Detection Of Antisperm Antibodies By Elisa System In The Cervical Mucus Of Women With Unexplained Infertility

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ABSTRACT

Background and Objectives: Unexplained infertility occurs in many couples of childbearing age, immune mechanisms have been postulated in this disorder for the last few decades. Circulating antibodies against spermatozoa present in serum and seminal plasma have been especially implicated. This autoimmunity against spermatozoa has been investigated in males, while the isoimmunity (in the females) has got low attention.

Methods: Fifty women with unexplained infertility and twenty fertile women were involved in this case-control study. ELISA system was prepared and used to detect antisperm antibody (ASA) in cervical mucus (CM) and serum specimens of both groups of women. CM was collected at mid-cycle period and dissolved mechanically (not by bromeline).

Results: Thirty percent of infertile women have IgG-ASA in their serum and 20% have IgA-ASA in the CM, while 22% of fertile women have IgG-ASA in their serum and no fertile women have any titer of IgA-ASA in their CM specimens. Only CM-IgA-ASA of infertile women showed significant statistical correlation with cellular property of CM, which was scored according to Insler score.

Conclusions: It is concluded that ELISA test is sensitive and specific test for detection of serum and secreted ASA. Also, secretory IgA-ASA are more indicative and have potential role in immunological infertility as iso-immunity than IgG-ASA. Therefore, it is strongly recommended that immunological infertility should be considered as an important cause of infertility and to be having a special interest by clinicians.

Key words: Antisperm Antibodies , Elisa System, Infertility.

INTRODUCTION:

Infertility affects one of six couples desiring children. The cause of childlessness is unknown in approximately 15% of these individuals[1,2]. Remarkable progress has been made in the management of the infertility, but there has been an increasing incidence of reproductive failure without demonstrable cause. Such cases are commonly referred as unexplained infertility[3]. Immunologic factors have been proposed to be involved in as many as 25% of women with unexplained infertility[1,4]. After a breach of the epithelial mucosal barrier of the female genital tracts, sperm antigens may gain access to subepithelial. B-lymphocytes with the resulting in the local production of IgA- Anti -Sperm Antibody (ASA) as isoimmunization in the female genital tract [4]. The value of testing Anti Sperm Antibody in Cervical Mucus (CM) of the women remains questionable and somewhat neglected in the investigation of immunological infertility. That may be because of difficulties in collection of CM and technical processing problems. One of these problems is liquefaction and dissolving of a naturally- viscose CM. The aims of the present study are; to study the correlation of the CM properties with systemic and secretory ASAs to the sperm antigens; and to test the mechanical method in dissolving the CM, not by bromeline, which was used by other's [5,6], and its relevancy to detect

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by ELISA method.

MATERIALS & METHODS:

The study is a case- control one, conducted upon fifty currently married women having primary unexplained infertility since at least one year. They were visitors of Basra Infertility Center from Oct. 2004 through Aug. 2005. Twenty fertile women have recently delivered without any assisted reproductive technology, considered as control group. The infertile women without evident cause for infertility, they have no ovulation disorder or pelvic and uterine factors neither any congenital anomalies using ultrasound (abdominal and/ or vaginal probe) with hysterosalpingogram. Both groups were with normal hormonal assay and not receiving clomiphene citrate or corticosteroids prior to sampling process.

Specimen collection: Preovulatory CM specimens were collected by sterile technique, they acquired by aspiration of endocervical canal using unlubricated speculum and 1 ml tuberculin syringe from both groups of women after they were refrain from sexual intercourse for 2-5days. A gynecologist collected all specimens, which have assigned the preovulatory period by examination of infertile and fertile women by ultrasound to detect follicles at the preovulatory period. This period ranged (12-15) day of the cycle in infertile women and ranged (12-14) for fertile women.

All women used clomiphine citrate were excluded and all specimens which contained blood were discarded. Five ml of venous blood specimens without anticoagulant were taken from all participants. The blood was allowed to clot and then centrifuged. The collected serum specimens were stored in sterile Epindroff tubes in deep freezing.

