

An Improved Indirect Enzyme-Linked Immunosorbent Assay (ELISA) Suitable for Mass Screening of Anti-Sperm Antibodies¹

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Abstract— An improved, indirect enzyme-linked immunosorbent assay (ELISA), which detects antisperm antibodies (ASA) in serum, which appears to be a sensitive and easy quantitative procedure for mass screening that is not available with conventional biologic assay systems. Using known positive human antisperm antisera, it was found that the ELISA method was quite reproducible at a wide spectrum of titers. When comparing the ELISA method and the gelatin agglutination (GAT) with the tray agglutination test (TAT) using heterologous mouse anti-human sperm antisera, it was found that the ELISA method was more sensitive than the GAT or TAT by more than a three fold dilution factor. The sera of 85 vasectomized subjects were studied with the ELISA, GAT and TAT to determine ASA. We concluded that, in busy laboratories with heavy workloads, this improved ELISA is a very sensitive, easily reproducible and efficient method of mass screening for sperm immunity.

Key words: *Antisperm antibodies (ASA), Indirect ELISA, Vasectomy*

INTRODUCTION

Over 1.2 million Korean men underwent vasectomies since the end of 1962 in Korea (Lee, 1986; Lee 1987). Vasectomy as a method of fertility control has been accepted so widely since the late 1960s that well over 50 million vasectomies have been performed all over the world (Linnet, 1983). Since 1959 when Rumke and Hellinga reported that a high rate of infertile men with antisperm antibodies (ASA) had genital tract obstructions, many prospective studies have clearly shown that vasectomies induce such circulating antibodies to homologous spermatozoa in the serum of 30-60% of the vasectomized men (Ansbacher 1973). In this case, the evaluation of the possible long-term effects of

ASA on health is of primary importance. Of secondary importance is that the factors affecting vasectomy reversal be clarified, since 1 reversal is performed for every 500 vasectomies (Lee, 1987).

Generally, even in the most skilled hands, about 1/3 of the vasovasostomized men are unable to bring about pregnancies later. The factors that interfere with pregnancy seem to be caused by the seminal presence of the antisperm antibody. An assay for ASA is necessitated as routine procedure in clinical laboratories in regions where vasectomy is employed as a major contraceptive tool, such as in Korea and China.

Commonly used assays for ASA like the gelatin agglutination test (Kibrick *et al.* 1952), the sperm immobilization test (Isojima *et al.* 1968), and the tray agglutination test (Friberg, 1974) require fresh donor semen of excellent quality and an experienced technical staff. Thus, due to the limited amount of donor ejaculation and the tedious reading of the results, only a small number of samples can be examined in one day. Acker-

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man *et al.* introduced an enzyme-linked immunosorbent assay (ELISA) for the detection of ASA in 1981, however, it was too laborious to be adopted as the routine procedure in busy laboratories.

Following study describes a modified indirect ELISA using whole spermatozoa as an antigen, coated to polystyrene microtiter plates and fixed by cytofix spray, in contrast to ELISAs used so far, thus yielding very stable and reliable assay results. Also, in this present work, another attempt was made to compare the antibody-detection methods of sperm agglutination in serum with regard to the reliability of each test result in vasectomized men.

MATERIALS AND METHODS

A. Subjects

Forty-two normospermic males, having no genital tract obstruction, were tested ASA in serum as a control group. Eighty-five vasectomized males were included to assess ASA by ELISA and various agglutination techniques. The duration of the marriages and of the genital tract obstructions in the patient group were 7.3 years and 5.2 years as their mean, respectively (Table 1).

B. Assessment techniques

Blood samples were drawn without anticoagulant and stored at -20°C after separating the serum by centrifugation. Sperm agglutination activity in the serum was determined by the Kibrick method (gelatin agglutination test, macro-

scopic sperm agglutination test, GAT), the Friberg method (tray agglutination test, microscopic sperm agglutination test, TAT) with minor modification, and an indirect ELISA test.

