https://doi.org/10.31925/farmacia.2023.2.7 **ORIGINAL ARTICLE**

SERUM ANTI - MULLERIAN HORMONE IN AZOOSPERMIA APPROACH

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Abstract

This study aimed to investigate the role of serum anti - mullerian hormone (AMH) determination combined with ultrasonography and semen parameters in the diagnosis of azoospermic patients. Fifty patients with azoospermia were selected for the azoospermia group and underwent an ultrasound examination, and another 50 patients with normal semen in the same period were selected as the control group. AMH was detected in seminal plasma and serum, and semen parameters, including inhibin B (Inh - B), testosterone (T), and follicle - stimulating hormone (FSH), were measured. Seminal plasma AMH levels were higher than serum AMH levels in the normal and azoospermia groups ($p < 0.05$). AMH levels in seminal plasma and serum were higher in the normal group than in the azoospermia group ($p < 0.05$). Serum AMH levels were positively correlated with total sperm count, sperm motility rate, sperm concentration, percentage of progressively motile sperm, Inh - B, and total T and negatively correlated with follicle - stimulating hormone. The thicknesses of the epididymis head, epididymis body, and cauda epididymis in the azoospermia group were higher than those in the control group ($p < 0.05$). AMH level can be used as one of the biomarkers reflecting the function of Sertoli cells, which are involved in the production and growth of sperm. Ultrasound can accurately locate the prostate, ejaculatory duct, seminal vesicle, and other lesions and can provide a reliable imaging basis for the localization diagnosis of lesions in azoospermic patients.

Rezumat

Acest studiu a urmărit să investigheze rolul hormonului anti - mullerian (AMH) combinat cu ultrasonografia și parametrii lichidului seminal în diagnosticul pacienților azoospermici. Au fost înrolați cincizeci de pacienți cu azoospermie și au fost supuși unei examinări cu ultrasunete, iar alți 50 de pacienți cu spermă normală au fost selectați pentru grupul de control. AMH a fost detectată în plasma și serul seminal și au fost măsurați parametrii seminali, inclusiv inhibina B (Inh - B), testosteronul (T) și hormonul de stimulare folicular (FSH). Nivelurile de AMH din plasma seminală au fost mai mari decât nivelurile de AMH seric în grupurile normale și cu azoospermie ($p < 0,05$). Nivelurile de AMH în plasma seminală și în serul seminal au fost mai mari în grupul martor *versus* grupul cu azoospermie ($p < 0,05$). Nivelurile serice de AMH au fost corelate pozitiv cu numărul total de spermatozoizi, rata de motilitate a spermatozoizilor, concentrația de spermatozoizi, procentul de spermatozoizi progresiv motili, Inh - B și T total și corelate negativ cu hormonul de stimulare folicular. Grosimea capului epididimului, a corpului epididimului și a epididimului caudat în grupul cu azoospermie au fost mai mari decât cele din grupul control ($p < 0,05$). Nivelul de AMH poate fi utilizat ca unul dintre biomarkerii care reflectă funcția celulelor Sertoli, care sunt implicate în producerea și dezvoltarea spermatozoizilor. Ecografia poate localiza cu precizie prostata, canalul ejaculator, vezicula seminală și alte leziuni și poate oferi o bază imagistică pentru diagnosticul de localizare a leziunilor la pacienții azoospermici.

Keywords: B - ultrasound; anti - mullerian hormone (AMH); azoospermia; semen; correlation

Introduction

Reproduction represents a complex health issue related to neuroendocrine balance, affecting both men and women [1-3]. Azoospermia is one of the important causes of male infertility, and obstructive azoospermia can account for up to 60% of cases [4-6]. Causes of obstructive azoospermia include congenital dysplasia of the vas deferens, inflammatory obstruction and iatrogenic factors. Obstructive azoospermia can be treated by surgery, such as percutaneous epididymal aspiration and testicular sperm extraction, with good clinical efficacy and prognosis [7-9]. Therefore, an accurate assessment of the location of obstruction and a diagnosis of the type of obstruction are critical for clinical diagnosis. Altinkilic *et al*. [10] found that transrectal ultrasound could clearly show structural abnormalities such as vas deferens and seminal vesicles. Hu *et al*. [11] showed that transscrotal ultrasound can provide a diagnostic basis for obstructive azoospermia of the proximal vas deferens.

