Chapter 2
Detection of Anti-sperm Antibody (ASA) in Women

Hiroaki Shibahara

Abstract Several assay methods were developed to detect anti-sperm antibodies (ASAs). There are two ways to test for ASA in infertile couples. One is the assays for infertile men that directly detect ASA on the sperm membrane. For this purpose, the mixed antiglobulin reaction (MAR) test and direct immunobead test (direct IBT) are recommended. Direct IBT is widely used as a screening test for ASA, and the inhibitory effects by ASA on sperm motion and fertilizing ability are evaluated to make a decision for the strategy of infertility treatments.

The other tests for ASA involve assays that indirectly detect ASA in serum, cervical mucus, follicular fluid, peritoneal fluid, or seminal plasma. For indirect ASA testing, serological tests, such as sperm-agglutination test (SAT) and sperm-immobilization test (SIT), have been usually performed for clinical purposes in infertile women.

It is obvious that the most important consideration is the selection of the method. It has been recommended that clinically specific test for female infertility should be chosen for detecting circulating ASA. ASA with bioactivities, including sperm-immobilizing antibodies, sperm-agglutinating antibodies and fertilization-blocking antibodies are suitable for choosing as initial testing for infertile women.

2.1 Methods for Anti-sperm Antibody Testing

Anti-sperm antibodies (ASAs) in serum or cervical mucus have been implicated as an etiological factor of infertile women. Although the incidence of such antibodies is increased in subfertile individuals, the exact nature of their effects on sperm function has yet to be fully elucidated.

There are two ways to test for ASA in infertile couples. One is the assays for infertile men that directly detect ASA on the sperm membrane. For this purpose, the

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mixed antiglobulin reaction (MAR) test and direct immunobead test (direct IBT) are recommended by the World Health Organization (WHO) [1]. Direct IBT is widely used as a screening test for ASA [2–4], and the inhibitory effects by ASA on sperm motion and fertilizing ability are evaluated to make a decision for the strategy of infertility treatments [5].

The other tests for ASA involve assays that indirectly detect ASA in serum, cervical mucus, follicular fluid, peritoneal fluid, or seminal plasma. For indirect ASA testing, serological tests, such as sperm-agglutination test (SAT) and sperm-immobilization test (SIT), have been usually performed for clinical purposes in infertile women [6].

In this chapter, the indirect tests for ASA for infertile women are described.

2.2 Available Tests for the Detection of Anti-sperm Antibodies (ASAs)

Several assay methods were developed to detect ASA. However, it is obvious that the most important consideration is the selection of the method. Currently available tests for detecting ASA are summarized in Table 2.1. They are classified into three groups according to the characteristics of each test.

Group I contains tests for the detection of bioactivity of ASA, including SAT, SIT, and fertilization-blocking test (FBT) that detects fertilization-blocking antibodies. Group II contains tests for the detection of ASA bound for motile sperm, including IBT or immunospheres (IS), MAR test, and panning test. Group III contains tests for the detection of ASA against sperm or sperm extract, including radiolabeled antiglobulin test, indirect immunofluorescent test, fluorescence-

Table 2.1 Available tests for detecting anti-sperm antibody (ASA)

Group 1. Tests for detecting the bioactivity of ASA
(a) Sperm-agglutination test (SAT)
(b) Sperm-immobilization test (SIT)
(c) Fertilization-blocking test (FBT)
Group 2. Tests for detecting ASA bound for motile sperm
(a) Immunobead test (IBT) or immunospheres (IS)
(b) Mixed antiglobulin reaction (MAR) test
(c) Panning test
Group 3. Tests for detecting ASA against sperm or sperm extract
(a) Radiolabeled antiglobulin test
(b) Indirect immunofluorescent test
(c) Fluorescent-activated cell sorter (FACS)
(d) Enzyme-linked immunosorbent assay (ELISA)
(e) Passive hemagglutination

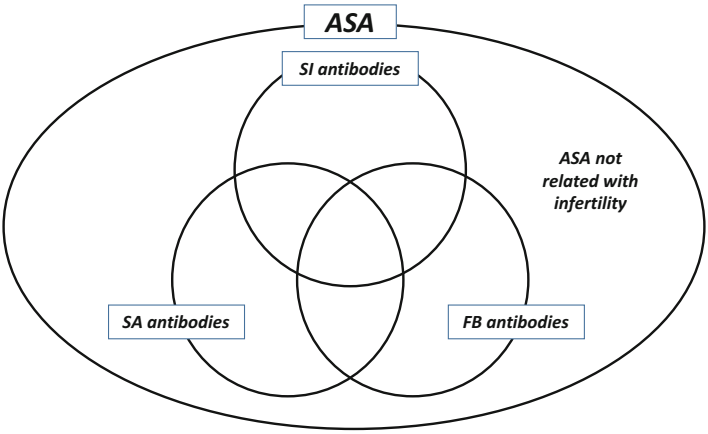

Fig. 2.1 The relationship of the various kinds of anti-sperm antibodies (ASAs) produced in immunological infertile women. There are various kinds of ASA that are produced in infertile women. They include at least two kinds of ASA. One is the ASA that only binds to sperm but not related with infertility. The other is the ASA, such as sperm-immobilizing (SI), sperm-agglutinating (SA), and fertilization-blocking (FB) antibodies, that is related with infertility to varying degrees. For example, some SI antibodies might have only SI activities (the zone that is not overlapped). Other SI antibodies might have all of SI, SA, and FB activities (the zone that is overlapped by the three circles)

activated cell sorter (FACS), enzyme-linked immunosorbent assay (ELISA), and passive hemagglutination for evaluation of ASA.

In these methods, it has been recommended that clinically specific test for female infertility should be chosen for detecting circulating ASA. The relationship between ASA with bioactivities, including sperm-immobilizing (SI) antibodies, sperm-agglutinating (SA) antibodies, and fertilization-blocking (FB) antibodies, and those without bioactivities is drawn in a scheme of Fig. 2.1. Therefore, the tests for detecting ASA that belonged to Group I are suitable for choosing as initial testing for infertile women.

