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NATURALLY-OCCURRING ANTISPERM ANTIBODIES IN MEN: INTERFERENCE WITH FERTILITY AND IMPLICATIONS FOR TREATMENT

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1. ABSTRACT

Naturally-occurring antisperm antibodies in men are a *relative* cause of infertility, being the fertility impairment related with the degree of sperm autoimmunization. The impairment of sperm penetration through the cervical mucus represents the best established mechanism of the antibody interference with fertility. Another mechanism may involve complement-mediated sperm injury and opsonizing effect through the female genital tract. Finally, sperm-bound antibodies can interfere with sperm functions involved in the fertilization process, mainly in the sperm-zona pellucida interaction. While some mechanisms of the antibody-interference with fertility depend only on the degree of sperm autoimmunization (e.g., inhibition of cervical mucus penetration), other mechanisms (e.g., interference with gametes interaction) could or could not occur depending on the relevance in the fertilization process of the specific antigen(s) recognized by antisperm antibodies, which are policlonal in nature. Intrauterine insemination is an effective treatment when sperm autoimmunization is low or moderate, mainly if combined with corticosteroid treatment and superovulated cycles. On the contrary, its effectiveness in cases of high degree of sperm autoimmunization is controversial. The resort to "high tech" procedures is mandatory when other less invasive approaches have failed or they may also be chosen as a first-choice method in cases of high degree of sperm autoimmunization. Since in most reports the fertilization rate with *in vitro* fertilization and embryo transfer (IVF-ET) was significantly lower in the presence of sperm-bound antibodies than in the case of other indications, the likelihood of fertilization is higher with intracytoplasmatic sperm injection (ICSI), where the reported fertilization rates are similar to those in other indications, or even higher.

2. INTRODUCTION: CONTROVERSIES ON THE SIGNIFICANCE OF ANTISPERM ANTIBODIES

The role of naturally occurring antisperm-antibodies (ASA) as a cause of male infertility was recognized since Rumke (1) and Wilson (2) reported the presence of serum sperm-agglutinating antibodies in infertile men in 1954. Following those early observations, a large amount of reports has been directed to the investigation of the significance of ASA in male infertility including the aetiology of ASA formation, sites and mechanisms of antibody action, and possible treatments. However, since published studies used varying approaches for the recognition and treatment of male immunologic infertility, such as different antibodies assays, different drug regimes and lack of well-designed and controlled studies, it resulted in confusion of the prevalence as well as the actual role of ASA in male infertility. With the development and the encouraged use of accurate and easy assays for the screening of ejaculates for sperm-bound antibodies, the development of sperm function assays, and

Table 1. Diagnostic strategy for the detection of ASA in the men

Test*	Application
IgG-MAR test[9] or SpermMAR[11,21]	For routine screening of all semen analyses
Direct immunobead binding test (IBT)[10]	To be performed in case of positive igg-MAR test, to determine whether iga-ASA are also bound to sperm surface
Sperm-agglutination test, expecially TAT[6]	For the titration of ASA in serum and seminal plasma in patients with a positive direct test, or as screening test when it is impossible to performe direct tests (e.g., Lack of motility)
Indirect antiglobulin tests[10,23,27]	As an alternative option to the sperm-agglutination test, with the same purpose

***: reference**

finally the progress in assisted reproductive medicine, the role of ASA in male infertility as well as the potential for their treatment is becoming better defined. In this article we will review current understanding of the interference of ASA with the male fertility and possible treatments, following a brief focusing on clinical aspects of the male immunologic infertility, including ASA assays, prevalence and prognostic evaluations.

2.1. Antisperm-antibody testing

Only ASA assays detecting antibodies directed towards surface antigens have a clinical significance, because subsuperficial antigens cannot be exposed to antibodies by living cells along the male genital tract (3). The first assays to be utilized were indirect tests detecting biological activities of circulating ASA, i. e., sperm agglutination techniques and complement dependent cytotoxicity techniques (see ref. 3 for review). Multicentric comparative studies (4,5) indicated that they determine largely the same antibody specificities but with different sensitivity which was higher for sperm agglutination techniques, especially for the Tray agglutination test-TAT (6).

However, circulating ASA may differ from sperm associated antibodies *"in vivo"* in their biological activity and affinity to sperm antigens, since locally produced secretory immunoglobulins occur in the genital tract in addition to serum-derived Ig (7,8). Therefore, during the last 2 decades widespread acceptance has been gained by direct tests developed for the detection of sperm-bound antibodies, including the mixed antiglobulin reaction (MAR) test (9), the immunobead test (IBT)(10) and the SpermMar test (11). Although MAR test detects only antibodies belonging to IgG class while IBT detects IgG,A,M antibodies, comparative studies have generally demonstrated that all these tests are suitable as effective screening direct tests (9, 11-18). In fact, sperm surface IgA are almost always found in association with IgG (12,15,19). Since MAR test and the commercially available SpermMAR are cheaper and quicker, they are generally considered as more suitable for routine screening of all semen analyses, with the IBT performed on samples with a positive former test (13,20), and indirect testing (especially TAT or indirect IBT) performed only in men with azoospermia or lack of sperm motility (table 1). Since IgA SperMar is now available and an its higher accuracy than IgA-IBT has been reported (21), this diagnostic strategy could be furter semplified; moreover, a rapid mixed immunobead screen has been recently proposed (22), but its accuracy must be confirmed.