C. Indirect enzyme-linked immunosorbent assay (ELISA)

Preparation of spermatozoa as antigens: Semen samples were obtained from healthy fertile donors. After liquefaction, the semen was suspended in 20 ml Dulbecco's phosphate buffered saline (PBS) for 30 minutes at room temperature to separate debris and clumps, and then the sperm was washed five times with 10 ml of PBS.

Coating of microtiter plates with spermatozoa: A 100 micro-litter aliquot of the sperm suspension (adjusted the concentration of 1×10^5 /ml) in PBS was dispensed into each well of the micro-ELISA plates (Dynatech, flat bottom). The plates were placed on a table at room temperature for 48 hours. The supernatant was aspirated and discarded using a 25-gauge needle connected to a vacuum pump. The plates were fan-dried, fixed by Cytofix pressure spray (Smith-Biolab) and left for 1 hour. They were washed three times at 10-minute intervals with 200 micro-liters of PBS containing 0.05% Tween 20 (PBS-Tween 20) per well. After a final wash with distilled water, the plates were air-dried completely prior to sealing and stored at 4°C until ready for use.

Procedure of the ELISA: The sperm-coated plates were incubated at 37°C in a 100 micro-litter well of 1% bovine serum albumin (BSA) in PBS Tween 20 for 1 hour before adding test sera, then washed. The test and control sera were diluted two-fold successively with 0.5% BSA in PBS-Tween 20. A 100 micro-litter aliquot of each diluted serum was added to each well in duplicate. The plates were then incubated for 1 hour at 37°C with a covering of aluminum foil. After incubation, the solution was discarded and the plates were washed three times at 10-minute intervals with PBS-Tween 20. The 100 micro-litter of peroxidase conjugated rabbit anti-human gamma globulins diluted PBS-Tween 20 was added to each well, and the plates were incubated at 37°C for 1 hour more with an aluminum foil cover. The solution was discarded and the plates were washed with PBS-Tween 20 three times at 10-minute intervals. The plates

Table 1. Clinical characteristics of study subject

Groups Parameters	Control	Vasectomy	Total
Number of patients	42	85	127
Age : Mean	29.0	35.3	34.3
: Range	22-43	25-61	22-61
Duration (yrs) of marriage			
: Mean	4.7	7.3	6.9
: Range	8-18	1-35	0-35
of obstruction			
: Mean		5.2	5.2
: Range		1-21	1-21

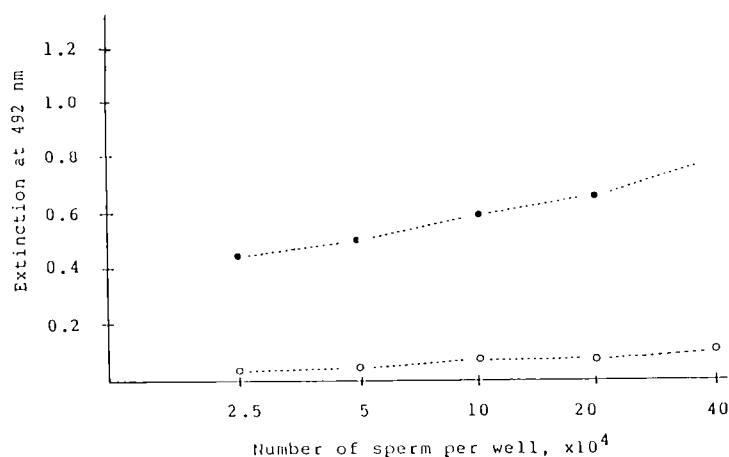


Fig. 1. Determination of optimal sperm concentration as antigen coated to the wells of a microtiter plate. (●—● = ASA (+) sera; ○—○ = ASA (-) sera).

were then washed three times with distilled water and air-dried. A 100 micro-liter of aliquot of peroxidase substrate, 5-amino-salicylic acid (5-AS) solution was added to each well, and the plates were incubated at 37°C for 30 minutes. The absorbance of the solution at 492 nm was monitored by the ELISA autoreader (Dynatech). When necessary, the reactions were stopped by adding 100 micro-liter of 4% sodium hydroxide solution, and the plates were read at a convenient time.