Anti - mullerian hormone (AMH) is secreted mainly by immature Sertoli cells of the testis and granulosa cells of the ovary in men. Secretion peaks during foetal life until puberty and declines to low levels

after adulthood. It is mainly involved in the regulation of cell differentiation and accelerates degenerate mullerian in male embryos [12-14]. Data have shown that AMH can play a regulatory function during sperm generation and promote sperm formation [15-17]. The relationship between AMH levels and semen parameters and the differences in AMH levels in different male reproductive system - related diseases are still hot topics in current clinical research. Zarén *et al*. [18] concluded that serum AMH levels were significantly positively correlated with sperm concentration; however, Witz *et al*. [19] concluded that serum AMH levels could not be used as an indicator to predict spermatogenesis and maturation, and serum AMH levels were not significantly correlated with parameters in testicular puncture results in azoospermic patients. The clinical data of 50 patients with azoospermia admitted to the hospital were analysed. Based on the diagnosis of orchiotomy and sperm extraction assisted by a light microscope, the sonographic findings of transrectal ultrasound in azoospermia were summarized to investigate the localization of transrectal ultrasound in azoospermia and the diagnostic value of obstruction types, as well as the AMH level in azoospermia patients. The correlation between serum AMH level and other sex hormones was explored to determine the diagnostic significance of serum AMH level for azoospermia and predict the spermatogenic function of the testis.

Materials and Methods

Patients

Fifty azoospermic patients admitted to Puren Hospital Affiliated with Wuhan University of Science and Technology, Wuhan, China, from March 2020 to October 2021 were included in the study. The patients were aged between 21 and 46 years, with a mean age of (31.82 ± 4.28) years, infertility for 2 - 7 years, and mean infertility for (4.21 ± 1.28) years. Another 50 patients with normal semen during the same period were selected as the control group, aged between 22 and 47 years, with a mean age of (32.84 ± 4.35) years, infertility for 2 - 6 years, and a mean infertility period of (4.53 ± 1.36) years. There was no significant difference in age, duration of infertility, and other clinical data between the two groups ($p > 0.05$). This study was approved by the Ethics Committee of Puren Hospital Affiliated to Wuhan University of Science and Technology, Wuhan, Hubei, China, and the patients and their families signed the informed consent form. Inclusion criteria were: (1) patients who met the diagnostic criteria for male azoospermia in the Department of Reproductive Medicine and healthy normal subjects; (2) patients with azoospermia, excluding other known genetic defects such as chromosomal abnormalities or Y chromosome microdeletions; (3) patients aged between 21 and 47 years; and (4) patients from whom complete follow-up data could be obtained. Exclusion criteria: (1) previous history of cryptorchidism, testicular tumours, and another surgical history; (2) genetic examination results of chromosomal structure and number of abnormalities; (3) patients younger than 21 years or older than 47 years; (4) patients from whom complete follow-up data could not be obtained; and (5) patients unwilling to join the trial.

Ultrasound examination method

A Color Doppler Ultrasound Diagnostic Apparatus (ACUSON S2000 Ultrasound System, Siemens Medical Solutions USA, Inc., Malvern, PA, USA) was used to examine the superficial linear array probe 9L4 (Siemens Medical Solutions USA, Inc., Malvern, PA, USA) through the scrotum at a probe frequency of 12 MHz. The patient was placed in a supine position, the penis was lifted, the scrotum was fully exposed, the probe was sleeved with a condom, and a coupling agent was externally applied to examine the testis, epididymis, spermatic vein and vas deferens to observe the external morphological structure and whether the duct was dilated, measure the size and preserve the sonogram.

For transrectal examination, a transrectal probe, EC9-4 (Siemens Medical Solutions USA, Inc., Malvern, PA, USA), was used with a probe frequency of 5 - 7.6 MHz. The patient was placed in the left lateral decubitus position, the knees were held with both hands flexed, the probe was sleeved with a condom, a coupling agent was applied, and the probe was slowly inserted into the anus. The prostate gland, ejaculatory duct, seminal vesicle, and vas deferens ampulla seminal vesicle were observed, and a sonogram was preserved. Testicular volume was calculated by the Lam - Bert equation.

