. Aktivita GCC byla v nedávné době nalezena také u lidí s primárním a metastazujícím karcinomem tlustého střeva, čímž by GCC mohl představovat selektivní marker pro metastázy kolorektálního karcinomu v extraintestinálních tkáních. Selektivní exprese GCC v normální střevní sliznici a v nádorových buňkách tlustého střeva může být využita k rozvoji diagnostických testů pro detekci nádorových buněk v normálních tkáních a v krvi pro stanovení stádia nemoci a pro sledování onemocnění[14].

Dalším markerem nezařazeným do uváděných skupin je např. hemoglobin, jehož průkaz ve stolici je asociován s kolorektálním karcinomem.

2.2.3. Detekce mutace genů jako rakovinný marker

Současný trend v oblasti diagnostiky rakoviny je spojen se studiem mutací na úrovni DNA. Jedná se především o tzv. tumor-asociované geny, které jsou zkoumány imunohistochemickými a molekulárními analýzami (PCR, chipové technologie) jako možné budoucí diagnostické, prognostické a terapeutické markery.

Jeden z nejčastěji mutovaných tumor-supresorových genů u lidských nádorů je gen p53. Mutace genu p53 vede ke vzniku nefunkčního proteinu p53, který tak nemůže kontrolovat genomovou integritu ani indukovat apoptózu, což má za následek množení poškozené buňky a tedy rozvoj rakovinného bujení. U nemalobuněčného typu rakoviny plic se mutace genu p53 nalézají až v polovině nádorů a častěji se vykytuje u nádorů skvamózních buněk než u adenokarcinomu[15]. O jeho využití jako rakovinného markeru se uvažuje především ve spojitosti s hodnocením prognózy u rakoviny prsu, plic a tlustého střeva. U rakoviny prsu byla prokázána spojitost mezi výskytem abnormálního p53 a zvýšenou agresivitou nádoru, zvýšeným rizikem recidivy i úmrtí[16].

V patogenezi lidských nádorů hraje důležitou roli také mutace *ras* genů. Mutací na kodonu 12,13 nebo 61 jednoho ze tří *ras* genů (K-*ras*, N-*ras*, H-*ras*) dochází ke vzniku aktivního onkogenu. Mutace *ras* genů se nalézá v různých typech nádorů. Nejvyšší incidence je u adenokarcinomů slinivky břišní, tlustého střeva, plic, nádorů štítné žlázy a u myeloidní leukémie. U jednotlivých druhů nádorů se může vyskytovat s vyšší četností jen jeden určitý mutovaný *ras* gen, např. mutace K-*ras* genu u adenokarcinomu plic, slinivky a tlustého střeva, oproti tomu N-*ras* u myeloidní leukémie[17].

V současnosti jsou mutace genů detegovány především pro predikci odpovědi pacienta na některá léčiva, popř. sledování účinnosti terapie (např. gen Her-2 u pacientů s rakovinou prsu) nebo pro odhalení pacientů s vysokým rizikem vzniku rakoviny prsu a vaječníků v rámci screeningu (např. BRCA 1 a 2)[6,18].

Genovou nestabilitu vedoucí k tvorbě nádorů způsobuje kromě tumor-supresorových genů i mutace genů zodpovědných za opravu chyb v DNA (tzv. mismatch repair gene – MMR). Nádory s defektním MMR mají odlišné molekulární a klinicko-patologické vlastnosti oproti nádorům s funkčním MMR a obvykle mají i příznivější prognózu. Mutace v těchto genech zabraňuje opravám delecí a inzercí vzniklých v mikrosatelitech a vzniká tak tzv. mikrosatelitní nestabilita (MSI), která se vyskytuje až u 90 % hereditárních nepolypózních kolorektálních karcinomů a u 10–15 % sporadických adenomů tlustého střeva. Široce využívaným markerem pro stanovení MSI v nádoru je poly(A)mononukleotidová repetice BAT26 (cit.[19]).

3. Závěr

Onkologická onemocnění jsou po nemocech srdce a cév nejčastější příčinou úmrtí v ČR[1]. Každý rok je diagnostikována a přijata k léčbě řada nových pacientů s nádorovým onemocněním, v roce 2009 – 55 696 pacientů, v roce 2010 – 55 157 pacientů[2,20]. Stanovení hladiny nádorových markerů je nedílnou součástí vyšetřovacích metod pro včasné zachycení nemoci, stanovení diagnózy a kontroly průběhu léčby. V současnosti není znám marker, jehož specifita a senzitivita by byla natolik vysoká, aby mohl být použit jako tzv. univerzální standard. Nicméně je známa řada specifických markerů, jejichž souběžné stanovení je dostatečně senzitivní pro záchyt velkého procenta onkologických onemocnění. Efektivní využití celé řady nádorových markerů v klinické praxi vyžaduje spolupráci praktických lékařů, onkologů, chirurgů a dalších specialistů. Důkladný popis, správná interpretace a odhalení či vyvarování se falešné pozitivitě výsledků u některých fyziologických nebo i patologických stavů nesouvisejících s rakovinou by mohlo přispět ke spolehlivému screeningu a volbě účinné terapie.

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I. Machová[a], A. Brázdová[b], M. Fusek[b], and J. Zídková[b] *([a] Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Prague, [b] Department of Biochemistry and Microbiology, Institute of Chemical Technology, Prague):* **Tumor Markers and Their Use in Clinical Practice**

Tumor markers known also as cancer or oncological markers are substances produced either by tumor or normal cells as a response to the presence of cancer . The markers are used for the detection, diagnosis, prognosis and control of treatment. There is no absolutely specific or universal cancer marker; therefore different markers for different cancer types are used in clinical practice. A problematic feature of cancer markers is their falsely positive results under physiological and non-cancer conditions.

14.1.2 Indoor long-term persistence of cypress pollen allergenic potency: a 10-month study

Cypress pollen represents one of the primary causes of late winter respiratory allergies, rhino-conjunctivitis and allergic asthma. Cypress pollen grains are very abundant during the pollinating season and are outdoor and/or indoor deposited where they may persist for a long time.

In this study, indoor persistence related to the allergenic potency of *Cupressus sempervirens* pollen grains was evaluated with regards to time (2, 6, 8, 10 months), temperature, light and humidity variations found in a living home regardless of storage conditions. Using enzyme linked immuno sorbent assay (ELISA) and immunoblotting, we proved the persistence of the entire collection of allergens, detected in an unexposed fresh pollen extract. We demonstrated that there was no change in the *in vitro* allergenic potency of *Cupressus sempervirens* pollen grains after a 10-month exposure period to an indoor environment representative of a home exposure site. Based on our results, we concluded that cypress pollen allergens might have induced the lasting symptoms in the sensitized patients.