Interpretation of the results: A threshold level of absorbance was pre-determined sera from a control group of healthy fertile males. The value of the threshold was set at the maximum absorbance (0.258) at titer of 1 : 64 obtained from these control sera. Test sera were considered positive if the absorbance value at a titer of 1 : 64 was greater than 30% above the threshold.

RESULTS

Antigen (spermatozoa) concentration coated to microtiter plates: As seen in Fig. 1, the sperm adsorption capacity of the polystyrene microtiter plate is high enough so that even in small amounts like 2.5×10^4 sperm/well yielded reasonable results. However, it is recommended that 1×10^5 spermatozoa be coated onto each microwell for ease of calculation, provided there is sufficient spermatozoa available.

Fixation of spermatozoa into microtiter plates: Spermatozoa fixed with Cytotfix pressure

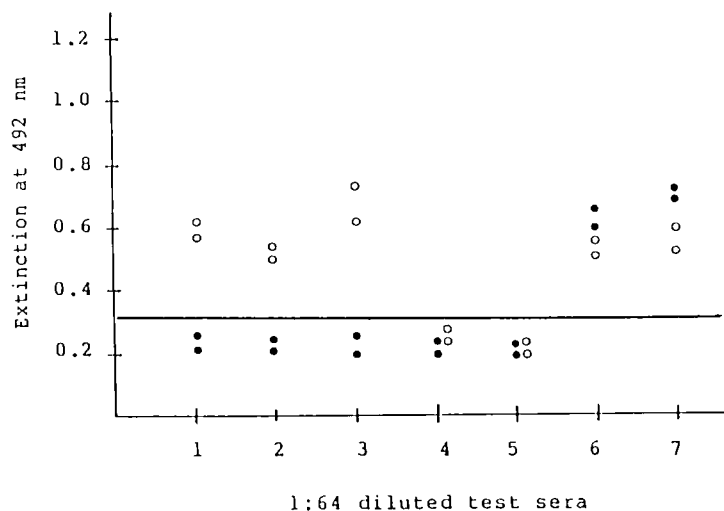


Fig. 2. Comparison of sperm fixed with Cytotprep and non-fixed in ELISA for ASA. (serum samples 1 to 5 were negative, 6 and 7 were positive. ● = Cytotprep; ○ = non-fixed)

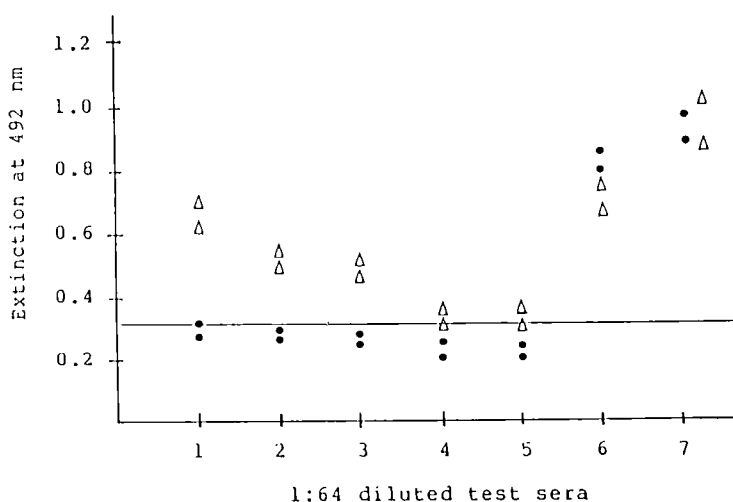


Fig. 3. Comparison of fixing reagents: Cytotprep and 0.01% glutaraldehyde in ELISA for ASA. (serum samples 1 to 5 were negative, 6 and 7 were positive. ● = Cytotprep; △ = 0.01% glutaraldehyde)

spray onto the wells of microtiter plates revealed more reliable results than non-fixed spermatozoa (Fig. 2) or spermatozoa fixed with 0.01% glutaraldehyde (Fig. 3).

