

EXPERT REVIEWS

Antisperm immunity and infertility

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Immunoinfertility is one of several causes of infertility in humans. Although progress on antisperm immunity and infertility has advanced during the past three decades, the nature of a real antisperm antibody (ASA) is still poorly understood. Dozens of sperm antigens have been isolated and characterized in association with infertility. However, it is difficult to identify a single predominant target antigen that could interact with all the ASAs. There are some protective mechanisms preventing ASA production in males and females. As chronic infection, vasectomy and vasovasostomy, heavy metals, and testicular cancer and torsion may induce the production of ASAs, they may be responsible for decreased motility and sperm penetration of cervical mucus, and the blockage of the acrosome reaction and the sperm–egg interaction. Many ASA assay methods have been developed, each with advantages and disadvantages. Efforts for the treatment of ASA-mediated infertility have been attempted. However, current therapy for ASA-associated infertility is almost empiric and largely unproven.

KEYWORDS: antisperm antibody • antisperm immunity • infertility • sperm antigen • treatment

On average, infertility occurs in one in every five couples of reproductive age [1]. Among whom, there is 10–20% of unexplained infertility. A subset of infertile patients has been found to possess antisperm antibodies (ASAs) in semen, blood, cervical mucus or follicular fluid. Clinically, these patients are classified as being immunologically infertile.

It is widely accepted that immunoinfertility is one of the major causes of infertility in humans [2]. Investigations on the potential role of ASAs in infertile couples have been performed extensively during the past three decades. However, questions about the underlying mechanisms of ASA in reproduction, testing methods, the relationship between ASAs and infertility, and the treatment of ASA-mediated human infertility still remain. Moreover, standardized assays and valid treatment methods for ASA-mediated infertility are so far lacking [3]. In fact, research on ASA-related infertility is limited.

Antisperm antibody

An ASA is defined as an immunoglobulin with antibody activity against a sperm antigen that plays a role in fertility, since not every antibody that binds to the sperm surface influences sperm function [4]. However, methods

currently used to detect ASAs in serum, seminal plasma or cervical mucus and on sperm only measure those immunoglobulins that bind to sperm components. Whether these immunoglobulins had antibody activity remains uncertain.

The production of ASAs is closely related to the stimulation of sperm antigen. However, numerous antigens on the sperm surface are shared with somatic cells. Evidence is presented for common antigens or cross-reactive antigens on human sperm and T lymphocytes [5]. ASA titers in sera, sperm extracts and the seminal plasma of the infertile subjects were reduced significantly after absorption with sperm, thymocytes or the T-cell line CCRF-CEM. Moreover, lymphocytes from the infertile patients, when stimulated with pokeweed mitogen *in vitro*, produced antisperm and anti-T-lymphocyte antibodies at significantly higher titers than normal controls.

Similarly, the cross-reactivity of ASAs with other autoantibodies, including antithymocyte antibody, antinuclear antibody, antiphospholipid antibody, was also confirmed [6]. The apparent cross-reactivity between ASAs and other autoantibodies suggests that a polyclonal B-cell activation [6], similar to that seen in autoimmune diseases, occurs in patients with ASAs. In addition, the immunoglobulin heavy

chain cDNA sequences of ASAs showed extremely high homology with previously reported immunoglobulin germline DNA sequences, also implying that ASA might be natural autoantibodies [7].

Therefore, a question arose, which of the sperm antigens could detect ASA. If we want to detect ASAs that cause infertility, we must get standard sperm antigens closely associated with fertilization. An ideal sperm antigen should be sperm specific, accessible to the antibody and play a key role in fertilization [8]. In addition, the sperm antigen, alone or after conjugation with an appropriate carrier protein, should be able to raise a high titer and a long-lasting antibody response both in circulation and locally in the genital tract. ASAs in men should be induced by epitopes that are specific to sperm and located on the sperm surface; but how do we get such sperm antigens? There are not any definite answers at present.

Infertility-associated sperm antigens

Antisperm antibodies are composed of numerous antibodies interacting with multiple sperm components. The number of identified sperm antigens has increased since the 1990s. Shetty *et al.* reported on their results achieved using a 2D-electrophoresis technique [9]: 98 sperm auto- and isoantigenic protein spots were recognized by sera with ASA from infertile males and females but not from fertile subjects. Dozens of sperm-specific antigens were reportedly associated with infertility, including lactate dehydrogenase-C4 (LDH-C4) [10], fertilization antigen (FA)-1 [11,12], SP22 [13,14], GP20 [15], SP-10 [16], YWK-II antigen [1], rSMP-B [17], BS63 [18], HSAP [19], YLP(12) [20], PH-20 [21], Sptrx [22–26], proteasomal subunits [27,28], nuclear autoantigenic sperm protein (NASP), BS17 [1] and HED-20 (zyxin) (TABLE 1) [1]. Each antigen is a distinct and separate entity and is produced by different cells in the reproductive tract, such as germ cells, epididymal epithelial cells and Sertoli cells [29]. Many of their genes have been cloned and their recombinant proteins have also been acquired. However, the exact identification of the biochemical nature of the cognate antigens of ASAs is possible only after reliable separation of the sperm membrane proteins and confirmation of their potency to bind ASAs. Sperm membrane proteins could undergo changes in the antigenic characteristics during sperm maturation and capacitation, possibly by increasing accessibility of some sialic acid residues and of the core peptide, particularly the GPI anchor [30,31]. In fact, it was difficult to separate a single predominant target antigen identified to interact with all the ASAs yet.

In addition to those sperm-specific antigens listed in TABLE 1, many other antigens were reportedly related to activation of zona pellucida (ZP) binding, such as trypsin, proacrosin, acrosin and rabbit sperm autoantigen I; involved in acrosome reaction (AR; e.g., M42); participants in sperm–egg membrane fusion (e.g., PH-30 and M29); or involved in ZP penetration, such as MS 207.