Other antiglobulin-based tests, such as radiolabeled antiglobulin assay (23,24), enzyme-linked immunosorbent assay (ELISA) (25-28) immunofluorescent test on living sperm suspensions (29,19), flow cytometry assay (30-35) has been proposed both as indirect and direct tests. Radiolabeled antiglobulin assay, although highly sensitive and specific, does not detect the regional specificity of antibody link. ELISA suffers from the same limitation, and lacks specificity for surface antigens because internal antigens are exposed by fixation; to overcome this limitation, a new type of ELISA without fixation has been proposed (36). Using living sperm suspensions instead of fixed smears makes the immunofluorescent test highly specific for surface antigens-directed ASA; it is usually utilized in the direct form (19) in our lab for a better evaluation of the regional specificity of the antibody link in all samples positive at direct IgG-MAR test. Flow cytometry assay is very promising for its potential to quantify the antibody load on each sperm cell (31).

2.2. Prevalence of antisperm antibodies

The reported prevalence of ASA varied depending on the modality of the immunological screening. Circulating ASA detected with indirect tests ranged from 8.1% to 30.3% in unselected men with infertile marriages (14,36-40). At low titres they were also reported in 2.4% to 10% of fertile men (36,40). Noteworthy, low titres of sperm-agglutinating activity can be due to non-immunological factors (41), representing false positive results. When stricter criteria were used (i.e., the occurrence of sperm-immobilizing activity in addition to sperm-agglutinating activity (14,37) and/or occurrence of sperm-agglutinating activity in seminal plasma (14), the prevalence of ASA in men with infertile marriages was 4.7% to 7.5%. Immunological screening by means of direct tests gave positive results in 7.8% to 20.1% (14-16,42-47), with the occurrence of strong positive results in about 6-7% of patients (14,16).

Some clinical conditions associated with a high prevalence of ASA have been recognized as identifiable causes of their development, mainly acquired genital tract obstructions. Among them, vasectomy is the most common, with a prevalence of ASA of 34% to 74% (40,44,48-50), and their persistence in 38% to 60% following successful vasovasostomy (49,51,52). On the contrary, it is not yet well established the association of ASA with obstructive azoospermia due to congenital causes, since conflicting data have been reported (53-57). Antisperm antibodies have also been associated with acute and chronic genitourinary infections (58-62). Some studies

have focused on the relationship between ASA and asymptomatic Chlamydia trachomatis infections. Although a high prevalence of ASA was reported in the presence of Chlamydia in genital secretion as detected by means of culture (63) or the polymerase chain reaction (64), circulating chlamydial antibodies were not associated (65) or only weakly associated (66) with the presence of sperm bound antibodies, whereas both a strong correlation (66) as well as no association (67) were reported between the presence of seminal chlamydial antibodies and ASA. A high prevalence of ASA has been reported in men with testicular carcinoma (68-70), in adults with a previous cryptorchidism and a late ochidopexy (71), in homosexual men (72), and in human immunodeficiency virus (HIV) - positive men (73,74). Finally, conflicting results have been reported on the association of ASA with varicocele (75-79) and spinal cord injury (80-84). In a recent multivariate analysis of men from infertile couples with and without ASA, only prior vas reversal and a history of genital tract infection were associated with the presence of sperm-bound antibodies (85).

2.3. Prognostic studies

Although the higher frequency of ASA in males with infertile marriages than in fertile controls could imply a negative effect of ASA on fertility, a cause-effect relationship needs to be validated by prospective studies of the relationship between the presence of ASA and subsequent fertility. Follow-up studies which compared negative and positive subjects for circulating ASA have produced conflicting results, since a significant association between antibody presence in the male and lower pregnancy rates was found in some studies (37,86,87) but not in others (39,88,89). A significant association was found when sperm bound antibodies were detected with direct tests (89,90). Furthermore, when the degree of the sperm autoimmunization was considered, a significant inverse correlation was found between eiher the titre of circulating ASA (37,91) or the percentage of sperm bound antibodies (92) and the incidence of pregnancies. A poor prognostic value of low to moderate levels of sperm-bound antibodies was also reported by Barrat *et al.* (93).

Altogether, the analysis of epidemiological and prognostic studies confirms the opinion of Bronson (2) that ASA are a *relative*, rather than absolute, cause of infertility. Fertility impairment is related with the degree of sperm autoimmunization, that is, the extent to which ASA are present in reproductive tract secretions and detected on sperm surface.

3. MECHANISMS OF FERTILITY IMPAIRMENT

Although the fertility impairment due to ASA is related with the degree of sperm autoimmunization, as suggested by epidemiological and prognostic studies, there are several evidences that also *qualitative* differences in the effects of ASA could have a role in the fertility impairment. In fact, firstly, different immunoglobulin isotypes of antibodies occurring on sperm surface can produce different biological effects (i.e., cytotoxic effects due to complement activation can be produced by IgG but not IgA sperm bound antibodies). Secondly, due to their polyclonal nature, ASA are directed against more than one sperm antigen, which may differ among patients and may be more o less relevant to fertility.

3.1. Effect on semen quality

With some exceptions (39,45), most epidemiologic studies did not find any significant difference in the principal semen parameters (sperm count, motility and morphology) between infertile patients with and without ASA (14,16,94,95). In any case, there is little evidence that suggests a cause/effect relationship between ASA and abnormality of the principal semen parameters. An antibody effect on semen quality should involve a complement mediated sperm cytotoxicity occurring within the male genital tract. However, anticomplementary activity has been reported in human semen (96,97), and it was recovered in the low molecular weight of the seminal plasma (20-60.000 Daltons) using gel filtration chromatography (96). This fraction inhibited total complement activity as well as the activity of the early C components C1 and C3. Afterwards, a potent inhibitor of C5b-7 complexes was identified in human seminal plasma, where it was found in 5- to 10 -fold higher concentrations than in serum. A sulphated glycoprotein termed clusterin was also found on ram sperm (98), and purified human seminal clusterin was shown to inhibit C5b-6 mediated hemolysis (99). Finally, D'Cruz and Haas (100) demonstrated the lack of a detectable product of C activation (SC5b-9) in the seminal plasma of men with sperm bound antibodies (IgG were present in most cases). Taken together, these findings suggest that human seminal plasma contains inhibitors for both the initial and the terminal portions of the C cascade thereby protecting sperm from C-mediated injury in the male reproductive tract.