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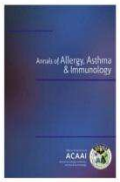

Letter

Indoor, long-term persistence of cypress pollen allergenic potency: a 10-month study

People living in industrialized countries spend 80% to 95% of their time indoors, and allergic disease triggered by indoor aeroallergens may cause disabilities that affect the quality of life in predisposed patients.[1] It is now well documented that various pollen airborne allergens may occur in the air of the home,[2] hospitals,[3] or work sites,[4] and a considerable amount of pollen allergens may remain indoors, even after the end of the pollen season.[5] In many regions worldwide, Cupressaceae trees release a large amount of allergenic pollen grains, some of which may penetrate indoors through ventilation systems, windows, or adherence to clothing.[5,6] Furthermore, Cupressaceae pollen–derived aerosols may be present in habitats as particles of smaller diameter than pollen grains.[7] In an indoor environment, areas that are more out of site, such as those below or above certain home furniture, are often miscleaned for a long period. In the present study, we aimed to investigate whether cypress pollen grains that have been placed several months in an indoor environment may preserve their allergenic potency.

Cypress pollen allergic patients were selected on the basis of seasonal symptoms, positive skin prick test results to cypress pollen, and their IgE binding patterns studied by immunoblotting on fresh cypress pollen extract. Serum samples were drawn after obtaining informed consent and pooled according to the 2 reactivity profiles previously described[8]: a first pool included 6 patients' serum samples with a heterogeneous IgE reactivity to high-molecular-weight (HMW; >30 kDa) allergenic glycoproteins, whereas specific IgE of the second set of 6 pooled serum samples mainly bound to a 14-kDa basic protein (BP14).

Pollen grains were freshly collected from *Cupressus sempervirens* trees growing in Southern France. Quadruplicates of 3 g of cypress pollen grains were deposited for 2, 6, 8, and 10 months in gauze-covered petri dishes (10 cm) placed on shelves at a height of 190 cm in a living room in the north of Marseille, France. For pollen protein extraction, 100 mg (1/10 wt/vol) of each pollen sample was incubated overnight under rotation in 1 mL of a 4% sodium dodecyl sulfate solution and centrifuged, and the collected supernatants were stored as aliquots at −20°C until use. Phosphate-buffered saline extracts were prepared as previously described by our group.[8]

Extracted proteins, obtained with similar yield for all samples, were separated by SDS-PAGE (8%–18% gradient polyacrylamide gel; ExcelGel; GE Healthcare, Uppsala, Sweden) as previously reported.[8] The separated proteins in gel were then transferred to a cyanogen bromide–activated nitrocellulose membrane for immunoblotting, and another part was stained by silver nitrate for protein detection. After electroblotting, the membrane was dried, blocked, and incubated with pooled sera as previously described.[8]

An inhibition enzyme-linked immunosorbent assay (ELISA) was also performed to quantitatively confirm the immunoblotting experiments. Microtiter plates were coated with a phosphate-buffered saline extract from fresh pollen (FPE; control not submitted to the indoor environment). Then patients' serum pool (1:20 dilution) preincubated without or with FPE (homologous inhibition) or the 16 (4 × 4) different indoor-exposed pollen extracts (heterologous inhibition) were applied to the FPE-coated plates. Bound IgEs were detected using an enzyme-conjugated goat anti-human IgE antibody followed by substrate. Heterologous inhibition curves were compared with the homologous inhibition obtained with FPE.

As shown in Figure 1, immunoblot experiments performed using either anti-HMW– or anti-BP14– serum pool revealed no obvious differences between IgE binding patterns to allergen extracts from unexposed fresh and exposed (2, 6, 8, and 10 m) cypress pollen grains to the indoor environment. The few minor qualitative differences observed were not meaningful because they also occurred between quadruplicates belonging to a same exposure group. The persistence of IgE reactivity was observed not only toward HMW allergens, which include the major allergen Cup s 1 at 45 kDa (Fig 1), but also toward a BP14 allergen, a marker of *C sempervirens* sensitization.[8]

ELISA inhibition experiments (not shown) confirmed immunoprint experiments. All experimental series (2, 6, 8, and 10 months of exposure to indoor environment) exhibited overlapping inhibition curves, and inhibition values were similar (±15%) to those obtained in the homologous inhibition using FPE.

In consequence, using ELISA, a quantitative technique measuring a global IgE reactivity, and immunoblot, a semiquantitative technique evaluating IgE reactivity to separated allergens, we found that a fresh pollen extract and an up to 10-month home-stored pollen could not be differentiated on the basis of the quantity and quality of the collection of IgE reactive allergens. Therefore, these home-stored pollen grains might potentially trigger the same allergenic symptoms than the fresh one when in contact with patient's mucosa.

In some regions cypress pollen grains could be detected in house dust sedimentations practically year-round. The present findings demonstrate that there is no change in the in vitro allergenic potency of *C sempervirens* pollen grains left for 10 months in an indoor environment representative of a home site. During this period, approximately from one pollinating season to another, the pollen was submitted to temperature, light, and humidity variations found in a dwelling house (ie, in uncontrolled conditions of storage). Ariano et al[9] reported that *Cupressus arizonica* pollen grains stored at room temperature in paper envelopes in a department at a university conserve their allergenic properties despite a loss of

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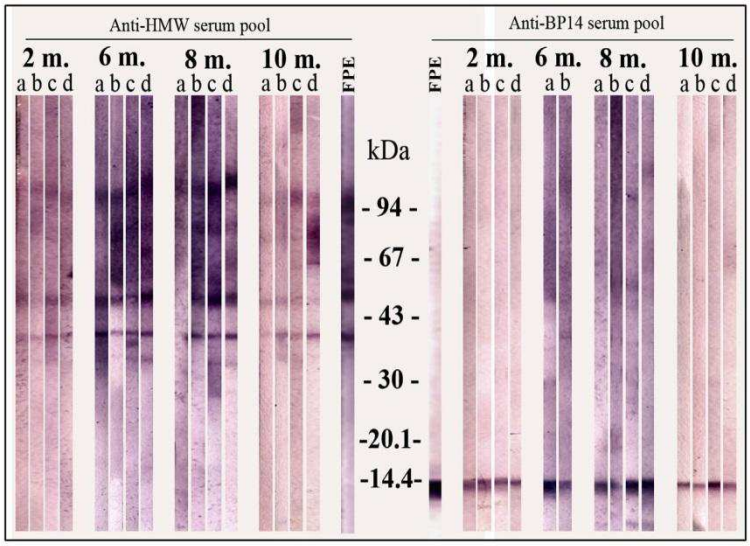

Figure 1. IgE immunoblots of quadruplicates (a, b, c, d) of 4 home-stored cypress pollen sodium dodecyl sulfate extracts separated by an 8% to 18% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. 2 m., 6 m., 8 m., and 10 m. indicate months of indoor exposure. Strips were incubated with either an anti–high-molecular-weight (HMW) serum pool or an anti-BP14 serum pool as indicated; the 6-month sample was performed in duplicate. FPE indicates fresh pollen extract (control extract not submitted to indoor exposure). Relative molecular masses in kilodaltons are indicated.

