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Anti-zona pellucida antibodies in infertile patients in relation to multiple puncture of ovaries and unexplained infertility

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Abstract

Background: Auto antibodies to zona-pellucida (AZA) seem to be important autoantibodies implicated in reproduction, with substantial role in both endocrine and reproductive functions of the human ovary. There are some debates on the relation of AZA with infertility, repeated In Vitro Fertilization (IVF) attempts, and outcome of it.

Objective: In this study, we assessed the presence of AZA in the follicular fluids (FFs) of women who underwent intra cytoplasmic sperm injection (ICSI), in relation to etiology of infertility and multiple puncture of ovaries.

Materials and Methods: In this prospective study, follicular fluids were evaluated from 96 infertile women, (19-40 years old, 31.5±5.1), who were candidates for ICSI based on the etiology of infertility. From these 80 women had explained infertility whereas 16 had unexplained infertility. All FFs were evaluated for presence of AZA by ELISA test.

Results: Twenty patients (20.8%) were positive for AZA in follicular fluid. In patients with unexplained infertility, AZA antibody in follicular fluid, was significantly higher than the group with proven etiology of infertility (p=0.001). In addition, 20.4 % of patients who had been punctured previously showed AZA in their FFs which is statistically similar to the patients who were punctured for the first time.

Conclusions: The high incidence of AZA in infertile women, especially women with unexplained infertility has to be considered. Relation of the presence AZA and repeated puncture of ovaries is still debatable. Determinations of AZA are highly recommended in evaluation of infertile couples especially in patient with unexplained infertility.

Key Words: *Anti Zona Antibody, Multiple puncture of ovaries,Unexplained infertility.*

Introduction

One in five of reproductive age couples suffer from infertility. Approximately 15% of them are diagnosed with unexplained infertility. Autoimmune abnormalities like AZA seem to be important immunological factor implicated in etiology of infertility (1-7) specially unexplained infertility (8-10)**.** The acellular zona pellucida (ZP) surrounds the egg at ovulation and remains in place until the implantation, it contains receptors (ZP1,ZP2,ZP3) for sperms which are ,with some exceptions, species-specific(11,12). ZP undergoes zona reaction in which it becomes impervious to other sperms once the fertilizing sperm penetrates and thus it provides a bar to polyploidy (13).The initial contact between the sperm and the oocyte is a receptor-mediated process. These receptors are glycoproteins and any alteration of these

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glycoproteins leads to a loss of receptor activity (14). Specific antibodies directed against these glycoproteins are able to inhibit sperm attachment and penetration into oocyte and may be the cause of natural or artificial fertilization (8). It is well documented that, AZA blocks fertilization of oocyte in- vitro by making it impenetrable to spermatozoa, so it can induce infertility and/or fertilization failure in IVF programs. (15-17). various studies have noted the incidence of AZA between 7.5-36.5 % in infertile patients (15, 16). There are some arguments about possible role of the antibody on fertilization failure in IVF programs (16, 19), however, some studies show the higher incidence (39-91%) of antibody in patient with IVF failure (8, 17). The possible association of AZA and unexplained infertility has been evaluated, and some studies suggest an etiologic association of the antibody with infertility (9, 17). Moreover, it may be argued that if anti-ovary antibodies like AZA, after attempts at human in vitro fertilization, induced by repeated hormonal stimulation and follicular puncture (8, 20, 21). The main purpose of this study was to evaluate the prevalence of AZA in follicular fluid of infertile women and the relation of the antibody and etiology of infertility to know if it is important to search these antibodies as a valuable diagnostic tool in infertile patient for appropriate decision making about the need for intra-cytoplasmic sperm injection (ICSI). Also we have also investigated the correlation between the number of previous punctures and the presence of antibody.

Materials and Methods

Investigated patients

This cross sectional study which takes one year was conducted in a prospective manner in Avesina Research Institute. All the women (n=96) who met the clinic, consecutively were considered in our study. The couples were assessed by history, physical exam, routine lab measurement (plasma FSH, LH and E2 at 3rd day of cycle, TFT, Prolactine, midluteal progesterone level),sperm analysis and imaging (sonography, HSG) to categorized their infertility conditions as explained (n=16) and unexplained (n=80).Women met the inclusion criteria, if 1) they were younger than 40; 2) they were the candidates for fresh ICSI, and 3) they didn't show the history of rheumatology and immunology diseases. The mean age (±SD) was 31.5±5.1 (range 19-39).