Since an increased sperm count in some oligozoospermic patients with ASA was reported in response to corticosteroid therapy, it was suggested that in those cases a cell-mediated immune reaction at the level of rete testis and/or epididymis responsive to the anti-inflammatory effect of corticosteroids might underlie the low sperm count (101). However, clinical and pathologic evidences of immune orchitis in men exhibiting natural autoimmunity to sperm has never been provided.

Sperm agglutination is the only semen alteration related to the presence of ASA. A significant increase in the proportion of motile sperm involved in agglutinations has been reported in the presence of ASA, whenever investigated (14,16,95,102). However, sperm agglutination, which is a time-dependent phenomenon, only rarely involves a large proportion of motile sperm soon after liquefaction, even when all ejaculated sperm are antibody-coated. Therefore, sperm agglutination, although extremely suggestive of sperm autoimmunization, does not represent an important mechanism of antibody-interference with fertility in most cases.

3.2 Interference with cervical mucus penetration

The impairment of sperm penetration through the cervical mucus represents the best known and established

mechanism of the antibody interference with fertility (2). Definitive clinical demonstrations of this impairment have been produced analysing the outcome of *"in vivo"* as well as *"in vitro"* tests of sperm cervical mucus interaction.

As far as the post coital testing is concerned, a significant association was reported between a poor sperm penetration into cervical mucus and sperm autoimmunization (103,104). Besides, the degree of impairment of sperm penetration into cervical mucus was found to correlate with the proportion of sperm exhibiting surface-bound antibodies (105), as well as with the titre of circulating sperm-immobilizing antibodies (104).

The outcome of the *in vitro* cervical mucus penetration test comparing men with and without ASA has largely confirmed this impairment (38,39,43). Finally, the demonstration of the actual responsibility of ASA in impairing cervical mucus penetration has been provided by matching donor sperm suspensions exposed to sera containing ASA against the same sperm suspensions exposed to control sera without ASA (106).

Although some reports suggested that IgA were more important than IgG in impairing sperm penetration of cervical mucus (107-109), other findings indicate that an abnormal interaction between the Fc portion of both IgA and IgG immunoglobulins bound to the sperm surface and constituents of the cervical mucus is responsible, almost in part, for the characteristic shaking phenomenon and the impairment of mucus penetration. Comparing the swimming ability of antibody-coated sperm within cervical mucus with that of sperm exposed to only the Fab fragments of the same antibodies, mucus penetration was abolished by complete antibody, whereas only reduced but not abolished by Fab (110). Bronson *et al.* (111) found an improvement of the ability of antibody-bound sperm to penetrate human cervical mucus *in vitro*, after exposure to a IgA_1 protease, which was expected to liberate Fc fragments of IgA_1 antibodies bound to the sperm surface. Moreover, this improvement varied inversely with the amount of remaining IgGs, not degraded by protease, indicating a role for both IgG and IgA sperm-bound antibodies in impairing cervical mucus penetration.

3.3 Complement-mediated cytotoxicity and opsonizing effect through the female genital tract

One mechanism of ASA-interference with fertility may involve sperm injury potentially mediated by complement and/or phagocytic cells in the female genital tract. While complement-mediated cytotoxic effect by complement-fixing ASA are prevented in semen, due to its anticomplementary activity (see 3.1), when antibody-bound sperm enter the female reproductive tract they might became liable to deleterious effects of complement activation, supposing that complement components are present in sufficient amount through the female genital tract. Full-complement component lytic activity has been documented in cervical mucus in amount enough to cause complement- dependent sperm immobilization (112). Also human follicular fluid exhibits complement activity, and IgG-antibodies bound to sperm were capable of activating follicular fluid complement as detected by their ability to deposit terminal complement complexes (MC5b-9) on human sperm (113). In an elegant study, D'Cruz *et al.* (114) provided the direct evidence for the involvement of complement-fixing ASA and complement activation in exerting sperm injury. Using flow cytometry to evaluate simultaneously the binding of antibody and autologous complement to sperm cells, they demonstrated that incubation of donor sperm with sera containing IgG-ASA resulted in the activation of autologous complement in vitro as assessed by the deposition of the initial (C3d) and the terminal (C5b-9) complement complex on the sperm surface. Antisperm antibodies and complement deposition resulted in a dramatic loss of sperm motility, as well as in activation and aggregation (rosetting) of polymorphonuclear leukocytes (PMN) to antibody- and complement-bound sperm. The inability of sera containing non-complement fixing IgG-ASA to promote sperm binding to PMN suggested that IgG alone is insufficient to initiate the interaction, that is, it would preclude a direct interaction between the Fc portion of sperm-bound Ig and the Fc-receptor on PMN.

An opsonizing effect exerted by IgG-ASA had been previously reported also by London *et al.*(115), who demonstrated that the incubation of donor sperm with sera containing IgG-ASA enhanced sperm phagocytosis and lysis by peritoneal macrophages. This effect had been hypothesised as mediated by Fc-receptor for IgG.

3.4. Interference with the fertilization process

A vast body of literature has focused on the possible interference of ASA with the fertilization process. Several experimental studies have demonstrated that antibodies raised against whole spermatozoa (116,117) or defined sperm antigens (118 for review) can interfere with sperm functions involved in the fertilization process, thereby blocking sperm-egg interactions. However, the actual role of naturally-occurring ASA in men in impairing sperm-egg interaction, as well as the level of this impairment, is not yet sufficiently known, because conflicting data have been produced. In this section we will attempt to analyse the reasons underlying these conflicting data, which could help the understanding of this debated matter.