their viability. This finding means that exposure of individuals to cypress pollen allergens may continue indoors even after the end of pollen season in agreement with the pollen counts that were in situ measured (data not shown). These results provide further support for the observation that some patients sensitized to a single pollen species have perennial symptoms.[10] Complementary experimental investigation is required to determine whether indoor air pollution (25%-62% greater than outside levels; http://www.arb.ca.gov/) could interact with cypress pollen allergens and might exacerbate the respiratory diseases as demonstrated for other inhaled allergens.[1]

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14.1.3Complementarity between microarray and immunoblot for the comparative evaluation of IgE repertoire of French and Italian cypress pollen allergic patients

Cypress pollen represents the primary cause of respiratory allergies in Mediterranean areas. In this paper, we compared 2 component-resolved explorative techniques in prior to specify the sensitization profiles of French and Italian patients suffering from a common cypress pollen allergy. Then, we described the IgE repertoires of French and Italian *Cupressus sempervirens* pollen allergies (CPA) probably related to the local environmental exposure. Patients allergic to *Cupressus sempervirens* pollen (*Cups*) can be discriminated on the basis of the IgE binding to a basic 14 kDa protein (BP14) or to high molecular weight (HMW) glycoproteins.

Specific IgE repertoire of two differentially exposed CPA cohorts, French and Italian, were investigated using the IgE microarray system and the individual IgE immunoblotting (IB) of whole *Cups* pollen extract separated by SDS-PAGE.

The prevalence of sensitization to BP14 was higher in French (37%) than in Italian patients (17%). Major differences were observed in IgE reactivities to Lipid Transfer Proteins (LTPs). Thirty percent of the Italian CPA (4% in the French group) had specific IgE against the *Parietaria* pollen LTP. Regarding peach LTP sensitization, all Pru p 3+ Italian CPA (10%) were in the HMW+ subgroup while Pru p 3+ French CPA (20%) were all included in the BP14+ subgroup. BP14 sensitization could be a marker of *Cups* exposure and is, in French CPA, significantly correlated to Pru p 3 sensitization. We proved that the IgE immunoblots and microarrays were efficient complementary tools to refine the sensitization profiles of French and Italian patients suffering from a common cypress allergy widely distributed around the Mediterranean basin.

COMPLEMENTARITY BETWEEN MICROARRAY AND IMMUNOBLOT FOR THE COMPARATIVE EVALUATION OF IGE REPERTOIRE OF FRENCH AND ITALIAN CYPRESS POLLEN ALLERGIC PATIENTS

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Running title: IgE repertoire in cypress pollen allergy

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Abbreviation: 1-DE SDS-PAGE - one dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis, 2-DE - two-dimensional gel electrophoresis, ANOVA - analysis of variance, BCIP - 5-bromo-4-chloro-3-indolyl phosphate, BP14 - 14 kDa basic protein, *Cupa* - *Cupressus arizonica*, *Cryj* - *Cryptomeria japonica*, *Cups* - *Cupressus sempervirens*, CPA - Cypress Pollen Allergic patients, HMW - high molecular weight, IB - immunoblotting, IgE - immunoglobulin E, ISAC® - immuno solid-phase allergen chip, LTPs - lipid transfer proteins, IUIS - International Union of Immunological Societies, Mr - relative molecular mass, NBT - nitro blue tetrazolium, NCa - cyanogen bromide activated nitrocellulose sheet, PBS - phosphate buffer saline, Tw - tween 20

Abstract. Cypress pollen represents the primary cause of respiratory allergies in Mediterranean areas. Patients allergic to *Cupressus sempervirens* pollen (*Cups*) (CPA) can be discriminated on the basis of the IgE binding to a basic 14 kDa protein (BP14) or to high molecular weight (HMW) glycoproteins only. Specific IgE repertoire of two differentially exposed CPA cohorts, French and Italian, were investigated using an IgE microarray system (some known major allergens from several allergenic sources) and individual IgE immunoblotting (IB) of whole *Cups* pollen extract separated by SDS-PAGE (all allergens from one allergenic source: cypress pollen). The prevalence of sensitization to BP14 was higher in French (37%) than in Italian patients (17%) and major differences were observed in IgE reactivities to Lipid Transfer Proteins (LTPs). Thirty percent of the Italian CPA (4% in the French group) had specific IgE against the *Parietaria* pollen LTP, independently of IB sub-groups. Regarding peach LTP sensitization, all Pru p 3+ Italian CPA (10%) were in the HMW+ subgroup while Pru p 3+ French CPA (20%) were all included in the BP14+ subgroup. BP14 sensitization is likely a marker of *Cups* exposure and is, in French CPA, significantly correlated to Pru p 3 sensitization. The IgE immunoblot and microarray are complementary tools that highlight differences in the subtle sensitization profile between groups of patients in comparative studies.

Key words. *Cupressus sempervirens;* pollen allergens; immunoblot; allergen microarray;

Introduction

Cypress pollen is considered the primary cause of rhino-conjunctivitis and allergic asthma in areas surrounding the Mediterranean basin (Bountin-Forzano et al., 2005). The cypress, *largo sensu* belongs to the Cupressaceae family in which 4 species have been studied in detail concerning their high allergenicity: *Cupressus arizonica* (*Cupa*, Arizona cypress), *Cupressus sempervirens* (*Cups*, Italian cypress), *Juniperus ashei* (mountain cedar) and *Cryptomeria japonica* (*Cryj*, Japanese cedar) responsible for well-studied allergic disorders in Japan (Okamoto et al., 2009). A study, performed on 23,077 Italian allergic patients using the Immuno Solid-phase Allergen Chip (ISAC®) microarray technique, showed that, among 75 allergens, Cup a 1 (the major allergen of *Cupa*), exhibited the highest prevalence of IgE sensitization (about 43%) (Scala et al., 2010).

Proteins belonging to pectate lyase, polygalacturonase, thaumatin-like protein and Ca-binding protein families currently constitute groups 1 to 4 of cypress-cedar allergens, respectively (www.allergen.org or www.allergome.org). In *Cups* pollen, only 2 allergens are officially classified in the data bank of the International Union of Immunological Societies (IUIS). The pectate lyase Cup s 1, represents the major allergen and is highly cross reactive with Cup a 1 and Cry j 1 (Arilla et al., 2004) whereas Cup s 3, identified using cDNA cloning and homology sequence analysis, seems to be absent or poorly expressed in *Cups* pollen grains (Togawa et al., 2006). Based on inhibition assays and specific IgE cross reactivity, *Cups* pollen extracts were also suspected to contain other allergenic components belonging to the profilin and β-galactosidase protein families (Bistoni et al., 2005, Barderas et al., 2004).

