Follicle-stimulating hormone autoantibody is involved in idiopathic spermatogenic dysfunction

Bing Yao¹, Jian Wang¹*, Wei Liang²*, Ying-Xia Cui¹, Yi-Feng Ge¹

¹Institute of Clinical Laboratory Medicine, Nanjing Jinling Hospital, Nanjing University, Nanjing 210002, China
²Department of Chinese Traditional Medicine, No.454 Hospital, Nanjing 210002, China

Abstract

Aim: To detect the anti-follicle-stimulating hormone (FSH) antibody in idiopathic infertile patients and fertile subjects in order to determine the role of this antibody in patients with spermatogenic dysfunction. Methods: The anti-FSH antibody in serum was detected by an enzyme-linked immunosorbent assay (ELISA). The functional and structural integrity of the sperm membrane was evaluated with hypo-osmotic swelling (HOS) test and the ultrastructure of the spermatozoa was investigated by transmission electron microscopy (TEM). Results: The extent of positive FSH antibody in the patients with oligozoospermia and/or asthenozoospermia was significantly higher than that in the fertile subjects and infertile patients with normal sperm concentration and motility, but it was significantly lower than that in the patients with azoospermia. The extent of anti-FSH antibody in the patients with azoospermia was significantly greater than that in patients with oligospermia and/or asthenospermia, infertile people with normal sperm density and motility and fertile people. The hypo-osmotic swelling test showed that the percentage of HOS-positive spermatozoa (swollen) was 45.1% ± 3.5% in the FSH antibody-positive group and 59.1% ± 6.2% in the FSH antibody-negative control group. The percentage of functional membrane damage to spermatozoa was significantly higher in the anti-FSH antibody-positive group than in the control group. TEM showed that the outer acrosomal membrane was located far from the nucleus, and detachment of the acrosome was found in the FSH autoantibody-positive group. Conclusion: These data suggest that the presence of anti-FSH antibody is strongly correlated with the sperm quantity and quality in idiopathic male infertility. Anti-FSH antibody may be an important factor causing spermatogenic dysfunction and infertility. (Asian J Androl 2008 Nov; 10: 915–921)

Keywords: follicle-stimulating hormone; antibody; reproduction; spermatogenic dysfunction

1 Introduction

Follicle-stimulating hormone (FSH) is one of the pituitary hormones that controls fertility in both males and females. For the male, FSH is essential for spermatogenesis [1] and many studies have demonstrated the biological role of FSH in rodent testes. For example, Singh and Handelsman [2] administered testosterone (T) and exogenous FSH to the gonadotrophin-releasing hormone (GnRH)-deficient mouse and found that when treated with testosterone they had normal spermatogenesis but the testicular size and germ cell numbers were reduced. In contrast, GnRH-deficient mouse treated with FSH had
FSH autoantibody is involved in idiopathic spermatogenic dysfunction

quantitatively normal spermatogenesis as well as normal size of the testis. The size of testis was increased by 43% after the mouse was treated with exogenous FSH [2]. Several other studies on gene mutations have confirmed this observation. For example, males with FSHβ gene mutations show the symptoms of azoospermia and their periods of puberty can be normal or absent. Furthermore, the testes of FSH null mutants were approximately half the normal size and the number of epididymal spermatozoa was reduced by 75% at the age of 6–7 weeks [3]. Further studies showed that injection of FSH-specific polyclonal antibody into pregnant hamsters on the 12th, 13th or 14th day of gestation or into newborn hamsters significantly reduced the number of primordial follicles. These results suggested that anti-FSH antibody inhibits the critical role of FSH on the formation of primordial follicle formation during fetal ovarian development [4]. Even though the role of anti-FSH antibody has been experimentally demonstrated in animals, it is still unclear whether FSH autoantibody is present and involved in the idiopathic infertilities with spermatogenic dysfunction. Therefore, in the present study, we investigated the presence of FSH autoantibody in idiopathic infertilities with spermatogenic dysfunction and in fertile people. In addition, we evaluated the functional and structural integrity of sperm membrane and sperm ultrastructure in FSH antibody-negative and -positive groups.