Measurements

All FFs were evaluated for presence of AZA by ELISA test to get the prevalence of AZA in infertile patients. Moreover, the prevalence of AZA were compared between patients with unexplained and explained infertility, and also compared according to the number of previous punctures.

Protocol of stimulation

GnRH- a (Buselin, Hoechst, 0.1mg/24 hours) was administered from day 21 of the cycle. Then all patients were treated with human menopausal gonadotropin (hMG; Merional) from day 3 of the next cycle. The initial dose of the administered gonadotropin was set at 150 IU / day and eventually increased by steps of 75 IU every 3-4 days while controlling follicular growth under ultrasound. Ovulation was induced by the intramuscular administration of 10000 IU of the human chorionic gonadotropin (hCG; Pregnyl) when the three leading follicles had reached a diameter of 18 mm. Oocyte retrieval was performed by transvaginal aspiration 36 hours after hCG administration and follicular fluids (FFs) were obtained following oocyte collection , and kept frozen until antibody evaluation .

ELISA test

1-Production of mouse anti-human zona pellucida antibodies: Murine anti human zona antibodies were prepared as described earlier with minor modifications (12). In brief, Balb/c mice were subjected to 4 intraperitoneal injections of separated zona pellucida from unfertilized oocytes or low quality embryos from IVF or microinjection procedures, after getting written consent from the respective couples. After the fourth injection, different dilutions of the mouse sera were tested by immunofluorescent staining on human oocytes or embryos using FITC conjugated goat anti mouse immunoglobulin (Sigma). Immunized mice were selected, sacrificed and their blood samples were collected and sera were separated by centrifugation.

2-Detection of human anti-zona pellucida antibodies in FF by a cell-based ELISA: To detect the presence of anti-zona antibodies in the FF of our subjects an oocyte/embryo based ELISA was developed. In each test, separated zona pellucida from unfertilized oocytes or low quality embryos was used. The cells were initially washed once with sterile phosphate buffered saline (PBS) under embryologic loop and by mouth pipetting. Separate drops of PBS each containing separated

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zona pellucida were laid in petridishes. The first drop that served as negative control received only PBS and cells. To the cells in the positive control drop 1:100 dilution of the mouse anti-zona immune serum was added. Cells in test drops received individual patient's FFs separately. After 30 minute incubation in the cold, cells in all drops were washed three times with cold PBS by mouth washing under loop microscope. The positive control drop was then incubated for 30 minutes with peroxidase conjugated Goat anti-mouse Ig (Sigma) on ice. All other drops received the same treatment but with peroxidase conjugated Goat anti-human Ig (Sigma). All cells were then washed three times with PBS and then were transferred in 50µl of TMB substrate solution into ELISA strip wells (Nunc) and incubated for 15 minutes in the dark followed by addition of 50µl of stop buffer (20% sulfuric acid). The optical density (OD) of the reactions was measured in an ELISA reader at 492 nm wavelength.

3-Validation of the ELISA results: All the FFs samples were also tested for presence of anti zona antibodies using a commercial Anti zona antibody ELISA kit (Bioserve Diagnostics, Rostock, Germany) and all the results were confirmed.

Statistics

Data were presented as mean ±SD for numerical variables and in percentage for categorized variables. The data analysis was performed with SPSS software. Statistical assessment of our results was performed using 1-sided and 2-sided Chi-Square test. A p-value of <0.05 was considered statistically significant.