We have recently reported that the use of detergent, chaotropic agents or saline conditions can solubilize additional allergens from *Cups* pollen (Shahali et al., 2010). Furthermore, a proteomic analysis using combinatorial peptide ligand libraries as selective purification techniques of extracted pollen proteins lead to the description of 10 unreported allergens (Shahali et al., 2012a). In particular, we have identified with >65% coverage in protein sequence the polygalacturonase of *Cupressus sempervirens* (putative Cup s 2) (data not shown, Shahali et al., 2012b) and we showed that the IgE reactivity to a 14 kDa basic allergen (BP14) in *Cups* pollen extracts could discriminate between 2 types of cypress pollen allergic patients (CPA). A first set of patients displayed a heterogeneous IgE reactivity to high

molecular weight (HMW, >30kDa) allergenic glycoproteins while specific IgE of a second set of patients mainly bound to BP14 (Shahali et al., 2012c).
The diagnosis of type 1 hypersensitivity is mainly based upon an evocative anamnesis and clinical history and *in vivo* skin prick tests. Then, *in vitro* detection of specific IgE against sensitizing molecular allergens can be performed using either commercial procedures in single or multi array (Mari et al., 2010) or immunoprint after separation of proteins from raw extract by electrophoresis in 1 or 2-dimensions (Le Mao et al., 1998, Poncet et al., 2010).
The aim of this study was to compare the IgE repertoire of CPA from France and Italy using, on one side, immunoblots with a *Cups* pollen extract and, on the other side, IgE microarray assay. Total IgE content as well as the specific reactivity towards a commercial *Cups* extract in classical immunoCAP (t23) assay were also evaluated. Results showed that the 2 analytical methods are complementary and suggest that co-sensitization and/or cross-reactivity and exposure play a role in shaping the subtle IgE repertoire in cypress pollen allergic patients.

Methods

Patient sera

This study has been approved by the institutional ethical committees (approval numbers: 2011-A00211-40 and 106-CE-2005) and written informed consent has been obtained from patients. Fifty-one CPA from Marseille, France, and 30 CPA from Rome, Italy, were selected according to their clinical symptoms (rhinitis, conjunctivitis and asthma) during cypress pollinating season. Mean age of French cohort was 40.3 years (range, 10-74) in a group of 28 females and 23 males and mean age of Italian cohort was 32.7 years (range, 11-62) in a group of 16 females and 14 males. For each immunoblot analysis, the serum from a healthy individual was used as a negative control.

Cypress pollen protein extractions

Cups pollen was supplied by Allergon AB (Angelholm, Sweden). One hundred mg of pollen was incubated for 18 h in a buffer containing 4% SDS. The pollen suspension was then centrifuged at 18,000 g for 20 min at 4°C, as previously described (Shahali et al., 2010). The supernatant was collected and stored as aliquots at -20 °C until use. The protein concentration in supernatants was measured with Bradford protein assay (Pierce, Thermo Scientific, Rockford, USA) on samples diluted to reduce the concentration of SDS <0.1% according to the manufacturers' recommendations in order to prevent interferences. Bovine serum albumin, used for protein calibration curve, was diluted in 0.1% SDS solution.

One-dimensional gel electrophoresis

For 1-DE SDS-PAGE separation, extracted proteins in a 38 $mmol.l^{-1}$ Tris buffer pH 6.8 containing 4% (w/v) SDS were applied to a thin 8-18% gradient polyacrylamide gel (ExcelGel, GE Healthcare, Uppsala, Sweden) and run on a flat-bed electrophoretic chamber (Multiphor II, GE Healthcare) at 12 °C. The gel was then either transferred onto a cyanogen bromide activated nitrocellulose (NCa, Demeulemester et al., 1987) sheet (Optitran®BA-S 83, Schleicher and Schuell, Dassel, Germany) for western blotting assays or stained for detection of the separated proteins. Molecular mass (Mr) markers (GE Healthcare) ranging from 14.4 to 94 kDa were used as comparative references.

Immunoblotting

Electroblotting of separated proteins was performed onto NCa sheets with a semidry Novablot apparatus (LKB, Uppsala, Sweden) following the manufacturer's instructions (1 h, 1 mA/cm^2). The membranes were then dried and blocked with PBS containing 0.3% (v/v) Tween 20 (Sigma-Aldrich) (PBS-Tw) for 1 h at 20 °C. For 1-DE screening, each NCa was then cut in

2.5 mm wide strips that were individually incubated with 1:10 diluted patient or control sera in PBS-Tw 0.1% (overnight at 20 °C). Each membrane was washed 3 times for 10 min in PBS-Tw 0.1 % (v/v) and incubated during 2 h at 20 °C with 1:700 dilution of alkaline phosphatase (AP)-conjugated goat anti-human IgE (Sigma-Aldrich). The AP activity was detected using 5-bromo-4-chloro-3-indolyl phosphate (BCIP, Sigma) and nitro blue tetrazolium (NBT, Sigma) in 0.1 mol.l $^{-1}$ Tris acetate buffer pH 9.5.

Total and specific IgE evaluation

Total IgE levels were assayed by nephelemetry (BNII, Siemens) and expressed in IU.ml^{-1}. Specific IgE to cypress allergen (Cupressus sempervirens, reference code t23) were quantified with the widely used fluorescence enzyme immunoassay (FEIA) ImmunoCAP® (ThermoFischer Scientific) on the ImmunoCAP 250 apparatus, as recommended by the manufacturer. The detection limit was 0.10 kU.l^{-1}. Specific IgE evaluation was performed on 46 French and on 30 Italian CPA using the commercial allergen microarray ImmunoCAP ISAC® 103 according to the manufacturer's instructions. The raw data were expressed as ISAC standardized units (ISU) and levels above 0.1 ISU were considered positive.

Statistical analysis

The differences in variables were evaluated using the analysis of variance (ANOVA) and Fisher's exact test.

Results

Immunoblot: IgE sensitization to BP14 is lower in prevalence in Italian patients

Sera from French (n=51) and Italian (n=30) patients were screened by 1-D immunoblots using SDS *Cups* pollen extract. In agreement with our previous results, 3 IgE reactivity patterns were observed. IgE from some patient sera bound BP14 with or without reactivity to HMW allergens (19 in French patients, i.e. 37% and 5 in Italian patients, i.e. 17%, see lines "Immunoblot" in table 1). IgE from other patient sera only bound to HMW and not to BP14 (41% and 60% of French and Italian CPA, respectively), and a third category was negative for all proteins of the extract (22% of the French patients and 23% of the Italian cohort). The results of 3 representative sera for BP14 and HMW sub-groups and one for the negative subgroup are depicted in Fig. 1 for French and Italian patients (Fig. 1A and 1B, respectively). These 3 groups will be referred as immunoblot (IB) sub-groups, BP14+, HMW+ and Neg in the rest of the study.