2 Materials and methods

2.1 Reagents

FSH and luteinizing hormone (LH) from human pituitaries were purchased from Sigma (St. Louis, MO, USA). FSH, LH, T and prolactin (PRL) radioimmunoassay kits were purchased from Beijing Fu Rui Biotechnology (Beijing, China). Polystyrene microplates were purchased from Combiplate 8, Biohit (Helsinki, Finland). Anti-FSH serum was purchased from Calbiochem (La Jolla, CA, USA). The anti-sperm antibody detection kit was produced by Rui Di Bioproduction (Nanjing, China).

2.2 Patients

The idiopathic infertility group included 150 patients who visited the Andrology Department at Nanjing Jingling Hospital between 2004 and 2007 (age 27.9 ± 4.6 years). A team consisting of urologists and andrologists performed detailed clinical investigations of all the patients and recorded the complete case history of each individual. All the infertile men were also subjected to karyotyping and endocrinological assays. The testicular volume of each patient was determined with punched-out elliptical rings (Takahara orchidometer). The volume of each testis was from 18.5 mL to 33.4 mL. Patients with non-obstructive azoospermia were confirmed with the observation of all phases of spermatogenic cells in testicular biopsy, thus excluding the obstruction in seminiferous tubule. The levels of serum FSH (1.42–15.2 U/L), LH (1.2–7.8 U/L), T (0.52–38.17 nmol/L) and PRL (1.3–3.5 mIU/L) in all the samples of this study were normal. Y chromosome microdeletions in the AZF (azoospermic factor) regions, as described by Imken [5], were not found. Anti-sperm antibody was not detected in all the patients. A control group included 50 men (age 28.7 ± 3.9 years) whose partners had borne their children within 1 year of marriage and had normal sperm concentration and motility.

2.3 Semen samples and analysis

Human ejaculated spermatozoa were obtained by masturbation after 3 days of sexual abstinence. Semen samples were liquefied at room temperature within 20–30 min of delivery. Semen samples were analyzed by placing a volume of 5 μL semen into a disposable semen analysis chamber (Cell-Vu; Fertility Technologies, Natick, MA, USA). Semen analysis was performed according to World Health Organization (WHO) criteria (1999) [6] All semen samples were analyzed by the same technician. Motility was analyzed for at least 200 sperm of each sample. The motility of each spermatozoon was graded as ‘a’, ‘b’, ‘c’ and ‘d’ according to the following criteria: (a) rapid progressive motility, (≥ 20 μm/s); (b) slow or sluggish motility; (c) non-progressive motility (< 5 μm/s); and (d) non-motile. Sperm motility was calculated as the number of spermatozoa with grade a + b motility divided by the total number of sperm (> 100). A repeat sperm motility assessment was performed on a separate aliquot of 5 μL from the same semen sample. If the two measures of sperm motility from the same specimen varied beyond the 95% confidence interval (CI) [6], two new slides were prepared and sperm motility was reassessed. When the two measures were within the 95% CI, the two measures were averaged and reported in this study. Patients were diagnosed with infertility and characterized as oligozoospermia, asthenozoospermia and azoospermia according to Layman [7]. Among 150 patients, 98 cases suffered from oligozoospermia (the
concentration of spermatozoa was less than 20 million/mL) and/or asthenozoospermia (the motility of the spermatozoa was less than 50%), 22 patients suffered from azoospermia (the absence of spermatozoa) and 30 patients suffered from infertility with normal sperm concentration and motility.

2.4 Enzyme-linked immunosorbent assay (ELISA)

The serum samples were acupunctured from the brachial vein and centrifuged for 30 min and immediately frozen at −20°C. Serum anti-FSH antibody level was determined by an antibody-captured ELISA using purified human pituitary FSH. A preliminary experiment was performed to determine the optimal conditions for FSH detection. After chessboard titration, we found that positive control serum diluted 1:100 and polystyrene microplates coated with 0.15 pmol purified FSH were the optimal conditions. After blocking, 100 μL of the diluted serum samples were added into the microplates, coated with FSH and incubated at 37°C for 30 min. After washing with phosphate buffered saline-Tween-20 (PBST) (pH 7.2), 100 μL of horseradish peroxidase (HRP)-conjugated goat-anti-human immunoglobulin G (IgG; 1 : 2 000) was added and incubated at 37°C for 30 min. The plates were then washed thoroughly with PBST and the absorbance (A) at 450 nm was measured after 100 μL of tetramethyl benzidine-hydrogen peroxide (TMB/H2O2) substrate was added and incubated at room temperature for about 10 min. A known negative control serum sample and a blank (0.01 mol/L PBS, pH 7.4) were included in each assay and the positive sample was judged as the value of (A of tested sample – A of blank)/(A of negative control – A of blank) ≥ 2.1 [8].