2.2.1 Tests for the Detection of Bioactivity of Anti-sperm Antibodies

2.2.1.1 Sperm-Agglutination Test (SAT)

Detection of ASA has evolved considerably since the first serum agglutination assays were used in the 1950s. In 1952, Kibrick et al. [7] described a macroscopic procedure for identifying antibodies to mammalian sperm in serum. This test is known as gelatin agglutination test (GAT). Since the work of Rumke [8] in 1954, numerous investigators have used this assay to demonstrate the presence of ASA in the sera of infertile women. The GAT is performed by suspending semen from a

donor without ASA with the complement-inactivated serum of the suspected subfertile patient in a gelatin mixture in a small glass tube. After incubation for 60 min at 37 °C, the formation of large agglutinated clumps of sperm in the upper portion of the tube indicates that ASAs were present in the serum sample. The GAT is a simple test to perform; however, it was suggested that agglutination may occasionally occur due to the factors that are unrelated with ASA.

In 1964, Franklin and Dukes [9, 10] introduced the tube slide agglutination test (TSAT).

In this assay, a mixture of donor semen and complement-inactivated patient serum is incubated at 37 °C for 60 min. An aliquot is pipetted onto a microscopic slide for observation of sperm-agglutination. They found that 20.1% of 214 women undergoing infertility investigations had detectable sperm-agglutinating activity in their serum. Women with unexplained infertility had a much higher incidence (72.1%) than women with organic causes for their infertility (8.4%) or fertile women (5.7%). It should be noted that this study found a very high incidence of ASA, and the results are not supported by the following reports. One of the reasons may lie in the difficulty of discriminating true antibody-specific sperm-agglutination from nonspecific agglutination.

After their reports, the relationship between sperm-agglutinating antibodies and infertility has been studied inconclusively by many other investigators. Tyler et al. [11] reported that the incidence of positive TSAT in 41 infertile women was 5%, while that in pregnant women was 8%. Isojima et al. [12, 13] reported that the sera from 38 (45.8%) of 83 pregnant women and those of 27 (37.5%) of 72 infertile women with unexplained cause gave a positive reaction with the use of Franklin-Dukes' TSAT [9, 10]. They also showed that the sera from 12 (37.5%) of 32 pregnant women and those of 10 (34.5%) of 29 infertile women with unexplained cause gave a positive reaction with the use of Kibrick's sperm-agglutination test. Isojima has shown that there are various types of sperm-agglutination by ASA as shown in Fig. 2.2 [14]. They include head-to-head (H-H, Fig. 2.2①), head-to-tail (H-T, Fig. 2.2②), and tail-to-tail (T-T, Fig. 2.2③) of sperm-agglutination patterns.

Rose et al. [15] suggested that sperm can also be agglutinated by some mycoplasmas, viruses, and non-antibody serum components. This means that control serum samples must always be included in each test series. Mandelbaum et al. [16] also reported that sperm-agglutination can occur with the presence of bacteria, fungi, or debris in seminal plasma and with non-immunoglobulin serum proteins or pregnancy-related steroids in serum. However, Franklin and Dukes' report was notable from a historical perspective in that it stimulated significant interest in the idea that female immunological responses to sperm could be involved in the development of otherwise unexplained infertility and in the concept of an anti-sperm contraceptive vaccine [17].

Later, Friberg [18] has introduced another microagglutination assay which is simpler to perform than the TSAT. This assay is called as the tray agglutination test (TAT). It uses microliter amounts of reagents and avoids the need for transfer of material from the tube to a microscope slide, which is required by the TSAT. In the TAT, donor sperm and patients' sera are incubated in a flat-bottom tissue-typing

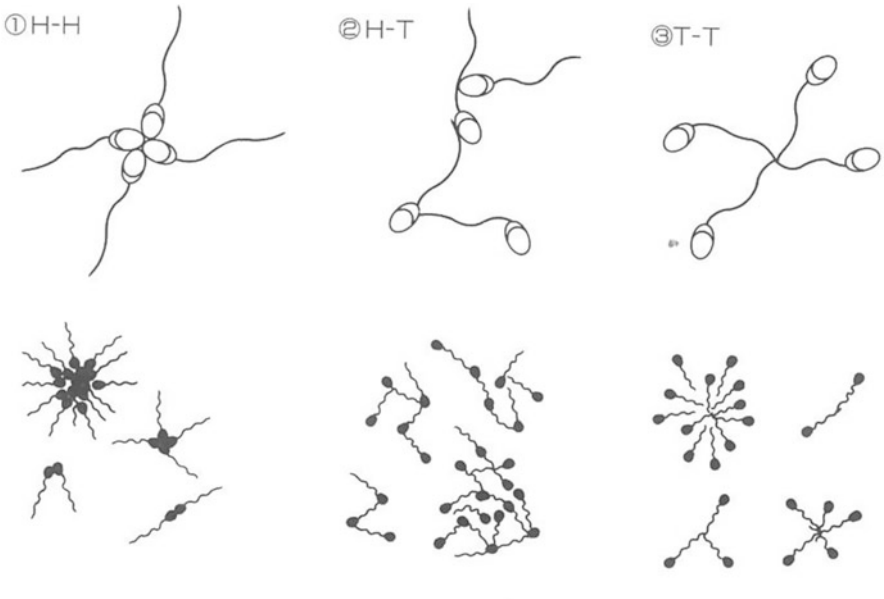

Fig. 2.2 Various types of sperm-agglutination by anti-sperm antibodies (ASAs) [14]. There are various types of sperm-agglutination by ASA. They include head-to-head (H-H, ①), head-to-tail (H-T, ②), and tail-to-tail (T-T, ③) of sperm-agglutination patterns

tray, and agglutination is observed using an inverted microscope. TAT enables a technician to test several hundred serum samples in a single day, using sperm from a single donor. The TAT is apparently more sensitive than the GAT technique, not only for detecting head-to-head agglutination but also tail-to-tail agglutination [19]. This difference in sensitivity is most prominent in the case of female sera, probably because the microscopic examination can detect small agglutinates commonly found with such sera [20].

2.2.1.2 Tests for the Detection of Sperm-Immobilizing Antibodies

2.2.1.2.1 Semiquantitative Sperm-Immobilization Test (SIT)

Because of the nonspecificity of the sperm-agglutination test (SAT), Isojima et al. developed a complement-dependent sperm-immobilization test (SIT) that detects sperm-immobilizing antibodies in the sera of infertile women [12, 13]. This assay utilized the function of sperm-immobilization in the presence of complement.