Microarray: a minority of cypress allergic patients are monosensitized

IgE immunoreactivity results are separately represented in Fig. 2 for French patients and in Fig. 3 for Italian patients. Demographic and clinical characteristics are given on the left sides of Fig. 2 and 3 (the correspondence letter code for molecular allergens and extracts are presented in table 2) and specific global percent reactivity to cypress allergens are shown in table 1. Most of the patients exhibited rhinoconjunctivitis, in both cohorts and, independently of the IB sub-groups, asthma was also observed in 35% of French and 10% of Italian CPA. With regards to microarray results (right side of both Fig. 2 and 3), the proportion of monosensitized versus polysensitized CPA was quite low both in French (13%) and Italian (10%) patients. The proportion of pollen-polysensitized patients was similar in French and Italian cohorts, 17% versus 20%, respectively. In contrast, the proportion of Italian patients allergic to multi-categories of allergens (i.e. all pollen plus other allergens) was high (63%) while it only reached 46% in French patients. Patients with a pollen sensitization limited to cypress along with a sensitization to non-pollen allergens represented 15% for French and 7%

for Italian CPA. These mono, pauci and polysensitizations seem to be independent of the 3 IB sub-groups.
Individual levels of specific IgE reactivity to Cup a 1, Cry j 1 and *Cups* as well as total IgE are graphically shown in Fig. 4 for each IB subgroup. Specific IgE to *Cups* (t23), Cup a 1 and Cry j 1 values were significantly higher in Italian patient group than in French one independently of IB subgroups ($p<0.05$) and total IgE levels showed a tendency to be lower in French than in Italian CPA patients ($p=0.1019$) (Fig. 4). While similar values of Cup a 1 and Cry j 1 specific IgE were observed in the Italian cohort (mean 18.6 and 20.4 ISU respectively), they were significantly lower for Cry j 1 than for Cup a 1 in the French patients independently of IB subgroups (mean 3.4 and 7.1 ISU respectively, $p=0.0129$). Interestingly, *Cups* (t23) specific IgE were significantly higher ($p<0.05$) in the BP14 sub-group as compared with HMW and Neg sub-groups in the French patients.

LTPs are the discriminating allergens
Data of Fig. 2 and 3 were also globally interpreted as histograms in Fig. 5 pointing out the percentages of positive sera for allergens or groups of allergens in order to compare French and Italian repertoires. The overall qualitative diversity was similar and 4 allergen sources showed quantitative differences. Grass pollen (35% vs. 80%), PR10 (2% vs. 17%), profilin (4% vs. 17%) and house dust mite (17% vs. 37%) sensitizations were higher in Italian than in French CPA. However, the most striking qualitative and quantitative difference was found for LTP sensitization. Italian CPA were significantly more sensitized to *Parietaria* pollen LTP as compared to French CPA (30% versus 4%, respectively, $p= 0.004$) whereas sensitization to peach LTP reached 20% in the French and 10% in the Italian CPA. This outcome prompted us to assess how these reactivities were distributed among each IB sub-group. While the reactivity of CPA to the main type of allergens - pollen, animals and house dust mites - was equally distributed among the 3 IB sub-groups, both in French and Italian patients (data not shown), the reactivity to peach LTP was strictly restricted to BP14 IB sub-group in French CPA. The 9 Pru p 3+ French patients were all included in BP14+ patient's sub-group while the 3 Pru p 3+ Italian patients were in the HMW+ sub-group (Fig. 6). This correlation is statistically significant ($p=0.01$) and is consistent with the anamnetic investigation revealing a higher proportion of fruit allergy in BP14+ French patients subgroup than in HMW+ subgroup (left side of Fig. 2). Interestingly, no preferential association was found for the reactivity to *Parietaria* pollen LTP Par j 2 in Italian patients since it was found in the 3 IB sub-groups (Fig. 6).

Discussion
Aside from the clinical symptoms collected by a physician in a traditional anamnesis, diagnosis is usually established with skin prick test and biological test evaluating the presence of specific IgE against a panel of allergens.
An optimized total extract has the advantage of containing the whole collection of components to which the individual has been exposed and is thus crucial to decipher the complete repertoire of a patient's IgE against a given source. Electrophoresis constitutes a useful tool to separate proteins from an allergenic extract and double dimension gel electrophoresis offers the best resolution (Poncet et al., 2010, Shahali et al., 2008, Le Mao et al., 1998). Coupled to immunoblotting with patient's IgE and mass spectrometry of recognized allergens, this immunoproteomic approach evaluate the diversity of sensitizing allergens as well as the diversity of the individual IgE immune response. However, since the technique is not yet miniaturized, it is tedious, time- and reagent-consuming when several allergenic sources have to be studied.

In contrast, a multiplexed microarray device, used for *in vitro* diagnosis, consumes low amount of serum, can be automated and delivers the results of IgE binding on more than 100 molecular native or recombinant allergens covering representative group markers of plant and non-plant allergenic sources (Mari et al., 2010). A large repertoire of allergen reactivities can thus be explored in selected physio-pathological models on already known and well characterized molecular allergens. One obvious drawback is, of course, the inability to evaluate reactivity to a molecular allergen that has not been previously reported. Non-hydrosoluble allergens, for example, represent one such class that remains poorly-studied until now (Godfrin et al., 2007).

Consequently, for purposes of research, these 2 techniques can be considered as complementary since immunoblots yield the complete allergen repertoire from a given allergenic source and the microarray gives a global reactivity overview against several allergenic sources.

We combined these 2 technologies to the study of cypress pollen allergy, a pervasive pollinosis in areas surrounding the Mediterranean basin. Very few studies are available on the IgE repertoire of cypress allergic French patients (Caimmi et al., 2013, Boutin-Forzano et al., 2005) whereas this pollinosis is more documented in Italy and Spain (Tordesillas et al., 2011, Papa et al., 2001, Corsico et al., 2000). We previously reported that CPA can be classified in 2 groups according to IgE immunoblot patterns after migration of a cypress extract in 1-D and 2-D gel electrophoresis: CPA with anti-HMW allergen reactivities and those expressing IgE against BP14, a basic protein of 14 kDa (Shahali et al., 2012b, Shahali et al., 2012c, Shahali et al., 2010). Proteomic analysis of cypress pollen extract in two-dimensional gel electrophoresis (2-DE) showed that the only protein found at 14 kDa in a PBS cypress extract is the cationic BP14 (Shahali et al., 2012b, Shahali et al., 2012c) making of the immunoblot after 1-DE a valuable and reliable method to assess CPA profile.

The comparison of the IgE immunoblot patterns of 2 cohorts of CPA, one from Rome, Italy and the other one from Marseille, France, showed that the anti-BP14 specificity was less represented in Italian patients. Together with microarray evaluation of sensitization patterns and the determination of Cup a 1, Cry j 1 and *Cups* specific IgE levels, our results point out some differences that might be attributed to variable exposure to local trees and/or other allergenic environments. Indeed, as compared to the Arizona cypress (*Cupressus arizonica*) and Japanese cedar (*Cryptomeria japonica*), the Italian cypress (*Cupressus sempervirens*) pollen is richer in BP14 (Shahali et al., 2012c) (with or without SDS in extraction buffer -unpublished data-) and is the most abundant species in the south of France, where it represents more than 80% of Cupressaceae. Conversely, the Arizona cypress is predominant in urban and peri-urban areas in Italy (Christian Pichot, INRA, France, personal communication). This might be one of the main reason explaining why patients allergic to cypress pollen and showing anti-BP14 IgE, are more frequent in the south of France.