Anti-FSH serum was also included in each assay. The performance of all antibodies was determined under an internal quality assessment scheme [9].

The positive samples were verified by another ELISA assay using 100 μL of synthetic peptide (0.5 ng/mL) synthesized by HD Biosciences (Shanghai, China) as the antigen. This peptide corresponds to the sequence of the 33-53 amino acid region of human FSH beta-chain and the sequence of the synthesized peptide is EECRFCISINTTWCAGYCYTR. Samples were accepted as positive when both ELISA assays indicated positive detection of anti-FSH.

2.5 Hypo-osmotic swelling (HOS) test

The HOS test was performed according to the method described by Jeyendran et al. [10]. Briefly, 0.1 mL sperm suspension was mixed with 1 mL hypo-osmotic solution (equal parts of 150 mmol/L fructose and 150 mmol/L sodium citrate) followed by incubation for 30 min at 37°C. After incubation, 200–300 spermatozoa were examined by phase-contrast microscopy at a magnification of 400. Gametes presenting a clear ballooning of their tail membranes were counted as swollen. Subclasses of swelling was not assessed.

2.6 Electron microscopy

Sperm samples were centrifuged at 2 000 × g for 10 min and the supernatant was discarded. The sperm pellet was fixed in cold Karnovsky fixative reagent (containing 2.5% glutaraldehye, 2% paraformaldehyde in a 0.1 mol/L [pH 7.3] sodium cacodylated buffer) and maintained at 4°C for 2 h. Fixed spermatozoa was washed in 0.1 mol/L cacodylate buffer (pH 7.2) for 12 h, postfixed in 1% buffered osmium tetroxide for 1 h at 4°C and dehydrated and embedded in Epon Araldite. Ultrathin sections were cut with a Supernova ultramicrotome (Reickert Jung, Vienna, Austria), mounted on copper grids, stained with 2% uranyl acetate in 50% methanol for 10 min, followed by 1% lead citrate for 7 min and then observed and photographed with a Philips CM10 transmission electron microscope (TEM; Philips Scientifics, Eindhoven, The Netherlands). For each patient, 300 ultrathin sperm sections were analyzed.

2.7 Statistical analysis

χ² or Fisher’s exact test were used to compare the extent of FSH autoantibody presence among the different groups. Coefficients of correlation were calculated by Spearman’s correlation analysis. All hypothesis tests were two-tailed with statistical significance assessed at the P-value < 0.05 level. The data are expressed as the mean ± SEM. Statistical analysis was conducted using SPSS 11.5 for Windows software (SPSS, Chicago, IL, USA).

3 Results

3.1 The precision of the ELISA method to detect anti-FSH antibody

The samples with high, middle and lower optical density were selected and each sample was detected 12 times at the same time. Each sample was repeated this experiment for 10 days and the detection result was analyzed to obtain the coefficient of variation (CV). The results
FSH autoantibody is involved in idiopathic spermatogenic dysfunction

showed that CV is less than 7% for both intra- and inter-variation (Table 1).

3.2 The specificity of the ELISA method to detect anti-FSH antibody

Positive serum from one patient (200 μL, 1:500 dilutions) was selected specifically and cultured with 20 ng, 10 ng and 5 ng FSH at 37°C for 1 h and then the mixture was centrifuged at 5 000 × g for 15 min at room temperature to remove the immuno-complex. The supernatant (100 μL) was added to FSH coated 96-well microtiter plates and detected in duplicate by ELISA. The absorbance was compared between the pre-cultured and post-cultured samples. Another assay was performed after samples were cultured with LH. The absorbance was significantly lower after the samples were cultured with 20 ng, 10 ng, 5 ng FSH compared with the samples that were not cultured with FSH. Although culturing with different concentrations of LH also decreased the absorbance, there was no significant difference (Figure 1).