Volume (mL) = diameter of testis up and down x diameter of testis left and right x diameter of testis front and back x 0.71, (1).

Sonographic analysis

Two sonographers with intermediate professional titles or above performed ultrasound examinations and image analyses of the patients. Ultrasonographic findings of the head of the epididymis were normal, showing only dilatation of the efferent tubules, inflammatory masses and dilatation of the efferent tubules accompanied by disorganized echoes. Ultrasonographic findings of epididymal body and cauda epididymis: normal, epididymal duct dilatation; cord - like changes, indicating thinning of epididymal body and cauda epididymis, narrowing of fibrosis, and increased echogenicity; truncation sign, indicating interruption of epididymal body and cauda epididymis; inflammatory mass, disorganized echogenicity.

Semen and serum collection

Semen collection: The subjects were abstinent for 2 - 7 days. All semen was collected in a disposable, sterile,

wide - mouth plastic container (purchased from Xuzhou Caihui Glass Products Co., Ltd.) by masturbation. The container was placed in a 37°C water bath for liquefaction. Routine semen parameters were measured. The remaining semen was centrifuged at 3,000 rotations *per* minute for 5 minutes, and the seminal plasma from the surface layer was taken and stored in an ultra - low temperature freezer at -70°C until AMH levels were measured.

Serum collection: 3 mL venous blood was collected from subjects between 8:00 and 11:00 am and allowed to stand at room temperature for 30 min. The venous blood was centrifuged at 3,000 rpm for 5 minutes, and 300 μL of the supernatant was transferred to an Eppendorf tube to test AMH levels.

Routine semen analysis

A sample of 8 μL of semen was placed in a Sefi medical sperm counting plate (Sefi - Medical Instruments, Haifa, Israel), and semen parameters were routinely analysed using a WLJY - 9000 Color Sperm Quality Detection System (WeiLi New Century Science and Technology Development Co. Ltd., Beijing, China), and the examination items included total sperm count ($\times 10^6$/mL), sperm concentration ($\times 10^6$/mL), sperm motility (%), and percentage of progressive motile sperm (%). Each semen specimen was controlled at or above 300 counts and analysed in triplicate, and the mean value was calculated as the sperm concentration and sperm motility.

Sperm morphology analysis

After being washed twice with normal saline, a sperm suspension was prepared. Then, 15 μL of the suspension was used to obtain a uniform smear, which was allowed to dry naturally. The smear was then stained using the Shorr method, which involved haematoxylin staining for 60 seconds. Next, the smear was immersed in running water for 5 minutes to blue the stain before being immersed in ethanolamine. Shorr staining was carried out for 3 - 5 minutes, and the smear was rinsed in running water before being dried. After these procedures, sperm morphology was assessed. The storage, dilution, sample addition, colorimetry, and other steps involved in the preparation of the samples were standardized to ensure the reliability of the results. All reagents used were purchased from Hebei Biology Science and Technology Co., Ltd. in China.

Serum reproductive hormone levels

AMH levels were measured by enzyme - linked immunosorbent assay (ELISA) (Beckman Coulter Ltd., Shangai, China), and the operation was performed in strict accordance with the instructions of the kit. Repeatability: coefficient of variation (CV) ≤ 15%; inter - batch difference: CV ≤ 15%.

Hormone levels were measured in samples collected prior to treatment. Venous blood was collected from patients under fasting conditions at 8:00 a.m. for testing. Total testosterone (T) (Abbott Architect 2nd generation total testosterone assay, Abbott Core Laboratories, Lake Forest, IL, USA), inhibin B (Inh-B) (Human INHB (Inhibin B) CLIA Kit, AMSBIO LLC, Cambridge, MA, USA), and follicle - stimulating hormone (FSH) (Elecsys FSH, Cobas e system, Roche Diagnostics, Indianapolis, IN, USA) were determined by electrochemiluminescence according to manufacturer specification.