Microarray experiments also revealed some differences in sensitization to LTPs. Thirty percent of Italian CPA reacted to *Parietaria* pollen LTP (Par j 2), independently of IB sub-groups and 20% of French patients reacted to peach LTP (Pru p 3) with a strong correlation with the BP14 sub-group. This correlation was not observed for the 3 Italian patients sensitized to Pru p 3 that fall in the HMW sub-group. LTPs constitute a group of heterogeneous allergens where no IgE cross-reactivities are found between *Parietaria* pollen and food LTP including peach (Tordesillas et al., 2011, Egger et al., 2010). We confirm these results since no co-sensitization Pru p 3/Par j 2 was observed, neither in French nor Italian CPA. This result is also in agreement with the absence of detected cross reactivity between cypress pollen allergens and plant-derived food in cypress pollen monosensitized Italian patients (Panzani et al., 2010). A relationship between cypress pollen and peach allergy has already been reported (Delimi et al., 2007, Hugues et al., 2006), in which the authors

suggested, upon immunoblot inhibition experiments, that proteins around 45 kDa might be the cross-reactive allergen (Hugues et al., 2006). The fact that Pru p 3 reactive CPA were found in the BP14 sub-group (in French patients only) was unexpected and raised the question of the nature of BP14. LTPs are small in molecular masses (6-14 kDa) and basic in terms of isoelectric point values. These characteristics are shared with BP14 and several experiments were performed to unravel the physico-chemical nature of BP14. These experiments included chromatographic methods specifically adapted to purify LTPs, immunoblots using specific anti-Pru p 3 rabbit antibodies and more than 25 trials of characterization by mass spectrometry analysis. The analysis of accumulated data led to the conclusion that there is no obvious direct immunochemical and structural relationships between known LTPs and BP14. Homology searches using a sequence of 9 N-terminal amino-acids of BP14 (unpublished data) confirmed that this protein is currently absent from data banks and displays no identical characteristics with the Cupressaceae pollen LTP described as an allergen in Japanese cedar (Ibrahim et al., 2010). A LTP has also been immunochemically described in *Cupressus arizonica* pollen (Sanchez-Lopez et al., 2011). Efforts are still ongoing in our group to structurally characterize BP14 whose IgE response seems to be a specific marker of sensitization to *Cups* pollen (Shahali et al., 2012b) and is associated with a Pru p 3 sensitization in French CPA only.

In conclusion, by combining 2 component-resolved explorative techniques, we highlighted some of the differences shaping the IgE repertoires of French and Italian CPA. These differences are related to the allergenic biodiversity of the local environmental exposure, either the species of cypress, *arizonica* versus *sempervirens* or the co-sensitization source, peach or *Parietaria* LTP. Using immunoblot, the IgE reactivity against numerous allergens in one allergenic source was revealed, and using microarray, some representative allergens of several allergenic sources were studied. These approaches have proven to be complementary and were used herein to efficiently decipher and refine sensitization profiles of French and Italian patients suffering from a common cypress allergy widely distributed around the Mediterranean basin.

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Figures

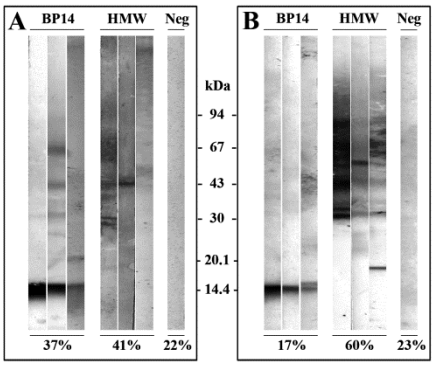

Fig. 1: Three immunoblot patterns obtained with representative patients' sera. An SDS protein extract of Cupressus sempervirens pollen separated in SDS-PAGE were blotted onto an NCa membrane. Each strip was individually incubated with sera from French (n=51) and Italian (n=30) CPA. Three representative patterns for

BP14 and HMW and one for negative (Neg) sera are shown for French (A) and Italian (B) CPA. Percentages in each sub-group and molecular mass (kDa) are indicated.

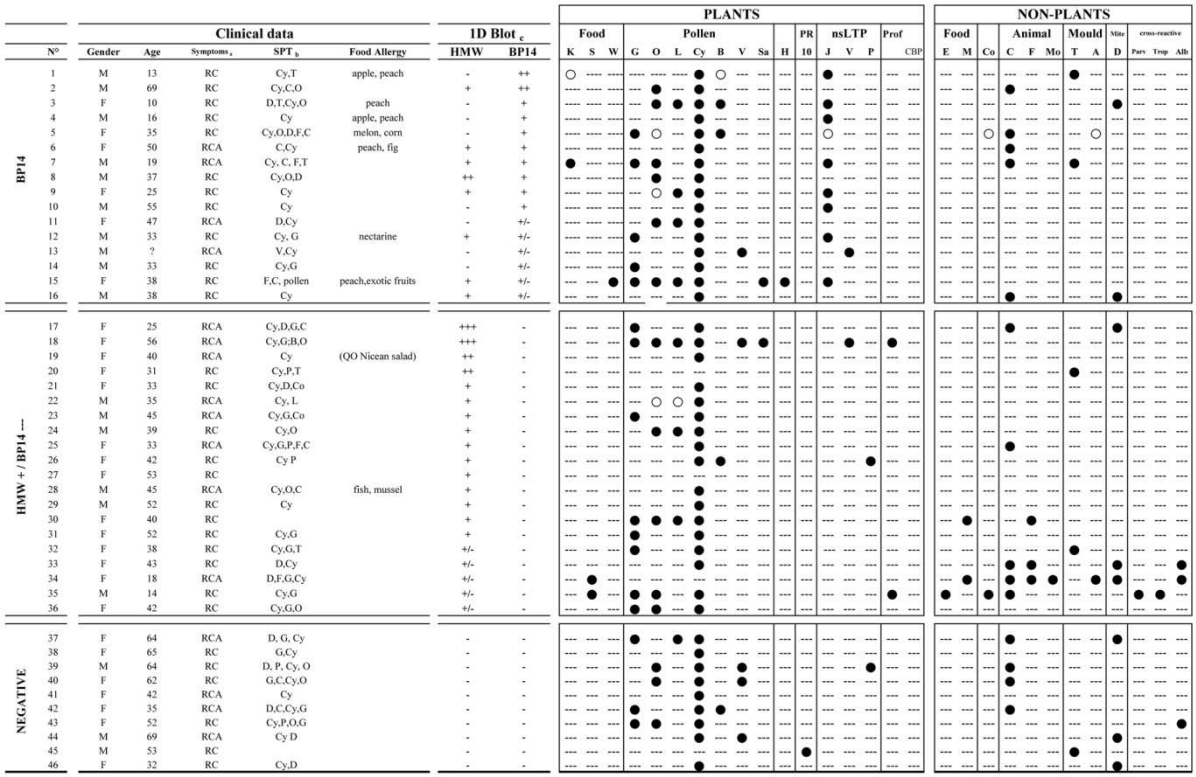

Fig. 2: Clinical and biological data of 46 French CPA. a: symptoms included R: rhinitis, C: conjunctivitis and A: Asthma. b: SPT: Skin Prick Test with standardized extracts. QO: Quincke oedema. c: results of IgE immunoblot are semi quantitatively expressed according to the intensity of the reaction. -: negative; +/-: low, +: medium; ++: high, +++: very high. Letter code for allergens or groups of allergens is similar for SPT and microarray results (see table 2). Close circle: positivity (ISU > 0.3). Open circle: low positivity (0.1<ISU<0.3). --- : negative (ISU < 0.1).