Effects of storage at -20°C on spermatozoa-coated microtiter plates: As seen in Fig. 4, the extinction of a spermatozoa-coated microtiter plate stored at -20°C did not change even, as long as 8 weeks after preparation. It seems to be a reasonable period for a routine batch assay of ASA.

Concentration of bovine serum albumin (BSA): The percent of concentrated bovine

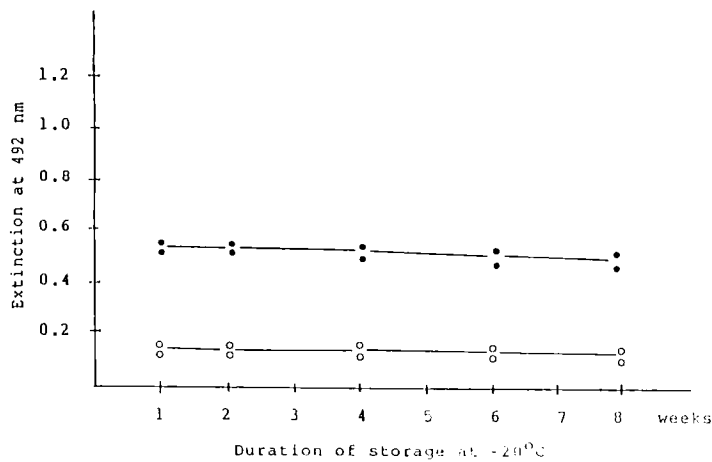


Fig. 4. The effect of storage at -20°C on sperm-coated microelisa plates. (● = positive serum; ○ = negative serum)

Table 2. Comparison of titers obtained from heterologous mouse anti-human sperm antisera with GAT, TAT, and ELISA

Sera*	GAT	TAT	ELISA
A	8	64	256
B	16	128	1,024
C	64	256	2,048
D	512	1,024	4,096

*WHO reference sera provided by Dr. T. Hjort, University of Aarhus, Denmark (A: #9, B: #7, C: #106, D: #29).

serum albumin in the detection of ASA was optimal in 0.5% and 1% as seen in Fig. 5.

Incubations: Optimal discrimination between antisperm antibody-positive and-negative samples was obtained with 1 hour incubations both for the test sample and the second antibody comparing 30 minutes and 90 minutes incubations.

Sensitivity: Four WHO-reference sera (kindly provided by Dr. T. Hjort, University of Aarhus, Denmark) were evaluated for ASA by different assays (Table 2). The ELISA method proved to be more sensitive than GAT or TAT, with a two-to-four-fold dilution factor, as shown in Fig. 6.

Reproducibility: ASA-positive sera were each diluted and dispensed into 80 wells of one-microtiter plates. As seen in Table 3, intra-assay reproducibility ranged from 94.8% to 96.0%, and inter-assay reproducibility ranged from 90.3% to

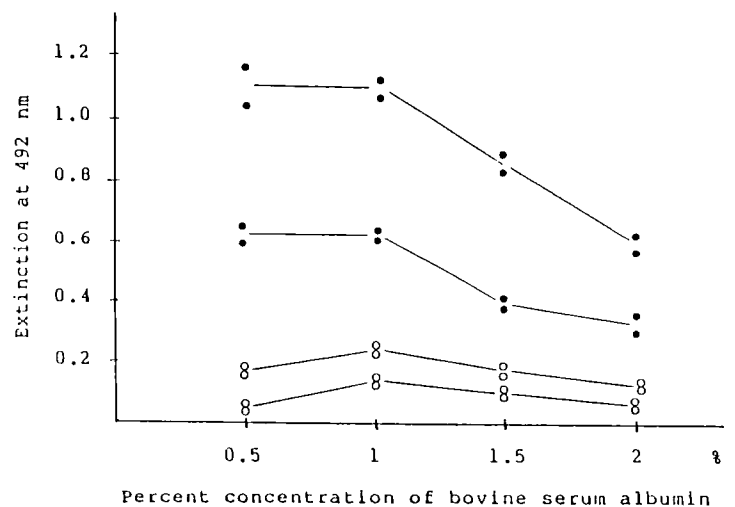


Fig. 5. The effect of bovine serum albumin concentration on sperm for ASA detection in ELISA. (● = positive sera; ○ = negative sera)

