Detection of anti-testicular antibodies among infertile males using indirect immunofluorescent technique

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(Received: July 2022 Revised: October 2022 Accepted: October 2022)

ABSTRACT

Introduction and Aim: The antinuclear antibodies (ANA) are unwanted molecules which bind and destroy certain structures within the nucleus. Immunofluorescence is a powerful technique that utilizes fluorescent-labeled antibodies to detect specific target antigens. The aim of this study was to detect the anti-testicular antibody among infertile males in Baghdad city and determine the most common type of infertility.

Materials and Methods: The study involved 73 male (53 infertile and 20 non-infertile) volunteers, at Kamal Al-Samarrai Teaching Hospital in Baghdad, Iraq. Serum collected from the study subjects was tested for steroid-cell antibodies (STC-Ab), anti-nuclear antibody (ANA) and anti-testicular antibodies (ATCA) by Indirect Immunofluorescence assay (IIFA). Data obtained was subjected to statistical analysis using the SPSS program.

Results: In the current study 52.9% of infertile men tested positive for testicular antibodies compared to the control group. The highest rate of testicular cell antibodies was observed in the serum of infertile patients aged between 30-39 years and the lowest in patients aged 50-59 years. The marriage duration among those showing the highest percentage of antibodies against testicular cells was 1-10 years. Study for the association of male infertility type to those positive for ATCA showed almost all (85.7%) patients with necrozoospermia to be positive for ATCA. This was followed by males with azoospermia (50%) and oligospermia (46.9%). The patients were negative for Addison’s disease while a few (28.6%) were positive for lupus erythematosus.

Keywords: Antitesticular antibody; infertile males; oligospermia; duration of marriage.

INTRODUCTION

Infertility is a condition with psychological, economic, and medical effects leading to trauma and stress, especially in a social group such as our society which mostly focuses heavily on the speed of reproduction after a few months of marriage. According to the International Commission for Monitoring Assisted Reproduction Technology, infertility is a disease caused by defects within the reproductive system (1). As a result of the scientific progress that has taken place these days, most medical sociologists agree that the concept of both health and disease is best understood, as decisions on what constitutes abnormalities, how to identify abnormality, and what steps, if any, should be taken to deal with all its circumstances, are taken in a social context (2). Infertility problems are generally estimated at 20-30% of cases in males, 20-35% in females, and approximately 25-40% due to combined problems in both sexes (3). The causes of infertility are different and multiple, which may be caused by anatomical disorder, physiological dysfunction, genetic or other acquired or environmental cause (4). In females the most common cause of infertility is due to ovulatory problems such as scanty or absence of menstrual periods (3) while in males the most common cause could be semen deficiency and quality (3, 5).

Scientific research has shown that immune causes of male infertility are common (6). According to the World Health Organization, any auto-immune response to sperm cells in the testicle has been
shown to be a cause of male infertility (7). Testicular has a peculiarity in the production of haploid germ cells that autoantigens appear newly after a long period of immune tolerance that occurs in the neonatal period, and under normal circumstances, these autoantigens will be protected by the blood testicular barrier formed by specific cells called Sertoli cells. Thus, the testicle is a distinct immune site where haploid cells have an important role to play in exposing autoimmune attacks by the immune system (8, 9). Anti-nuclear antibodies (ANA) are considered as a general term for autoimmune antibodies against various nuclear components and an important part in the research of clinical and immunological medicine (10). Studies with human and mice cells have shown that the nuclei of normal body cells are attacked in case of inflammation and autoimmune diseases which makes the detection of these cells difficult by cytochemical staining techniques (11). Hence, the use of techniques for simultaneous detection of multiple antigens through immunochemistry is highly desirable in order to investigate spatial interrelationships between different molecules, which employ two important methods, the Elution and the non-Elution technique. The use of these two techniques is simple and at the same time very useful for multiple staining of a wide range of antigens (12). The aim of this study is to detect the anti-testicular antibody among infertile males in Baghdad city and determine the most common type of infertility within the study sample.

MATERIALS AND METHODS

Subjects

Data was collected from patients admitted to Kamal Al-Samarrai Hospital in Baghdad city for treatment and medical consultation for infertility. The study included 73 male (20 fertile and 53 infertile) subjects aged 20-60 years. Following consent from the participants, blood was drawn from each subject and serum used in testing for steroid-cell antibodies (STC-Ab), anti-nuclear antibody (ANA) and anti-testicular antibodies (ATCA). Semen was also collected for analysis. All participants were asked to fill-in a simple questionnaire, which consisted of questions pertaining to their age, period of marriage, type of infertility according to the final diagnosis by the infertility specialist and tests conducted.