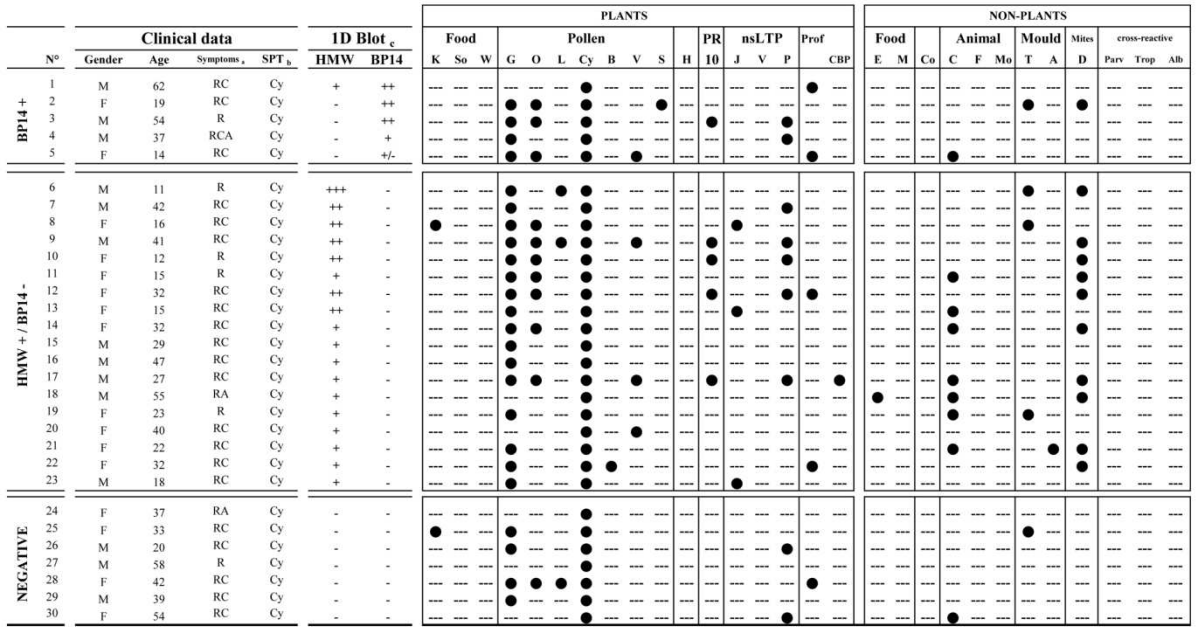

Fig. 3: Clinical and biological data of 30 Italian CPA. a: symptoms included R: rhinitis, C: conjunctivitis and A: Asthma. b: SPT: Skin Prick Test with standardized extracts. c: results of IgE immunoblot are semi quantitatively expressed according to the intensity of the reaction. -: negative; +/-: low, +: medium; ++: high, +++: very high.

The correspondence of letter code with molecular allergens for microarray is given in table 2. Close circle: positivity (ISU > 0.3). Open circle: low positivity (0.1<ISU<0.3). --- : negative (ISU < 0.1).

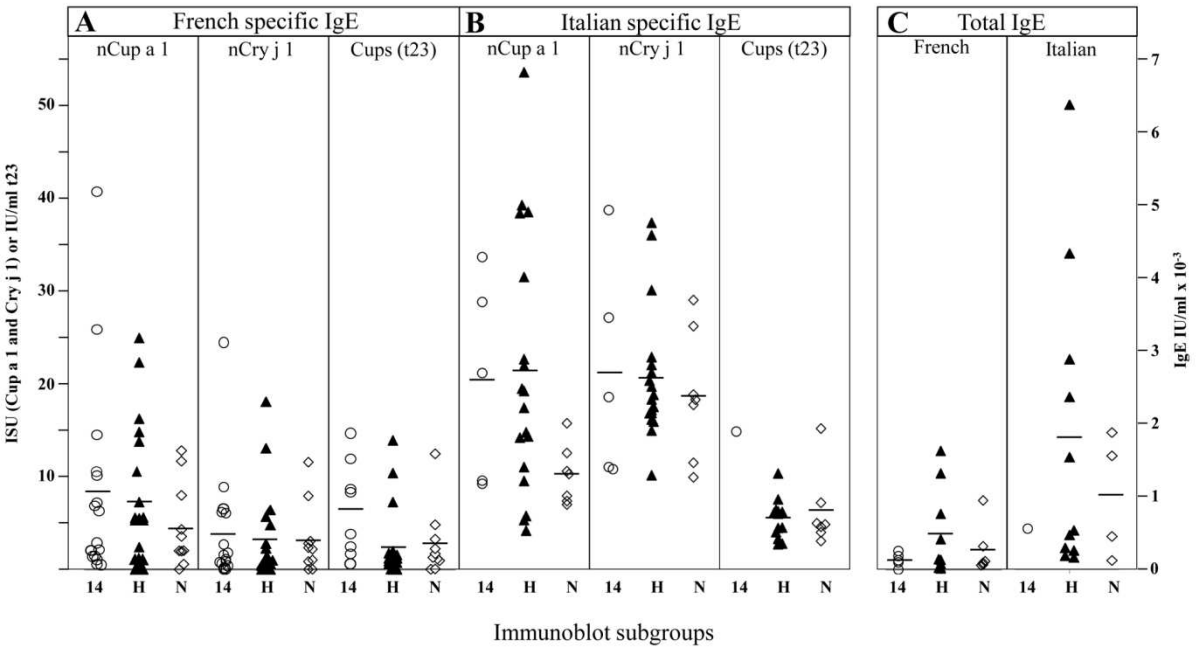

Fig. 4: Individual distribution plots of specific (A and B) and total IgE (C) levels in French and Italian CPA and for each IB sub-group. (14) open circles: BP14 sub-group, (H) close triangles: HMW subgroup, (N) open diamond: Neg sub-group. Horizontal bars in each sub-group represent the mean value. Total IgE was quantified by nephelemetry and expressed in IU.ml-1. ImmunoCAP specific IgE levels to the whole *Cups* pollen extract (t23) were indicated in IU.ml-1. The level of specific IgE to nCup a 1 and nCry j 1 was determined with ImmunoCAP ISAC and expressed in ISU.