In their reports, positive reactions in the SIT were given by sera of 17.2% of the patients with infertility of unexplained cause and not by those of normal pregnant and unmarried women. Later, this observation was also confirmed by other studies [21–25]. Therefore, among the several assay methods detecting ASA, SIT has been

Table 2.2 Comparison of the incidence of sperm-immobilizing antibodies between two institutes in Japan

Institute	No. of patients		Incidence (%)
	Tested	Positive	
Jichi MU (*May 1999 to May 2008*)	1339	31	2.3*
Hyogo CM (*Jan. 1991 to Dec. 1995*)	1676	46	2.7*
Total	3015	77	2.6

*$P > 0.05$

shown to be the most reliable assay for aiding in the determination of female infertility because of its specificity.

The original assay method for the SIT [12] was slightly modified to improve the specificity and sensitivity [26]. In the modified test, a volume of 0.25 mL of heat-inactivated (56 °C, 30 min) test serum, 0.025 mL of human sperm suspension (40×10^6/mL), and 0.05 mL of guinea pig serum (ca. 10 C'H_{50} units as complement) are mixed in a small test tube and incubated with gentle shaking at 32 °C for 60 min. One drop of the mixture is put on a glass slide, and the number of motile sperm is counted under the microscope (×200). The sperm-immobilization value (SIV) is calculated by dividing the percentage of sperm motility in the control (C%) by that in the test serum with complement (T%). The ratio C/T is designated as the SIV. When the SIV is 2 or more, the test serum is judged as positive for sperm-immobilizing antibodies. The higher the SIV, the stronger the sperm-immobilizing antibody activity; however, it should be noted that this result is only to be semi-quantitative. The recent incidences of the SIT in the two institutes that this author belonged were 2.3–2.7% (Table 2.2).

As the ordinary tube assay method is unsuitable for the examination of sperm-immobilizing antibodies in a small volume of specimens like cervical mucus, a microtechnique of the SIT was developed [27, 28]. To improve its specificity, sensitivity, and feasibility, a slightly modified method was developed. As shown in Fig. 2.3, 10 μL of inactivated patient's serum, 1 μL of human sperm suspension (40×10^6/mL), and 2 μL of guinea pig serum as complement source (C'$H_{50} = 200$) or heat-inactivated guinea pig serum as control are mixed on a Terasaki microplate and incubated at 32 °C for 60 min with shaking on a plate mixer. A 10 μL of inactivated unmarried women's serum with complement and 10 μL of inactivated standard patient's serum containing sperm-immobilizing antibodies with complement are used as negative and positive controls. The sperm motility is measured directly in the plate under the microscope (×200).

2.2.1.2.2 Quantitative Sperm-Immobilization Test (SIT)

In the qualitative sperm-immobilization method, the very high titer of SIV introduces errors, and high titers of SIV are not quantitative. As the SIV cannot quantify the antibody exactly, Isojima et al. developed the quantitative method for this antibody [29].

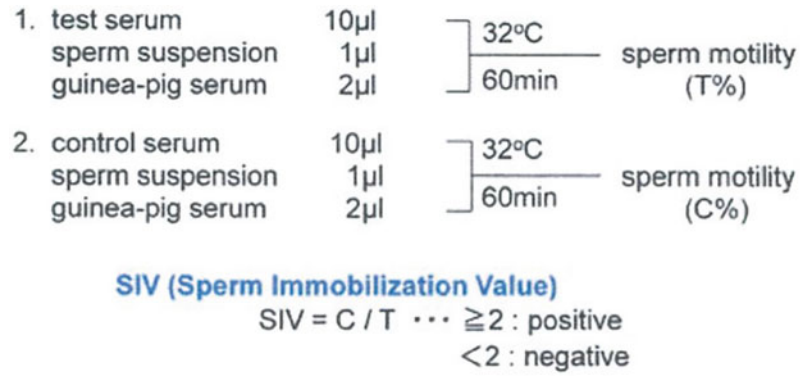

Fig. 2.3 Sperm-immobilization test (micro-method). 10 μL of inactivated patient's serum, 1 μL of human sperm suspension (40×10^6/mL), and 2 μL of guinea pig serum as complement source ($C'H_{50} = 200$) or heat-inactivated guinea pig serum as control are mixed on a Terasaki microplate and incubated at 32 °C for 60 min with shaking on a plate mixer. 10 μL of inactivated unmarried women's serum with complement and 10 μL of inactivated standard patient's serum containing sperm-immobilizing antibodies with complement are used as negative and positive controls. The sperm motility is measured directly in the plate under the microscope (×200)

For quantitative assay for SIT, each test serum is serially diluted twofold with control serum. The sperm motility in the test serum (T%) and in the control serum (C %) were calculated according to the semiquantitative assay method. The antibody activity for sperm-immobilization is calculated by the formula (C-T/C × 100), and the value is plotted against the dilutions of the test serum on a semi-logarithmic chart to obtain a sigmoid dose-response curve. The dilution of the test serum at which the sigmoid curve crossed the value of 50 for the antibody activity is determined on the dose-response line and designated as 50% sperm-immobilization unit (SI_{50}), as shown in Fig. 2.4. Thus, the amount of sperm-immobilizing antibodies in various sera can be compared. This method is very useful for routine examination of sperm-immobilizing antibody in the sera of infertile women.

Isojima and Koyama found by using the scanning electron microscope that the sperm-immobilizing antibodies opened holes at the acrosome lesion of sperm head and also around the midpiece in collaboration with the complement (Fig. 2.5, unpublished data). This phenomenon is the reason for the sperm-immobilization due to the lysis of sperm membrane by the antibodies and the complement.