Statistical methods

Statistical data analysis was performed using the SPSS 23.0 (IBM, USA) software package and the Excel software (Microsoft, USA). Measurement data conforming to a normal distribution were expressed as mean ± standard deviation (±s), and those not conforming to a normal distribution were expressed as median (25% ~ 75%). An analysis of variance and a correlation test were performed; when there was a difference in F values, a q test was used to compare the two groups, and $p \leq 0.05$ was considered statistically significant.

Results and Discussion

Basic parameters for azoospermic patients

The basic parameters of azoospermic patients are shown in Table I. Median age: 28 years (26 - 34); median progressive motile sperm: 31.21% (17.29 - 41.35); median abstinence time: 4 (3 - 7) days; median morphologically normal sperm: 5.37% (4.35 - 5.76); median semen volume: 3.5 mL (2.4 - 4.6); median AMH: 8.39 ng/mL (5.38 - 12.5); median total sperm count: 104.3×10^6/mL (38.4 - 183.6); Inh-B: 146.53 ± 47.29 ng/L; median sperm concentration: 35.41×10^6/mL (12.38 - 54.39); median total T: 13.62 ng/mL (12.52 - 26.34); median total sperm motility: 37.29% (21.38 - 48.72); median FSH: 4.2 mIU/mL (3.1 - 6.3).

Comparison of seminal AMH and serum AMH levels between the two groups

Frequency distribution plots of seminal and serum AMH concentrations are presented in Figure 1 and Figure 2, respectively. The comparison of seminal AMH with serum AMH was conducted for both groups and is shown in Figure 3.

The seminal plasma AMH level in the normal group was significantly higher than the serum AMH level ($p < 0.05$). The seminal plasma AMH level in the azoospermia group was significantly higher than the serum AMH level ($p < 0.05$). AMH levels in seminal plasma and serum were significantly higher in the normal group than in the azoospermia group ($p < 0.05$).

Table I

Basic parameters of azoospermia patients (n = 50)

Variable	$\bar{x}$±s or median (25% ～ 75%)	Variable	$\bar{x}$±s or median (25% ～ 75%)
Age (years)	28 (26 - 34)	Progressive motile sperm (%)	31.21 (17.29 - 41.35)
Abstinence time (d)	4 (3 - 7)	Morphologically normal sperm (%)	5.37 (4.35 - 5.76)
Semen volume (mL)	3.5 (2.4 - 4.6)	AMH (ng/mL)	8.39 (5.38 - 12.5)
Total sperm count (× 10^6/mL)	104.3（38.4 - 183.6）	Inh - B (ng/L)	146.53 ± 47.29
Sperm concentration (× 10^6/mL)	35.41（12.38 - 54.39）	Total T (ng/mL)	13.62 (12.52 - 26.34)
Total sperm motility (%)	37.29（21.38 - 48.72）	FSH (mIU/mL)	4.2 (3.1 - 6.3)