Results

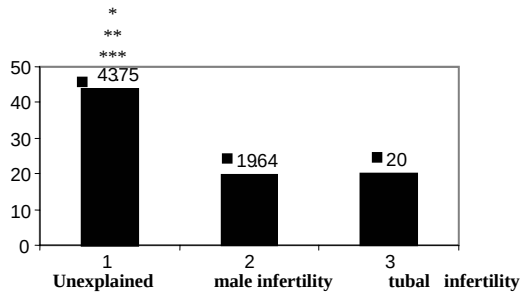
The patients' infertility conditions were categorized as explained and unexplained. According to the etiology of infertility, 80 (83.3%) of women had explained infertility including 56 male factor (58.3%), 10 tubal factor (10.4%), 3 tubal and male factor (3.1%), 7 ovulatory factor (7.2%), 4 ovulatory and male factor (4.1%), whereas 16 were diagnosed with unexplained infertility (16.6%). In FFs, according to the result of the ELISA test 20.8% of patients (n=20) presented AZA. Table-I shows the prevalence of AZA with regard to the etiology of infertility in the patients undergoing ICSI.

We evaluated the relation of age and frequency of AZA. No statistically significant relation between age (mean ± SD) and frequency of AZA (%) were apparent (31.4± 5.06 years for AZA negative group and 32.55 ± 5.1 for AZA positive group). Frequencies of positive AZA in FFs of unexplained and explained groups and their

Table I. AZA regarding to the etiology of infertility in patients undergoing ICSI

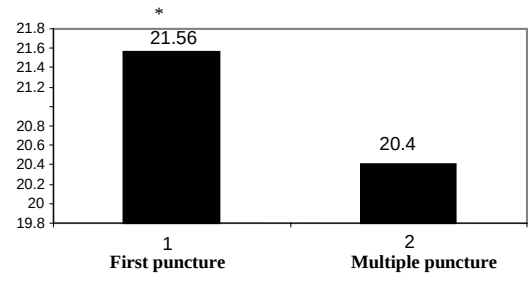
	Total (n = 96)	Positive AZA (n = 20)
Male factor (%)	56 (58.3)	11 (19)
Tubal factor (%)	10 (10.4)	2 (20)
Ovulatory factor (%)	7(7.2)	_
Tubal and male factor (%)	3 (3.1)	_
Ovulatory and male factor (%)	4 (4.1)	_
Unexplained (%)	16 (16.6)	7 (43)

Figure1- Comparison of frequencies of AZA (%) between unexplained, tubal and male factor infertility



** p= 0.001 vs Explained infertility*
*** p= 0.045 vs male factor infertility*
**** p= 0.004 vs tubal factor infertility*

Figure2- Comparison of frequencies of AZA (%) according to the number of punctures



** p vs multiple puncture= not significant*

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statistical comparison are shown in figure 1. In patients with unexplained infertility, AZA antibody in FFs was significantly higher than the group with proved etiology of infertility (43.75% vs. 16.25% respectively).

The frequencies of positive AZA were compared between unexplained and tubal factor groups. Overall, higher proportion of antibody was observed in patients with unexplained infertility, than the group with tubal factor infertility (43.75% vs. 20% respectively).

We have also compared the frequencies of positive AZA between unexplained and male factor groups. In patients with unexplained infertility, AZA antibody in FFs, were significantly higher than the group with male factor infertility (43.75% vs. 19.64% respectively).

Figure 2, shows the comparison of frequencies of positive AZA, according to the number of punctures. As shown, 20.4 % of patients who had been punctured previously showed AZA in their FFs which is statistically similar to the patients who were punctured for the first time (21.56%).

Discussion

The ovary can be the target of an autoimmune disease involving many different auto antigens. Different reports suggest possible immuno-pathological mechanism for reproduction failure in patients with organ-specific auto antibodies (1-7). These auto antibodies such as anti thyroid and anti ovarian antibodies may serve as possible markers for reproductive failure (22). Presence of anti-zona activity in infertile women may be an autoimmune response, possibly due to absorption and degradation of the ova into the peritoneal cavity or in the reproductive tract and the subsequent exposure of the degradation products to the immune system. This is similar to the mechanisms described by Mhaskar et al. (1984) who demonstrated the presence of antibodies, specific to zona antigens, in tubectomized women (23).

Presence of AZA in human sera and their characterization have been studied extensively (17, 18). However, there are few reports regarding the incidence of AZA in FF of infertile patients. In this study we assessed the rate of AZA in FFs of infertile women (20.8%) in comparison with the previous findings in human sera. Nishimoto et al. (1980) found that, AZA may be produced during the aging process (18), but we didn't show any relation between age and frequency of AZA in our study.

