Shenjing Guben Wan promotes sperm development by increasing the activity of seminiferous epithelium Sertoli cells

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Background: Infertility is an important social problem. Asthenozoospermia (AZS) is a common pathological cause of male infertility, but its pathogenesis is unclear. Shenjing Guben Wan (SJGBW), a traditional Chinese medicine, has shown remarkable effects during the clinical treatment of oligozoospermia or AZS.

Methods: In this study, clinical evaluations were carried out on 184 AZS patients receiving SJGBW treatment, including sperm count, sperm quality, and pregnancy rate. Also, ornidazole was used to build an AZS mouse model, and SJGBW treatment was administered. The sperm quantity and fertility of mice in different groups were evaluated; a cholecystokinin octapeptide-8 (CCK-8) experiment was carried out to test the activity of seminiferous epithelium Sertoli cells, and immunohistochemistry and the Terminal Deoxynucleotidyl Transferase-mediated dUTP Nick-End Labeling (TUNEL) method were employed to test the pathological information and expression of the Sertoli cell surface marker in the testicular tissues of mice in each group.

Results: The sperm vitality, progressive sperm motility, and sperm morphology of patients who received SJGBW treatment were all improved (P<0.05). In the AZS group, the average sperm count, sperm vitality, pregnancy rate, and female mouse litters were all lower relative to mice in the control group. Following SJGBW treatment, the average sperm count, sperm vitality, pregnancy rate, and female mouse litters of mice in the AZS group were all significantly improved. The cytobiological experimental results showed that compared with the serum of normal male mice in the control group, the drug serum containing SJGBW could improve the cell vitality and proliferative ability of seminiferous epithelium Sertoli cells in AZS mice. Furthermore, the TUNEL results showed that the seminiferous tubule Sertoli cells and mesenchymal cells of the AZS mice exhibited the most significant apoptosis, which was alleviated following SJGBW treatment. Moreover, the levels of Sertoli cell marker, SOX9, and anti-apoptosis protein, Bcl2, in SJGBW-treated mice were both higher than that in AZS mice.

Conclusions: SJGBW can promote the development and maturation of germ cells by facilitating the proliferation of Sertoli cells in AZS patients, thereby improving the fertility of these patients.

Keywords: Asthenozoospermia (AZS); Shenjing Guben Wan (SJGBW); oligozoospermia; sperm development

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Introduction

Infertility is an important social problem affecting around 8–12% of married couples, with 50% of cases resulting from various factors in males (1). Asthenozoospermia (AZS) is related to poor sperm quality (especially decreased sperm vitality), which is a common pathologic cause of male infertility (particularly in young men) (2). AZS has numerous etiological agents but its pathogenesis remains unclear, and there are currently no effective clinical treatments for this problem.

Spermatogenesis is a complicated biological process. The blood-testis barrier (BTB) is a big junctional complex consisting of closely connected adjacent Sertoli cells in the seminiferous tubules of the testicles and is one of the tightest blood barriers in mammals (3). The periodic opening and reproduction of the BTB enables anterior epithelial spermatocytes to transfer from the outer to the inner compartments of seminiferous tubules, so maintaining the BTB is critical to spermatogenesis and male fertility (4). Functional disorder of the seminiferous epithelium Sertoli cells will lead to the failure of timely movement in undeveloped germ cells, thereby resulting in sperm developmental problems and ultimately infertility. Meanwhile, these germ cells must maintain steady attachment to Sertoli cells via the special intermediate filaments (i.e., desmosome connection) and actin (i.e., exoplasm specialization) of the testicles, to prevent the “falling off” of immature germ cells from the seminiferous epithelium (5,6).

Sertoli cells interact directly with germ cells, and provide morphological and nutritional support to spermatogenesis, and are therefore critical (7,8). Yokonishi et al. (7) used benzalkonium chloride (BC) to remove the host Sertoli cells, and observed a loss of germ cells following BC administration. They speculated that the loss of germ cells was secondary to the loss of nutritional support from Sertoli cells. Zhao et al. (9) employed single-cell ribonucleic acid (RNA) sequencing to compare the developing testicles of patients with different types of non-obstructive azoospermia and those of healthy subjects, and observed that the most common defect in non-obstructive azoospermia patients was Sertoli cells with functional disorders.