Some new specific epitopes of sperm antigens were found recently by using phage-display technology instead of the recombinant antigens being researched. These new epitopes have higher specificity than recombinant antigens, and lower cross-reactivity with other shared antigens. Naz *et al.* [32] used this technology to delineate the peptide sequences that are involved in human immunoinfertility, and the peptide sequences present on human sperm cells that are involved in binding to human ZP. They used the antibody-positive sera from immunoinfertile men to screen seven clones that had dodecamer sequences in a random order, designated #2, PSAL-GRFTRGPL; #22, SLIFVTISSEWG; #69, LSLSLDLLTFRT; #77, PDIRHYFIQNRG; #81, GCRIVYRRPLHL; #84, RTAGFDIKLIDT; and #95, RIQYQAISTVSL. All seven peptides reacted strongly with the sera from infertile males, but not with the sera from fertile men. Due to the difficulty in identifying ASAs, our group screened phage libraries using specific ASAs from rabbits immunized with human sperm. Four dodecamer sequences closely associated with fertility were obtained. These specific epitopes have been found to be superior to other known sperm-specific antigens in the diagnosis of ASA (LU J-C *ET AL.*, UNPUBLISHED DATA).

In general, multiple antigens react with ASAs. These sperm antigens, which are involved in fertilization are still poorly understood. ASAs can only inhibit a single sperm epitope and function. No single sperm antigen has yet been found that can completely block the effect of ASAs.

Relationships of the level, subtype & *in vivo* distribution of ASA with infertility

Level of ASAs & infertility

Antisperm antibody concentration may be of importance in affecting sperm function. As the level of antibodies increases, sperm function could be impaired, and the chance of spontaneous pregnancy declines, since it is well known that men may father children in the presence of lower or equivocal titers of ASA [33].

Although sera from women, in general, have been demonstrated to have higher titers of autoantibodies than their male counterparts, levels of ASA titers were significantly lower in women than in men [6]. High titers of ASA measured in the seminal plasma or serum of men are believed to be the main cause of their infertility.

Subtypes of ASAs & infertility

It was reported that the subtypes of ASA focused on IgG, IgA and IgM. An elevated IgE response in subjects with significantly elevated ASA titers could be observed.

Both men and women with ASAs showed elevated total IgG levels compared with those of normal control subjects. IgG and IgM were primarily present in serum from infertile men and women. IgM was produced in the early stage when the human immune system contacted sperm antigens, and IgG arose approximately 2 weeks after the interaction between

Table 1. Features & antifertility effects of infertility-associated sperm antigens.

Sperm antigens	Features	Antifertility effects	Ref.
LDH-C4	140 kDa	Anti-LDH-C4 antibody enters the female reproductive tract and prevents sperm from fertilizing the egg	[10]
FA-1	Monomer (~23–24 kDa) or dimer (~47–53 kDa); its cDNA has been cloned and sequenced from the human testis	Antibodies to FA-1 can completely block sperm–ZP interaction	[11,12]
SP22	Located on the anterior-ventral surface of the equatorial segment of the sperm head, and on clear cells of the epididymal epithelium in rat	An antibody to SP22 prevented cauda epididymal sperm from fertilizing the oocytes in rat	[13,14]
GP20	A 20 kDa glycoprotein; it may be a sperm coating protein from the epididymis	Anti-GP20 antibody could block sperm penetration of zona-free hamster oocytes	[15]
SP-10	A sperm intra-acrosomal protein	A mAb to SP-10 inhibited sperm–oolemma binding	[16]
YWK-II antigen	Located on the equatorial sector of human sperm; belongs to the APP family; consists of 191 amino acid residues	The anti-YWK-II antibody can agglutinate human sperm, interfere with sperm-egg interaction, and arrest the growth and development of zygotes or embryos	[1]
BE-20 protein	It may be a proteinase inhibitor from the human epididymis; consists of 123 amino acid residues	Anti-BE-20 antibody agglutinated and immobilized human sperm and blocked their capacity to penetrate <i>in vitro</i> zona-free hamster eggs	[1]
rSMP-B	A sperm tail component of 72 kDa; hSMP-1 is coded by the HSD-I gene located on human chromosome 9, region p12-p13	Anti-rSMP-B antibody may immobilize sperm and block the <i>in vitro</i> penetration and fertilization of zona-free hamster eggs by human sperm	[1,17]
BS-63	A 66 kDa polypeptide containing 608 amino acid residues; located on chromosome 2 and at locus 2q11-q12	It is hypothesized that BS-63 is a testis-specific nucleoporin and possibly acts as a docking site and a cotransporter of Ran and transportin	[18]
BS-17 (calpastatin)	A 17.5 kDa glycoprotein; located primarily on the surface of the acrosomal region of human sperm, and slightly in the tail	Anti-BS-17 antibody blocks the <i>in vitro</i> penetration and fertilization of zona-free hamster eggs by human sperm	[1]
HED-20 (zyxin)	A 20 kDa glycoprotein produced by Sertoli cells	Anti-HED-20 antibody can immobilize and agglutinate human sperm, and inhibit the <i>in vitro</i> penetration and fertilization of zona-free hamster eggs by human sperm	[1]
HSAP	A sperm-specific protein consisting of 328 amino acids, and first cloned from a human testis; located in the acrosomal compartment	The anti-r-HSAP antibodies bind with human live sperm, and inhibited sperm binding/attachment in zona-free hamster penetration assay	[19]
YLP(12)	A dodecamer sequence (YLPVGGLRRIGG) described by Naz <i>et al.</i> ; located primarily on the acrosome and tail regions of human sperm; recognizes the ZP3 component of human ZP	Antibodies to synthetic YLP(12) peptide inhibited human sperm–human zona binding	[20]
NASP	A testicular histone-binding protein consisting of 787 amino acids	Most vasectomized men developed autoantibodies of NASP	
PH-20	A cognate antigen of ASA in the humans	PH-20 is involved in zona penetration	[21]
Sptrx	Sperm-specific thioredoxins, including Sptrx-1, -2, -3; located in the spermatocytes, the tail of spermatids and sperm, and the cytoplasmic droplet of ejaculated sperm	A postobstruction potential autoantigen	[22,25, 26]
Proteasomal subunits	ζ-chain and component C2 of proteasome, sperm membrane proteins	Antisperm antibodies in infertile men could bind with them	[27,28]

APP: Amyloid precursor protein; LDH: Lactate dehydrogenase; mAb: Monoclonal antibodies; NASP: Nuclear autoantigenic sperm protein; ZP: Zona pellucida.

sperm antigens and immune cells. However, IgG could present in serum for a long period of time, whereas IgM levels decrease gradually.

In the genital tract, IgA was primarily present in semen, cervical mucus or follicular fluid, since the production of IgA is closely associated with mucus immunity. It was reported that the infertile men had more IgA and other secretory components in semen than controls, and that IgA was considered responsible for the reduced penetration of spermatozoa in cervical mucus and for the 'shaking phenomenon' (jittering *in situ*).