Methods

Immunological tests

The serum of each subject was tested for antitesticular antibody (IgG). Sera positive for IgG was further tested for anti-adrenal cortex and anti-nuclear antibodies by using the Indirect Immunofluorescence assay (IIFA). The IIFA is a two-step serological technique that involves the use of two antibodies wherein, the first unlabeled primary antibody specifically binds to the target molecule and the secondary antibody carrying the fluorophore recognizes the primary antibody and binds to it (Betterle and Zanchetta 2012). For IIFA assay, the patients’ serum was incubated with a substrate for a period of 30 min, after which washed to remove the unbound protein, followed by addition of the second fluorescein-conjugate antibody. The bound complex was visualized using a fluorescent microscope (Lielix CO. Germany).

Detection of anti-testicular antibody

The serum anti-testicular antibodies were tested on Binding site EIA plates (The Binding site Ltd., Germany) pre-coated with antigen and performed as per the manufacturer instructions. Briefly, 40µl of serum was diluted with 160µl of PBS. 50-100µl of diluted serum was added to pre-coated wells and incubated for 30 minutes in a moist chamber at room temperature (approximately 18-24°C). After incubation the plates were removed, rinsed with PBS for 15 mins to remove excess serum. The slides were blot dried and to each well a drop (~20-30 µl) of the fluorescent conjugate was added and the slides further incubated in the moist chamber at room temperature for 20 minutes. Following incubation the slides were rinsed, dried and visualized under fluorescent microscope.

Detection of IgG anti-adrenal cortex antibody

The same procedure of (ATCA) was applied for detection of anti-adrenal cortex IgG antibody. Each slide (The Binding site Ltd., Germany) used for detection this isotype consisted of five wells, which contained monkey adrenal cortex sections as substrate. Control and patient serum was added to the enzyme substrate. The colour developed was used in measuring the concentration of IgG antibodies in the samples.
Detection of anti-nuclear antibody (ANA)

For detection of ANA in serum samples a slide consisting of 24 wells was used. To each well pre-coated with human epitheliuma cancer (HEp-2) cells (Biomerieux, Germany), 25µl of the serum sample diluted with 975µl of PBS was added and incubated. After incubation fluorescent anti-Ig was added, and the fluorescence developed was visualized using a fluorescent light.

Statistical analysis

The data obtained was entered into the Microsoft Excel software and subjected to statistical analysis using the Statistical Packages Program for Social Sciences (SPSS). The Chi-square test was used to determine the level of significance (p-value). A p-value <0.05 was considered as statistical significant.

RESULTS

The frequency of anti-testicular antibodies (ATCA) detected among the study subjects is shown in Table 1. As seen from Table, the frequency (52.9%) of ATCA among patients was higher in comparison to healthy individuals in the control group with no statistical significance observed between the groups (p=0.681). Cells positive for anti-testicular antibody by immunofluorescence is depicted in Fig. 1.

Table 1: Frequency of anti-testicular antibodies detected among the study subjects

<table>
<thead>
<tr>
<th>Samples (n)</th>
<th>Number of positive cases (%)</th>
<th>Statistics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients (53)</td>
<td>28 (52.9%)</td>
<td>Chi-square = 0.170</td>
</tr>
<tr>
<td>Control (20)</td>
<td>0</td>
<td>p-value= 0.681 (NS)</td>
</tr>
</tbody>
</table>

Fig. 1: Fluorescent Leydig cells positive for anti-testicular antibody

Table 2 shows the distribution of male infertile patients and control group based on their age groups. Most of the patients (60%) significantly positive to ATCA belonged to the 30-39 years age group, while the least (33.33%) positive for ATCA was seen among males aged 50-59 years.

Table 2: Distribution of infertile males positive for ATCA in different age groups

<table>
<thead>
<tr>
<th>Age groups in years</th>
<th>No. of patients (%)</th>
<th>No. of positive ATCA cases (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20-29</td>
<td>15(28.30)</td>
<td>7(46.66)</td>
</tr>
<tr>
<td>30-39</td>
<td>20(37.74)</td>
<td>12(60)</td>
</tr>
<tr>
<td>40-49</td>
<td>15(28.30)</td>
<td>8(53.33)</td>
</tr>
<tr>
<td>50-59</td>
<td>3(5.66)</td>
<td>1(33.33)</td>
</tr>
<tr>
<td>Total</td>
<td>53(100.0)</td>
<td>28(52.83)</td>
</tr>
</tbody>
</table>

Statistics

<table>
<thead>
<tr>
<th>Chi-square</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>11.830</td>
<td>0.008</td>
</tr>
</tbody>
</table>

NS: Non-significant, S: Significant, HS: Highly significant

The study results also showed the highest percentage of infertile patients (56.52%) significantly positive for ATCA to be married for a duration of 1-10 years. With increasing marriage duration (11-20 years), ATCA cases were observed to decrease among infertile males (Table 3).