CM evaluation : The condition of preovulatory CM was evaluated according to Insler score[7] (Table A). Insler score took in to account five properties of CM known to affect sperm-CM interaction. Each score of 15 indicate favorable CM and less than 10 represents unfavorable CM while less than 5 is a hostile CM.

Solubilization of CM:

To reduce high viscosity, CM was diluted 1:1 in phosphate buffered saline (PBS) (pH 7.4) (DiaSorin S.A., StillWater, MN 55082). The mixture was liquefied by repeatedly passage through a sterile needle with syringe in to centrifuge tube until the mucus was dissolved completely (mechanical method) [8,9]. Centrifugation was followed for 10 min. at 3000 rpm, and the resultant supernatant fraction was aspirated and stored in small- sterile tubes (Epindroff tubes) in deep-freezing for immunological processing.

Detection of ASA by ELISA ELISA procedure was used to detect secretory CM-IgA- and serum-IgG-ASAs according to Wolff and Schill,1988 [9]. Briefly, swimming up technique[10] was used to obtain sperm antigens. The collected sperms were washed five times by PBS (pH 7.4), and the total resulted washed sperms were adjusted to 200x 10^6 sperm/ ml. Five cycles of thawing and freezing was used to fractionation of sperm cells, and the resultant extract was centrifuged (3000 rpm for 10 min.) to remove the unfractured cells which sediment in the bottom, while the supernatant was aspirated and stored in deep freezing. 400µl of this suspension was dissolved in 2 ml of coating buffer to be used as antigen for ELISA runs, which was performed on microtiter polystyrene plates. The sperm-coated wells were washed three times with PBS-Tween 20 and 100 µl of CM and serum specimens were added in double dilution. The addition of Tween 20 eliminated nonspecific immunoglobulin binding to the wells. After 60 min. incubation at 37°c, the wells were washed three times with PBS-Tween. One hundred µl of 1:100 dilution in PBS of alkaline phosphatase-cojugated goat anti-human immunoglobulin of classes IgA and IgG was added. After 60 min. incubation at 37° c, the wells were washed three times with

was added in dark for 45 min. Finally, stop solution (3N NaOH) was added at 100 µl/ well. The optimum dilution of the CM and serum specimens and sperm antigen to be used for conducting ELISA technique, were measured by Chequar- board titration for control positive specimens. Then cutoff value on the resulted optical density of absorbance measured by spectrophotometer (Backman Coulter) at 450 nm of control negative specimens was calculated.

Statistical methods

The analysis of data was conducted by using available software of Statistical Package for Social Sciences (SPSS) version 10.1 to get results of descriptive and Spearman correlation statistics. For cut off values, the equation of mean + 2 X SD was used [11].

RESULT:

The age of infertile women ranged from 18-40 (29.04±5.21) years, compared to 20-35 (29.5±3.44) years in the fertile women, with no significant difference (p>0.05). No significant difference between both groups in the period of marriage which ranged 1-10 (4.0±1.79) years in infertile women and ranged 2-11 (4.62±2.17) years in fertile women (t=1.133). Descriptive and differences in CM properties. Table(1) shows the frequency and percentages of CM properties (amount, Ferning, viscosity, spinnbarkeiting and cellular) of infertile and fertile women. Chi-square (X^2) test was between the graded score (0-1) and (2-3) of each property. All of these properties showed significant differences (P<0.05) with exception to the amount of CM. The range of pH of the CM of infertile women was 7.1-7.5 (7.32±0.32) and in fertile women it was 7.2-7.8 (7.54±0.34), with significant difference (P<0.01).

Insler score:

No significant difference (p>0.05) between infertile and fertile women in the total number of Insler score was found. In infertile women it ranged from 7-11 (8.72±2.11) and in fertile women it was 8-14 (8.0±1.60).

Table A: Insler Score.