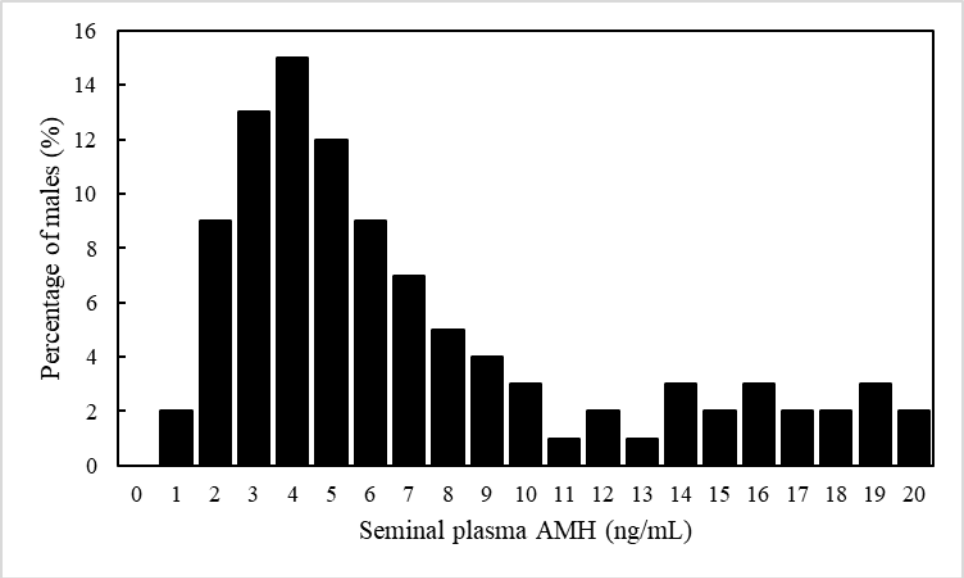

Figure 1.
Frequency distribution of seminal plasma AMH concentration

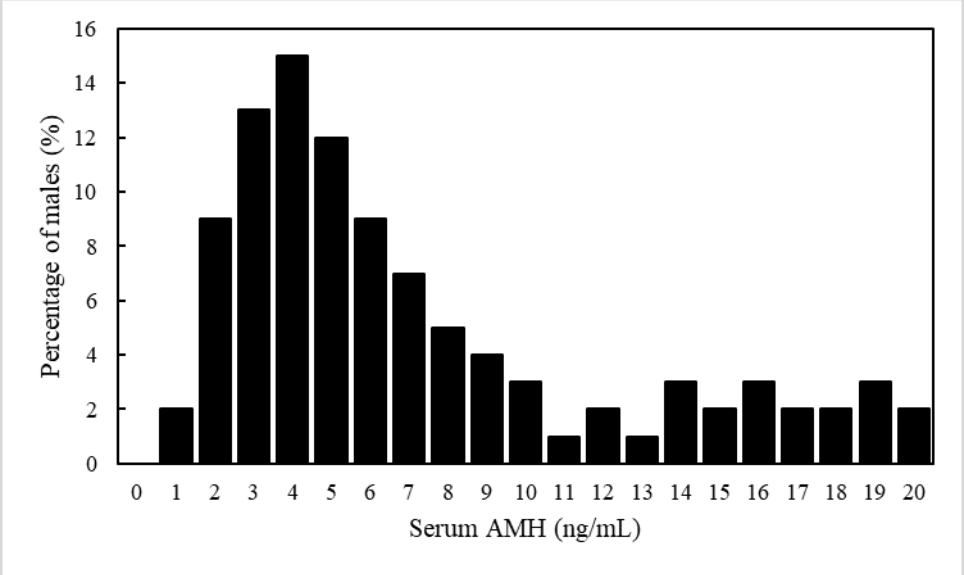

Figure 2.
Frequency distribution of serum AMH concentration

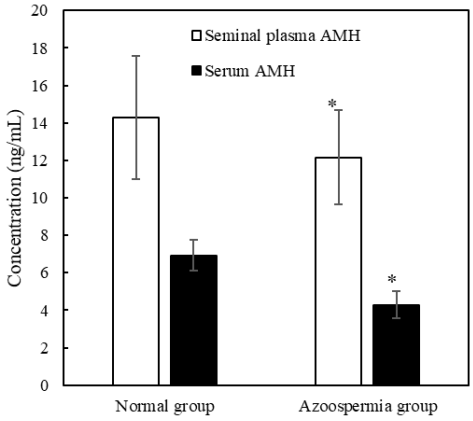

Figure 3.
Comparison of seminal plasma AMH and serum AMH between the two groups
* Compared with the normal group, $p < 0.04$

Correlation analysis of serum AMH levels with semen parameters and reproductive hormone levels
The correlation between serum AMH levels and sperm parameters and reproductive hormone levels is shown in Table II. There was no significant correlation between serum AMH level and age, abstinence time, percentage of normal sperm morphology and semen volume ($p > 0.05$). Serum AMH levels were positively correlated with total sperm count ($r = 0.241$, $p < 0.001$), sperm concentration ($r = 0.247$, $p < 0.001$), sperm motility ($r = 0.131$, $p = 0.024$), percentage of progressively motile sperm ($r = 0.125$, $p = 0.001$), Inh - B ($r = 0.352$, $p < 0.001$), and total T ($r = 0.175$, $p = 0.002$); Serum AMH levels were negatively correlated with FSH ($r = - 0.246$, $p < 0.001$).