Antisperm antibodies can also influence the outcomes of *in vitro* fertilization (IVF) and the specific effect is dependent upon the subtypes of ASAs. Several authors reported that the presence of both IgG and IgA, IgM alone or IgA alone was associated with a reduced rate of fertilization [33]. In the meantime, investigators found that IgA only had an impact when a high level of binding was detected on the sperm head, and that IgM was the immunoglobulin isotype that significantly affected the fertilization rates when located at the sperm head and sperm-tail tip [33].

In vivo distribution of ASAs & infertility

Antisperm antibodies can be detected in serum, semen, cervical mucus, oviductal washings and follicular fluid. It was observed that there was no relationship between antisperm activity in the oviductal washings and that in the sera, and that the presence of ASAs in sera was not necessarily reflected in individual's genital tract secretion and *vice versa*. For infertile men and women, it appeared reasonable to test locally produced ASAs for their contribution to conception failure.

ASAs & semen parameters

There were contradictory data on the relationship between the presence of ASAs and semen parameters, such as sperm concentration, motility, viability and morphology. Most published results showed that there was no definite relationship between the presence of ASAs and sperm concentration, motility, viability and morphology [34]. However, serum titers of ASAs were associated with decreased sperm counts and normal forms [35]. The immobilizing ASAs were able to decrease sperm motility and viability.

Mechanisms of immune response against sperm & ASA-induced infertility

The human immune system is trained during the early postnatal period. In men, at puberty when the sperm first appear in the testis and epididymis, the human immune system will have the chance to contact sperm antigens. Similarly, when women become sexually active, their immune system will inevitably contact sperm antigens. Therefore, once sperm, as an autoantigen, activates the human immune system, an autoimmune response against human sperm will occur.

Protective mechanism preventing ASA production

The blood–testis barrier and the epididymal blood–epithelium barrier in humans are important structures in preventing sperm antigens from contacting immunocompetent cells, due to the tight junctions of Sertoli and epithelial cells [36]. This creates favorable conditions for spermatogenesis and sperm survival in the testicular fluid, and sperm maturation in the epididymal fluid. It also prevents the occurrence of autoimmunity after puberty. Therefore, alteration of the blood–testis barrier and the blood–epithelium barrier allows the production of ASAs and, hence, may lead to infertility.

Various protective mechanisms besides the blood–testis barrier and the blood–epithelium barrier have also been identified. Immunosuppressive substances have been found in semen and follicular fluid. Moreover, suppressor T lymphocytes in the human immune system, which partially mediate the normal state of immunologic unresponsiveness toward sperm autoantigens, also play an important role in preventing the autoimmune response. In addition, the cervix is a site of sperm filtration, and uterine fluid has significantly high concentrations of IgG and IgA, which lead to increased phagocytosis of intact or defective sperm, and corrupted sperm debris. Moreover, components of seminal plasma and polymorphonuclear neutrophils in semen could eliminate nonviable sperm or debris [37].

Recently, three complement-regulatory proteins, decay-accelerating factor, membrane cofactor protein and CD59, have been found on spermatozoa and in the extrafetal tissues [38]. It is likely that these inhibitors are essential for normal reproductive function.

A soluble form of Fc γ RIII (CD16) has been isolated from seminal plasma, which may modulate immunosuppression of antisperm immune responses in both male and female reproductive tracts. Individuals who were ASA positive had lower detectable levels of Fc γ RIII compared with those who were ASA negative, indicating that the inhibition of CD16 plays an important role in preventing ASA production [39].

Factors leading to ASA production

During maturation of spermatogenesis, new antigens are expressed on developing spermatocytes and spermatids. When these antigens come into contact with immunocompetent cells, or spermatozoal antigens are exposed to the mucosal and systemic immune systems, ASA formation occurs. Developmental abnormalities of the formation of the blood–testis barrier, traumatic disruption, infection or unilateral focal cryptic obstructions could lead to immunogenic sperm antigens being exposed to the immune system thus initiating an immune response to produce ASAs [40]. Gastrointestinal exposure to sperm was also associated with the development of ASAs in homosexual men. In addition, in some cases, immunity to sperm cells may be the result of altered sperm antigens.

Chronic infection

A chronic infection, such as *Chlamydia trachomatis*, which is capable of eliciting an immune response has been demonstrated in male and female genital tracts. Several authors reported that

Hsp60 in human seminal fluid was associated with the formation of ASAs [41]. Heat-shock protein (Hsp)60 is associated with a humoral immune response to *C. trachomatis* or other infection agents, indicating that genital infection could be an important factor causing ASA production.

The possible relationship between ASA production and *Ureaplasma urealyticum* infection was also investigated. It was confirmed that *U. urealyticum* and human sperm membrane proteins share cross-reactive antigens (61, 50 and 25 kDa) [42]. Among the cross-reactive antigens, the urease complex component UreG of *U. urealyticum* was determined. Furthermore, the cross-reaction between human NASP and UreG was verified. Both anti-rUreG antibodies and antiserum against synthetic peptide NASP393–408 inhibited mouse sperm–egg binding and fusion. After immunization with rUreG or synthetic peptide, 81.2 and 75% of female mice became sterile, respectively [42]. These findings proved that the cross-reactive antigens shared in sperm and microorganisms induce ASA production and infertility.

Vasectomy & vasovasostomy

Vasovasostomy has become a popular and highly successful method for restoring fertility to those who have undergone a vasectomy. It was reported that there was a high correlation between vasostomy and ASA production. More than 50–70% of men develop ASAs after a vasectomy, and there is limited success in the regain of fertility, even after successful surgical reanastomosis in vasovasostomy attributed to the presence of ASAs [43]. The highest incidence of titers is 1 year after vasectomy, but titers can be found as early as 6 months or as late as 20 years post-operatively. These ASAs are causative factors of infertility, because disappearance of ASAs causes regain of fertility.

However, considerable disagreement remains as to whether antibodies are the primary causative agent. No significant changes were observed in the prevalence of the antibodies over the period following vasectomy, and in patients with and without postoperative sperm granulomas [44]. Sperm antigens are in abundant supply in vasectomized men as a result of the continuous resorption of spermatozoa after vasectomy. Therefore, it is possible that undetectable antibody titers reflect high levels of ASAs circulating as immune complexes.