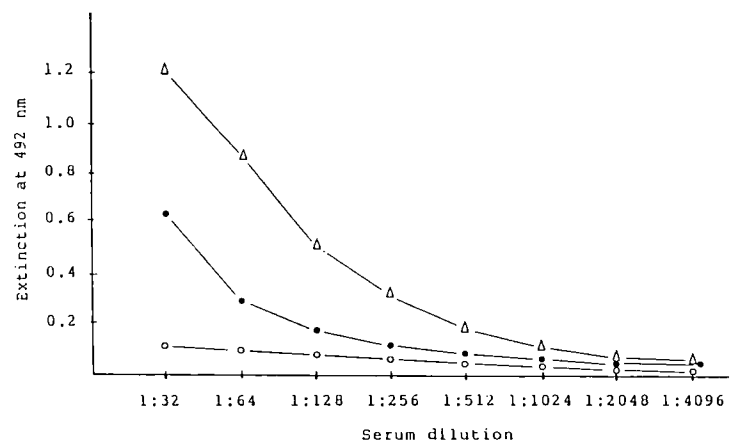


Fig. 6. Typical serum ASA titration curves in ELISA. (△ = WHO reference serum #106, 1 : 2048; ● = vasaectomized subject, 1 : 512; ○ = control fertile man, negative)

91.4%.

Threshold and "cut-off" levels of absorbance adjusted in ELISA: The value of the threshold was set at the maximum absorbance (0.258) at a titer of 1 : 64 obtained from these control sera. The sera were considered positive if the absorbance value at a titer of 1 : 64 was greater than 30% above the threshold (0.335) as shown in Fig. 7.

Comparison of GAT and TAT to ELISA: The correlation of ASA detected by ELISA to those of GAT and TAT on sera of eight-five vasaectomized subjects is seen in Table 4. The concordance rates were 62% between GAT-positive and ELISA-positive sera and 58% between TAT-positive and ELISA-positive sera.

Table 3. Reproducibility of ELISA for the detection of antisperm antibodies at various titers

Titers	Intra-assay ^a , n=4		Inter-assay ^b , n=4	
	Absorbance, ×1,000(Mean±S.D)	Reproducibility ^c	Absorbance ×1,000(Mean±S.D)	Reproducibility
1 : 32	218±11.3	94.8%	248±24.0	90.3%
1 : 64	169± 8.1	95.2%	172±17.2	90.0%
1 : 256	190± 7.6	96.0%	186±16.0	91.4%

a: Evaluated by analyzing 4 duplicated results from same plate.

b: Evaluated by analyzing duplicated results of 4 different days over a 1 month period.

c: 100% minus coefficient of variation.