3.3 Detection of anti-FSH antibody in clinical samples

The serum from both the patients and control groups were assayed for the presence of anti-FSH antibody by the ELISA method described above. The percentage of anti-FSH antibody-positive patients with oligozoospermia and asthenozoospermia was 22.4% (22/98), which was significantly higher than that in fertile subjects (4%, 2/50, P < 0.05) and than that in infertile patients with normal sperm concentration and motility (6.7%, 2/30, P < 0.05). However, it was significantly lower than that in the patients with obstructive azoospermia (54.5%, 12/22, P < 0.05). Furthermore, the concentration of anti-FSH antibody represented by absorbance in the patients with azoospermia was significantly higher than that in oligozoospermia and/or asthenozoospermia (0.51 ± 0.10 vs. 0.27 ± 0.11) (P < 0.05), infertile people with normal sperm concentration and motility (0.51 ± 0.10 vs. 0.21 ± 0.12) (P < 0.05) and fertile people (0.51 ± 0.10 vs. 0.19 ± 0.07) (P < 0.05) (Table 2).

3.4 Effects of FSH autoantibody on the percentage of HOS cells

Forty-four patients with oligozoospermia and asthenozoospermia were selected. Twenty-two of the patients were positive for FSH-autoantibody and the remaining 20 patients were negative. There was a statistically significant increase in the percentage of functional membrane damage to spermatozoa in the FSH antibody-positive group in comparison with the values in the control group (Figure 2). The average percentage of HOS-

<table>
<thead>
<tr>
<th>Sample</th>
<th>Inter-assay variation</th>
<th>Intra-assay variation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>Higher</td>
<td>1.211</td>
<td>0.071</td>
</tr>
<tr>
<td>Middle</td>
<td>0.816</td>
<td>0.057</td>
</tr>
<tr>
<td>Lower</td>
<td>0.459</td>
<td>0.018</td>
</tr>
</tbody>
</table>

Table 1. The value of optical density and the coefficient of variation of three samples with higher, middle and lower anti-follicle-stimulating hormone (FSH) detected by enzyme-linked immunosorbent assay (ELISA). CV, coefficient of variation.

![Figure 1](http://www.asiaandro.com; aja@sibs.ac.cn)

Figure 1. Adsorption of serum containing follicle-stimulating hormone (FSH) antibody with FSH and luteinizing hormone (LH). The different absorbances are shown on the y-axis and the incubation time is shown on the x-axis. The result shows that FSH autoantibody detection decreased significantly only when serum was cultured with FSH but not by LH, indicating the specificity of enzyme-linked immunosorbent assay (ELISA).

<table>
<thead>
<tr>
<th>Absorbance (χ² ± SEM)</th>
<th>Cases</th>
<th>Positive (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azoospermia</td>
<td>22</td>
<td>12 (54.5) a</td>
</tr>
<tr>
<td>Oligozoospermia and/or asthenozoospermia</td>
<td>98</td>
<td>22 (22.4) b</td>
</tr>
<tr>
<td>Infertile men (with normal sperm concentration and motility)</td>
<td>30</td>
<td>2 (6.7) c</td>
</tr>
<tr>
<td>Proven fathers</td>
<td>50</td>
<td>2 (4) d</td>
</tr>
</tbody>
</table>

Table 2. The prevalence of anti-follicle-stimulating hormone (FSH) antibody (%) and the optical density at 490 nm in fertile and infertile male, respectively. P < 0.05 (a vs. b; c vs. b; d vs. b; A vs. B; A vs. C; A vs. D); P > 0.05 (c vs. d; B vs. C; B vs. D; C vs. D).

http://www.asiaandro.com; aja@sibs.ac.cn
positive spermatozoa (swollen) was 45.1% in the FSH antibody-positive group and 59.1% in the FSH antibody-negative control group. This difference was statistically significant (Figure 3).