Table II
Correlation of serum AMH levels with sperm parameters and reproductive hormone levels

Variable	Serum AMH (ng/mL)	
	r	p
Age (years)	- 0.029	0.162
Abstinence time (d)	- 0.015	0.532
Semen volume (mL)	0.035	0.524
Total sperm count ($\times 10^6$/mL)	0.241	< 0.001
Sperm concentration ($\times 10^6$/mL)	0.247	< 0.001
Sperm motility (%)	0.131	0.024
Progressive motile sperm (%)	0.125	0.001
Morphologically normal sperm (%)	- 0.032	0.563
Inh - B (ng/L)	0.352	< 0.001
Total T (ng/mL)	0.175	0.002
FSH (mIU/mL)	- 0.246	< 0.001

Comparison of testicular volume and thickness of the head, body, and tail of the epididymis between the two groups
There was no significant difference in testicular volume between the two groups ($p > 0.05$) (Figure 4). The thickness of the head, body, and tail of the epididymis in the azoospermia group was significantly higher than that in the control group ($p < 0.05$) (Figure 5).

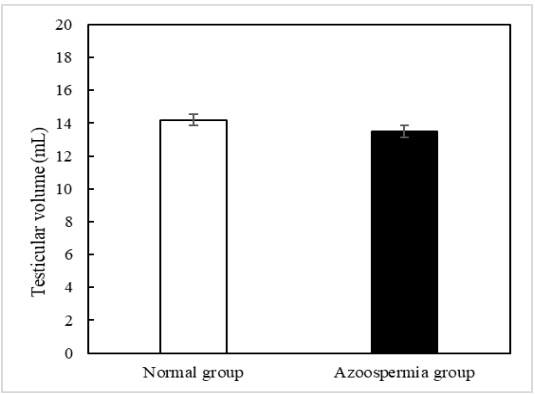

Figure 4.
Comparison of the testicular volume between the two groups

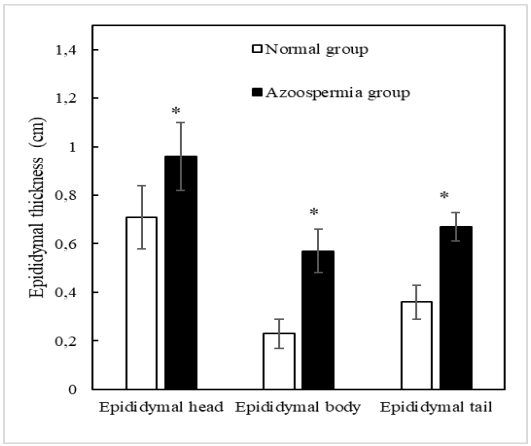

Figure 5.
Comparison of the thickness of the head, body, and tail of the epididymis between the two groups.
*Compared with the normal group, $p < 0.05$

Results of a B ultrasound examination
Of the 50 azoospermic patients, transrectal ultrasonography revealed 13 midline prostatic cysts, 12 ejaculatory duct calcifications, and 9 chronic seminal vesiculitides. Scrotal ultrasonography showed vas deferens dilatation in 24 cases, an abnormal epididymal mass in 16 cases, local thickening of the epididymal body with reticular

changes in 18 cases, and testicular rete dilatation or cyst in 3 cases.

As a member of the transforming growth factor β superfamily, serum AMH is a dimeric glycoprotein composed of more than 500 amino acid residues [20-22]. In male patients, AMH is mainly secreted by Sertoli cells and is negatively regulated by androgens as well as positively regulated by FSH. From 56 days of gestation, male Sertoli cells begin to produce and secrete AMH, which plays a key role in the development of male reproductive organs. Measuring serum AMH levels can distinguish whether the neonatal disease is cryptorchidism or bilateral anorchidism. High levels of AMH can be detected in the serum of new-borns aged 3 months - 1 year; during puberty, Sertoli cells are not yet mature and secrete high levels of AMH [23-25].