Other factors

Heavy metals can also adversely influence reproduction since in sensitive individuals they are able to alter the immune responses including autoantibody production, this can then cause infertility [45]. For example, after mercury stimulation, less IFN- γ and more ASAs were produced by the lymphocytes of patients [46]. This suggested that the release of metal ions from dental materials can be one of the stimulating factors that may adversely affect fertility.

Testicular cancer and testes torsion patients also have a markedly high incidence of ASAs [47], which may be related to radiation therapy and severe damage of seminiferous

tubules. However, it was observed that no rabbits had detectable levels of antisperm IgG after testes torsion compared with controls.

In addition, a high ASA rate (43.1%) was observed among prostitutes [48]. It may be related to repeated inoculations with multiple sperm antigens and/or microorganisms.

Mechanisms of ASA-induced infertility

Immunologic infertility is a consequence of the combined actions of multiple ASAs [1]. ASAs can be present at different sites, can react against different antigens, and can impair fertility in various ways. ASAs can negatively affect sperm motility, cervical mucus penetration, the AR, ZP recognition and the fusion of gametes, by immobilizing and/or agglutinating sperm, blocking sperm–egg interaction, preventing implantation, and/or arresting embryo development [1,49]. The ASAs only have functional significance when they are fixed to their antigens. ASA directed against the sperm head were of primary importance, whereas those against the tail were involved in poor motility. Moreover, ASA-coated sperm may be more vulnerable to phagocytosis in the female reproductive tract [33].

ASA decreased motility

Modifications of sperm motility were the main cause of infertility in some patients, resulting in blockage of transport to, and union with, the oocyte. However, how do ASAs interfere with sperm motility? ASAs can bind to antigens of sperm membranes, but subcellular structures can not be reached by ASAs in living cells. Therefore, it is speculated that the function of transmembrane proteins may be altered by the ASAs concerned. Another possible explanation is via complement-mediated membrane damage.

ASAs could inactivate human sperm motility in the presence of complement, showing that complement-dependent inactivation of sperm motility might be the biological mechanism of female infertility [38]. IgG ASAs were capable of activating complement and depositing MC5b-9 on human sperm. Meanwhile, the concomitant detection of sperm-bound IgG and the initial (C3d) and terminal (C5b-9) complement components on the surface of human sperm could be confirmed using a flow cytometric assay [50]. IgG- and C3-bound motile sperm adhered to human neutrophils *in vitro*. According to scanning electron microscopy, most of the sperm were linked to the neutrophil by the acrosomal region of the sperm head. This adhesion potentiated the localized release of oxygen radicals at the site of neutrophil/sperm membrane contact. The deposition of activated C3 fragments, the assembly of terminal membrane attack complexes (C5b-9) and oxygen radicals could lead to C3-mediated sperm binding to neutrophils or C5b-9-mediated sperm-motility loss [51], since incubation of motile sperm with complement-fixing immune sera resulted in a significant loss (43–87%) of motility, which was associated with characteristic C5b-9-induced alterations in sperm morphology ultimately leading to sperm lysis [52].

Only the coincubation of neutrophils with sperm in the presence of complement-fixing ASA⁺ sera resulted in marked neutrophil aggregation (20.5 ± 0.26 vs $2.4 \pm 1.6\%$; $p < 0.0001$), a concomitant increase in neutrophils containing ingested sperm (71 ± 5.8 vs 3.5% ; $p < 0.0001$) and maximum expression of CD11b antigen. CD11b upregulation correlated with a significant ($P < 0.05$) increase in tyrosine phosphorylation of neutrophil proteins during sperm phagocytosis. Only the combination of monoclonal antibodies directed to the $\beta 2$ -integrin, CD11b (D12/SHCL-3), or CD18 (MHM23) subunits maximally inhibited ASA- and complement-mediated sperm adhesion to neutrophils (by 70%) or sperm phagocytosis (by 75%), as well as neutrophil aggregation (by 96%) [53]. These findings strongly implicate the CD11b/CD18 glycoprotein complex (CR3) in the adhesive events involved in ASA- and complement-mediated immune destruction of motile sperm by neutrophils. It also substantiated the presence of a receptor on sperm involved in susceptibility to immobilization [31].

ASA-affected sperm penetration of cervical mucus

The presence of ASAs in cervical secretions is not frequent but nevertheless when present it is a severe cause of infertility [54]. ASAs may influence sperm penetration of cervical mucus by the immobilization and shaking phenomenon of sperm in cervical mucus. Surface antigens of the acrosome and sperm tail principal piece appear to be recognized by circulating sperm-immobilizing and -agglutinating antibodies.

Impairment of sperm penetration into the cervical mucus appears to be of consequence in the following mechanisms: the activation of the complement cascade by immunoglobulins attached to the sperm surface, at the end of which cell lysis and initiation of the phagocytotic process may take place. The complement induced cell lysis depends on the immunoglobulin class of the antibody. IgM is far more effective than IgG, while some IgA subclasses are unable to interact with the early complement components. During their residence in the cervical mucus, sperm are exposed to complement activity. The complement activity in cervical mucus is approximately 12% of that of serum. Thus, it may take longer for sperm immobilization to occur.

In addition, binding can also occur between the Fc region of the antibody and the cervical mucus [55]. This process could contribute to the binding of spermatozoa to the antibody and the immobilization of this complex by the cervical mucus that is seen in immunological infertility.

ASA-affected AR

There is a large data pool on antigens involved in the AR and antibodies to these antigens [1]. ASA binding to the sperm head influences the AR [49]. A number of spontaneously occurring ASAs were shown to enhance the number of acrosome-reacted sperm [28]. An antiactin monoclonal antibody significantly inhibited the ZP-induced AR, the ionophore A23187-induced

AR and hyperactivation of sperm in medium [56]. Other ASAs or antigens inhibiting the spontaneous AR include TSA-1, FA-1 and calpastatin [57].

The action of mononuclear cells in ASA production leading to infertility

Antibody-coated sperm may activate lymphocytes, thus leading to the production of ASAs. This may be an additional mechanism that leads to infertility. Mononuclear cells include lymphocytes, monocytes and macrophages, which may play a role in infertility caused by ASAs. Cytokines secreted by these mononuclear cells may also have roles, however, these are unclear at present.