Shenjing Guben Wan (SJGBW), a kidney-reinforcing and blood circulation-activating formula in traditional Chinese medicine, has shown remarkable effects during the clinical treatment of oligozoospermia or AZS. It has been approved by the China Food and Drug Administration (CFDA) for clinical use, but there have been no specific discussions of its mechanism of action. Therefore, by summarizing the clinical effect of SJGBW and the research on AZE mouse model, this study preliminarily confirmed that SJGBW can promote the vitality and proliferation of testicular seminiferous epithelial cells, promote spermatogenesis and improve the fertility of AZE. We present the following article in accordance with the ARRIVE reporting checklist (available at https://tau.amergroups.com/article/view/10.21037/tau-22-381/rc).

Methods

Patient recruitment and conventional semen analysis standard

In this study, 184 AZS patients who received infertility treatment at the Second Hospital of Hebei Medical University from May 2018 to July 2020 were recruited. Before inclusion in our study, the participants underwent standard clinical and laboratory evaluation, including measurement of the testicle size and volume, conventional semen analysis, and assessment of the hydrocele of tunica vaginalis, varicocele, and secondary sex characteristics.

The inclusion criteria were as follows: (I) males aged between 22 and 45 years; (II) those diagnosed with male infertility and AZS; (III) those with normal sexual function and regular sex life; and (IV) patients who were willing to participate in this study and signed the informed consent. The exclusion criteria were as follows: (I) patients with serious spermatogenesis functional disorders triggered by congenital causes or specific factors such as drinking, drug abuse, smoking, or substance abuse; (II) those with multiple obvious flagella with abnormal morphology; (III) patients with abnormal androgen, biochemical results, or elastase of seminal plasma; (IV) those with reproductive system infections; (V) patients with anti-sperm antibody detected in mixed antiglobulin reaction test (+); and (VI) those with mental diseases, malignant tumors, or serious organic diseases.

The diagnostic standard of AZS infertility was based on the “World Health Organization (WHO) Laboratory Manual for the Examination and Processing of Human Semen” (5th edition) (Issue 67, 2010) (10). The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Medical Ethics Committee of Hebei Medical University (No.180313). Informed consent was taken.
from all the patients.

**Drug preparation**

Through the pharmacological experimental scheme, the adult body weight was 60 kg and the daily dose of SJGBW was 15-g crude drug, which was converted to the intervention dose of SJGBW in the mouse experimental group was 200 μL after decocted and concentrated, and the total crude drug content was 20 g/kg. Two equal prescriptions were added to purified water according to a mass-to-volume ratio of 1:10 and soaked for 30 min. After being boiled under high heat (1,000 W), the preparation was boiled for 15 min under gentle heat (500 W), and the liquid was then filtered using three layers of gauze. The drug residue was boiled a second time and added to purified water according to a mass-to-volume ratio of 1:8. After being boiled under high heat, the preparation was boiled for 10 min under gentle heat and filtered, and the two filtrates were combined. Next, the filtrates were concentrated to 0.5 g of crude drug every milliliter and then stored below 4 °C for future use.

**Construction of an animal model and drug intervention**

Thirty C57BL6N male mice and 60 C57BL6N female mice [aged 8–12 weeks (19–24 g)] were purchased from Vital River (Beijing, China). After adaptive feeding for 1 week, the 30 male mice were randomly divided into three groups, with 10 mice in each group: a control group (Ctrl), an AZS group, and an SJGBW group. Mice in the control group were fed conventionally. The AZS and SJGBW groups received intragastric administration of ornidazole (ORN) at a dose of 400 mg/kg, which was provided for 14 days to construct an AZS mouse model.