Retrospective or prospective analyses of fertilization data from *in vitro* fertilization and embryo transfer (IVF-ET) programs provide a potential means of assessing possible effects of ASA on human gametes interaction. However, this model of study cannot give information about the level of this possible interference. Several studies in the last two decades have tried to determine the level as well as the actual occurrence of this interference. The hamster egg penetration test has mainly been employed to investigate the effect of ASA on sperm functions involved in the fusion with the oolemma, that is, the capability to complete capacitation and to exhibit the acrosome reaction as well as the fusogenic properties involved in the interaction with the oocyte membrane. Some studies have also been carried out to directly test the effect of ASA on each specific sperm function (i.e., capacitation or acrosome reaction). Other studies have

Table 2. Fertilization rate in IVF-ET programs in the presence of antisperm antibodies (ASA) in the male.

	ASA +		ASA -
Reference	**Fertilized/total ova (Fertilization Rate)**		**Fertilized/total ova (Fertilization Rate)**
Clarke *et al.*(1985)[128]	65/131 (50%)	IBT >20%	
	18/66 (27%)	IgA >80%	
	47/65 (72%)	IgA <80%	
Mandelbaum *et al.*(1987)[125]	8/14 (57%)	IBT >20%	118/180 (65%)
Matson *et al.*(1988)[119]	37/70 (53%)	IBT >20%	156/201 (78%)
	15/39 (38%)	IgG+IgA	
	22/31(71%)	IgG or IgA	
De Almeida *et al.*(1989)[130]	70/175 (40%)	IBT >10%	
	6/43 (14%)	>70%(IgG+IgA)	
	31/52 (60%)	<70%	
Palermo *et al.*(1989)[132]	132/273 (48%)	MAR test >10%	
	33/80 (41%)	>90%	
	99/193 (51%)	<90%	
Chang *et al.*(1993)[120]	17/59 (30%)	IBT >10%	654/984 (66%)
Lahteenmaki (1993)[131]	98/355 (28%)	MAR test >10%	
	28/170 (17%)	>90%	
	40/113 (35%)	>40% and <90%	
	30/72 (42%)	<40%	
Rajah *et al.*(1993)[121]	53/105 (50%)	MAR test >20%	93/128 (73%)
Acosta *et al.*(1994)[122]	(42%)	MAR test >10%	(73%)
Sukcharoen & Keith (1995)[126]	124/165 (75%)	IBT >20%	978/1412 (69%)
Ford *et al.*(1996)[123]	209/544 (38%)	IBT >20%	380/558 (68%)
Vazquez-Levin *et al.*(1997)[124]	46/104 (44%)	MAR test >20%	65/77 (84%)
Ombelet *et al.*(1997)[188]	153/283 (54%)	MAR test >50%	
Culligan *et al.*(1998)[127]	(66%)	IBT >15%	(63%)
Total	**1012/2278 (44%)**		**2444/3540 (69%)**

focused on the interference of ASA with the sperm-zona pellucida (ZP) interaction.

Some considerations could be helpful in analyzing the results, often conflictual, which have been reported. The effects of natural ASA on fertilizing ability of human sperm have been studied either by matching donor sperm

suspensions exposed to sera from patients with circulating ASA against the same sperm suspensions exposed to control sera, or using spermatozoa coated "*in vivo*" with ASA. In both cases the results must be interpreted with caution. In fact, circulating ASA may differ from sperm associated antibodies "*in vivo*" in their biological activity and affinity to sperm antigens, since locally produced secretory immunoglobulins occur in the genital tract in addition to serum-derived Ig. On the other hand, using spermatozoa coated "*in vivo*" with ASA, the concomitant presence of non-immunological sperm abnormalities raises doubts about the responsibility of ASA in affecting sperm functions. Using antibodies eluted from autoimmune ejaculates instead of circulating ASA is another and potentially more demonstrative approach. Although used in some studies, its feasibility is hindered by the difficulty in eluting sufficient amounts of antibodies.

3.4.1. *In vitro fertilization* (IVF) as model of study

Table 2 shows the fertilization rates reported in series including couples with sperm autoimmunization in the male partner. In most reports the fertilization rate was significantly lower in the presence of sperm-bound antibodies than in the case of other indications for IVF (119-124). However, in some other reports no significant difference was found (125-127). Although in a early report sperm head directed IgA- more than IgG-antibodies seemed to be associated with a reduced fertilization rate (128), in subsequent reports a significant reduction of the fertilization rate was found when sperm were covered both with IgG- and IgA-ASA (119,129, 130, and table 2). However, trying to analyse routine IFV results to determine the actual effect of sperm-bound antibodies on fertilization process is difficult because of some serious reasons. Firstly, nonimmunological sperm abnormalities may bias the results. Only in some reports the conventional semen parameters were taken into account in the comparison between patients with and without ASA (121,122,124,131). In these series, an independent impairment by sperm-bound antibodies was reported in the presence of normal semen parameters (121,124,131) as well as in the presence of asthenozoospermia (131) or teratozoospermia (122). Secondly, the criteria employed to define the occurrence of immunological infertility were different among series, and often inadequate. Also patients with low or moderate sperm autoimmunization were included in most series. The proportion of antibody-free sperm could be the determining factor of the fertilization outcome in those patients. Therefore, the inclusion of patients with low or moderate sperm autoimmunization could account for the normal overall fertilization rate found in some series, as well as for the variability of the fertilization outcome among