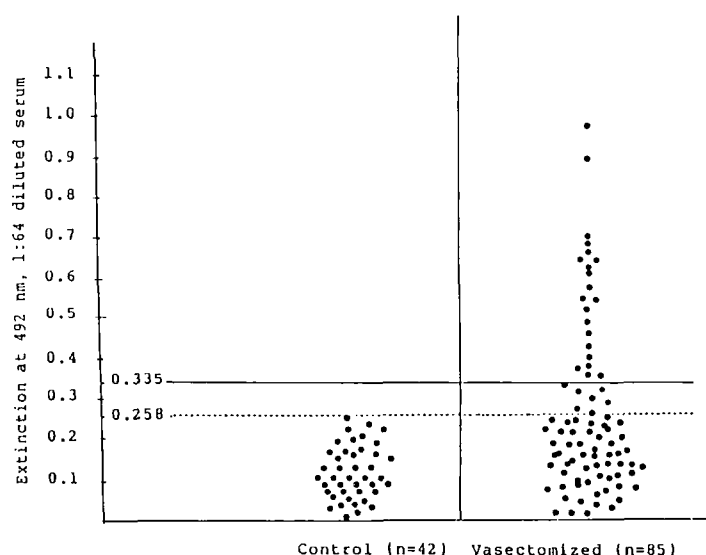


Fig. 7. Threshold and "cut-off" levels of absorbance adjusted in ELISA to detect ASA in the control and vasectomized groups.

Prevalance of the ASA detected by GAT, TAT and ELISA: As seen in Table 5, ASA were positive in 7% by ELISA in the control subjects and 25% in vasectomized subjects when establishing the positive at a dilution of more than 1 : 64.

Mode of sperm agglutination: Among fifty-three TAT-positive subjects, the mode of sperm agglutination in the ELISA-positive group was tail-to-tail (73%) and ELISA-negative group was mostly head-to-head agglutination (84%) as shown in Table 6.

DISCUSSION

Vasectomy clearly results in ASA (Shulaman *et al.*, 1972; Jones, 1982), and this production is related to the autoimmune potential of spermatozoa and provoked in connection with the continuous disposal of sperm (Kibrick *et al.*, 1952).

Table 4. Correlation of antisperm antibodies detected by ELISA to those of GAT and TAT on sera of eighty-five vasectomized subjects

Groups	GAT		TAT	
	Positive	Negative	Positive	Negative
ELISA				
Positive : 21(25%)	13 (62%)	8 (38%)	12 (58%)	9 (42%)
Negative : 64(75%)	40 (63%)	24 (37%)	39 (60%)	25 (39%)
Total : 85(100%)	53 (62%)	32 (38%)	51 (60%)	34 (40%)

Criteria of positive titer. GAT: titer > 1 : 4; TAT: titer > 1 : 4; ELISA: titer > 1 : 64 (threshold of absorbance value : 0.335)

Table 5. Prevalance of antisperm antibodies in sera of study groups by detection technique

Groups Tests	Control	Vasectomy
TAT (>1 : 4)	7 (17%)	53 (62%)
TAT (>1 : 4)	5 (12%)	51 (60%)
ELISA (>1 : 64)	3 (7%)	21 (25%)
Total No. pts(%)	42 (100%)	85 (100%)

The consequences of this ASA production are the resultant effect on fertility after vasovasostomy and the potential systemic effect on other organ systems (Choi and Lee, 1984).

To estimate antisperm antibodies, different techniques, such as the gelatin agglutination test (GAT), tray agglutination test (TAT), tube slide agglutination test (Franklin and Dukes, 1964), sperm immobilization test (SIT), mixed antiglobulin reaction test (Meinertz and Hjort, 1986), cellular radioimmunoassay (Erickson, 1972), and the enzyme-linked immunosorbent assay (Wolff and Schill 1985; Ackerman *et al.*, 1981), have been used.

Regarding the established techniques for detection of agglutinating surface antibodies, the most widely used tests are the gelatin agglutination test and the tray agglutination test. The two agglutination tests yield comparable titers, but the advantage of the TAT test is that very few donor sperm are needed, and the mode of agglutination can be recorded, i.e., head-to-head, tail-to-tail or mixed agglutination. But the ELISA technique has a number of advantages compared to agglutinations tests (Wolff and Schill, 1985). There is no need for excellent donor semen, and even cryopreserved spermatozoa of average quality may be used as antigens. Furthermore, as shown in the results of the present study (Table 2), the ELISA technique proved to be more sensitive than the GAT and TAT by exhibiting the highest ASA tiers.