Subsequently, the AZS group mice received intragastric administration of normal saline (NS) for 6 weeks (11), and the SJGBW group mice had intragastric administration of prepared SJGBW solution for 6 weeks. After the last drug administration, the mice were euthanized using 1% pentobarbital sodium, and their epididymides were quickly removed for further examination. Successful construction of the AZS mouse model was determined by evaluating the epididymal sperm count and vitality of the mice.

This study was carried out in strict accordance with “The Guide for Care and Use of Laboratory Animals” formulated by the National Institutes of Health (NIH). The animal use plan was approved by the Animal Care and Use Committee of Hebei Medical University (No. 180313). A protocol was prepared before the study without registration.

**Detection of sperm count and vitality**

The sperm counts of patients were observed under a microscope (CX43, Olympus corporation, Tokyo, Japan) according to the standard operating procedure, and a computer-aided semen analyzer (SAS-II system, Beijing Precise Instrument Co., Ltd., Beijing, China) was used to measure sperm vitality. The sperm count and vitality tests were carried out using the testing method for human patients as a reference.

**Mouse fertility evaluation**

Each male mouse was placed in the same cage with two female mice. There were 30 male mice and 60 female mice in total. The pregnancy rate after mating and the number of mice given birth to by each female mouse were recorded.

**Separation of seminiferous epithelium Sertoli cells in mouse testicles**

The method described by Puri et al. (4) was employed to separate Sertoli cells in 20-day-old mice, and these cells were cultured in a serum-free medium. The specific process was as follows: the testicle was digested in a bicarbonate buffer containing collagenase for 12 min below 33 °C and was then washed in a petri dish three times to separate the seminiferous tubules. The separated seminiferous tubules were digested for 12 min using trypsin (0.5 mg/mL), and Dulbecco’s Modified Eagle Medium (DMEM) of an equivalent volume containing 10% fetal calf serum was then added to terminate digestion. After precipitation and re-suspension, the seminiferous tubules were cultured in a petri dish or coverslip coated with Matrigel (Solarbio, Beijing, China) under 32 °C and 5% CO₂. On the second day, after being washed using PBS, the specimens were further cultured in a serum-free medium, and the obtained culture generally had a purity of >95%.

**Immunohistochemistry**

Immunostaining of testicular tissues with paraffin embedding was also carried out. After deparaffinization and permeabilization, the specimen was heated for 20 min in citrate buffer to recover the antigens. After blocking and incubating the antibody, the specimen was washed
Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) apoptosis assay

The testicular tissues with paraffin embedding were permeabilized and washed, and after preparing the TUNEL reaction mixture, 100 μL of TUNEL reaction mixture was added to react for 1 h under 37 °C. Next, the tissues were soaked in 2× SSC (saline sodium citrate buffer) for 15 min, and after closed soak cleaning, 100 μL streptavidin-fluorescein isothiocyanate (FITC) (diluted with PBS according to a ratio of 1:500) was added to react for 30 min. The cell nuclei were re-dyed using propidium iodide. Next, the specimen was closed using antifade mounting medium. Finally, a fluorescence microscope (IXplore Pro, Olympus, Japan) was used to take photos for analysis.

Cell vitality and proliferative ability assay

A cholecystokinin octapeptide-8 (CCK-8) system (Dojindo Laboratories, Japan) was used to evaluate the influence of SJGBW-positive serum on the vitality of Sertoli cells according to the operating instructions. 5-ethynyl-2'-deoxyuridine (EdU, Abcam, Shanghai, China) was employed to detect the impact of SJGBW-positive serum on the proliferation of Sertoli cells according to the operating instructions.

Statistical analysis

All data were analyzed using GraphPad Prism 8.0 (GraphPad, San Diego, USA). All results were presented with the standard deviation of the mean, and all comparisons and analyses were based on variance analysis.