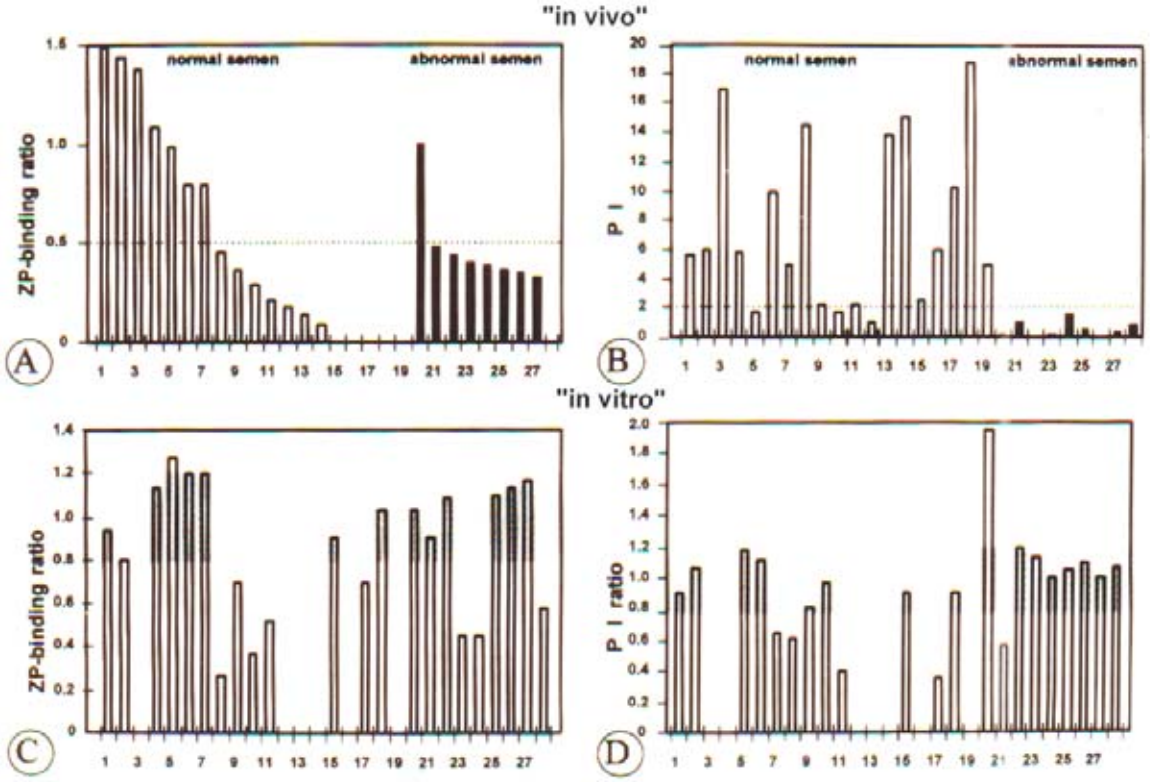

Figure 1. Results of the ZP-binding test **(A)** and the HEPT **(B)** exhibited by ejaculated sperm coated "in vivo" with antisperm antibodies from patients with normal and abnormal semen; PI = penetration Index (n° penetrations/oocyte); (----) indicates the cut off values for normal results; **(C)** and **(D)**, effect of circulating antisperm antibodies from the same patients on the ZP-binding test **(C)** and on HEPT **(D)**; the results are expressed as ZP-ratio and PI ratio, respectively, between antibody-exposed and non-exposed sperm suspensions from the same donor; *shaded area* represents the intra-assay variation. From Francavilla *et al.* (143) with permission.

individual cases of the same series. Indeed, when the degree of sperm autoimmunization was taken into account, it was always inversely correlated with the overall fertilization rate (130-132, and table 2). But, noteworthy, in some individual patients, a high fertilization rate was achieved even in the presence of a high degree of sperm autoimmunization (121,123,124,128).

In conclusion, the analysis of human IVF results seems to indicate that also on fertilization process sperm bound antibodies exert a *relative* impairment, which, to some extend, is related with the degree of sperm autoimmunization. However, the degree of autoimmunization does not completely explain the variability of the antibody impairment. Seemingly, at the level of gamete interaction, more than at other levels (i.e., cervical mucus penetration) the interference of ASA exhibits *qualitative* apart from quantitative differences among patients, suggesting that this interference depends on the relevance of the specific antigens, targets of natural ASA, to the fertilization process.

3.4.2. The hamster egg penetration test (HEPT) as model of study

Conflicting results have been reported when the effect of circulating ASA on HEPT results was tested. In fact either inhibition (133-135), or enhancement (136), both inhibition and enhancement (106,137), or no effect were found (138,139). Some discrepancies may be explained by procedural differences. Generally, when inhibitory effects were reported, donor sperm were exposed to ASA after capacitation or directly into the insemination medium: in any case, free antibodies were not removed before sperm/oocytes coincubation (106,134,135). Using this procedure, an interaction of ASA with internal sperm antigens, which can be revealed after the acrosomal loss, has been supposed to explain the inhibitory effect (140). However, although this possible interference may occur in the presence of ASA in the female, it cannot occur when ASA are detected in the male. When donor sperm were washed following exposure to ASA and then capacitated (procedure which is more suitable for studying the effects of naturally occurring ASA in the male), generally no effect (138,139) or even an enhancement of penetration was found (136).

Zouari and De Almeida (141) reported that antibodies eluted from eight autoimmune ejaculates and transferred onto donor sperm, reduced sperm penetration in three cases and in a case a modest increasing effect was exhibited. All samples with inhibitory effect contained both

IgG and IgA. The elimination of one of the two isotypes restored the ability of the sperm to penetrate the hamster oocytes.