Koyama et al. applied this quantitative method to a follow-up study of the SI_{50} titers in the sera of infertile women [30]. When the SI_{50} titers were followed over 3 years in infertile women with sperm-immobilizing antibodies, the SI_{50} titers were found to be unstable and undulated over a period of several months (Fig. 2.6). Later, Kobayashi et al. reported that they found the SI_{50} titers associated with pregnancy rates [31]. Patients with high SI_{50} titers (Fig. 2.6, Group A, greater than 10 units) did not conceive by intrauterine insemination (IUI) except when they were treated with in vitro fertilization-embryo transfer (IVF-ET). Patients with relatively low SI_{50} titers (Fig. 2.6, Group C, less than 10 units) could conceive by IUI, though the

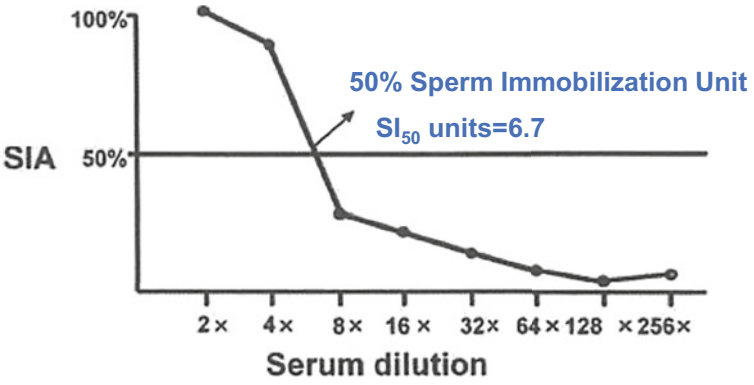

Fig. 2.4 Sperm-immobilization test (quantitative method). Sperm-immobilization in various dilutions of a standard human serum with the sperm-immobilizing antibody is presented. A certain standard patient's serum was serially diluted twofold with control serum. The sperm motility in the test serum (T%) and in the control serum (C%) were calculated according to the semiquantitative assay method. The sperm-immobilization activity (SIA) was calculated by the formula (C-T/C × 100), and the value was plotted against the dilutions of the test serum on a semi-logarithmic chart to obtain a sigmoid dose-response curve. The dilution of the test serum at which the sigmoid curve crossed the value of 50 for the antibody activity was determined on the dose-response line and designated as 50% sperm-immobilization unit (SI_{50}). The SI_{50} titer of the standard serum was calculated as 6.7 on this dose-response line

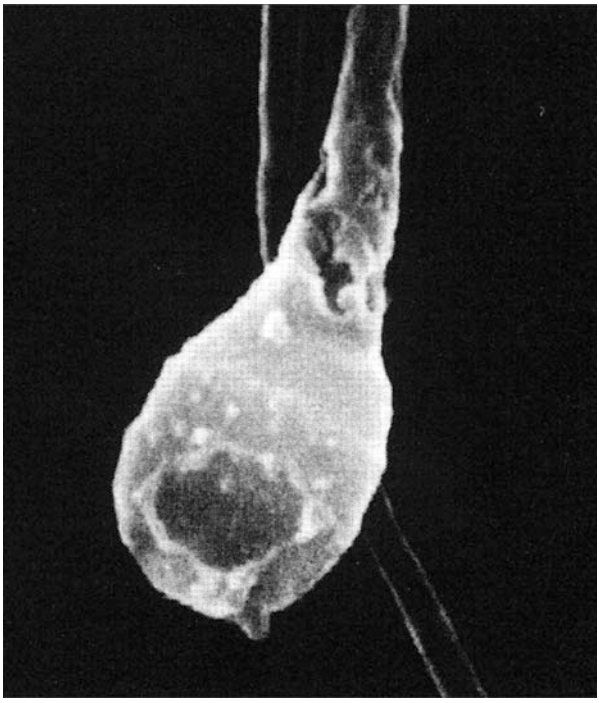

Fig. 2.5 Electron microscopic findings of immobilized human sperm reacted with sperm-immobilizing antibodies and complement. By using the scanning electron microscope, it was found that the sperm-immobilizing antibodies opened holes at the acrosome lesion of sperm head and also around the midpiece in collaboration with the complement (Fig. 2.5, unpublished data). This phenomenon is the reason for the sperm-immobilization due to the lysis of sperm membrane by the antibodies and the complement

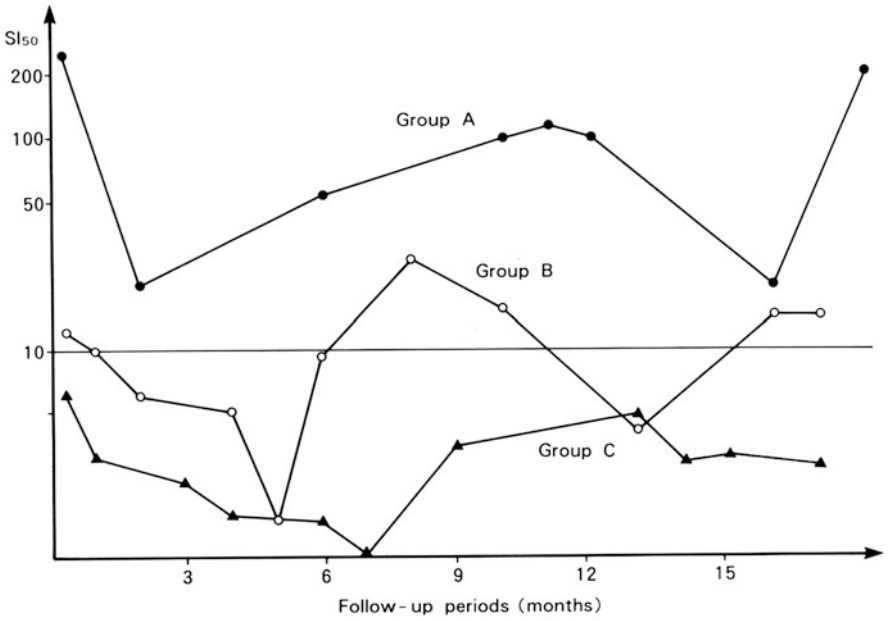

Fig. 2.6 Three undulation patterns (Groups A, B, and C) of SI_{50} titers in the sera of infertile women [31]. Patients were retrospectively divided into three groups (A, B, and C) according to their SI_{50} titers, and the typical patterns of SI_{50} titers in these three groups are shown. Group A included patients whose SI_{50} titers were constantly >10 units, Group B included patients whose SI_{50} titers varied around 10 units, and Group C included patients whose SI_{50} titers were constantly <10 units

success rates were lower than by IVF-ET. They concluded that it is important to assess the SI_{50} titers by the quantitative method to select treatments for infertile women with sperm-immobilizing antibodies.