The ELISA provided very good reproducibility (90 to 96%) because many microtiter plates can be coated by one ejaculation in order to perform numerous tests with identical antigens. The coated microtiter plates can be kept frozen from eight weeks to nine months without any loss of quality. As is in large hospitals in Korea, several

Table 6. Comparison of mode of sperm agglutination in ELISA-positive and-negative groups among fifty-three TAT positive subject

Groups Mode of Agglutination	ELISA(+)	ELISA(-)
Tail-to-tail	16 (73%)	3 (10%)
Head-to-head	5 (23%)	26 (84%)
Mixed	1 (4%)	2 (6%)
Total	22 (100%)	31 (100%)

tens of samples are submitted daily to clinical laboratory to assess ASA.

Most of the samples are from subjects who underwent vasovasostomy and epididymovasostomy but failed to impregnate their wives. Lee (1987) reported that 43% of the Korean men who continued to be sterile even after patient vasovasostomy, had positive serum sperm-agglutinating antibodies.

Inclusion of the antisperm-antibody test in the vasovasostomy program should be necessary to determine the longevity of ASA following vasovasostomy, in order to correlate prevasovasostomy antibody titers with the success of the procedure and to develop more sensitive assays for other sperm antigens that may play a role in fertilization (Lee, 1987). Thus, a rapid and highly-standardized assay is required for routine tests in clinical laboratories in the hospitals of Korea. It is possible to screen hundreds of test samples objectively within one day by an automatic microtiter plate reader using the ELISA technique.

The ELISA is able to detect not only agglutinating or immobilizing ASA but also antibody altering gamate interaction e.g., by reducing the ability of spermatozoa to attach and penetrate the zona pellucida (Bronson *et al.*, 1982).

Prevalance of ASA in sera of eighty-five vasectomized subjects revealed that 21 (25%) subjects tested positive for ASA by ELISA, 53 (62%) by GAT, and 51 (60%) by TAT, respectively. Comparing the ELISA to the agglutination tests, it was revealed that among 21 ELISA positive subjects, 13 showed positive for GAT (62%) and 12 tested positive for TAT (58%). These results suggest that the ELISA method detected a

different, though often overlapping, spectrum of sperm antibodies compared with the other two agglutination techniques.

Of the ELISA-positive subjects, 73% had tail-to-tail agglutination by TAT, whereas 84% of ELISA-negative subjects had head-to-head agglutination, as in the present study, as shown in Table 6. Friberg (1981) suggested that head-to-head agglutination in male serum indicates a reduced chance for the presence of sperm agglutinins in semen. However, it is uncertain yet what the mode of agglutination means in terms of the nature of the antibodies and surface distribution of the sperm antigens involved.

In contrast to other antisperm antibody-ELISA techniques, we used cytopreparative spray instead of glutaraldehyde to fix spermatozoa to the wells of the microtiter plates, thus enabling them to yield reproducible results. It is also possible to determine the immunoglobulin class of the ASA when specific antihuman immunoglobulin antisera is used. Wolff and Schill (1985) reported that IgM-ASA detected by ELISA caused sperm agglutination in the GAT in 75% of the cases, in contrast to 45% with IgG-ASA.

A threshold level of absorbance was predetermined by sera from a control group of healthy fertile males. Test sera were considered positive if the absorbance value at a titer of 1 : 64 was greater than 30% above the threshold in the present work. This guideline was fairly stable even under lesser-skilled hands in this ELISA method.

We concluded that, in busy laboratories with heavy workloads, this improved ELISA can be a very sensitive, easily reproducible, and efficient method of mass screening for antisperm antibodies.