3.5 Characterization of sperm ultrastructure

We further characterized the ultrastructure of the spermatozoa from the 44 samples described above. TEM micrographs of longitudinal and cross-sections of spermatozoa showed that those in the negative group were characterized by regular nuclei with condensed chromatin (Figure 4A). For the spermatozoa in the positive group, the outer acrosomal membrane was located far from the nucleus and detachment of the acrosome was observed (Figure 4B). The axonemes showed normal structure with visible dynein arms (Figure 4A, B).
4 Discussion

Mammalian spermatogenesis is a finely tuned and complex process involving intimate interactions among cells in the two compartments of the testis. In this highly organized event, the development of an undifferentiated diploid germ cell into a fully differentiated and mature spermatozoon is orchestrated in a period unique for each species. FSH, which is one of the two glycoprotein hormones produced by the pituitary gland in response to the stimulus from the hypothalamic GnRH [11], is involved in the process. Understanding the mechanisms by which FSH regulates spermatogenesis has become a focus for investigators working in the area of male reproductive endocrinology, infertility and contraception. The critical role of FSH in maintaining qualitative and quantitative spermatogenesis has also been a topic of debate, with different interpretations depending on the species [12].

A large body of evidence suggests that FSH is required for primate spermatogenesis [13]. However, there are different views about the role of FSH in regulating spermatogenesis in rodents, particularly in the adult [14]. The role of anti-FSH antibody in regulating spermatogenesis in humans is still unknown. We established an ELISA method to detect anti-FSH antibody using purified FSH and specific peptides as antigens. This ELISA assay showed tight inter- and intra-variation. Meanwhile, we used purified FSH and LH to perform an immune neutralization assay. Even though FSH and LH share a common α-subunit, the absorbance neutralized by LH was little decreased, suggesting that the method possesses good precision and specificity.

Because FSH shares the common glycoprotein alpha subunit with LH, human chorionic gonadotrophin and thyroid-stimulating hormone etc., results will be positive if the antibody to FSH is reactive to the common alpha-subunit. For this reason, the experiment was conducted twice using purified FSH and peptide specific to beta-subunit as antigens to obtain a positive result specific to the anti-FSH antibody. It is worth noting that the presence of antibodies might interfere with FSH assays. We are trying to find a way to exclude this effect, e.g., separating the antibody from the immune compound before measuring the concentration of FSH.

Reimand et al. [9] hypothesized that production of autoantibody might be initiated by the inflammation induced by viral and bacterial infection because there is no evidence of ovary tissue directed autoantibodies being detected in their patients. It is unclear whether the production of anti-FSH antibody is developed as a consequence of immune system activation by specific auto antigens or by viral and bacterial inflammation. Our laboratory is currently analyzing the epitope of FSH purified from the patients with anti-FSH antibody to investigate the mechanism of anti-FSH antibody production.

The results of ELISA detection showed that the prevalence of anti-FSH antibody in the patients with azoospermia, oligozoospermia and asthenozoospermia was significantly higher than that in the fertile patients with normal sperm concentration and motility. Moreover, the level of anti-FSH antibody was higher in the patients with azoospermia than in other patients. Moudgal et al. [15] demonstrated that continuous bioneutralization of endogenous FSH in bonnet monkeys by specific immunization produces oligospermia and infertility. The poor quality of the sperm ejaculated by the FSH-immunized monkeys was indicated by a variety of parameters including decreased viability, motility and gel penetrability, as well as acrosin and hyaluronidase activities [16]. Therefore, it is possible that the anti-FSH antibody could decrease the biological activity of serum FSH leading to the poor support of spermatogenesis process by the Sertoli cells.

In the present study, injury to the plasma membrane in the tail region of spermatozoa, as assessed by the HOS test, correlated significantly with the presence of FSH autoantibody. Furthermore, damaged acrosomal membranes, analyzed by TEM, were found to be associated with the presence of FSH autoantibodies. However, the mechanisms that caused the damage of membranes should be further explored.

Acknowledgment

This work was supported partly by Natural Scientific Foundation from Jiangsu Province, China (BK2006135).

References

3 Abel MH, Wootton AN, Wilkins V, Huhtaniemi I, Knight PG,


Edited by Dr Ralf Henkel and Dr Trevor G. Cooper