2.2.1.2.3 Objective Measurement of the Sperm-Immobilization Test (SIT)

Because the judgment of sperm motility is subjective, the results may vary depending on the experience and ability of the operator. To improve it, Komori et al. [32] applied and found the usefulness of Sperm Quality Analyzer (SQA, United Medical Systems Inc., Santa Ana, CA, USA) [33–39] to estimate objective sperm motility for the detection of sperm-immobilizing antibodies. They showed that there was a significant correlation in comparison with SI_{50} titers between the traditional method and the digital method using the SQA. The SQA is a simple and inexpensive commercial unit; they concluded that the results obtained by the method were in good agreement with those obtained by the conventional method.

Recently, Wakimoto et al. [40] applied the computer-aided sperm analysis (CASA) (SMAS, DITECT CO., Tokyo, Japan) for the detection of sperm-immobilizing antibodies. CASA systems that can identify and track human sperm

have been developed to revolutionize the research of the movement of human sperm [37, 39, 41–46]. The CASA has the advantages of providing objective semen analysis data on sperm kinetics. Our group has been using CASA to predict sperm fertilizing ability in IVF [43], comparing its functions with other devices such as SQA [34], and analyze the association with multinucleate formation in IVF [44]. We have also shown that three semen parameters including normal morphology before sperm separation and rapid and VCL after sperm separation were identified as predictors of pregnancy by IUI. These variables would be helpful when counseling patients before they make the decision to proceed with in vitro fertilization (IVF)/intracytoplasmic sperm injection (ICSI)-ET [46].

Wakimoto et al. developed a novel method using CASA, and the results were compared with those obtained by the traditional method. The results were identical, and 25 of 78 samples tested were positive and 53 samples were negative for sperm-immobilizing antibodies based on both methods. For the SIT-positive samples, the values of SI_{50} obtained using the two methods correlated closely with high co-efficiency. They concluded that the novel method using CASA will make it possible to objectively evaluate SIT and SI_{50} data at several clinical facilities and will increase the convenience of using the SIT as a clinical indicator. Furthermore, we recently developed a modified method of SIT using CASA by substitution of cryopreserved sperm in case fresh sperm are unavailable [47].

2.2.1.3 Tests for the Detection of Fertilization-Blocking Antibodies

There are various stages of the normal fertilization process including sperm capacitation, sperm-zona pellucida binding, acrosome reaction, sperm penetration through zona pellucida, sperm-egg fusion, and post-fusion events. Several diagnostic tests were developed to predict the fertilization potential of sperm, including the hemizona assay (HZA) for sperm-zona pellucida tight binding [48], zona pellucida penetration assay for sperm penetration through zona pellucida [49], and zona-free hamster egg-sperm penetration assay for sperm-egg fusion [50]. It has been shown that the sperm-immobilizing antibodies can exert inhibitory effects on sperm-egg interaction at the level of the acrosome reaction [51] and zona pellucida recognition and penetration [52–60].

2.2.1.3.1 Test for the Detection of Anti-sperm Antibodies that Inhibit the Hemizona Assay (HZA)

In 1988, Burkman et al. developed the hemizona assay (HZA) to predict the fertilizing potential of sperm in human [48]. The HZA uses the matching halves of a human zona pellucida from a non-fertilizable and non-living oocyte, providing an internal control on zona-to-zona variability. Maximal binding of human sperm to the hemizona usually occurred after 4–5 h of coincubation. Sperm from fertile men exhibited significantly higher binding capacity to the hemizona compared with

sperm from men who had fertilization failure during IVF treatment. The hemizona index (HZI) is calculated as follows: (bound sperm from subfertile male) ÷ (bound sperm from fertile male) × 100. They concluded that the HZA may be a useful diagnostic tool in male infertility evaluations (Fig. 2.7).

We applied the procedure of the HZA in human to detect the inhibitory effects of sperm-immobilizing antibodies on sperm-zona pellucida tight binding [5, 56, 59–61]. Briefly, human oocytes obtained from excised ovarian tissues were stored at −70 °C in a 2.0 M dimethyl sulfoxide solution in phosphate-buffered saline. The frozen oocytes were thawed and cut almost in half using the micromanipulators (Narishige, Tokyo, Japan) mounted on a phase-contrast microscope (Nikon, Garden City, NY, USA). After discarding the degenerated ooplasma, the two matched hemizona were placed overnight at 4 °C in a droplet of medium under mineral oil. Swim-up human sperm were incubated with patient's serum or test solution containing monoclonal anti-sperm antibodies against human sperm before exposure to hemizona at 37 °C for 1 h. One hemizona was placed in a 100 μL drop of swim-up sperm suspension with test sample, while the matched hemizona was placed in a drop of control serum or diluent of solution containing monoclonal anti-sperm antibodies confirmed to be non-inhibitory to sperm-zona binding. After 4 h of coincubation, each hemizona was removed and rinsed vigorously to detach loosely associated sperm. Then the number of sperm tightly bound to the outer hemizona surface was counted. Each zona and each sample were tested twice. The HZI was the number of sperm bound to the hemizona in the test sample divided by that in the control, all multiplied by 100 (Fig. 2.8). When the HZI was 50% or less, the sperm binding to zona pellucida was considered to be inhibited in the test sample as compared with the control sample, according to the criterion of Mahony et al. [57, 58].

To evaluate the in vitro effects of sperm-immobilizing antibodies on sperm-zona pellucida (ZP) tight binding, the HZA was used to study the inhibitory effects of infertile women's sera with and without sperm-immobilizing antibodies on sperm-zona pellucida tight binding. These results were compared with those of monoclonal sperm-immobilizing antibodies. Sera from 40 infertile women (24 with and 16 without sperm-immobilizing antibodies) and 2 postpartum women as control were used. Of 24 patients' sera with sperm-immobilizing antibodies, 23 (96%) showed significant inhibitory effect, whereas none of 16 patient's sera without sperm-immobilizing antibodies exhibited any inhibitory effect. However, there was no correlation between the antibody titers of sperm-immobilizing antibody and the hemizona index. The vast majority of sperm-immobilizing antibodies reduce sperm-zona pellucida binding even without the presence of complement [59].