T lymphocytes bearing the $\alpha\beta$ - and $\gamma\delta$ -antigen receptors were present in semen samples. Moreover, the presence of ASA was associated with elevated concentrations of both $\gamma\delta$ and $\alpha\beta$ T cells [58]. The $\alpha\beta$ and/or $\gamma\delta$ T lymphocytes and/or monocytes/macrophages in semen might be a source of Hsp60, since a large fraction of $\gamma\delta$ T lymphocytes are specifically activated by Hsp60. Moreover, $\gamma\delta$ T cells could induce Hsp60 expression in reverse [59]. Thus, the interaction of T lymphocytes with Hsp60 may be responsible for ASA production.

However, not all men with ASAs were positive for seminal Hsp60. It has been reported that no significant difference was found in the concentration of leukocytes or subpopulations of these cells (monocytes, lymphocytes and granulocytes) between fertile, sterile without ASAs and immunological sterile groups [60]. These contradictory data indicated that the association between chronic infection of the male genital tract, $\gamma\delta$ T-cell activation, antisperm autoimmunity and Hsp60 expression requires further exploration. It is also possible that both the presence of ASAs and the increase of leukocytes in semen are manifestations of an immunological response or a common etiological factor of genital tract infections, and they are not interrelated.

In addition, a significant increase in large granular lymphocytes (LGL) was detected in semen samples from immunological sterile patients when compared with fertile ejaculates and sterile ejaculates without ASAs. The LGLs form the population responsible for antibody-dependent cellular cytotoxicity (ADCC). An expansion of these cells is reflected by an increase in ADCC effector cells. Immunoregulation by ADCC induces the disappearance of the antigens recognized by each antibody. Thus, the appearance of ASAs in an ejaculate could generate the expansion and activation of LGLs to decrease ASA production by B lymphocytes. Nevertheless, LGL activity may be a specific cellular mechanism operative in limiting the fertilizing ability of these spermatozoa.

Antisperm cell-mediated immunity (CMI) has been associated with infertility in men and women. Sperm antigens can specifically induce CMI factors that have detrimental effects on sperm motility and preimplantation embryos. Products of activated lymphocytes and macrophages (lymphokines and monokines) affect the fertilizing ability of human spermatozoa

in the zona-free hamster egg-penetration test [61], indicating that soluble factors produced by activated lymphocytes and macrophages in reproductive tissues could be significant mediators of immunologic infertility.

However, antisperm CMI responses have been observed in all individuals of the following groups: infertile men and women, fertile women, virgin women, oligozoospermic men, and men with andrological disorders. Therefore, the role of antisperm CMI in reproductive processes also remains enigmatic.

In general, mechanisms by which ASAs inhibit fertility are unclear and may be unique to each individual's antibodies. Antisperm immunization has only a relative effect on impairment of fertilization, rather than an absolute effect. In any case, the interference of ASAs at the level of gamete interaction is more than that at other levels [33].

To what extent did ASAs affect fertility?

It was reported that the fertilization rate in the antisperm group (61.3%) was significantly lower than that in the control group (76.8%). However, the implantation rate per embryo transferred (23.5%) and, consequently, the modified pregnancy rate per oocyte recovery procedure (34.4%) in immunologically infertile women were significantly higher than those in the control group (7.9 and 17.8%, respectively). Although sperm-immobilizing antibodies prevent sperm–egg interactions, they do not seem to have any adverse effects on the likelihood of pregnancy. Moreover, the occurrence of a cellular or humoral immune response against sperm seems to augment the uterine receptivity for the implantation of fertilized ova or blastocyst, and to be favorable for successful pregnancy by the IVF–embryo transfer (ET) procedure [62].

Similarly, antibodies to several sperm molecules inhibit sperm–oocyte interaction/fertilization *in vitro*. Interestingly, active immunization with many of these molecules does not inhibit fertility *in vivo*. In addition, gene knockout animals of many of these molecules do not inhibit fertility. For example, although antibodies to fertilin/PH-30 inhibit fertilization *in vitro* [63], active immunization with fertilin/PH-30 does not affect fertility *in vivo*; although antibodies to sperm integrins $\alpha 6$ and $\beta 1$ inhibit sperm–oocyte fusion *in vitro*, gene knockouts of these molecules do not affect the fertility *in vivo* [64].

In spite of the difficulty to explain these contradictory results, it was generally accepted that most autoantibodies occurring in biological fluids do not cause autoimmune diseases. For ASAs, if the cognate antigen is not involved in the process of fertilization or the antibodies do not bind to the functional domain of the antigen, these sperm autoantibodies will not alter sperm function. Therefore, different effects between *in vitro* and *in vivo* may be a result of ASA–sperm antigen binding *in vitro* and ASAs untouchable to sperm antigens *in vivo*. Moreover, the antibody-binding region of the antigen may be different from the region being active in metabolic processes [4]. Several authors reported that there was no statistically

significant difference in the pregnancy rate between those women with and without ASAs [65]. Of course, conflicting opinions about the effects of ASAs on fertilization could be due to inadequate experimental approaches in evaluating antisperm immunity [66].

Another challenge is to establish an appropriate animal model to investigate the efficacy of a sperm antigen. The commonly used animal model is the mouse. However, until now, no one has reported a 100% block in fertility after immunization with any single antigen in a murine model. Even immunization with the whole sperm or their solubilized preparations does not cause a total block in fertility of male or female mice. The maximum reduction in fertility after immunization with any antigen/sperm preparation is up to 70–75%. Very few, if any, knockouts of a single gene have made mice totally infertile. The recently reported Izumo gene knockout and the double knock out of oocyte proteins CD9 and CD81 that could interact with sperm Izumo did make the male mice almost totally infertile [67,68]. It remains to be seen whether the 70–75% reduction in fertility in a mouse model could be translated to a 100% reduction in humans. Promisingly, after active immunization or deleting a single gene, a few mice were found totally infertile.

In conclusion, sperm antigens and ASAs appear to have both adverse and favorable effects, with some even having no effect on fertility. But when do they act on fertility, and to what extent do they affect fertility? All these questions are yet to be answered.

Evaluation of ASA-assay methods

In clinical andrology, an ASA assay has been considered to be a necessary step for the assessment of male infertility. This practice is generally accepted even though there is still some disagreement surrounding the true meaning of antisperm immunity, and there remains a good deal of controversy about the test, which is regarded as the most suitable for the detection of antibodies directed against sperm antigens. There are many ASA assay methods, each with advantages and disadvantages. However, until now, it was difficult to address which test is optimal.