REFERENCES

- Ackerman SB, Worthan JWE, Swanson RJ.** An indirect enzyme-linked immunosorbent assay (ELISA) for the detection and quantitation of antisperm antibodies. *Am. J. Reprod. Immunol.* 1981, 1: 199-206.
- Ansbacher R.** Vasectomy: Sperm antibodies, *Fertil. Steril* 1973, 24: 788-790
- Bronson R, Cooper G, Rosenfield D.** Sperm-specific isoantibodies and autoantibodies inhibit the binding of human sperm to the human zona pellucida, *Fertil. Steril.* 1982, 38: 724-729
- Choi BG, Lee HY.** Detection of antisperm antibody in vasectomized and infertile males. *Korean J. Urol.* 1984, 25: 511-516
- Erickson RP.** An antiglobulin test for the detection of surface antigens of mouse spermatozoa. *J. Reprod. Fertil.* 1972, 28: 109-115
- Friberg J.** A simple and sensitive micromethod for demonstration of sperm-agglutinating activity in serum from infertile men and women. *Acta. obstet. gynec. scand. suppl.* 1974, 36: 21-29
- Friberg J.** Post-coital test and sperm-agglutinating antibodies in men. *Am. J. Obstet. Gynecol.* 1981, 141: 76-83
- Granklin RR, Dukes CD.** Antispermatozoal antibody and unexplained infertility. *Am. J. Obstet. Gynecol.* 1964, 89: 6-12
- Isojima S, Li TS, Ashitaka Y.** Immunological analysis of sperm-immobilizing factor found in sera of women with unexplained sterility. *Am. J. Obstet. Gynecol.* 1968, 101: 677-683
- Jones WR.** Assessing immunity to sperm. *Clin. Reprod. Fertil.* 1982, 1: 3-12
- Kibrick S, Belding DL, Merrill B.** Methods for the detection of antibodies against mammalian spermatozoa. *Fertil. Steril.* 1952, 3: 340-438
- Lee HY.** A 20-year experience with vasovasostomy. *J. Urol.* 1986, 136: 413-417
- Lee HY, Kim JQ, Kim SI.** An evaluation of an immunological consequence of post-vasectomy azoospermias in Korean males. *J. Nation. Acad. Sci. Korea Natu. Sci.* 1987, 26: 177-200
- Lee HY.** Vasectomy and its reversal. In: *Andrology*, Seoul. Nation. Univ. Publ., Seoul, Korea 1987, pp. 113-173
- Linnet L.** Clinical immunology of vasectomy and vasovasostomy. *Urol.* 1983, 22: 101-107
- Meinertz H, Hjort T.** Detection of automimmunity to sperm: mixed antiglobulin reaction (MAR) test for sperm-agglutination. A study on 537 men from infertile couples. *Fertil. Steril.* 1986, 46: 86-89
- Rumke P, Hellinga G.** Autoantibodies against spermatozoa in sterile man. *J. Clin. Pathol.* 1959, 32: 357-361
- Shulaman S, Zappi E, Ahmed U, Davis JE.** Immunologic consequences of vasectomy. *Contraception* 1972, 5: 269-272
- Wolff H, Schill WB.** A modified enzyme-linked immunosorbent assay for the detection of antisperm antibodies. *Andrologia* 1985, 17: 426-439

= 국문초록 =

항정자 항체의 검출을 위한 간접 ELISA법의 고안

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항정자 항체를 검출하기 위한 간접 효소면역측정법(Indirect enzyme-linked immunosorbent assay)을 고안하였다. 본 방법은 기존 생물학적 제 방법에 비하여 간편하며 자동화가 가능하여 통상적 임상병리검사나 집단 선별검사에 적합하다고 생각된다. 검사 예민도는 젤라친 응집법(GAT)과 평판응집(TAT)에 비하여 회석배수 이상으로 높았으며 검사중 재현성(Intra-assay precision)은 95% 이상, 검사간 재현성(Inter-assay precision)은 90% 이상으로 우수하였다. 본 방법에 의한 항정자 항체의 양성 판정은 피검 혈청 1:64의 회석배수에서 정상대조군의 최대흡광도(0.258)보다 30% 이상인 경우(0.335)로 정하였다. 본 방법으로 측정된 항정자 항체의 발현율은 정관절제술을 받은 군(85명)에서 25%이었다. ELISA법에 양성되었던 군에서의 정자응집의 양상은 꼬리대 꼬리(Tail-to-tail)의 응집이 많았다(73%).