2.2.1.3.2 Test for the Detection of Anti-sperm Antibodies that Inhibit the Zona Pellucida Penetration Assay (ZPA)

To assess the fertilizing capacity of human sperm, the zona pellucida penetration assay (ZPA) was developed by Yanagimachi et al. [49]. We used the ZPA to test the

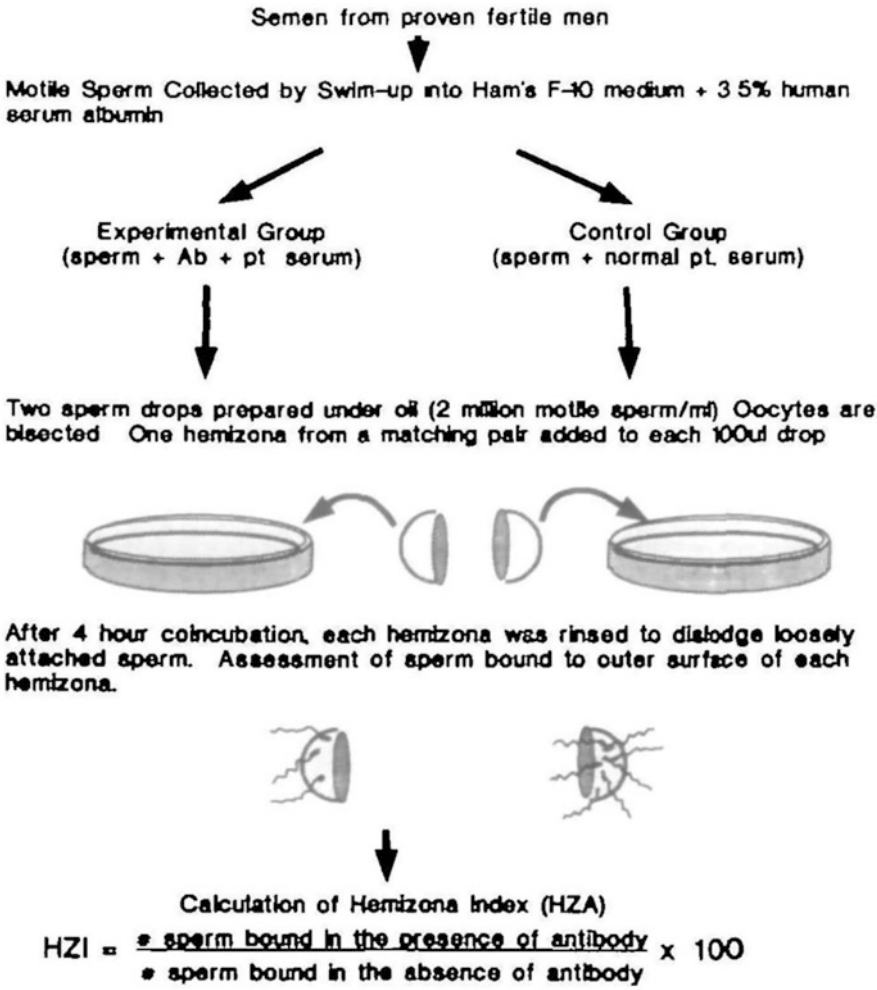

Fig. 2.7 Procedure for the hemizona assay (HZA) to detect the inhibitory effects of anti-sperm antibodies on sperm-zona pellucida tight binding [5]. The frozen human oocytes were thawed and cut almost in half using the micromanipulators mounted on a phase-contrast microscope. After discarding the degenerated ooplasma, the two matched hemizona (HZ) were placed overnight at 4 °C in a droplet of medium under mineral oil. Swim-up human sperm were incubated with patient's serum or test solution containing monoclonal anti-sperm antibodies (ASAs) against human sperm before exposure to HZ at 37 °C for 1 h. One HZ was placed in a 100 μL drop of swim-up sperm suspension with test sample, while the matched HZ was placed in a drop of control serum or diluent of solution containing monoclonal ASA confirmed to be non-inhibitory to sperm-zona binding. After 4 h of coincubation, each HZ was removed and rinsed vigorously to detach loosely associated sperm. Then the number of sperm tightly bound to the outer HZ surface was counted. Each zona and each sample were tested twice. The HZI was the number of sperm bound to the HZ in the test sample divided by that in the control, all multiplied by 100 (Fig. 2.8). When the HZI was 50% or less, the sperm binding to zona pellucida was considered to be inhibited in the test sample as compared with the control sample

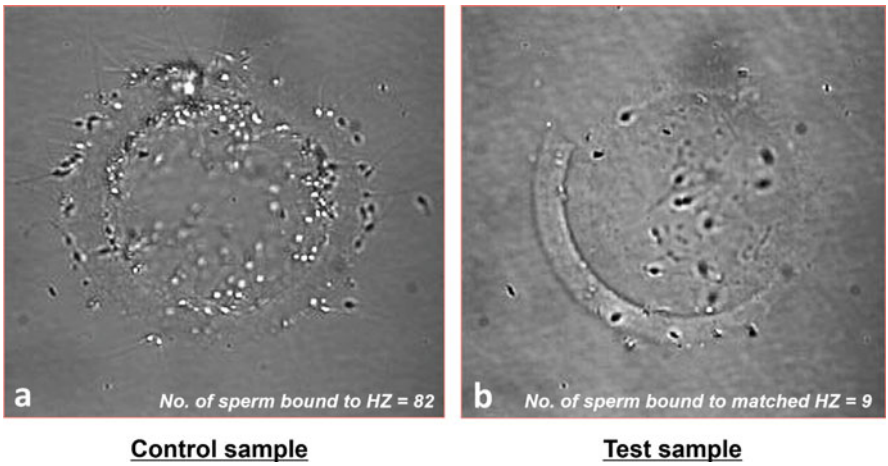

Fig. 2.8 Inhibitory effect of sperm-immobilizing antibodies in the sera of infertile women on hemizona assay (HZA) [5]. The hemizona index (HZI) was the number of sperm bound to the hemizona (HZ) in the test sample divided by that in the control, multiplied by 100. When the HZI was 50% or less, the sperm binding to the zona pellucida was considered to be inhibited in the test sample as compared with the control sample. The number of sperm bound to the HZ was (**a**) 82 when inseminated with control sperm and (**b**) 9 when inseminated with test sperm with ASA. The HZI in this patient was 11.0