The requisite of an ASA assay is the preparation of sperm antigens. Sperm antigens used in the assay were prepared by different methods:

- Antigen extract was made by treatment of freeze-thawed sperm pools with 0.3 mol/l lithium 3,5-diiodosalicylate or detergents, such as Tween-100 and NP-40;
- Sonicated sperm antigens: whole spermatozoa were disrupted with sonic bursts on ice, and the supernatants were obtained by supercentrifugation;
- Cavitated sperm antigens: sperm pellets were placed in the chamber filled with nitrogen, and the cavitated sperm suspension was made by the sudden release of pressure;

- Various recombinant sperm antigens prepared by gene engineering;
- Sperm antigen epitopes prepared by phage-display techniques.

The sperm pool used in the first three methods should also be obtained by Percoll technique, Ficoll or swim-up so as to prevent leukocyte contamination. It appears to be accepted that outer sperm-membrane antigens are of relevance, for ASAs are not able to penetrate the outer cell membrane of vital sperm [69]. The exception to this rule concerns acrosomal antigens, for the inner acrosomal membranes of the spermatozoon become externalized and antigens are presented during the AR, which are otherwise not recognized [4]. Owing to sperm antigens from different individuals varying widely the method to select adequate sperm donation is yet to be fully elucidated.

Sperm agglutination tests

Sperm agglutination tests include glass agglutination test, the tray agglutination test and the gelatin agglutination test. Unfortunately, this assay requires fresh motile spermatozoa. Moreover, individual sperm may vary in their antigenic expression.

In agglutination tests, agglutinates could be formed due to bacteria and amorphous material within semen, nonspecific immunoglobulin, as well as nonimmunoglobulin proteins in serum, leading to a false-positive reaction. The regional specificity of antibody binding limits the formation of agglutinates of a relatively large size. In addition, agglutination tests could not be used to identify the classes of immunoglobulins implicated. Therefore, these tests are not recommended for routine analysis.

Sperm immobilization test

Sperm immobilization test (SIT) involves counting motile sperm under the microscope, and is subjective as it relies on the judgement of the examiner with respect to sperm motility. Komori *et al.* analyzed motile sperm by a digital method using Sperm Quality Analyzer instead of the conventional method [70], which may be of more value in the measurement of motile sperm in determining sperm-immobilizing antibodies. However, because complement-mediated sperm-immobilization assays are limited in their usefulness for the detection of IgA, since IgA does not fix complement; and many sperm head-binding antibodies do not lead to a loss of sperm motility, which might give false-negative results, SIT is also not recommended.

Immunobead test & mixed antiglobulin reaction

Immunobead test (IBT) and mixed antiglobulin reaction (MAR) methods are very easy, inexpensive, and rapid, such as with similar sensitivity and specificity, detection mechanism and operation and therefore, were recommended by the WHO as the 'standard' laboratory technique for the determination of sperm-bound ASAs [71]. They can be used to identify the classes of ASAs and the proportion of sperm-binding antibodies, but are not able to identify specific antigens, the number of antibody molecules bound to the sperm surface and the place of fixation because the

relatively large 'labels' (immunobeads, latex particles and erythrocytes) limit resolution. Moreover, the spermatozoa from different donors were found to differ significantly in the degree and location of immunobead binding after exposure to the serum sample containing ASAs.

In addition, these two methods have different standards judged for positive, in general, for 10%, but also 20 or 50%. Moreover, it is impossible to discriminate which antibodies are of clinical relevance and which are not [4].

Immunofluorescence assays

Immunofluorescence assays (IFAs) seem to be valuable tools in the diagnosis of immune infertility, in particular when impairment of the acrosome function is suggested [49]. However, internal sperm antigens exposed after plasma membrane damage or the use of methanol fixation may lead to false-positive results. Moreover, the determination of regional specificity of sperm-binding antibodies is limited by the low resolution of these methods.

IFAs can only detect the immunoglobulin class of the antibody concerned and the proportion of sperm-binding antibodies, but are not able to detect the number of antibody molecules or antigens involved in the binding. Moreover, it is impossible to discriminate which antibodies are of clinical relevance and which are not [4], and results based on IFAs are unreliable and lacking in correlation with continuing infertility. Thus the routine use of IFAs is not recommended. The use of an immunogold assay, which is similar to IFA, has the same shortcomings.

Enzyme-linked immunosorbent assay

ELISA could be used to detect ASAs by the antigen absorbed in the solid phase, and antihuman globulin conjugated to alkaline phosphatase or horseradish peroxidase as a developing reagent. The ELISA assay enables detection of IgM, IgG and IgA ASAs in serum, cervical mucus and seminal plasma samples. Moreover, ELISA is more sensitive, less subjective, does not need fresh sperm and easier to perform than other commonly used microscopical methods (SIT, IFA, IBT and MAR). However, ELISA requires fixation of whole sperm or use of membrane extracts. Fixation of sperm may lead to denaturation of sperm antigens or membrane damage, resulting in false-positive or false-negative results. Membrane extracts may not include relevant antigens associated with the process of fertilization. Recently, our group investigated the variations of the results on the detection of ASAs among four different brands of ELISA kits, with up to ten-times the difference of positive rates (LU J-C *ET AL.*, UNPUBLISHED DATA), indicating that the detection methods of ASAs urgently need to be standardized, especially the preparation procedures for sperm antigens.

Radiolabeled antiglobulin assays

Radiolabeled antiglobulin assay (RIA) is another means of identifying ASAs. Similar to ELISA, RIA provides no information about the proportion of antibody-bound sperm or the regional specificity of ASAs. Both RIA and ELISA were hampered by the fact

that the antigens were not biochemically defined and those relevant for the process of fertilization might not be included in the antigen preparation. In addition, seminal plasma itself interferes with the antigen–antibody reaction in such an immunoassay [72].

Flow cytometry

Hjort [73] thought that flow cytometry seemed to be a promising technique to determine the exact amount of IgA and IgG on individual sperm. However, this method does not always provide objective and reproducible results [74]. Moreover, few medical centers and university laboratories have access to a flow cytometer, and the practical use of flow cytometry has been limited by the need for a skilled operator and expensive instrumentation [75].

Other assays

Immunoblotting and affinity chromatography were reportedly the minors methods for the detection of ASA [76]. However, antigens may be denaturated during the antigen preparation. In addition, a lymphocyte proliferation assay was used to detect CMI. However, Focacci *et al.* [77] observed no relationship between serum ASAs, and sperm CMI, indicating that the lymphocyte proliferation assay did not appear to be useful in the diagnosis of immunological infertility.