Using spermatozoa coated *in vivo* with ASA Haas *et al.* (142) reported a variable degree of impairment in penetrating hamster oocytes, but the concomitant presence of astheno- and/or-teratozoospermia may bias the results. More recently, using the TEST-yolk buffer enhanced HEPT, our group (143) found that 12/28 patients with all ejaculated sperm coated with IgG- or IgG/IgA-antibodies exhibited a penetration index (PI = penetrations per oocyte) < 2 (the lowest value exhibited by fertile controls). But excluding from the analysis 9 patients exhibiting abnormal semen in terms of oligo/astheno-and/or-teratozoospermia, only in 16% of cases the PI was slightly less than 2 (figure1B). Moreover, only 24% of the sera from the patients produced a reduction of the PI (figure1D), when absorbed on donor sperm suspensions, and in no case was the inhibitory effect great enough to cause a poor HEPT. It could be argued that after preincubation in TEST-yolk buffer antibody-coated sperm acquire the ability to fuse with oolemma by virtue of the spermatozoa being made highly fusogenic in spite of the presence of antibodies on their surface. This is not the authors opinion, who previously reported that ASA do not interfere also with the results of conventional HEPT (138).

3.4.3. Effects on capacitation and acrosome reaction

An interference of ASA with the acrosome reaction (AR) either as promoting or inhibitory effect was hypothesized since early experimental and clinical data suggested an antibody interference with gametes interaction. In some studies the effect of ASA on spontaneous acrosome reaction rate under capacitation conditions was evaluated. In most of them, no effect was found (137,138,144,145). In the most extensive of these studies (138), our group demonstrated that the exposure to ASA of all isotypes, even in association, from sera or seminal plasma samples, did not modify the spontaneous acrosome reaction rate of donor sperm used for HEPT, whose outcome similarly was not affected. On the contrary, testing ASA from female sera, an inhibitory effect was found in two reports (146,147). However, in the first report (146) the mean AR rate was 2.8% in antibody-exposed sperm and 5.8% in control sperm. The biological significance of this difference, although reported as significant, is doubtful. In the second report (147), the omission of vitality assessment, which permit to differentiate true from degenerative AR, may bias the results. Finally, in a recent report (148) *in vivo* antibody-coated sperm from infertile patients exhibited a massive acrosome loss not only in capacitating conditions but also in native preparations. The omission of vitality assessment and the inexplicable high rate of AR, which was also found in the control group, make these results hard to be accepted. However, high levels of spontaneous AR had also been reported by Lansfort et al.(149) in most patients with sperm coated in vivo with IgG plus IgA antibodies. However, neither in this report vitality assessment was carried out. Indeed, there is a general agreement that in human sperm, spontaneous AR represents a sporadic event (150) with little biological efficacy, whereas, the sperm ability to undergo a complete AR in response to ionophore challenge has been reported as significantly related to the fertility status (151) and to the human IVF rates (152,153). Since the ionophore challenge bypasses the biological signalling that initiates the AR, and uncapacitated sperm respond poorly (154), this test may measure the capacitation status and the integrity of the chain of events between entry on calcium and exocytosis. In light of these considerations, in some studies the effect of ASA on AR induced by ionophore challenge was evaluated, but conflicting results were reported. Mahony *et al.* (144) reported an inhibitory effect exhibited by two sera with ASA also inhibiting ZP-binding, and Zouari et al. (145) reported an inhibitory effect exhibited by sperm-eluted antibodies. On the contrary, in a report of our group (139), circulating and seminal ASA caused a slight but constant and significant AR increase in response to ionophore challenge compared with that of the same donor sperm suspensions exposed to control sera. However the clinical relevance of this effect was considered as questionable, since it was not reflected, in most cases, in the results of HEPT also performed after ionophore challenge.

The effect of ASA on other aspects related to the capacitation process, such as hyperactivated motility (144,145) or calcium uptake after stimulation with follicular fluid (144) was also studied with inconclusive results. An interference with the capacitation-associated expression of sperm surface mannose receptors was reported by Benoff *et al.* (147) using ASA contained in female sera. This effect was related to an inhibition of the reduction of membrane cholesterol content associated with sperm capacitation, which prevented the membrane fluidity changes needed for mannose receptors expression.

3.4.4. Effects on the zona pellucida (ZP) interaction

There is general agreement that ASA can interfere with ZP-interaction. Circulating ASA have been shown to reduce sperm binding to (144,155-157) and penetration through (158) the ZP. Using spermatozoa coated *in vivo* with ASA, Liu *et al*.(159) found a reduced binding to salt-stored human ZP as compared to donors' spermatozoa. However the low number of patients and the presence of concomitant non-immunologic semen abnormalities (even men with 5% normal sperms were included in the study) make difficult to infer the responsibility of ASA in this impairment. Zouari and De Almeida (141) reported that antibodies eluted from autoimmune ejaculates uniformly reduced the ZP-binding of spermatozoa from donors. Although this demonstration indicates the responsibility of sperm-coated antibodies in this impairment more appropriately, the very low number of samples (number=5) again does not permit the inference of information about the actual occurrence of this impairment. More recently our group (143) tested 22 patients with all ejaculated spermatozoa coated with antibodies against the sperm head, taking into account in the evaluation of the results the concomitant presence of non-immunological abnormalities in the conventional semen profile. An impairment of the ZP-binding was demonstrable in 50% of patients with normal semen profile (figure1A). Since a normal ZP-