Some studies showed an anti –gamete antibodies (AGA) including AZA in high percentage of patients with unexplained infertility versus patients with proven etiology of infertility (9, 24). Nishimoto et al. (1980) detected AZA in human sera, by fluorescence , in 7.4% of patients with unexplained infertility, in 1.6% of patients with proven etiology of infertility, but in none of age-matched control subjects (fertile women and men) (18). Moustafa et al. (1994) found AGA in 45% of patients with unexplained infertility and suggested it as a possible cause of infertility (25). Also, AZA were found in 3 of 10 patients' sera with low response to ovarian stimulation in Hovav study, which showed an important negatively influence of AZA on both endocrine and reproductive function of the human ovary (24). Our study supports the results of previous studies and showed comparable high incidence of AZA in women with unexplained infertility in our patients. These results may suggest an etiologic association of antibody with infertility.

Some studies propose trigger of an autoimmune process due to micro trauma induced by repeated punctures of ovarian follicles, which can result in the production of auto-antibodies in women subjected to IVF. Gobert et al. (1992) showed anti-ovary antibodies (AOA), especially AZA, after attempts at human IVF were induced by follicular puncture rather than hormonal stimulation (26). These antibodies have been detected in serum samples of women undergoing in IVF. High concentrations of these antibodies have been reported in women who have experienced several IVF attempts and additionally they appear to correlate with reduced chances of pregnancy (4). In the above mentioned study, serum was obtained 8 days after the beginning of ovarian human menopausal gonadotrophin (hMG) stimulation, then 15 days after follicular puncture. Significantly higher concentrations of IgG ($p<0.0001$) of AOA were observed in the second series of samples than in the first series, suggesting that ovarian trauma - and not hormonal stimulation - is responsible for triggering antibody production (26). Ulcova-Gallova and Mardesic (1996) analyzed and compared the levels of zona pellucida antibodies in 250 women with recurrent IVF failures (from 1 to 4 times) with the results of a control group of 211 unexplained infertile women that were never treated by IVF. Their results showed increased occurrence of zona pellucida antibodies in women after repeated IVF. Zona pellucida antibodies were found in 20% of the patients with one unsuccessful IVF, in 64% after two, in 91% after three and in 4

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of 5 cases after four IVF failures. The above mentioned results show evolution of autoimmune process due to repeated ovarian intervention during oocyte collections. (8). Barbarino-Monnier et al. (2003) assessed anti-ovarian antibodies (AOA) in serum samples at various times of IVF attempts to determine whether ovarian stimulation could result in the production of such autoantibodies in women. They showed the absence of influence in endogenous or exogenous ovarian stimulation by gonadotropins on anti-ovarian autoimmunity (20). In an earlier study they mentioned that micro trauma induced by repeated puncture of ovarian follicles can result in the production of auto - antibodies in women submitted for IVF (21). In contrast, Hovav et al. (1997) study about the presence of antizona pellucida autoantibodies in IVF patients in relation to low ovarian response, multiple IVF attempts and unexplained infertility suggested an association between antizona pellucida antibodies and suboptimal response to gonadotrophins. None of patient with multiple IVF attempt, demonstrated measurable level of anti zona antibody in the study group. It was indicated that repeated stimulation and puncture of ovaries in IVF procedures do not elicit autoimmunity to gametes (14). The present study showed no correlation between previously repeated punctures and positive AZA in serum and follicular fluid and it suggest no respective role of repeated puncture of ovaries to induce such an autoimmune response to produce AZA higher than patients who were punctured for the first time.

Conclusion

Anti-ovarian auto-antibodies especially AZA play an important role in both endocrine and reproductive function of the human ovary and can influence them negatively. High incidence of AZA and long-term resistance to treatment in women with unexplained infertility suggests an etiologic association of antibody with infertility which may be closely correlated with inhibition of sperm-egg interaction by AZA, that is produced in these women .Correlation between previously repeated punctures and measurable level of AZA in follicular fluid is still debatable. In conclusion, determinations of AZA are highly recommended in evaluation of infertile couples especially in patients with unexplained infertility.

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