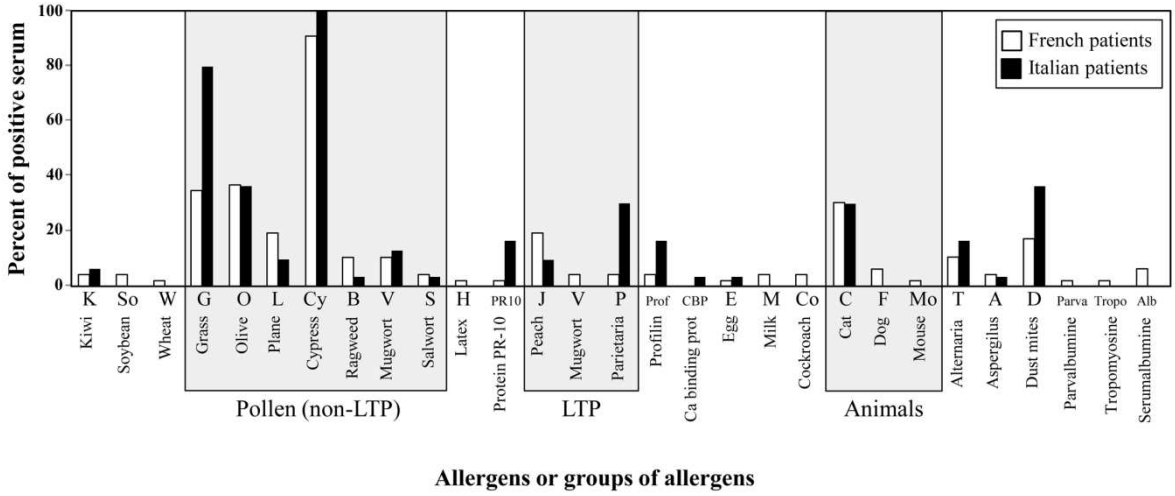

Fig. 5: Comparative distribution of IgE reactivities in French and Italian CPA patients. Data from the commercially available microchip array ISAC® (103 allergens) are presented as percentages of positive sera for each allergen or group of allergens. See table 2 for letter code correspondence. White histograms: French CPA, black histograms: Italian CPA. Pollen allergenic sources, LTP and animal-derived allergens are grouped in boxes to facilitate the comparisons.

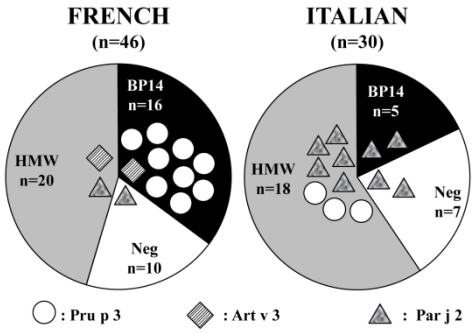

Fig. 6: Distribution of LTP sensitization obtained by microarray ISAC® (103 allergens) within IB subgroups in French and Italian patients. Each subgroup, HMW, BP14 and Neg, is indicated with the number of patient. 0 : patient sensitized to peach LTP (Pru p 3), ◆: patient sensitized to mugwort LTP (Art v 3) and ▲ : patient sensitized to *Parietaria* LTP (Par j 2).

Table 1: IgE reactivity of French and Italian cohorts to allergens or specific extract from *Cupressus sempervirens* using different techniques

Technique	Cypress allergen or extract	French$_a$	Italian$_a$
Microarray	Cup a 1	91	100
Microarray	Cry j 1	85	100
FEIA$_b$	Cup s (t23)	89	100
Immunoblot	Cup s HMW	41	60
Immunoblot	Cup s BP14	37	17

$_a$ Results are expressed in % positive patient serum.
$_b$ Fluorescence Enzyme Immuno Assay

Table 2: Correspondence letter code for molecular allergens and extracts used for figure 2 and 3. For SPT reported in figure 2 letter code represents total extract source.

	code	source	species	*Molecular Allergen*		
plant food	K	Kiwi		*nAct d 2*		
	S	Soybean		*nGly m 5, 6*		
	W	Wheat		*nTri a 18*	*nTri a gliadin*	
Pollen	G	Grass pollen	Bermuda	*nCyn d 1*		
			Timothy	*rPhl p 1, 2, 5 , 6, 11*		*nPhl p 4*
	O	Olive pollen		*nOle e 1*		
	L	Plane pollen		*rPla a 1*	*nPla a 2*	
	Cy	Cypress pollen	Arizona cypress	*Cup a 1*		
			Japanese cedar	*Cry j 1*		
	B	Ragweed pollen		*nAmb a 1*		
	V	Mugwort pollen		*nArt v 1*		
	S	Saltwort pollen		*nSal k 1*		
	H	Latex		*rHev b 5, 6*		
	PR10	PR10 family	Birch pollen	*rBet v 1*		
			Alder pollen	*rAln g 1*		
			Hazel pollen	*rCor a 1.11*		
			Hazelnut	*rCor a 1.41*		

			Apple	*rMal d 1*		
			Peach	*rPru p 1*		
			Soybean	*rGly m 4*		
			Peanut	*rAra h 8*		
LTP family	J	Peach		*nPru p 3*		
	V	Mugwort		*nArt v 3*		
	P	Wall Pelitory	*(Parietaria)*	*rPar j 2*		
Profilin family	Prof	Profilins	Birch	*rBet v 2*		
			Olive	*nOle e 2*		
			Latex	*rHev b 8*		
			Mercury	*rMer a 1*		
			Timothy	*rPhl p 12*		
	CBP	Ca binding	Birch	*rBet v 4*		
		protein	Timothy	*rPhl p 7*		
	E	Egg		*nGal d 3 (ovotransferrin)*		
	M	Milk		*nBos d lactoferrin*		
	Co	Cockroach		*rBla g 5*		
	C	Cat		*rFel d 1, 4*		
	F	Dog		*rCan f 1, 2*		
	Mo	Mouse		*nMus m 1*		
Mold	T	Alternaria		*rAlt a 1, 6*		
	A	Aspergilus		*rAsp f 4*		
	D	House Dust	*D. pteronyssinus*	*nDer p 1, 2*		
		Mite	*D. farinae*	*nDer f 1, 2*		
	Parva	Parvalbumin	Carp	*rCyp c 1*		
			Cod	*rGad c 1*		
	Tropo	Tropomyosin	Shrimp	*rPen a 1*	*nPen i 1*	*nPen m 1*
			Mite	*rDer p 1*		
			Cockroach	*Bla g 7*		
			Anisakis	*rAni s 3*		
	Alb	Animal serum	Cow	*nBos d 6*		
		albumin	Cat	*nFel d 2*		
			Dog	*nCan f 3*		
			Horse	*nEqu c 3*		