Results

SJGBW can improve the fertility of AZS patients

We analyzed the sperm count, sperm quality, and pregnancy rate to evaluate the effects of using SJGBW to treat AZS patients, and the results are presented in Figure 1A. The average sperm counts of AZS patients were significantly increased after SJGBW treatment [(7.36±4.63) ×10^6 before treatment vs. (50.57±36.53) ×10^6 after treatment), P<0.05]. Furthermore, sperm quality was evaluated in terms of sperm vitality, forward movement sperm rate, and morphology, and the results are shown in Figure 1B-1D. After treatment, the sperm vitality, forward movement sperm rate, and morphology of patients all showed remarkable improvement (P<0.05).

According to the follow-up visit, among the 184 patients participating in our clinical experiment, the pregnancy rate reached 74.46% in 6 months, and patients who could get their spouses pregnant within 9 months accounted for 63.05% (Figure 1E).

SJGBW can improve the fertility of AZS mice

We built the AZS mouse model using ORN to further investigate the ability of SJGBW in improving fertility. The results showed that the average sperm count of the AZS group was (3.85±2.56) ×10^6, which was significantly lower than that of the Ctrl group [(11.85±2.22) ×10^6], while the average sperm count of the SJGBW treatment group was (8.68±2.35) ×10^6 (Figure 2A). The above results indicated that after SJGBW treatment, the sperm counts of AZS mice experienced significant growth (P<0.05). Also, the average sperm vitality of the AZS group was 25.84%±6.07%, which was markedly lower than that of the Ctrl group (57.42%±5.03%). Meanwhile, the average sperm vitality of the SJGBW treatment group was 41.92%±6.89%, which showed remarkable growth compared to the AZS group (P<0.05) (Figure 2B).

All 20 female mice in the Ctrl group were pregnant, while only 14 female mice in the AZS group got pregnant, and 19 female mice in the SJGBW group were pregnant (Figure 2C). By counting the births of mice in each group, we found that in the Ctrl group, the mated female mice gave birth to 5–8 mice in each litter (5.95±0.88 on average). In the AZS group, the female mice gave birth to 2–5 mice in each litter (2.64±1.08 on average). In the SJGBW group, the female mice gave birth to 3–9 mice in each litter (4.32±0.75 on average) (Figure 2D). Based on the above data, we believe that SJGBW can improve the sperm count and fertility of AZS mice.

SJGBW can improve the vitality of seminiferous epithelium Sertoli cells and promote fertility

To further analyze the mechanism of action of SJGBW in improving the fertility of AZS patients, we prepared a drug...
serum containing SJGBW and separated the seminiferous epithelium Sertoli cells from the mice testicles. Next, CCK-8 and EdU experiments were carried out to verify the influence of SJGBW on the seminiferous epithelium Sertoli cells of mice. The cell vitality test results showed that compared to the serum of normal male mice in the Ctrl group, the drug serum containing SJGBW could improve the vitality of the seminiferous epithelium Sertoli cells of mice (Figure 3A). Moreover, the EdU experimental results showed that compared to the serum of the Ctrl group, the drug serum containing SJGBW could promote the division and proliferation of mouse seminiferous epithelium Sertoli cells (Figure 3B, 3C). Therefore, we speculate that SJGBW can improve the fertility of AZS mice by promoting the vitality and proliferation of seminiferous epithelium Sertoli cells.

**SJGBW can inhibit the apoptosis of testicle Sertoli cells in AZS mice**

To further verify the inference based on the cytological

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Figure 1 SJGBW intervention can improve the fertility of AZS patients. (A) Average sperm count of patients before and after treatment with SJGBW; (B) sperm vitality of patients before and after treatment with SJGBW; (C) forward movement sperm rate of patients before and after treatment with SJGBW; (D) sperm morphology of patients before and after treatment with SJGBW; (E) pregnancy rate of patients. **, P<0.01; ***, P<0.001. AZS, asthenozoospermia; SJGBW, Shenjing Guben Wan.
In experiment, we employed immunohistochemistry and the TUNEL method to analyze the pathological information of testicular tissues of mice in each group. The hematoxylin-eosin (HE) results showed that compared to the Ctrl group, the seminiferous tubules of AZS mice had scarce sperm cells, there was a severe lack of seminiferous epithelium Sertoli cells, and the structures of seminiferous tubules were seriously damaged. Even though the seminiferous tubules of mice were still damaged after the SJGBW intervention, relatively complete Sertoli cells and rich sperm cells were recovered following the intervention (Figure 4A).