Table 3: Distribution of infertile males according to their marriage duration

<table>
<thead>
<tr>
<th>Duration of marriage</th>
<th>No. of patients (%)</th>
<th>No. of positive ATCA cases (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-10 years</td>
<td>46 (86.79%)</td>
<td>26 (56.52%)</td>
</tr>
<tr>
<td>11-20 years</td>
<td>7 (13.21%)</td>
<td>2 (28.57%)</td>
</tr>
<tr>
<td>Total</td>
<td>53 (100.0%)</td>
<td>28 (58.09%)</td>
</tr>
</tbody>
</table>

Statistics

<table>
<thead>
<tr>
<th>Chi-square</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>28.698</td>
<td>0.000</td>
</tr>
</tbody>
</table>

NS: Non-significant, S: Significant, HS: Highly significant

Serum testing of infertile males (n=53) with anti-testicular antibodies 32 (60.38%) patients to be oligospermic, and 24 (46.9%) to be azoospermia and 7 (13.21%) to be necrozooospermia (Table 4). Study for the association of male infertility type to those positive for ATCA showed almost all (85.7%) patients with necrozooospermia to be positive for ATCA. This was followed by males with azoospermia (50%) and oligospermia (46.9%).

Table 4: Association of male infertility type to ATCA

<table>
<thead>
<tr>
<th>Type of infertility</th>
<th>No. of patients (%)</th>
<th>No. positive for ATCA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oligospermia</td>
<td>32 (60.38)</td>
<td>15 (46.9)</td>
</tr>
<tr>
<td>Azoospermia</td>
<td>14 (26.42)</td>
<td>7 (50.0)</td>
</tr>
<tr>
<td>Necrozooospermia</td>
<td>7 (13.21)</td>
<td>6 (85.7)</td>
</tr>
<tr>
<td>Total</td>
<td>53</td>
<td>28</td>
</tr>
</tbody>
</table>

Statistics

<table>
<thead>
<tr>
<th>Chi-square</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>18.830</td>
<td>0.000</td>
</tr>
</tbody>
</table>

NS: Non-significant, S: Significant, HS: Highly significant
In order to rule out Addison's disease among infertile positive for ATCA, the sera was further used in the detection of anti-adrenal cortex IgG antibodies. All samples were negative for the test. A representative figure for the negative adrenal antibody detection by immunofluorescence assay is shown (Fig.2).

![Fig. 2: Immunofluorescence assay for adrenal antibody detection](Image)

Further, in order to rule out system lupus erythematosus (SLE) among males positive for ATCA, we subjected the sera for the detection of antinuclear antibody (ANA) using human epithelial cell line (Hep-2) as a substrate. Results showed 8 among the 28 (28.6%) tested to be positive in the ANA test.

![Fig. 3: Hep-2 cells positive for anti-nuclear antibody test](Image)

DISCUSSION

Worldwide, 30% of infertile couples suffer from unexplained or idiopathic infertility, defined as infertility with no obvious cause (14). Male infertility most likely involves issues with sperm count and quality and to other nutritional and environmental considerations (15). Among the several investigations involved in checking fertility in men, the semen analysis is still the most important clinical procedure of potential reproductive men. This analysis determines the ability of the semen, movement, sperm count, and morphology. In this study, we analysed serum of infertile males for autoantibodies against testicular cells and found 52.9% of them to be positive for these antibodies with no significant difference from controls. Our results are in agreement with a previous study (16), wherein the authors report a low incidence of anti-sperm antibodies among 49 infertile couples studied. Similar results were obtained for a survey of antisperm antibodies in infertile couples in Iran (17) that reported the percentage of anti-testicular antibody in infertile males to be low. The reason has been attributed to the presence of testicular cytokines that are pro-inflammatory (18). Presence of high levels of interleukin-1α (IL-1α), IL-6 and activin A in testicular cells has been previously reported (19, 20) and the cytokines shown to control testicular function and regulate sperm development by controlling cell division and its survival.

Majority of patients (60%) significantly positive to ATCA in this study were in the age group of 30-39 years, which is in agreement with previous studies (21,22). Contradictory report exists for the presence of anti-sperm antibodies (ASAs) that affect fertility (7). In this study, more than half of infertile males who were significantly positive for ATCA had marriage duration of 1-10 years, and being in the age group of 30-49 years. Probably this is an active age for men where the period represents an active immune system and the presence of high levels of antibodies against testicular antigens (23). Yet another study, reporting the male infertility factor showed the marriage time of 1-10 between years could be considered as a potential risk factor for poor sperm production in the patients diagnosed as infertile patients (24). Most male infertility patients are significantly affected by oligosperma (low sperm count) (25, 26). However, in this study we report necrozoospermia to be the associated with male infertility followed by azoospermia and oligospermia (Table 4). Scanty or absence of sperms has been linked to genetic factors leading to chromosomal abnormalities and mutations in the Y chromosome which could be inherited (27). Other factors associated to male infertility are sexually transmitted diseases, erectile dysfunction and hormonal issues, testicular overheating, obesity, alcohol intake and medications all of which leading to reduction in sperm production.

CONCLUSION

Young patients and those whose marriage period ranged from 1-10 years significantly showed positive results for ATCA. Anti-testicular antibodies have not been significantly identified in male infertile patients.

DOI: https://doi.org/10.51248/v42i5.1963
CONFLICT OF INTEREST
Authors declare that there is no conflict of interest.

REFERENCES