Properties	Degree of score			
	0	1	2	3
Amount (ml)	0	0.1	0.2	> 0.2
Ferning	No	Primary	Secondary	>Secondary
Viscosity	Thick	Moderate	Thin	Watery
Spinnbarkitig (cm)	< 1	1-4	5-8	≥ 9
Cellularity (cell/HP)	≥ 11	10-6	5-1	0

Positive ASA: The designation of positive ASA was used for the high titers detected by the absorbance of optical density (OD) in spectrophotometer. Cutoff value calculated for serum specimens was 0.994 and for CM was 0.816.Therefore, any higher OD reading was considered as positives. Fifteen out of fifty (30%) infertile women have serum IgG-ASA, compared to four (20%) fertile women (Table-2). Cervical Mucus- IgA-ASA was detected in 11 out of 50 (22%) in infertile women but none of the fertile women was with this secreted immunoglobulin. As shown in (Table-3), only infertile women have both serum- and secreted- ASAs at the same time. Whereas 20% fertile women have IgG-ASA in their serum with no secreted ASA There were 22% of infertile women with serum-IgG-ASA alone, and other 18% (9/50) have CM-IgA-ASA alone (without

Correlations of ASAs with CM properties:

As shown in Table-4, there were no correlations between ASAs and CM properties except for cellularity of CM which showed significant correlation (P<0.01) with secreted IgA-ASA in CM of infertile and fertile women, but not with serum IgG-ASA.

Table 1: Descriptive comparison of CM properties according to Insler score.

CM properties	Infertile women N= 50 (%) №	Fertile women N=20 (%) №	X^{2*}	P
Amount (ml)				
1-0	12 (24)	4 (20)		
3-2	38 (76)	16 (80)	0.853	NS
Ferning				
1-0	17 (34)	2 (10)		
3-2	33 (66)	18 (90)	9.88	0.05>
Viscosity				
1-0	30 (60)	2 (10)		
3-2	20 (40)	18 (90)	15.37	0.01>
Spinnbarkeiting(cm)				
1-0	29 (58)	3 (15)		
3-2	21 (42)	17 (85)	10.713	0.05>
Cellular (cell/HPF)				
1-0	27 (54)	3 (15)		
3-2	23 (46)	17 (85)	13.773	0.01>
Total	2.11±8.72 (11-7)	1.60±8.05 (14-8)	t=1.273	NS
PH	0.32±7.32	0.34±7.54	t=2.522	0.01>

Table 2: Positives(> cutoff values) of serum IgG- and CM IgA-ASAs in infertile and fertile women.

ASA	Infertile women (50)		Fertile women (20)	
	№	(%)	№	(%)
Serum-IgG	15	(30)	4	(20)
CM-IgA	11	(22)	0	

Table 3: Presence of serum - with and without secretory-ASAs.

-ASA	Infertile women		Fertile women	
	№	(%)	№	(%)
IgG- alone	11	(22)	4	(20)
IgA- alone	9	(18)	0	
IgG-with IgA-	6	(12)	0	

Table 4: Correlations of ASAs with CM properties.

CM prop.	Infertile women		Fertile women	
	serum IgG	CM-IgA	Serum IgG	CM-IgA
Amount (ml)	0.052	-0.034	-0.112	-0.284
ferning	-0.064	-0.088	-0.261	-0.101
viscosity	-0.058	-0.005	-0.233	-0.056
spinn. (cm)	-0.151	-0.025	-0.345	-0.391
Cellular (HPF)	0.222	0.649*	0.063	0.596*
Total	-0.079	-0.068	-0.262	-0.263
PH	0.584	0.079	0.002	0.211

***correlation is significant at level of 0.001**

DISCUSSION:

In the present study CM of infertile and fertile women was evaluated according to the dependant [12] Insler score [7]. Any women receiving Clomiphene citrate was excluded from the study, because the known effect of this drug on the quality of CM [13]. It is well known that the CM properties depends on hormonal status, and since the normality of hormones was an inclusion criteria for infertile and fertile women, thus, all the significant difference in CM properties may refers to causes other rather than hormones.Ferning refers to the degree and pattern of crystallization observed when CM dried on a glass surface. The significant difference between infertile and fertile women may be due to the presence of leukocytes in the CM of infertile women. Leukocytes affects directly the crystallization of CM [14]. The significant both groups of women in their CM viscosity (consistency) and spinnbarrkeiting (elasticity), might result from the local immunological events which may lead to alteration in protein, ions and water concentrations and influence the molecular arrangement of the CM [15]. Thus, the CM of infertile women may loss it's elasticity and increase it's consistency, that is clear in significant difference between the two groups of women. The cellularity was significantly different as well, reflecting immunologic process or subclinical infections. The latest one may be excluded, since all the included women in the study were healthy (with no clinical features of infections). These results agreed with many studies [16].The pH of CM, as consequently, might be affected by all the previous factors. Although, pH was not considered in the Insler score, it has important role in viability of the