The TUNEL method was adopted to detect the apoptosis of mouse seminiferous tubules in various groups. The results showed that the AZS mice had the most significant apoptosis of seminiferous epithelium Sertoli and mesenchymal cells. As for mice in the SJGBW group, even though their seminiferous tubules also suffered from apoptosis, the severity was markedly lower than that of the AZS group (Figure 4B).

Furthermore, immunohistochemistry was employed to examine the expression levels of Sertoli cell marker and apoptin in the seminiferous tubules of mice in each group. Figure 5A showed that the expression level of the Sertoli cell marker, SOX9, was consistent with the HE results. In the seminiferous tubules of AZS mice, we found that only a small amount of Sertoli cells were marked as brown by the SOX9 antibody. For mice in the SJGBW group, even though the number of marked Sertoli cells in seminiferous...
In this study, we investigated the effects of SJGBW in treating AZS according to the related clinical data as well as animal and in vitro experiments. The results of both clinical
Figure 4 Pathological examination results of mouse testicular tissues in the various groups. (A) HE staining of testicular tissues of mice in the various groups; (B) detection of seminiferous tubule apoptosis of mice in the various groups. AZS, asthenozoospermia; Ctrl, control; HE, hematoxylin-eosin; SJGBW, Shenjing Guben Wan.

Figure 5 Examination of the expression levels of Sertoli cell marker and apoptin in the seminiferous tubules of mice in each group using immunohistochemistry. (A) Expression level of the Sertoli cell marker; (B) expression level of apoptin Bax; (C) expression level of IAP Bcl2. AZS, asthenozoospermia; Ctrl, control; IAP, inhibitor of apoptosis protein; SJGBW, Shenjing Guben Wan.
and animal experiments showed that SJGBW significantly increased the sperm count and vitality of AZS patients, and improved the pregnancy rate. In the in vitro experiment, we used the drug serum containing SJGBW for intervention in the testicle seminiferous epithelium Sertoli cells, and the results showed that SJGBW increased the vitality and improved the proliferation of seminiferous epithelium Sertoli cells. Therefore, we speculate SJGBW can improve the fertility of AZS patients by improving the state of Sertoli cells.

To further verify our speculation, we carried out HE staining and apoptosis assay of the testicle seminiferous tubules of AZS mice, and found that the structures of Sertoli cells in the seminiferous tubules of AZS mice exhibited significant damage due to increased apoptosis levels. This result was consistent with the findings of Yokonishi et al. (7), which showed that after completely depleting the Sertoli cells, the mice lost their fertility and no mature sperms were formed. However, SJGBW intervention could significantly improve this phenomenon and promote the vitality and proliferation of Sertoli cells, and the results of the apoptosis assay performed in this study also indicated that SJGBW could inhibit the apoptosis of Sertoli cells.

In conclusion, SJGBW can facilitate the development and maturation of germ cells by promoting the Sertoli cells of AZS patients, thereby improving the fertility of AZS patients.

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Footnote

Reporting Checklist: The authors have completed the ARRIVE reporting checklist. Available at https://tau.amegroups.com/article/view/10.21037-tau-22-381/rc

Data Sharing Statement: Available at https://tau.amegroups.com/article/view/10.21037-tau-22-381/dss

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tau.amegroups.com/article/view/10.21037-tau-22-381/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Medical Ethics Committee of Hebei Medical University (No. 180313). Informed consent was taken from all the patients. The study was carried out in strict accordance with “The Guide for Care and Use of Laboratory Animals” formulated by the National Institutes of Health (NIH). The animal use plan was approved by the Animal Care and Use Committee of Hebei Medical University (No. 180313).

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