Identity of different assays

The ASAs detected by different methods are not identical. Comparative investigations using various assays to detect levels of antibodies to human spermatozoa have produced widely varying results. A possible explanation for the observed wide variation in results from different methods to detect ASAs may be the different target antigens used in assays. Comparison of methods between laboratories could be improved only if equivalent target sperm preparations were used. It is important to validate assays, such as the ELISA, before widespread application to patient screening particularly. In general, no single test to detect the presence of ASAs has correlated precisely with subsequent fertility.

In conclusion, although there is reason to accept antibody-mediated antisperm immunity as a cause for subfertility, the routinely used methods are not reliable for the detection of specific ASAs. The methods described so far have been hindered by codetection of undesired nonspecifically bound antibodies. Moreover, the lack of a standardized and universally accepted assay for the determination of ASAs may be the reasons why physicians are not inclined to test ASAs in subfertile couples [4]. At the moment, it is difficult to consider the routinely used ASA tests as essential procedures in the fertility work-up. It is even more difficult to justify a treatment on the basis of such tests.

Treatment of ASA-mediated human infertility

The identification of functionally relevant ASA antigens is a prerequisite for treatment options. Currently, no antibody-specific treatment of autoimmune diseases is possible, but the

treatment is based on the suppression of antibody production in general.

Several methods have been attempted for the treatment of ASA-mediated infertility [2], including immunosuppressive therapies, assisted reproductive technologies, laboratory techniques and integrative Chinese and Western medicine (ICWM) treatment [78]. However, currently used therapy for ASA-associated infertility is empiric and largely unproven. The results are usually disappointing. It justifies a therapeutical approach in the presence of high levels of antibodies.

Condom therapy

Reduction of antigenic load is advised for infertile women. From an immunologic point of view, condom therapy, although it may work, is illogical. Although the antibody activity declined from 1:256 to 1:8 after 7 months of condom therapy, antibody titer will rise when no condom is used. In fact, results of condom therapy were disappointing. Therefore, condom therapy is now less used.

Immunosuppressive therapies

Steroids, such as corticosteroids, hydrocortisone, prednisolone or ciclosporin, have been used to treat those patients with ASAs. Corticosteroids suppress antibody levels and thus improve certain spermatozoan parameters and enhance fertility in such patients. Hydrocortisone may inhibit the production of ASA, in cervical mucus. However, the overall results of all the steroid-treated patients showed significant differences in performance. The results of the patients who were treated with prednisolone for three cycles showed a clear reduction of sperm-bound IgG antibody levels in three out of 11 (27%) patients, while only IgA was reduced in two out of 11 (18%) patients, and in one patient the level of sperm antibody even increased [79]. Also, high-dose corticosteroid therapy was ineffective in achieving pregnancies induced by infertile men positive for ASAs [80]. Moreover, there was no relationship between the decline of antibody titer and the occurrence of pregnancy. The data suggested that steroid therapy may be ineffective for immune infertility. The fluctuations of antibody levels in some individuals may be the result of drug administration.

Most importantly, side effects of corticosteroids should not be underestimated in healthy men [80]. A total of 63% of patients complained of weight gain, dyspepsia, irritability, acne or tingling sensations. Several reports showed that the side effects were less marked with β -methasone than with methylprednisolone.

Assisted reproductive technologies

Assisted reproductive technologies include intrauterine insemination (IUI), gamete intrafallopian transfer, IVF, and intracytoplasmic sperm injection (ICSI). Each patient who will undergo the various techniques of insemination or IVF should have the ASA assay performed as a preliminary screening [33]. While IUI can diminish the level of sperm-bound immunoglobulins and can improve the chance of conception in a

selected group of couples with a diagnosis of immunological infertility, insemination can induce the production of ASAs and increase the ASA titer in other groups. With intraperitoneal insemination, the immediate and intense effect of direct microtraumatic contact of the pool of sperm with peritoneum could cause ASA production, which can block sperm passage through the female genital tract and interfere with the fertilization process, and this can reduce the chance of a successful assisted reproductive treatment. In this case, only ICSI seems able to overcome the problem [33,81,82], for fertilization and pregnancy rates of ASA-positive patients undergoing ICSI maintains in the same range as ASA-negative patients [83]. Moreover, in assisted reproduction, when there are several oocytes to be inseminated, the chance of fertilization rises. These data indicate that ASAs did not hamper embryonal development and implantation, but further investigations on the development of fetuses are necessary.

Laboratory techniques

In cases of male immune infertility, the use of various techniques for semen manipulation, such as sperm washing, swim-up, dilution, cryopreservation, immunomagnetic sperm separation and proteolytic enzyme treatment, has been proposed in order to elute the ASAs bound to sperm and obtain ASA-free sperm pools.

Sperm washing can diminish the level of sperm-bound immunoglobulins and thus improve the chance of conception in a selected group of couples with a diagnosis of immunological infertility. The percentage of spermatozoa bound to IgG decreased significantly by rapid dilution and washing of the semen containing ASAs. Cumulus/oocyte complex washing and/or enzymatic cumulus removal are also considered as selective interventions in the case of antisperm immunity. However, because of the difficulty to elute the antibodies from the sperm surface by any method, these techniques are discouraging. Moreover, the rate of fertilization was not improved [84]. Treatment of ASA-positive seminal fluid using Percoll gradient separation is ineffective for removing ASAs bound on sperm [33]. Another approach was reported to elute ASAs bound to sperm, that is, modifications of sperm membrane taking place during capacitation of sperm [33]. Indeed, this approach changed the nature of antigens, declining their reactivity with ASAs. If so, the detection of ASAs is unnecessary. Moreover, whether there was new ASAs or not remains unclear.

FA-1 treatment

FA-1 can remove autoantibodies from the surface of sperm cells of immunoinfertile men by immune adsorption and permit an increased AR [85]. Moreover, the IUI of FA-1 antigen-adsorbed antibody-free sperm caused normal pregnancies and healthy babies, indicating that the antigen treatment does not have deleterious effects on implantation or on embryonic and fetal development. Similarly, can other sperm antigens that are involved in immunoinfertility be applied in the therapy of immunoinfertility? If the antigen has a real effect on fertility, it

can be applied not only to the specific diagnosis of immunoinfertility but also to the treatment. More research is needed to confirm this approach.