blocking effects of anti-sperm antibodies on sperm penetration through the human zona pellucida. Human immature oocytes obtained from excised ovarian tissue were incubated in Menezo's B2 medium (France) containing 1% bovine serum albumin (BSA) for 48 h and then stored at 4 °C in a highly concentrated salt solution containing 0.5 M ammonium sulfate (Wako-junyaku, Osaka, Japan), 1 M magnesium chloride (Wako-junyaku), and 0.1% dextran (Wako-junyaku) until use. Swim-up sperm were incubated with test solution containing monoclonal antibodies against human sperm or control solution containing NS-1 (mouse myeloma cells) before exposure to salt-stored human zona pellucida at 37 °C for 24 h. Three eggs were used in each assay, and each monoclonal antibodies was tested three times. The zona pellucida penetration index (ZPI) was calculated by dividing the number of sperm penetrated into the perivitelline space in the test sample by that in the control sample and multiplying by 100. A value <50% was considered to show inhibition of zona pellucida penetration by sperm.

Four monoclonal antibodies (2C6, 1G12, 3B10, and H6-3C4) against human sperm were used to investigate the diversity of the ASA on fertilization by using the sperm functional assays including HZA, ZPA, and the zona-free hamster egg-sperm penetration assay (SPA), as described below. Both of the two monoclonal antibodies, 2C6 and 1G12, had strong sperm-immobilizing activities and showed significant inhibitory effects in all the three assays. One monoclonal antibody, 3B10, which did not have sperm-immobilizing activity, never inhibited sperm binding to the zona pellucida but showed blocking effects on sperm penetration

through both the zona pellucida and the ooplasm. A human monoclonal antibody, H6-3C4, had strong sperm-immobilizing activities but did not show any inhibitory effects in any of the assays [60].

2.2.1.3.3 Test for the Detection of Anti-sperm Antibodies that Inhibit the Zona-Free Hamster Egg-Sperm Penetration Assay (SPA)

To assess the fertilizing capacity of human sperm, the zona-free hamster egg-sperm penetration assay (SPA) was developed by Yanagimachi et al. [50]. We used the SPA to test the blocking effects of anti-sperm antibodies on sperm penetration through zona-free hamster egg. Female golden hamsters at the age of 8 weeks were induced to ovulate by an intraperitoneal (i.p.) injection of 30 IU of pregnant mare serum (PMS) on the morning of post-estrous vaginal discharge. At 48 h later, each animal received an i.p. injection of 30 IU human chorionic gonadotropin (HCG, Mochida Pharmaceutical Co., Ltd., Tokyo, Japan). Each hamster was killed 15–17 h later, its oviducts were removed, and the cumulus mass was freed; 0.1% hyaluronidase (Sigma) and 0.1% trypsin (Sigma) were used to free the ova from the cumulus and to remove the zona pellucida. From 10 to 20 ova were added to 0.1 mL of capacitated human sperm plus patient's serum, covered with mineral oil, and allowed to incubate for 3 h at 37 °C. The ova were then mounted on a slide, compressed with a coverslip, and examined at ×400 magnification with a phase-contrast microscope. Penetration was determined by the presence of a swollen sperm head and attached tail within the cytoplasm (Fig. 2.9) [62]. Sperm samples with no serum added served as a control and an indicator of the integrity of the SPA on any given day. Each sample was tested twice in different experiments. The sperm penetration index (SPI) was calculated by dividing the number of eggs penetrated in the test sample by that in the control sample and multiplying by 100. A value <50% was considered to show inhibition of sperm penetration.

We compared the hemizona index and sperm penetration index in infertile patients to study the diversity of the blocking effects of ASA on fertilization in human. Sera from 22 infertile women with sperm-immobilizing antibodies and 13 women with unexplained infertility without the antibodies were tested for their effects on sperm-zona pellucida binding by using HZA and on sperm penetration into the ooplasm by using SPA. Of 22 patient sera with sperm-immobilizing antibodies and 13 patient sera without the antibodies, 21 (95.5%) and none showed inhibitory effects on HZA, respectively. Of 22 patient sera with sperm-immobilizing antibodies and 13 patient sera without the antibodies, 19 (86.4%) and 8 (61.5%) inhibited SPA, respectively. There was no correlation between the HZI and the SPI in either group. No statistical correlations were found between SI_{50} titers and HZI, nor between SI_{50} titers and SPI [59].

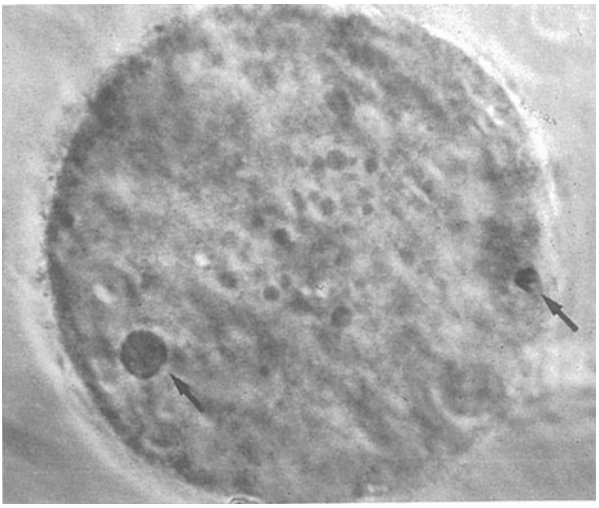

Fig. 2.9 Assessment of the swollen human sperm head in the zona-free hamster egg-sperm penetration assay (SPA) [62]. The zona-free hamster egg-sperm penetration assay (SPA) was used to test the blocking effects of anti-sperm antibodies on sperm penetration through zona-free hamster egg. The oviducts were removed from a hamster, and the cumulus mass was freed with hyaluronidase, and the zona pellucida was removed with trypsin. The zona-free oocytes were added to capacitated human sperm plus patient's serum, covered with mineral oil, and allowed to incubate for 3 h at 37 °C. Penetration was determined by the presence of swollen sperm heads (arrow) and attached tail within the cytoplasm

2.2.2 Tests for the Indirect Detection of Anti-sperm Antibodies Bound for Motile Sperm

The identification of patients with immunological infertility has traditionally depended on tests for ASA in circulating blood serum. However, some individuals possess antibodies in local secretions such as cervical mucus or seminal plasma without the presence of detectable systemic antibodies [63]. Therefore, assays such as the IBT have been developed to allow for the demonstration of membrane-bound antibodies, reflective of local immunity to sperm antigens [2–4]. The IBT, used directly, can detect and characterize sperm membrane-bound antibodies; indirectly (see Chap. 8), it can identify and provide semiquantitative data on ASA in reproductive tract secretions.