binding was observed even when all ejaculated spermatozoa were coated with both IgG- and IgA-antibodies, neither Ig class, even combined, appears to affect unavoidably this sperm function. Noteworthy, all normozoospermic patients with low ZP-binding showed circulating IgG-ASA with inhibitory effect, when transferred on donor sperm, while no patient with normal ZP-binding showed circulating ASA with inhibitory effect (figure1C). Therefore, IgG-ASA transuded from the blood into the genital tract can exert the inhibitory effect on ZP-binding *in vivo*. Zouari and De Almeide (141) reported that the removal of either IgG or IgA antibodies eluted from autoimmune ejaculates did not change the inhibitory effect on ZP-binding. Altogether, these observations suggest that both humoral and local sperm autoimmunizzation exhibit the same behaviour in impairing or not the ZP-binding. The fact that ZP-binding is not unavoidably inhibited by ASA, could explain the high fertilization rate in IVF programs, which was reported in some cases even in the presence of a high degree of sperm-autoimmunization (121,123,124,128). However, the ZP-binding is only the first step of the more complex interaction between spermatozoa and zona. Zona pellucida also triggers the AR of bound sperm, that is required by sperm for ZP penetration and fertilization. We recently demonstrated that ASA can interfere with sperm-ZP interaction not only by inhibiting ZP-binding but also by inhibiting the induction of AR by ZP (160). While the inhibition of ZP-binding is always associated with the inhibition of ZP-induced AR, this last interference can also occur in the absence of the inhibitory effect on ZP-binding. However, neither inhibitory effect may occur. The occurrence of different effects is not related with the ASA-titre, and it can be explained by the polyclonal nature of the naturally occurring ASA in men. Sperm-antibodies inhibiting ZP-binding could mask or prevent the expression of specific receptors on the plasma membrane over the sperm heads for the ZP3-O-linked oligosaccharides. Since ZP3 serves as both a ligand for sperm binding and a trigger for acrosome reaction (161), ASA, which inhibit ZP-binding, inhibit ZP-induced AR too. When an inhibition of ZP-induced AR occurs in the absence of interference on ZP-binding, ASA could interfere with cross-linking of several antigenic sites recognized by ZP3 resulting in a blockade of their aggregation which triggers the acrosome exocitosis (162). Another possibility is that ASA affect the fusogenic response to the biological signalling that triggers the AR. We are in favour of the first hypothesis since in our hands ASA did not affect the induction of AR by calcium-ionophore (139).

3.5. Postfertilization effects

Although a reduction in cleavage rate in the presence of ASA in the female was reported in one early IVF series (125), most clinical data indicate that sperm-bound antibodies do not interfere with postfertilization events. No reduction of cleavage and pregnancy rates have been generally reported in IVF programs where the presence of sperm-bound antibodies was associated to a reduced fertilization rate (120,121,131,132). However, in disagreement with previous data, a detrimental effect on early embryonic development was more recently claimed by Vazquez-Levin *et al.* (124), who reported a significant reduction both in the cleavage rate and pregnancy rate in the presence of sperm-bound antibodies.

Data from intracytoplasmatic sperm injection (ICSI) available so far, confirm that ASA are not associated with a reduction of cleavage and pregnancy rate (163-165), even if a poorer embryo quality (163) and a higher rate of pregnancy loss (164) were reported. However, neither a poorer embryo quality nor a higher rate of pregnancy loss were born out by Clarke *et al.* (165).

Since in animals embryos share epitopes with sperm antigens (166), antibodies occurring in the female against sperm antigens could interact with embryonic antigens, providing an attractive reason to hypothesize that ASA could adversely affect embryonic development, when they occur in the female. Antibodies against the cleavage signal (CS-1), sperm derived protein which should function as an extranuclear cleavage signal for early division of fertilized zygotes, could represent an attractive explanation of a possible postfertilization effect of ASA when they occur also in the male (167). However the actual role and occurrence of these antibodies have to be determined.

4. IMPLICATIONS FOR TREATMENT

In the evaluation of possible therapeutic modalities for infertile patients with ASA, it is important to keep in mind two previous analysed considerations: 1) ASA are a relative cause of infertility, which is related to the degree of autoimmunization; 2) some mechanisms of the antibody-interference with fertility depend only on the degree of autoimmunization (e.g., sperm-agglutination, inhibition of cervical mucus penetration), while other mechanisms could or could not occur depending on the specific antigen(s) involved in the immune response (e.g., interference with gametes interaction); 3) It is not possible prevent or disrupt antigen/antibody complexes on sperm surface by means of "in vitro" sperm processing tecniques unless methods inconsistent with sperm vitality (heating at 56°C or lowering the pH to <3) are utilized (168,169)

4.1. Corticosteroid therapy

The rationale for this treatment is to reduce the production of ASA, thereby obtaining a proportion of antibody-free sperm sufficient for fertilization. Either long-term low dose treatment (e.g., prednisolone, 5 mg three times daily for at least 6 months)(170), even following soppression of spermatogenesis with testosterone (171), or intermittent high-doses of metilprednisolone (96 mg/day for 7 days) (172) have been widely used, with better results claimed with the latter approach (170). Unfortunately, most studies lack a placebo control (173 for review). In a double-blind, placebo-controlled study, intermittent high-doses of metilprednisolone did not produce a favourable effect over placebo on the men's subsequent fertility (174). Because of the risk of serious adverse effect of high doses of corticosteroid treatment (175) an intermediate-dose cyclical regimen was evolved. In a double-blind crossover trial, prednisolone treatment , at 20 mg twice daily on days 1-10 of the female partner menstrual cycle, followed by 5 mg on days 11 and 12, was associated with a cumulative

pregnancy rate of 31% during 9 months, which was significantly higher than the rate of 9.5% for placebo (176). Unfortunately, with the same treatment and the same study design, no pregnancy was achieved during three months in a subsequent report (177). In both studies, the circulating ASA-titres were not significantly modified by steroid treatment, while a significant fall in antibody titres in seminal plasma was found in the former. With intermittent high-doses of metilprednisolone, a significant reduction of sperm-associated IgG had been reported using radiolabeling antiglobulin assay, with no effect either on sperm-associated IgA or on circulating IgG-ASA levels (174). Finally, in a placebo-controlled flow cytometric study, the antibody levels measured before and after treatment with low-dose prednisolone (20 mg/day) or with placebo were not statistically different, but in 2/10 patients treated with prednisolone, a marked decrease in the proportion of spermatozoa positive both for IgG and IgA was observed (178).

In conclusion the efficacy of corticosteroid treatment has not been definitively proven.