Soluble complement receptor 1 treatment

It was reported that soluble complement receptor (sCR)1 can treat complement-mediated sperm injury, for sCR1 abrogated the binding of C3 fragments to human sperm and fully protected sperm from C5b-9-mediated sperm immobilization. Moreover, CR1 inhibited ASA- and complement-mediated neutrophil aggregation by 46% and sperm phagocytosis by 57% [51]. These findings suggested the therapeutic potential of sCR1 to prevent complement-dependent sperm dysfunction.

Fab fragments treatment

In vitro treatment of sperm with antisperm Fab fragments had a protective effect from isoimmune attack *in vivo*. Successful use of Fab fragments for reversal of ASA-mediated infertility observed in the isoimmune rabbit model offers the prospect of a new means of restoring fertility in some isoimmune women [86].

Chymotrypsin treatment

It was shown, by immunobead binding, that immediate exposure of spermatozoa to D-galactose in the presence of chymotrypsin resulted in a considerable decrease or total loss of bound ASAs in males who had previously had a high titer of antibodies [55]. This pretreatment regimen for the ejaculate is suggested as a form of therapy for infertility related to the presence of ASAs.

Plasmapheresis & hemosorption

The effects of plasmapheresis and hemosorption on the efficacy of the treatment of autoimmune male infertility (AMI) were studied in 289 AMI males with oligoasthenozoospermia aged 19–37 years [87]. Patients of group 1 with high ASA levels in the blood but low in ejaculate received a course of plasmapheresis. Patients of group 2 with high ASA levels, both in the blood and ejaculate were subjected to hemosorption and plasmapheresis in one contour. Group 3 patients with high levels of ASAs in ejaculate but low in blood received efferent therapy only after medication and photomodification of blood. The treatment reduced elevated levels of ASAs in blood and ejaculate, normalized free-radical oxidation, cellular and humoral immunity and antioxidant defense. This resulted in improvement of spermogram parameters, efficacy of assisted reproductive technologies, and a higher probability of natural pregnancy in the patients' wives. However, similar reports are few.

Chinese medicine treatment

Although it remains a mystery, the combined therapy of acupuncture with herbal drugs has reportedly definite therapeutic effects on male immune infertility [88]. Chinese medicine Yiqi-huoxuetang (YQHXT) could inhibit ASAs by maintaining the balance of T-lymphocyte subpopulations in immunoinfertile

men [89]. Herb medicine could treat immune infertility accompanied by chronic seminal vesiculitis. The results showed that ASA tests became negative in 85.6% of the patients after treatment, and the pregnancy rate of their wives was 49.1% [90]. In addition, ICWM therapy could effectively regulate autoimmunity and endocrine function [2]. Since most of the available techniques, such as the aforementioned methods, have side effects, are invasive and expensive and have low efficacy, or have conflicting results, Chinese medicine treatment may be a relatively adequate choice, although their action mechanisms remain unclear.

Except the aforementioned methods, monoclonal antibodies were used in the treatment of autoimmune diseases [4] and may be adapted for the treatment of autoimmune infertility. In addition, zinc therapy (250 mg twice daily for 3 months) has a role in reducing the levels of ASAs and TNF- α and increasing that of IL-4 [91].

Expert commentary & five-year view

It is difficult to delineate the appropriate fertility-related antigens. Although ASAs were found in the sera of infertile couples, and reportedly have correlation with poor fertility rates, ASAs may also occur in men proven fertile. Further exploration on screening of appropriate fertility-related antigens is necessary in the future. The identification of specific sperm-antigen epitopes and recombinant proteins should be the main direction in the field.

There is wide variability among individuals. This may be the reason why the detection of ASAs has low predictive value and its clinical significance in infertility is uncertain [92], and does not influence the fertility prognosis. Moreover, ASAs have been shown to be directed against several different antigens, and each have different effects on sperm function, with some even having no effect on sperm function. Are there some common fertility-related antigens among individuals? Their identification

may be very important for the diagnosis of immunoinfertility.

The mechanism by which infertility is generated was unclear. Although various factors influenced fertility and possible mechanisms of action have been reported, it is difficult to explain why some antigens could block *in vitro* fertilization but not fertility *in vivo*. Every mechanism may play a small role in infertility, or one mechanism may operate in a small proportion of infertile couples. Therefore, the exact mechanism causing infertility remains unknown. The characterization of sperm antigens relevant to fertility will help to clarify the mechanism of ASA-induced infertility.

There is no standardized ASA assay. Although the WHO recommended IBT and MAR as the standard methods for ASA assay, these two methods are actually rarely used in clinical andrology laboratories, and were challenged by many other methods. Moreover, there were widely variable results among different methods. Recently, specific sperm epitopes have been identified with a phage display technique. Mixed epitopes as specific antigens used for ASA assay may be a choice for establishing a standard method for ASA assays, since the antigens are not altered during analysis.

The availability of effective treatment means depends on the extent of our understanding of ASA-associated infertility. Thus, in the study of immunoinfertility, the most important field may be its treatment of immunoinfertility. This should be the focus of research in the future.

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Key issues

- Dozens of sperm-specific antigens were isolated and characterized in association with infertility. It is difficult to find a particular sperm antigen that could detect real antisperm antibodies (ASAs). Moreover, it was confirmed that there was no a single predominant target antigen identified to interact with all the ASAs so far.
- There are some protective mechanisms that prevent ASA production in men and women, while chronic infection, vasectomy and vasovasostomy, heavy metals, and testicular cancer and torsion may induce the production of ASAs.
- ASA-induced infertility may be responsible for decreased motility and sperm penetration of cervical mucus, and blockage of the acrosome reaction and sperm-egg interaction. Moreover, mononuclear cells may play an important role in ASA-caused infertility.
- Many ASA assays were developed, including sperm agglutination tests, sperm immobilization test, immunobead test, mixed antiglobulin reaction, immunofluorescence assay, ELISA, radiolabeled antiglobulin assay, flow cytometry and immunoblotting, each with advantages and disadvantages. However, comparative investigations on various assays to detect and quantitate levels of antibody against human spermatozoa showed widely varying and unreliable results.
- Efforts for the treatment of ASA-mediated infertility were attempted, including immunosuppressive therapies, assisted reproductive technologies, laboratory techniques and integrative Chinese and Western medicine treatment. However, current therapy for ASA-associated infertility was almost empiric and largely unproven.

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