The IBT is considered the gold standard of sperm antibody assays. This test uses polyacrylamide beads labeled with antiglobulins (anti-IgG, anti-IgA, and anti-IgM), which bind to the corresponding antibody on the sperm surface. The indirect IBT can be performed on serum, cervical mucus, and follicular fluid and also on seminal plasma of semen with too few motile sperm for a direct IBT [64]. Samples for the indirect IBT may be stored frozen until analyzed. Before use, the sample is heated to 56 °C for 30 min to inactivate the complement. Aliquots of the sample are incubated

with donor sperm previously proven negative for membrane-bound antibodies by the direct IBT. These sperm are washed to eliminate unreacted immunoglobulin and mixed with immunobeads, as in the direct IBT. ASA in the test sample will bind to the donor sperm, which then can interact with the anti-human immunoglobulin on the immunobeads.

We collected serum samples from 23 infertile patients with sperm-immobilizing antibodies and 1 pregnant patient to screen sera to determine whether they contained factors to inhibit sperm-zona pellucida tight binding by using the HZA to test for this binding [56]. The HZA showed that all 23 serum samples inhibited sperm-zona pellucida tight binding. The HZI ranged from 3 to 53 with a mean of 18.1 compared to a normal HZI of 100. All 23 serum samples bound to the surface of sperm plasma membrane after 1 h coincubation as evidenced by the fact that they all demonstrated 50% IgG beads bound. Further the results of the indirect IBT for IgG showed that positive sera significantly inhibited binding more than negative sera (HZIs = 12.4 vs 24.4, $p < 0.05$). Yet serum with positive I-IBT for IgM did not affect sperm-zona binding (HZIs = 17.1 vs 19.4, $p > 0.05$). No association existed between HZI and site of immunobead binding. These results might indicate that sera with both sperm-immobilizing antibodies and antibodies recognized by indirect IBT for IgG and IgA may play a significant role to inhibit the sperm-zona pellucida tight binding. In conclusion, physicians should expect patients with low HZI to have more problems conceiving than those with normal HZI. IVF using heat-inactivated human cord serum or donor serum may help them to conceive.

In our laboratory, we have been adopting immunospheres (IS) assay since the IBT was discontinued of its production [65]. The IS uses color-coded latex beads of uniform 3.0 micron size coated with the antiglobulins which can be viewed with bright-field light microscopy. Centola GM et al. compared the IBT and IS in an indirect test using human serum specimens ($n = 42$), which were tested for the presence of antibody isotypes IgG, IgA, and IgM. Donor sperm was washed in BWW with 5% BSA and diluted to a final concentration of 50×10^6 motile sperm/mL. The sperm were incubated with a 1:10 dilution of test serum for 30 min to 1 h at 37 °C and then washed by three cycles of centrifugation. The sperm and beads (IBT, IS) were mixed on a glass slide, covered with a coverslip, and observed within 5 min. At least 100 motile sperm were counted and scored for bead binding. A specimen was considered positive if 20% or more of the sperm were coated with one or more beads. The IS was able to detect 94% of IgG antibodies, 91% of IgA antibodies, and 100% of IgM antibodies. One serum specimen was IgG negative by IS (14% binding), but positive by IBT (20%). A second serum specimen was IgA negative by IS (16%) yet positive by IBT (29%). There were no false positives with the IS assay. Of the IgM positives (five of six) occurred alone and not with IgG or IgA, suggesting the necessity for testing all specimens also for IgM. They concluded that the ASA test results obtained by the IS assay are in agreement with the results obtained with the IBT test [65]. The IS are monodispersed and color-coded and can be visualized with bright-field microscopy.

2.2.3 *Tests for the Detection of Anti-sperm Antibodies Against Sperm or Sperm Extract*

The ELISA technique has been adapted to quantitatively assess the presence of ASA. ELISA combines the specificity of the antigen-antibody reaction with the continuous degradation of chromogenic substrate by an enzyme to amplify the sensitivity of the reaction. Numerous materials and methods have been used as variations for the ELISA procedure: solid-phase materials (silicone rubber, glass, polyvinyl chloride, polystyrene), carriers (test tubes, beads, disks, microtitration plates), enzymes (alkaline phosphatase, horseradish peroxidase, glucose oxidase, galactosidase), substrates (*p*-nitrophenyl phosphate, *o*-phenylenediamine), and a wide range of wash solutions and incubation conditions [66]. Many different methods are used, differing mainly in the type of antigen. Most researchers use whole sperm as antigen, while some use a sperm membrane extract. Other variables include sperm concentration, type of sperm fixation, blocking agents, serum, and seminal plasma dilutions [66]. Said et al. described that the complexity, instrumentation, and expense of the ELISA have prevented its widespread use in the workup of immunological infertility [67].

The use of flow cytometry has been reported to detect sperm-bound antibodies and to quantitate the sperm antibody load (antibody molecules/spermatozoa). Following staining of the washed sperm samples, dead sperm are excluded with fluorescein isothiocyanate-conjugated F(ab')2 fragments of anti-IgG and IgA antibodies by the use of calibration standards. Flow cytometry has the potential reliability and objectivity to quantitate sperm antibodies; therefore, the sperm antibody load can be used to compare different patients or to follow up the progression of the same patient [68]. Similar to ELISA, flow cytometry is not currently widely used for the detection of ASA due to its complexity, expense, and instrumentation requirement [69].

In the same context, the agglutinin radiolabeled antibody assay for the detection and quantitation of ASA is of limited use. This method is also limited by an inability to determine specific ASA location, expense, and reliance on highly skilled labor [69].

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