4.2. Intrauterine insemination

Since the most established interference of ASA with fertility is represented by the impairment of cervical mucus penetration by antibody-coated sperm, intrauterine insemination (IUI) has been widely used for the treatment of the male immunological infertility. However, the usefulness of IUI for this indication remains controversial because conflicting results have been reported. In studies, which included patients with variable degree of sperm-autoimmunization (179-183), the reported pregnancy rate/couple ranged from 0%(181) to 37% (182). Indeed, there are two questions to be answered. The first one is: does *overcoming the cervical mucus barrier remove the clinical interference of sperm-coated antibodies with fertility?* In a study designed with this purpose(184), our group did not obtain any pregnancy with 110 IUIs in 19 couples, where all ejaculated spermatozoa were antibody-coated, while a pregnancy rate/couple of 25.6% (5.6% cycle fecundity) was obtained in the control group (n°=86) without ASA. The responsibility of ASA for the failure of IUI was inferred from the significant difference obtained by comparing the results in patients with and without ASA, homogeneous for both epidemiological data and seminal parameters, having excluded teratozoospermic patients (with and without ASA) from the analysis, since teratozoospermia had been proved as strongly impairing the outcome of IUI (185). The opsonizing effect through the female genital tract and/or the effects on fertilization process could account for this failure. In disagreement with this report, 6 out of 9 patients with ASA, whose wife conceived following IUI, exhibited a strong positive (>90%) IgG-MAR test in a crossover, randomized trial, where IUI (3 cycles/couple) was significantly more effective than cyclic, low dose prednisone treatment (1 pregnancy) in 40 couples (186).

The second question is: *Can IUI improve the chance of achieving a conception when used as an adjuvant therapy to steroid treatment?* In a randomized, cross-over study comparing IUI in superovulated cycles with natural intercourse in men receiving cyclical intermediate dose steroid therapy for immunological subfertility (immunobead binding levels ≥ 50% in either seminal plasma or serum), a cumulative pregnancy rate of 39.4% over four cycles of IUI was achieved, compared with only 4.8% over four cycles of timed intercourse with the same regimen of steroid therapy (187).

In conclusion the analysis of the reported data indicates that IUI is an effective treatment for low or moderate sperm autoimmunization, mainly if combined with corticosteroid treatment and superovulated cycles. On the contrary, its effectiveness in cases of high degree of sperm autoimmunization is controversial. In a recent pilot non-randomized study (188), where the effectiveness of IUI with ovarian stimulation was compared with that of IVF in 29 couples where the male partner had a positive (>50%) direct MAR test, 64.3% of patients conceived after a maximum of three IUI cycles, while 46.6% of patients conceived during the first IVF cycle. Cost benefit analysis favoured a course of four IUI cycles, indicating this treatment as a valuable first-choice method to use before starting more invasive and expensive techniques of assisted reproduction.

4.3. IVF-ET vs ICSI

The resort to IFV procedures (IVF-ET or ICSI) is mandatory when other less invasive approaches have failed or they may also be chosen as a first-choice method in cases of high degree of sperm autoimmunization. The question is: IVF-ET or ICSI ? Since in most reports the fertilization rate was significantly lower in the presence of sperm-bound antibodies than in the case of other indications for IVF-ET (119-124; see also section 3.4.1.), the likelihood of fertilization is higher with ICSI, where the reported fertilization rates were similar to those in other indications (164,165), or even significantly higher (163). The inability of antibody-coated sperm to bind to the zona pellucida is apparently the main obstacle to fertilization (see section 3.4.), but it not unavoidably occurs (143). Therefore, the outcome of the ZP-binding test could identify those patients with immunological infertility who profit by conventional IVF and those who need micromanipulation techniques applied to IVF to achieve fertilization. However, the demonstration that ASA can inhibit the induction of AR by ZP in the absence of an inhibitory effect on ZP-binding (160) indicates that the ZP-binding test does not completely explore the interference of ASA with the sperm-ZP interaction. Therefore, testing AR-induction by ZP could be usefully included in the diagnostic screening before IVF. But simpler tests should be developed, and recombinant human ZP3 would be a convenient tool in such development.

5. PERSPECTIVE

Although many aspects of the infertility due to naturally-occurring ASA in men has been clarified, some controversies remain. Greater standardization of antibody testing, further development of sperm function assays, and more precise criteria to define the occurrence of

immunological infertility could help to throw light on the remaining debated aspects. Furthermore, the identification of the sperm antigens recognized by natural ASA, as well as the recognition of their relevance in the fertilization process, could shed new light upon this matter. Although a few definite sperm antigens relevant to fertilization have been characterized fairly well (189 for review), a role in human immunoinfertility has been suggested by some evidences only for two of them, fertilization antigen 1 (FA-1)(190-192) and cleavage signal (CS-1) protein,(167; see also section 3.5.) .

The development of immunocontraceptive strategy has renewed the interest toward the study of clinical infertility mediated by ASA. Antisperm antibodies develop in post-vasectomized men or they spontaneously occur in infertile men without physiological complications, despite their persistence for years. Thus, ASA induced by immunization of men or women with antigens involved in natural immunoinfertility might similarly be without side effects. With this assumption, the study of clinical infertility due to ASA, regarded as "experiments of nature" in fertility reduction, has been approached to identify candidate sperm antigens for immunocontraceptive development (193,194). Numerous investigators have reported the identification of specific antigens using sera from WHO reference bank or from independent collections (106, 195-206). However, characterization of sperm antigens was limited to SDS-Page in most studies, and results varied widely due to the methods and reagents employed. With another approach, a two-dimensional protein database of human sperm proteins has been created as a means to identify sperm-surface proteins: vectorial labelling of the cell surface by biotinylation and iodination identified 98 dual-labelled sperm surface proteins (207). Only 3 of them was recognized by postvasectomy sera in a preliminary observation (208).

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