So far, it is controversial whether AMH in male serum can be used as a biomarker for spermatogenesis and maturation. Dević Pavlić *et al.* [26] reported that AMH concentrations in the serum of patients with obstructive azoospermia (OA) were significantly higher than those in the serum of patients with non-obstructive azoospermia (NOA). Aksglaede *et al.* [27] stated that AMH concentrations in serum were significantly higher in normal, healthy men than in azoospermic patients. However, Kien Nguyen *et al.* [28] concluded that AMH levels in the serum of patients with azoospermia or oligozoospermia were not statistically different from concentrations in normal healthy subjects. The above studies indicate that there are inconsistent conclusions about the correlation between AMH levels in serum and semen parameters. In the current study, the correlation between AMH level and semen parameters was analysed. The results showed that serum AMH level was positively correlated with total sperm count, sperm concentration, sperm motility rate and percentage of progressively motile sperm, and negatively correlated with FSH. This is similar to the findings of Papanikolaou *et al.* [29]. That study suggests that the maturation of spermatogenesis may be related to the functional relationship of Sertoli cells, and the special structure and function of Sertoli cells make them directly involved in the support of spermatogenic epithelial structure, the influence of germ cells and the process of spermatogenesis. Therefore, the advantages and disadvantages of Sertoli cell function in the testis can be reflected by detecting the number of sperm [30]. In conclusion, serum AMH may serve as a biomarker of Sertoli cell function as well as spermatogenesis. Histological data showed that AMH was produced only in seminiferous tubules and immature spermatids in the testis, further confirming this view.

Inh-B, an important evaluation index of adult male fertility, is directly involved in the negative feedback regulation of body fluids. The results showed that there was a significant positive correlation between AMH levels secreted by Sertoli cells and Inh-B and a significant negative correlation between serum AMH levels and FSH, which was consistent with the conclusions of Urrutia *et al.* [31]. Serum AMH levels were significantly positively correlated with total T, indicating that serum AMH is involved in regulation through the hypothalamic - pituitary - gonadal axis.

Sperm pipelines include the seminal vesicle, vas deferens, epididymis, ejaculatory duct and prostate, and azoospermia is induced when a site is hypoplastic or obstructed. Ultrasonography is a common method for the clinical diagnosis of azoospermia and has the advantages of being non-invasive, non-X-ray, repeatable and having high accuracy. Transrectal ultrasound and transscrotal ultrasound were performed in both groups. The transrectal ultrasound probe is tightly attached to the rectal wall, the examination process is interfered with by a few factors, the probe frequency is high, the image quality is good, and the seminal vesicle gland, prostate, vas deferens ampulla, and other structures can be displayed. Moreover, ultrasound is easy to perform, has a low cost, no radiation, and no wound, and can be used as the first choice of detection method for azoospermia. The results showed that there was no significant difference in testicular volume between the two groups ($p > 0.05$). The thickness of the epididymis head, epididymis body, and cauda epididymis in the azoospermia group was significantly higher than that in the control group ($p < 0.05$). Sonograms showed epididymal lesions that mainly manifested as increased epididymal thickness and epididymal duct dilatation; the floating phenomenon could be observed; wall echogenicity was enhanced; and significant inflammatory masses could be observed in the epididymis, which may be produced by the accumulation of exfoliated epithelial cells or dead spermatogenesis.

In summary, there was a positive correlation between serum AMH levels and total sperm count, percentage of progressively motile sperm, sperm motility, sperm concentration and total T. Serum AMH levels were higher in the normal group than in the azoospermia group, predicting a correlation between AMH levels and spermatogenesis. Serum AMH levels were significantly positively correlated with Inh-B and negatively correlated with FSH, indicating that AMH can be used as a biomarker to reflect Sertoli cell function.

Conclusions

Our study aimed to evaluate the utility of transrectal ultrasound in localizing azoospermia and identifying the type of obstruction, as well as to investigate the relationship between serum AMH levels and other sex hormones in patients with azoospermia. AMH level can be used as one of the biomarkers reflecting Sertoli cell function; serum AMH level is correlated with sperm production and maturation; transrectal

combined scrotal B ultrasound can accurately locate the prostate, ejaculatory duct, seminal vesicle and other lesions and can provide a reliable imaging basis for the localization diagnosis of lesions in azoospermic patients.

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Conflict of interest

The authors declare no conflict of interest.

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