mucus immobilized spermatozoa, whereas alkaline mucus may enhance motility. Excessive alkalinity of mucus (>8.5) may, however, adversely affect the viability of spermatozoa [17]. The optimum pH value for sperm migration and survival in CM is between 7.0 and 8.5 which represent the pH range of normal midcycle CM [12].

CM evaluation (Insler score):

In spite of statistical difference between some CM properties of both groups, the Insler score was not different in the two groups, may be due to the small number included in control group. In the present study a significant correlation (<0.01) was found between Insler score and CM properties. That may explain the appearance of high ferning, low viscosity with low number of leukocytes in CM (favorable) of fertile women, in contrast, hostile CM may have unfavorable properties. In the absence of typical human CM, the validity of using threshold value to compare between those two[(12)] may be unreliable[18], thus the cut off point mentioned by some researchers [19] may be useless. According to the previous information, both fertile and infertile women in the present study have relatively favorable CM, therefore, the hostility of CM, as a cause of infertility in this population was not expected.

Correlation of ASAs with CM properties:

The results of the present study show clearly that only IgA-ASA has correlation with cellularity in CM. That is may be due to presence of activated B- lymphocytes in CM, which produce IgA against sperm antigens[20,21]. Determination the cellular basis for the major first-line specific defense system by the fact that secretory mucosa contain most of the body's activated B cells, particularly the lamina propria where at least 80% of immunoglobulin- producing cells were found[22]. The present results more than other studies[6,19,23] whom used Tray Agglutination Test (TAT) in detection of ASA in CM. This superiority in result of the present study may be due to different repeatedly passage of CM specimens through the needle of syringe (mechanical method)[(8,9)] was used in the present study in order to dissolve and liquefy of CM before ASAs measurement, while the others used bromeline to dissolve CM. The bromeline may lead to degradation of antibodies to lower molecular weight fragments. VanKooij,1984 [8] showed that both IgG and secretory IgA bands of electrophoresed mucus had disappeared after treatment with bromeline, indicating an extensive breakdown of the immunoglobulin structure. Nevertheless, there were resulted rates in researches used bromeline, so there is one possible explanation of this discrepancy. A low specific mode of action of bromeline, which in these cases possibly did not have papin-like effect (where immunoglobulin degrading to Fc+Fab+Fab)[24], but instead displayed pepsin- like activity (degrading to Fc+ Fab_2). Fab_2 fragments obtained by pepsin digestion are still capable of sperm-agglutination resulting in a positive TAT (lower than the real), and so, mechanical method might have results more than bromeline dissolving of CM. Furthermore, because the absence of α- and γ- chain bearing Fc parts, which are the recognized antigens for the second antibody in the ELISA, the sperm-agglutinating Fab_2 fragments are not detectable in this assay. Therefore, ELISA results in the present study could be more than those who have to use bromeline or any mucolytic in processing of CM. Surprisingly, the results of iso- immunity may be strongly indicated that local secretory immunity against spermatozoa was independent from systemic immunity against the same antigens[(20)]. That is may be because of mucosal immune system have a number of features that differentiate it from systemic lymphoid system. These include; the T-cells within mucosa have specific regulatory properties and/ or effecter capabilities; and a mucosa- oriented cell-homing system that allows lymphocytes initially activated in the mucosal follicles to

the epithelium (secondary lymphoid organs, mucosa- associated lymphoid tissue MALT). This last feature leads to the partial segregation of mucosal cells from systemic cells and thus qualifies the mucosal immune system as a somewhat separate immunologic entity [25]. This selective homing ensures that lymphoid cells that become activated in mucosal follicles preferentially migrate to effecter sites in the lamina propria. In addition, it accounts for the fact that antigen contact at one mucosal surface (eg. the intestine) can lead to the production of specific IgA at other mucosal surfaces [26].

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